

Leigh syndrome – new variants,
new perspectives in a
heterogeneous
neurodegenerative disease.

Manuela Schubert Baldo

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Manuela Schubert Baldo



MANUELA SCHUBERT BALDO

**LEIGH SYNDROME – NEW VARIANTS, NEW PERSPECTIVE
IN A HETEROGENEOUS NEURODEGENERATIVE DISEASE.**

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Programa Doutoral Da Universidade Do Porto
(Instituto De Ciências Biomédicas Abel Salazar)

Orientadora – Doutora Laura Vilarinho
Categoria – Investigadora
Afiliação – Instituto Nacional de Saúde Doutor
Ricardo Jorge

Co-Orientadora - Doutora Esmeralda Martins
Categoria – Professora catedrática convidada
Afiliação - Instituto De Ciências Biomédica Abel
Salazar Da Universidade Do Porto,
Centro Hospitalar da Universidade do Porto

Co-Orientadora - Doutora Sílvia Vilarinho
Categoria – Professora associada
Afiliação – Universidade de Yale, USA

À minha família que sempre me impulsionou adiante em meus sonhos e acreditou no meu potencial profissional e aos meus amigos que, mesmo por vezes sem compreenderem, também estiveram ao meu lado em todas as minhas decisões.

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Resumo

A Síndrome de Leigh, atualmente nomeada Espectro da Síndrome de Leigh, descrita pela primeira vez por Denis Archibald Leigh em 1951, é uma doença mitocondrial neurodegenerativa mais frequente na faixa etária pediátrica, afetando classicamente crianças até aos dois anos de idade potencialmente levando à sua morte. A sua fisiopatologia baseia-se na deficiência da fosforilação oxidativa mitocondrial com comprometimento da produção de energia secundariamente manifestos clinicamente, radiologicamente e metabolicamente. A avaliação laboratorial e por neuroimagem permitiu avanços diagnósticos relevantes, porém restritos devido entraves como a heterogeneidade clínica e a confirmação do diagnóstico. Com os avanços recentes de novas metodologias de sequenciação do genoma humano, o diagnóstico desta patologia tornou-se mais eficiente ainda que se trate de uma patologia potencialmente fatal e sem tratamento curativo.

Este trabalho objetivou a caracterização molecular de uma coorte (n=82) altamente suspeita clínico-laboratorialmente de Espectro de Síndrome de Leigh, na qual foi confirmado o diagnóstico em 40 doentes, 28 revisitados e 12 novos. A variante mais frequentemente encontrada foi a m.8993T>G no gene *ATPase6* suportada pelo perfil de aminoácidos, o qual revelou poder ser extremamente sugestivos de doença mitocondrial e até mesmo de mutações específicas, como a m.8993T>G e a m.9176T>G. Em alguns doentes, identificamos variantes de significado indeterminado e em função dos dados bioquímicos e imagiológicos, sugerimos que sejam provavelmente patogénicas.

Na coorte de doentes sem diagnóstico confirmado (n=42), reavaliámos os resultados genéticos e foram estudados do ponto de vista populacional, sem correlação entre o haplogrupo populacional e a gravidade do fenótipo. Em virtude dos resultados obtidos, foi desenvolvido e proposto um algoritmo de abordagem diagnóstica ao Espectro da Síndrome de Leigh validado na coorte diagnosticada, o qual prevê a integração de dados clínicos, bioquímicos e moleculares de forma a maximizar o potencial de diagnóstico.

Os avanços nos estudos de biologia molecular permitiram revelar a etiologia molecular de muitas doenças mitocondriais e concretamente do Espectro da Síndrome de Leigh. Pese embora todos estes desenvolvimentos e melhorias nos

fluxogramas de diagnóstico, vários casos permanecem ainda sem caracterização.

Abstract

Leigh Syndrome, now referred to as Leigh Syndrome Spectrum, was first described by Denis Archibald Leigh in 1951. It is classified as a mitochondrial disease and is recognized as the most prevalent neurodegenerative disorder in the pediatric population, primarily affecting children under the age of two and potentially leading to death. The pathophysiology of Leigh Syndrome is characterized by a deficiency in mitochondrial oxidative phosphorylation, which results in impaired energy production, manifesting clinically, radiologically, and metabolically.

Significant advancements in diagnostic capabilities have been achieved through laboratory and neuroimaging evaluations; however, these advancements are constrained by challenges such as clinical heterogeneity and the confirmation of diagnoses. Recent developments in human genome sequencing methodologies have enhanced the efficiency of diagnosing this condition, despite its potentially fatal nature and the absence of curative treatments.

This study aimed to molecularly characterize a cohort (n=82) with a high suspicion of Leigh Syndrome Spectrum based on clinical and laboratory findings. The diagnosis was confirmed in 40 patients, comprising 28 previously evaluated and 12 new cases. The most frequently identified variant was m.8993T>G in the ATPase6 gene, which was supported by the amino acid profile indicative of mitochondrial disease, alongside specific mutations such as m.8993T>G and m.9176T>G. In three patients, variants of uncertain significance were identified; based on biochemical and imaging data, we propose that these variants are likely pathogenic. In the cohort of patients without a confirmed diagnosis (n=42), we re-evaluated the genetic results and analyzed them from a population perspective, finding no correlation between population haplogroups and the severity of the phenotype.

Consequently, a diagnostic algorithm for Leigh Syndrome Spectrum was developed and proposed, validated within the diagnosed cohort, integrating clinical, biochemical, and molecular data to optimize diagnostic potential. Advances in molecular biology have elucidated the molecular etiology of numerous mitochondrial diseases, particularly Leigh Syndrome Spectrum. Despite these advancements and improvements in diagnostic workflows, several cases remain uncharacterized.

Keywords

Leigh Syndrome Spectrum

Next generation sequencing

Mitochondrial disorders

Neurodegeneration

Infantile subacute necrotizing encephalopathy

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Publications

1. Baldo, M.S. and Vilarinho, L. **Molecular basis of Leigh Syndrome Spectrum: a current look**. Orphanet J Rare Dis. 2020 Jan 29;15(1):31. doi: 10.1186/s13023-020-1297-9.

2. Baldo, M.S.; Nogueira, C.; Pereira, C.; Janeiro, P.; Ferreira, S.; Lourenço, C.M.; Bandeira, A.; Martins, E.; Magalhães, M.; Rodrigues, E.; et al. **Leigh Syndrome Spectrum: A Portuguese Population Cohort in an Evolutionary Genetic Era**. Genes 2023, 14(8):1536. doi: 10.3390/genes14081536.

3. Baldo, M.S.; Azevedo, L.; Coelho, M.P.; Martins, E.; Vilarinho, L. **A Comprehensive Approach to the Diagnosis of Leigh Syndrome Spectrum**. Diagnostics 2024, 14(19), 2133

List of Abbreviations

A

ACMG - American College of Genetics and Genomic Medicine

ADP - Adenosine Diphosphate

ANT - Adenine Nucleotide Translocator

ATP - Adenosine Triphosphate

B

BBGD - Biotin responsive Basal Ganglia Disorder

BCKDH - Branched Chain α -Ketoacid Dehydrogenase Complex

BER - Base/clipping Excision Repair

C

CES - Clinical Exome Sequencing

CSF - Cerebrospinal Fluid

CoQ10 - Coenzyme Q10

COX - Cytochrome c oxidase

CNS - Central Nervous System

CVB - Chorionic Villus Biopsy

D

DARS2 - Aspartate aminoacyl-tRNA synthetase gene

DNA - Deoxyribonucleic acid

dNTPs - Deoxyribonucleotide triphosphates

DRP1 - Dynamin-related protein 1

E

EARS2 - Glutamyl-tRNA synthetase gene

ETC - Electron Transport Chain

F

FAD - Flavin-Adenine Dinucleotide

FGF-21 - Fibroblast Growth Factor 21

FARS2 - Phenylalanyl tRNA synthetase gene

G

GDF-15 - Growth and Differentiation Factor 15

I

ICBAS - Instituto de Ciências Biomédicas Abel Salazar

INSA - National Institute of Health Doutor Ricardo Jorge

iPSCs - Induced Pluripotent Stem Cells

K

KSS - Kern-Sayres Syndrome

L

LDH - Lactate Dehydrogenase

LBSL - Leukoencephalopathy, Brain stem and Spinal cord involvement and Lactic acidosis

LS - Leigh Syndrome

LLS - Leigh-Like Syndrome

LSS - Leigh Syndrome Spectrum

LHON - Leber Hereditary Optic Neuropathy

15-LO - 15-lipoxygenase

LRPPRC - Leucine Rich Pentatricopeptide Repeat Containing

M

MDs - Mitochondrial disorders

MELAS - Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes

MEGDEL - 3-methylglutaconic aciduria with Deafness, Encephalopathy, and Leigh-like syndrome

MERFF - Myoclonic Epilepsy with Ragged Red Fibers

MFN1 - Mitofusin 1

MFN2 - Mitofusin 2

MILS - Maternally Inherited Leigh Syndrome

MIS - Mitochondrial Intermembrane Space

MitoGCEP - Mitochondrial Experts Group

MMR - Mismatch Repair

MRC - Mitochondrial Respiratory Chain

MRI - Magnetic Resonance Imaging

mtDNA - mitochondrial DNA

mtDNAssb - mitochondrial single-stranded DNA binding protein

N

nDNA - nuclear DNA

NADH - Nicotinamide Adenine Dinucleotide Hydride
NAD⁺ - Nicotinamide Adenine Dinucleotide oxidized
NARP - Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa
NDVD - Neurodevelopmental Delay
NDUFV2 - NADH-ubiquinone oxidoreductase flavoprotein 2
NER - Nucleotide Excision Repair

O

OXPHOS - Oxidative Phosphorylation
OPA1 - mitochondrial dynamin-like GTPase
OTC - Ornithine Transcarbamylase

P

PCR - Polymerase Chain Reaction
PEO - Progressive External Ophthalmoplegia
PDHc - Pyruvate Dehydrogenase Complex
POLG - DNA Polymerase Gamma

R

RARS2 - arginyl tRNA synthetase
RNA - Ribonucleic acid
RNA^t - transporter RNA
ROS - Reactive Oxygen Species

S

SDH - Succinate Dehydrogenase
SOD - Superoxide Dismutase
SURF1 - Surfeit locus protein

U

UOA - Urinary organic acids

V

VUS - Variant of Uncertain Significance

W

WES - Whole Exome Sequencing
WGS - Whole Genome Sequencing

Chapter I. Introduction

1. The mitochondrion

Eukaryotic organisms evolved from the need to produce energy more efficiently. One evolutionary conquest was achieved by oxidative phosphorylation (OXPHOS), inside the mitochondria, using oxygen as a final electron acceptor. This allowed the generation of adenosine triphosphate (ATP), from different substrates could dramatically increase. The most important part of this efficient energy production occurs through the mitochondrial respiratory chain (MRC) process and electric transport chain (ETC) to generate ATP using OXPHOS, which happens in the inner membrane changing its potential through ion flux along the complexes¹. The last complex (complex V) merge ADP to inorganic phosphate into ATP as shown in figure 1².

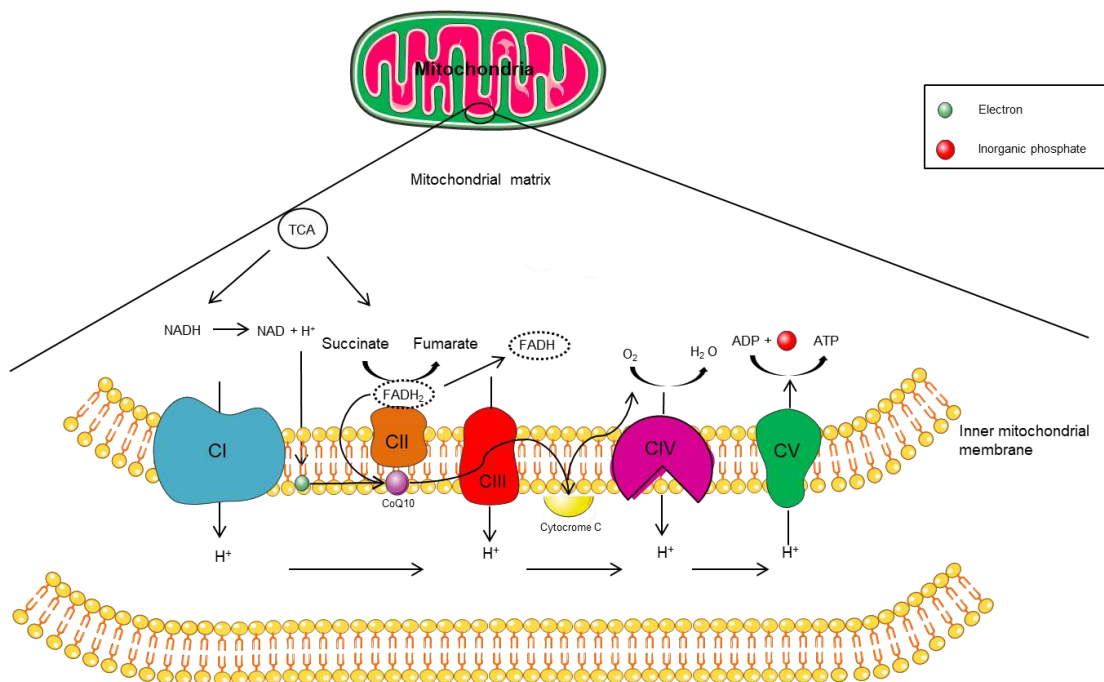


Figure 1. Representation of OXPHOS system in mitochondria. Energetic metabolism produces intermediates that can be used by cytochromes and be submitted to oxidation-reduction states producing electrons and in the last step promoting ADP association to an inorganic phosphate resulting in ATP. Elaborated with Servier Medical Art.

Mitochondria are unique organelles which have their own genetic material - mitochondrial DNA (mtDNA) – able to carry on their own protein production. The mtDNA is composed of thirty-seven genes detailed in 16,569 base-pair (figure 2), encoding thirteen subunits of four MRC. The mtDNA organization follows a double-strand circular shape where the strands are named heavy and light,

respectively, and host translation process. Besides the structural and RNAs materials (transporter and ribosome), the control regions are associated with populational tracking since harboring gene groups transmitted through generations³. Any disruption in the circular shape interferes directly with the availability of DNA material. The remaining components are encoded by nuclear DNA (nDNA).

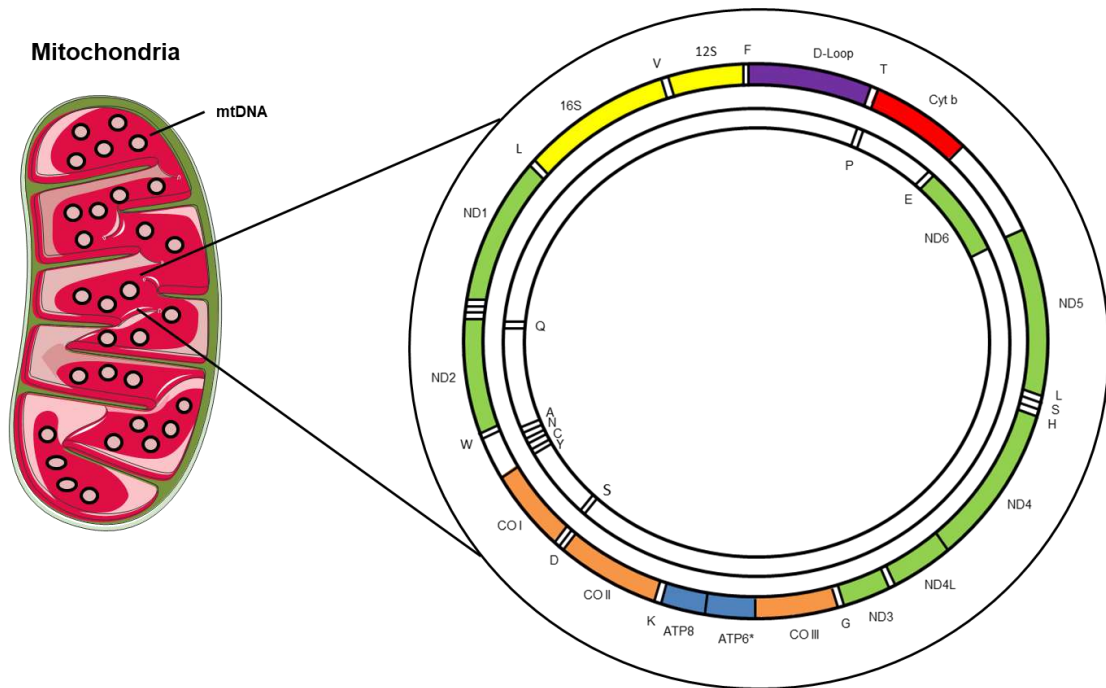


Figure 2. Representation of mitochondrial DNA (mtDNA) and respective genes. Elaborated with Servier Medical Art.

In each cell there are thousands of mitochondria and in each mitochondria several mtDNA molecules. The mtDNA pool is dynamically conserved and replicated harboring normal and mutated molecules. So, the mitochondrial population is formed by normal mtDNA coexisting with mutated mtDNA, a concept named heteroplasmy⁴.

As both genomes control the OXPHOS process, any interference in their homeostasis affects direct or indirectly the energy production, potentially triggering variable symptoms⁵.

Mitochondrial disorders are the most frequently inherited metabolic disorders, estimated to 1:5000 in population⁶. The clinical manifestations are unpredictable in age and heterogeneity resulting in a diagnostic challenge. Mitochondria are almost universally present in human cell lines and therefore in several organs in the human body whose function is pivotal. In specific energy

dependent organs, such as central nervous system (CNS) and muscle tissues, more prone to energy deficit, symptoms may arise first and more pronounced than others.

1.1 MtDNA characteristics

The majority of mtDNA changes are considered non-pathological and frequent in the population, which enables them to trace human territorial spreading³. In the late 80's, the first DNA mutation was identified⁷⁻⁹ and since then, several of them followed to be identified counting over hundreds of them comprising point mutations and rearrangements¹⁰. They result in diverse clinical syndromes with growing overlap between them. These changes are inherited differently than nDNA disorders and don't obey the Mendelian rules. Particular features are involved in mtDNA inheritance such as: *i*) threshold effect that requires a minimum amount of mutations to clinically express into a clinical phenotype¹¹; *ii*) the mitotic segregation which shows random distribution of mutated and normal mitochondria into offspring challenging the prediction of mutated mitochondria in them¹²; *iii*) clonal expansion leading to higher mutated rates in post-mitotic tissues by replicating the mutated DNA over the normal DNA^{13,14} possibly due to the mtDNA replication characteristics¹⁵, and *iv*) the bottleneck effect which explains how the heteroplasmy percentage can evolve to homoplasmy after replicating through the descendants generations¹⁶⁻¹⁹. The mechanisms of this latter effect are still under debate and hypotheses are controversial.

1.1.1 MtDNA point mutations

The point mutations are maternally inherited, occurring in proteins and RNA genes, especially tRNAs, implicating damaging MRC or mitochondrial translation²⁰. They are mostly heteroplasmic, exception made to isolated-organs phenotypes²¹⁻²³. As far as point mutations go, the mutation load and the clinical symptoms may vary. The coexistence of mutated and normal mtDNA is most frequently seen in variable degrees of organ involvement, resulting in mitochondrial cytopathies²⁴. Despite being commonly multisystemic and diverse, classical phenotypes of this group of diseases are recognized, such as: Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS), Myoclonus epilepsy associated with ragged red fibers (MERFF), Neuropathy, Ataxia and Retinitis Pigmentosa (NARP) and Maternally Inherited

Leigh Syndrome (MILS) are classic phenotypic examples of variable heteroplasmic mutations where muscle tissue and CNS are deeply involved and host dominant symptoms²⁵. On the other hand, less frequently, the mutation load can be high up to 100% in specific organ which produces an isolated deficiency such as Leber Hereditary Optic Neuropathy (LHON) where the high heteroplasmic rates, usually above 95%, preferably manifest in one organ instead of simultaneous different tissues²⁶.

1.2.2 MtDNA rearrangements

Mitochondrial rearrangements are secondary to changes mtDNA shape causing interruption in its replication. They may be partial deletions, duplications and even triplications²⁷. The most frequent deletion is the 4977bp 8344-13447del yet ranging from 1,3 to 10kb size and containing several genes²⁸. In this type of rearrangement, the deletion size presented in all affected tissues is the same²⁹. Moreover, the cells hosting the defect are heteroplasmic. The single-large deletion syndromes are known to be a *continuum* under the same genetic background, manifesting as Pearson syndrome, Kearns-Sayre syndrome (KSS), and Progressive external ophthalmoplegia (PEO). They are rare and the chance of inheritance by the affected individuals' offsprings is minimum (usually less than 1%)³⁰ suggesting the process might remain in maternal oocytes²⁹. Additionally, mtDNA might suffer from multiple deletions of variable sizes. These several losses are due to nDNA mutations that control the stability and replication of mtDNA. Therefore, the balance between normal and mutated DNA and the number of DNA copies inside mitochondria are relevant to normal functioning^{31,32}.

2. Mitochondrial genome interactions

2.1 Nuclear genome

The nuclear genome is responsible for the majority of the mitochondrial proteosome, such as OXPHOS, mitochondrial genome replication and maintenance³³. When the mitochondrion, a prokaryotic organ, was incorporated into eukaryotic cell, it abdicated several functions by losing parts of its DNA to nuclear DNA³⁴. The nDNA exome (protein codifying part) has thousands of genes, several of them interacting with mitochondrial bioprocesses.

2.2 Mitochondrial maintenance

Despite having their own DNA, most of the mitochondrial material to maintain their DNA pool is under nuclear control³³. These proteins are key to mitochondrial replication, expression, and repair, and must be imported by them into their matrix. Their own pack of proteins and specific machinery is called “nucleoid”, the responsible complex for mtDNA maintenance³⁵. Mutations in these nuclear genes produce mitochondrial maintenance defects, responsible for imbalance in quality and quantity of mtDNA. These disorders are autosomal dominant, recessive or X-linked inherited³⁶. They manifest in four phenotypes, basically: myopathic, encephalomyopathic, hepatocerebral and neurogastrointestinal forms³⁶. The heterogeneity and unpredictability of onset until molecular confirmation reinforces the difficulties in diagnosing.

2.2.1 mtDNA replication

The mtDNA replication (figure 3) obeys its own command and is processed autonomously³⁷. The D-loop region is a non-coding region that sets the time and the pace of replication of both DNA strands in a mutual interactive mechanism³⁸⁻⁴⁰. Under *POLG* (polymerase gamma) control, the heavy-strand begins to replicate until achieving the light-strand initial site of replication, which follows the opposite direction until the whole molecule has undergone the entire process⁴¹. This complexity is sustained by the replisome, a set of enzymes that ensure the process to be protected, stabilized and safe such as *TWINKLE* (DNA helicase), Topoisomerase I and mitochondrial single-stranded DNA binding protein (mtDNAssb), the binding protein to prevent denaturation of the new single-stranded mtDNA⁴². Additionally, the process is beyond the machinery to control, adjust and correct eventual mistakes, where *POLG* is one of acting enzymes (lyase function)⁴³. The amino acid supply held by deoxyribonucleotide triphosphates (dNTPs) is fundamental, especially to keep a balanced pool of them⁴⁴, and not only their cytosolic synthesis but their uptake and import by mitochondria⁴¹. They are produced in a pre-form, the dMTPs, and after phosphorylated to dNTPs to be later imported using adenine nucleotide translocator (ANT) and mitochondrial inner membrane protein MPV17 (MPV17) as transporters⁴⁵.

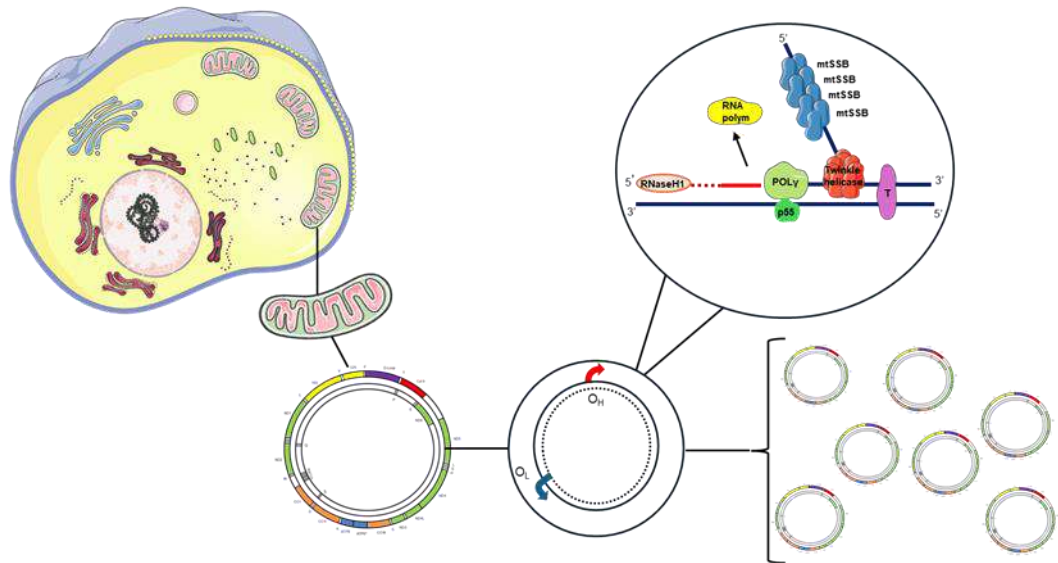


Figure 3. mtDNA replication system. The illustration of an eukaryotic cell hosting several mitochondria and several mtDNA copies and the replisome machinery. Abbreviations: Oh: heavy strand; Ol: light strand; RNaseH: Ribonuclease H1; RNA polym: RNA polymerase; mtSSB: mitochondrial single-stranded DNA binding protein; Topo: Topoisomerase I; Poly: Polymerase gamma. Adapted from Kasiviswanathan et al⁴² figure.

2.2.2 mtDNA dynamics and repair

The dynamic processes are also regulation parameters to this mtDNA balance, keeping enough pool to replicate and recover material in major damaging states⁴⁶. The fusion/fission processes represent another measure to keep organization and integrity of mitochondrial content and overall function⁴⁷. The fission process divides mitochondria into several smaller mitochondria by dynamin-related protein 1 *DNML1* which strangles the outer membrane in a circular shape and after following site marks done, *DNML1* cuts the membranes; and the fusion process merges mitochondrion through inner and outer membranes under mitofusins 1 and 2 (*MFN1*, *MFN2*) merging the outer membranes and *OPA1* regulates inner membrane merging and rebuilding the cristae^{48,49}.

For mtDNA, the recovery and repair mechanisms are contrasting to nDNA who undergoes nucleotide excision repair (NER). Alternatively, fewer options are available such as base/clipping excision repair (BER), the most important of them⁵⁰, mismatch repair (MMR), direct reversal (DR), and double strand break repair (DSB)⁵¹. Unfortunately, the amount of mtDNA molecules per cell are several times higher than nDNAs which can be high-handed for the repairing system and the injured material must be removed⁵².

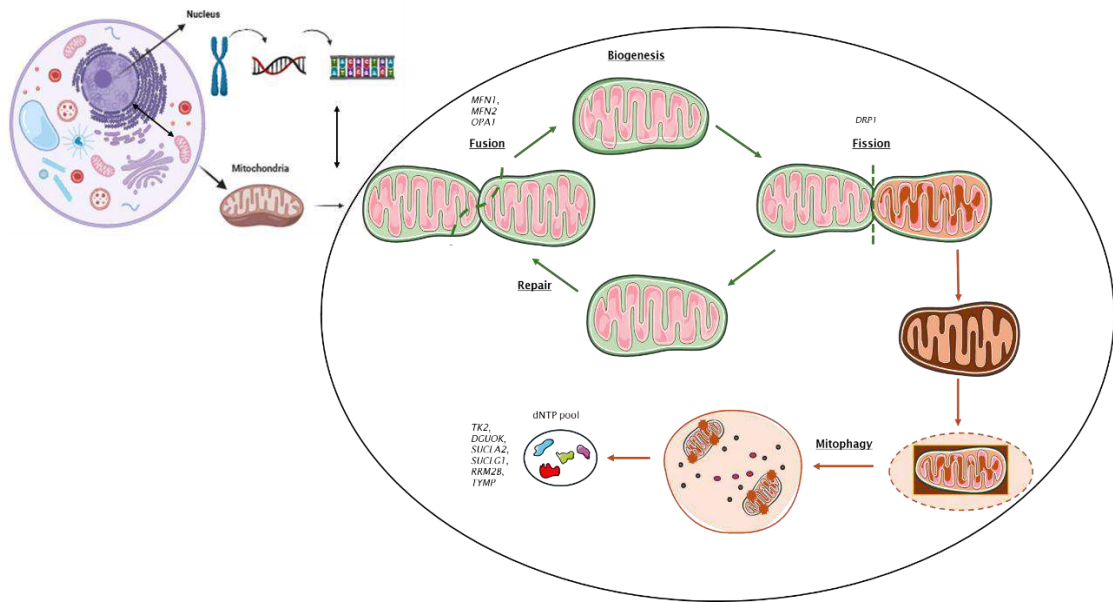


Figure 4 :Interaction between both genomes in mitochondrial dynamic processes. The interactions between nDNA and mtDNA by regulating mitochondrial functions and balancing the dNTP pool to ensure effective replication process.

2.3 Heterogeneous phenotypes

The heterogeneity of inheritance and degrees of poor interaction between both genomes for MDs justify the correspondent heterogeneity in clinical spectrum of phenotypes due to varied inheritance modes and defects. In childhood, MDs are most frequently manifested as Leigh Syndrome Spectrum (LSS), an early-onset potentially fatal neurodegenerative disorder. This syndrome presents as severe neurological impairment, hyperlactacidemia, ophthalmological impairment leading to premature death. Despite biochemical screening and imaging support, the diagnosis is still a challenge.

3. Leigh Syndrome Spectrum

Leigh Syndrome Spectrum (LSS), previously separated into Leigh syndrome (LS) and Leigh-like syndrome (LLS), also known as necrotizing leukoencephalopathy, was first described by Sr. Denis Archibald Leigh in 1951 when evaluating a seven-month-old boy during acute infectious intercurrent with loss of motor and cognitive milestones, spasticity, deafness, blindness and hyperlactacidemia. The baby died three days after initial evaluation and *postmortem* investigation remarked symmetric lesions confined to basal ganglia and brain stem⁵³.

Classically, it presents early onset neurological features usually before the age of two years⁵⁴. This syndrome comprises neurological delay or regression, hypotonia, epilepsy, ophthalmological abnormalities, central apnea, typical symmetric neuroimaging changes with elevated serum biochemical markers such as lactate. Organs involvement beyond the central nervous system (CNS) may be seen such as cardiac, hepatic, renal and endocrine systems⁵⁵.

The features in LSS rise due to failure in ATP availability to high-energy demand neuronal circuits⁵⁶. These neurons activate a cascade of gliosis when ATP levels are imbalanced translating into symmetric hyperintensities in basal ganglia, *thalamus* and/or brainstem associated to a lactate peak in spectroscopy in brain magnetic resonance imaging (MRI) T2-weighted acquired sequences⁵⁷. Additional brain areas might be involved such as cerebellum, spinal cord and the white matter.

Since the most common presentation was in the first decade of life, only infants were investigated for LSS. However, during the decades more evidence was supportive of adolescents and adults meeting criteria for LSS. Souza *et al* assessed patients through whole exome sequencing (WES) from 3 months to 69 year-old undiagnosed neuropsychiatric patients with exacerbated symptoms during illnesses with metabolic acidosis and hyperlactacidemia⁵⁸. From an important global hypotonia in a 3-month-old baby to a slowly progressive ataxia in a 69-year-old, the diagnosis was obtained in genes previously associated with mitochondrial functions and compatible biochemical support. This report reassured the importance in looking carefully to other symptoms besides the classic phenotype described supported by the fact that LSS updated criteria include psychiatric manifestations⁵⁹. McKelvie *et al* also reported resemblance to multiple sclerosis⁶⁰.

The outstanding progress in molecular assessments brought a wider comprehension of this entity, and the classic phenotype is no longer enough to raise diagnostic suspicion. Asymmetric lesions in neuroimaging, normal biochemical assessment or incomplete clinical, laboratorial, radiological evaluation of the patients are insufficient to fulfill the LS diagnosis. These situations were attributed to a Leigh-like syndrome (LLS) nomenclature⁶¹. Currently, after consensus of Mitochondrial Experts Group (MitoGCEP)⁵⁹ the nomenclature was unified into LSS considering well defined criteria for diagnosis published in the last consensus^{59,62} presented in Table 1.

Table 1. Criteria for diagnosing LSS.

	Leigh Syndrome Spectrum (LSS)
Clinical features	<u>Metabolic decompensation</u> (elevated lactate levels in blood and/or cerebrospinal fluid) and/or characteristic symptoms during acute illness (episodic). <u>Neurologic manifestations:</u> hypotonia, spasticity, movement disorders (dystonia, chorea), cerebellar ataxia, peripheral neuropathy, deafness, blindness, apnea, Parkinson and Multiple Sclerosis symptoms and loss of previously acquired abilities. <u>Psychiatric manifestations:</u> hallucinations, psychosis <u>Other systems manifestations:</u> hypertrophic cardiomyopathy, anemia, renal tubulopathy, liver involvement, ptosis, muscle weakness.
Radiological features	Bilateral symmetric T2-weighted hyperintensities on MRI or hypodensities in CT scan in basal ganglia and/or brainstem <i>With or without</i> Bilateral T2-weighted hyperintensities on MRI or hypodensities in CT scan in thalamus, cerebellum, subcortical white matter and/or spinal cord

The main symptoms are exemplified by motor and/or cognitive disability, clinical signs of basal ganglia or brainstem dysfunction featured by symmetric damage in correspondent neuroanatomy, ineffective aerobic metabolism reflected in high lactate serum and/or in CSF supported by a molecular background confirmation. The importance of early recognizing these symptoms is proceeding with the investigation, diagnosis and offering the proper clinical support to these patients especially to treatable causes.

3. Biochemical studies

They comprise complete blood work, serum, urine and CSF analysis such as:

3.1 Lactate

This metabolite is formed from pyruvate during anaerobic metabolism, in a reaction characterized by the recovering of NAD⁺. In individuals with mitochondrial dysfunctions due to MRC impairment, there is an inability to recover NAD⁺ from its reduced form (NADH) which translates into high lactate levels and high lactate/pyruvate ratios⁶³.

3.2 Amino acid profile

Amino acids profile may display important information when investigation of metabolic disorders. Hyperlactacidemia may be reflected in hyperalaninemia, since it correlates to lactate levels. Low citrulline levels are a marker of the deficiency ornithine transcarbamylase (OTC). However, It was observed that patients with mitochondrial DNA mutations, specifically m.8993T>C/G and m.9176T>C, exhibited remarkably low citrulline levels in approximately 80% of these patients⁶⁴. It is hypothesized that the underlying deficiency of ATP synthase is particularly impactful in small bowel enterocytes interfering with citrulline availability⁶⁵.

3.3 Urine organic acids studies

The identification of urinary organic acids (UOA) might be elucidative in specific pathways such as methylmalonic aciduria or even intermediates of Krebs cycle such as fumaric acid associated to 2-oxoglutarate, succinate, fumarate, malate and citric acids. 3-methylglutaconic acid (3-MGA) constitutes another metabolite associated to *SERAC1* deficiency causing MEGDEL syndrome. These are frequently related to mitochondrial disorders⁶⁶ although there is no universal metabolic signature for MDs.

3.4 Mitochondrial respiratory chain (MRC) enzyme assays

The test of mitochondrial MRC might be performed in fibroblasts or muscle tissue enabling identification of isolated or combined complexes deficient activities. The muscle sample should be rapidly processed or conserved ice-frozen and have the complexes studied isolatedly⁶⁷. The test is performed by measuring the complexes activity against a control enzyme⁶⁸.

Frequently muscle biopsies as well as neuroimaging investigation require anesthetic procedure which may imbalance ATP adding risks to metabolic patients⁶⁹. In case of rapidly progressive symptoms, manifestations confined to muscle tissue, inability to reach a specialized Center simultaneous molecular study and muscle biopsy tests are indicated. Otherwise, deep tissue collection should be carefully considered. In small children, fibroblast tissue is an alternative but still might fail the diagnosis⁷⁰. Moreover, the identification of biochemical defects can be helpful leading molecular investigation.

3.5 Serum biomarkers: FGF-21 (fibroblast growth factor 21) and GDF-15 (growth and differentiation factor 15)

Attempts to validate these metabolites as screening to muscular group of disorders are ongoing. Lehtonen *et al* raised the possibility of a positive correlation between FGF-21 levels in mitochondrial translation defects and mtDNA deletions with disease severity, suggesting using this biomarker to follow up patients⁷¹. Since GDF-15 is widely expressed in CNS, it can be interesting to screening for neurodegenerative disorders⁷².

Most reports claim statistical significance firmly correlating GDF-15 to mitochondrial translation and deletions defects^{73,74}. In non-mitochondrial early degenerative diseases like vanishing white matter, and mitochondrial hepatopathies GDF-15 is also characteristically elevated^{72,75}.

Occasionally, these markers still elevated in different medical conditions requiring a more detailed validation for including in the routinary screening for MDs.

4. Neuroimaging studies

Imaging studies are key to diagnosis. Included as obligatory criteria for LSS, a detailed study of CNS is mandatory. The brain and spinal cord acquired imaging are the chosen topography to be studied since major lesions are in these two structures. Currently, MRI and CT scan are accepted as valid methods for scanning the CNS⁵⁹. MRI has a more detailed acquisition of the lesions, demonstrating acute events blood flow variations and detailing damaged structures. Additionally, MRI enables local metabolite studies through spectroscopy. Each compound represents a local metabolic condition of the brain parenchyma resulting in a peak at its site. The resultant imaging groups into a graphic these compounds⁷⁶.

For MDs, lactate and/or pyruvate peaks are the most important serving as biochemical proof of mitochondrial inefficient glycolysis. Lactate is the most frequent of them appearing in spectroscopy twice, one of them inverted. This reflects the accumulation of lactate in cerebral tissues. Yet, it is not clear the minimal amount of lactate to suggest a mitochondrial disorder^{77,78}. The radiologic criteria for LSS are bilateral and symmetric hyperintense signal in the basal ganglia, *thalamus*, brainstem, cerebral white matter, and/or spinal cord on T2-weighted sequence acquired images as shown in figure 5.

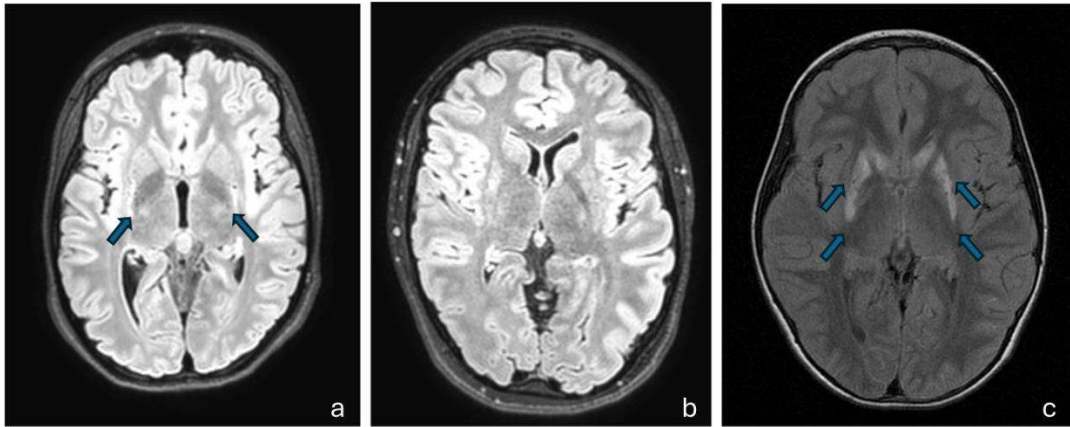


Figure 5. Brain MRI images in axial T2 FLAIR-weighted acquisition (a, b,c) in three different patients in our cohort demonstrating abnormal signs in bilateral basal ganglia (blue arrows), which is a common finding of Leigh Syndrome.

Additionally, neuroimaging studies can reproduce radiological features that are known to be related to a specific molecular defect. Complexes I and IV are habitually similar in topography such as basal ganglia, stroke-like lesions and leukoencephalopathy. Complex V usually affects brainstem area and cerebellum^{79,80}.

The translation defects (reading of mitochondrial RNA by the ribosomal machinery resulting in amino acids) are responsible for generation of thirteen respiratory complex subunits⁴. They present cortical and cerebellar atrophy with basal ganglia calcification and stroke-like lesions, contrasting to maintenance defects displaying cortical and cerebellar atrophy with stroke-like lesions in *POLG*⁸¹, and global brain atrophy for *SUCLA2/SUCGL*⁸². According to MRI, cortical atrophy and leukoencephalopathy with major white matter changes are seen in mitochondrial arginyl tRNA synthetase gene (*RARS2*) or phenylalanyl tRNA synthetase gene (*FARS2*) deficiencies⁸³. The aspartate aminoacyl-tRNA synthetase gene (*DARS2*) displays leukoencephalopathy, brain stem, spinal cord involvement and lactic acidosis (LBSL), a variable phenotype⁸⁴. Moreover, mutations in mitochondrial glutamyl-tRNA synthetase gene (*EARS2*) present an early leukoencephalopathy with major symmetrical white matter changes⁸⁵.

5. Molecular assessment

The major field of breakthrough in diagnosis for mitochondrial disorders is the human genome approach. Since MDs were diagnosed by proving the biochemical defect in affected tissue, a risky practice for the patients, the genetic

evaluation ensured less invasive diagnosis and the counseling for affected families. The interruption in MRC resulting in ATP deficit is a consequence from a direct interference in OXPHOS (OXPHOS defect) or from an external interference in this process (non-OXPHOS defect, combined defects and maintenance defects) as shown in tables 2 and 3. Therefore, these defects can be responsible for MDs in general and LSS.

Table 2. Nuclear and mitochondrial genes involved in LSS

Biochemical defect	Associated nuclear genes
Complex I	<i>NDUFA1, NDUFA2, NDUFA9, NDUFA10, NDUFA12, NDUFS1, NDUFS2, NDUFS3, NDUFB8, NDUFS4, NDUFS7, NDUFS8, NDUFV1, NDUFV2, MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5, MT-ND6</i>
Complex II Deficiency	<i>SDHA</i>
Complex III Deficiency	<i>UQCRCQ, Cyt b</i>
Complex IV Deficiency	<i>NDUFA4, COX8A, MT-COI, MT-COII, MT-COIII</i>
Assembly factors deficiency	
Complex I	<i>NDUFAF2, NDUFAF4, NDUFAF5, NDUFAF6, C17ORF89, FOXRED1, NUBPL</i>
Complex II	<i>SDHAF1</i>
Complex III	<i>BCS1, TTC19, UQCRC, UQCRC2</i>
Complex IV	<i>SURF1, COX10, COX15, SCO2, PET100, PET117,</i>
Cofactors and biosynthesis	
Pyruvate Deshydrogenase Complex	<i>PDHA1, PDHX, PDHB, DLAT, DLD</i>
Biotinidase Deficiency	<i>BDT</i>
Thiamine Deficiency	<i>TPK1, SLC19A3</i>
Lipoic acid	<i>LIPT1, LIAS, BOLA3, MECR</i>
Amino acid	<i>HIBCH, ECHS1, SUOX, SQOR</i>
Coenzyme Q10 Deficiency	<i>PDSS2, COQ9</i>
Other functions	
Carnitine acyltransferase	<i>CRAT</i>
Meiotic nuclear division	<i>RMND1</i>
Posttranslational RNA modification	<i>PUS1</i>
ROS Detoxification	<i>NAXE</i>

Table 3. Nuclear genes involved in other mitochondrial functions causing LSS

Mitochondrial function	Associated genes
Mitochondrial DNA maintenance	<i>FBXL4, POLG, POLG2, SUCLA2, SUCLG1, TWNK, SLC25A4, MPV17</i>
Mitochondrial translation	<i>GTPBP3, TRMU, EARS2, FARS2, IARS2, GFM1, GFM2, LRPPRC, TACO1, MTFMT, C12Orf65, MRPS34, MRPS39 (PTCD3), NARS2, TSFM</i>
Mitochondrial dynamics	<i>SLC25A46, DNM1L, MFN2, RRM2B, AIFM1; MFF</i>
Mitochondrial material import/export	<i>SLC25A19, TIMM50, TIMMDC1</i>
Membrane phosphocomponents	<i>SERAC1</i>
Sulfur dioxygenase	<i>ETHE1</i>
AAA oligomeric +/- ATPase	<i>CLPB, SPG7</i>
RNA import	<i>PNPT1</i>
RNA-specific adenosine deaminase	<i>ADAR, RNASEH1</i>
Nuclear translocation system	<i>RANBP2</i>
Nuclear pore complex	<i>NUP62</i>
Manganese transportation	<i>SLC39A8</i>
Aminoacylase deficiency	<i>ACY1</i>
Mitochondrial peptidase	<i>PMPCB</i>

5.1 OXPHOS defects

5.1.1 Complex I (ubiquinone oxireductase - NADH)

The first and the largest complex of ETC, associated to several mutations responsible for MDs. This complex is under control of both genomes, where the mtDNA encodes for 7 out of the 45 subunits and the remaining of them are codified by nDNA⁸⁶. In several species, CI is a mostly conserved L-shape structure where the OXPHOS process begins by transferring electrons from NADH form to Coenzyme Q10 (CoQ10)⁸⁶. The electron transference through changes in membrane potential generates a specific gradient that culminates in reactive oxygen species (ROS), an inconvenient fact due to the closeness to inner mitochondrial membrane (IMM), where the process takes place.⁸⁷ Consequently, they represent a potential danger to OXPHOS by threatening the integrity of IMM.

This electro gradient generation is pivotal for OXPHOS where CI is responsible for 40% of the contribution to ATP production⁸⁸.

The impaired CI destabilizes all the following steps as the formation of the respirasome. Usually, it represents an early onset, being frequently identified in children. The hallmark of CI deficiency is leukoencephalopathy followed by white matter cavitations⁸⁹. Still, the phenotypes linked to CI deficiency are heterogeneous ranging from fatal neonatal lactic acidosis to later or isolated forms as cardiomyopathy and LHON.

Besides the structural components of CI, there associated chaperones (assembly structures) to assist the modules inside the complex forming the total holoenzyme. Until date, these enzymes are exclusively under nDNA control⁹⁰.

While mtDNA mutations are more commonly expressed in LSS, MELAS and LHON, nDNA mutations result in lactic acidosis, cardiomyopathy and LSS²⁰.

5.1.2 Complexes II and III

These complexes are the least affected. Complex II, the SDH (succinate dehydrogenase) complex is a small complex system with 4 nuclear-encoded units performing simultaneous functions in MRC and TCA are performed. After succinate oxidation, the electrons are transferred to CoQ10 through flavoprotein (FAD)⁹¹. The most important subunit is codified by *SDHA* and *SDHB*, being *SDHA* responsible for most of the cases of CII deficiency⁹². The clinical presentation varies from early-onset LSS to late-onset epileptic syndromes, cognitive disability and KSS⁹³.

The third step is complex III (ubiquinol: cytochrome c oxidoreductase), the rarest of them, complex III transfers electrons from CoQ10 to Cytochrome c⁹⁴. Basically, three nuclear genes are mostly implicated to LSS: *UQCRCQ* family gene, *TTC19* and *BCS1L*. The last one is the GRACILE-like/LLS syndrome, a constellation of features which are low birthweight, hepatorenal involvement, delay in milestones and hypotonia⁹⁵. *TTC19* is relevant in Portuguese population since reported by Nogueira and colleagues in Portuguese families presenting cerebellar and psychiatric symptoms in adults, and motor developmental delay and neurological regression in children^{96,97}.

5.1.3 Complex IV (Cytochrome c oxydase – COX)

Complex IV is the most implicated to LSS which also attends for COX complex, composed by 14 subunits from which only three of them are under mtDNA control⁹⁸. Complex IV reunites the electro gradient produced by prior

steps including oxidation of *cytochrome c* and reduction of the molecular oxygen to water, enhancing the protons collection in mitochondrial intermembrane space (MIS)^{99,100}. The protons stored in this space will be reversely pumped by Complex V to produce ATP. The mutations interfering with CIV functioning are habitually disruptive by diminishing COX activity and causing the enzyme to lose its assembly⁹⁸.

COX deficiency is resultant from related genes mutations. *SURF1*, a surfactant locus protein integrant may account for one third of all mutations⁴⁸. *SURF1* is as implicated as *LRPPRC*, a protein involved in posttranscriptional regulation of mitochondrial gene expression⁴⁹. *LRPPRC* is known to participate in many cellular functions, more importantly integrating COX functioning. From infections to cancer¹⁰¹, *LRPPRC* hosts a founder-effect mutation c.1119C>T causing LSS with stroke-like episodes and severe lactic acidosis, which has been reported in different populations¹⁰².

SURF1 usually displays similar clinical and biochemical phenotypes with typical LSS neuroradiological images⁵¹, but heterogeneous phenotypical patterns are seen in clinical sphere¹⁰³ and in imaging, such as leukodystrophy and hypertrophic olivary degeneration¹⁰⁴. *SURF1* is the current target of gene therapy through adenovirus delivery with studies in ongoing phase 2/3 giving the frequency of CIV deficiency and its implication in its function¹⁰⁵.

5.1.4 Complex V (ATP synthase)

ATP synthase is a 17-subunit protein that uses all the proton gradient generated by ETC to convert ADP to ATP^{106,107}. The last two incorporated proteins to the complex are exclusively under mtDNA control, *MT-ATP6* and *MT-ATP8*. It is known the mammalian CV plays an impressive damaging role in effective glycolytic energy production since the resultant phenotypes are severe^{108,109}.

Despite the majority of nDNA surveillance, the m.8993T>G/C on *MT-ATP6* seems to be most prevalent point mutation impacting CV function due to an amino acid substitution¹¹⁰. Heteroplasmy rates between 60% and 85% are seen in NARP syndrome and above 85% usually results in MILS presentation. Nonetheless, m.8993T>C at heteroplasmy rates over 90% presents a milder NARP phenotype also resulting in late-onset slowly progressive disease while the m.8993T>G results in severe MILS¹¹¹⁻¹¹³. The aggressiveness of m.8993T>G might be consequence of a non-polar amino acid substitution for a charged amino acid, arginine producing a major instability of the catalytic site of the Complex V¹¹².

5.2 Combined deficiencies

The combined deficiencies are resultant of an interference in mtDNA maintenance reflecting in deletion or depletion of mtDNA by nDNA or mtDNA mutations¹¹⁴. *SUCLA2* and *SUCLG1* are most seen causes of mtDNA depletion syndromes. They affect the succinyl-CoA synthetase step of TCA generating variable levels of methylmalonic acid (MMA) in UOA analysis. This metabolite is reliable as in investigation as to surveillance of the disease⁷⁰. When the MMA is associated to an encephalomyopathy, the probability of a mitochondrial depletion syndrome due to succinyl-CoA ligase deficiency (*SUCLA2*) enhances. *SUCLA2* defects present neuromuscular symptoms and failure to thrive. MRI displays cortical and basal ganglia atrophy¹¹⁵. *SUCLG1* manifests a severe fatal phenotype with marked hyperlactacidemia and liver dysfunction.

MMA is a metabolic hint for *SUCLA2/SUCLG1*, other organic acids may be guiding the investigation. 3-Methylglutaconic acid (3-MGA) is found in MEGDEL syndrome, a *SERAC1* deficiency. MEGDEL is defined by 3-Methylglutaconic aciduria, Deafness, Encephalopathy and LLS. *SERAC 1* is involved in cholesterol transport and remodeling cardiolipin¹¹⁶.

Less frequently, mutations in mitochondrial tRNA genes can also manifest in combined deficiencies. First was *MTFMT* c.626C>T related to LSS, and sequentially other genes were proved to be implied with LSS⁵⁴. After, m.3243A>G *MTTL1* identified in MELAS and m.8344A>G *MTTK* related to Myoclonic Epilepsy with Ragged Red Fibers (MERRF) were part of LSS genotype^{117,118}. Several mutations related to MELAS, MERRF and other mitochondrial syndromes are part of LSS genotypic spectrum.

5.2 Non OXPHOS defects

5.2.1 PDHc deficiency

The pyruvate dehydrogenase complex (PDHc) is a protein formed by 3 subunits. The first two are thiamine-pyrophosphate dependent on the production of Acetyl-CoA, a major substrate to electron transport chain (ETC). The conversion of pyruvate to lactate interrupts ETC by limiting glycolysis. The most frequent form is the X-linked *PDHA1* deficiency (*PDHA1*) classically displays changes in *corpus callosum* and basal ganglia associated to variable neurological impairment^{119,120}. Thiamine supplementation associated with ketogenic diet are current adopted measures for treatment with satisfactory results¹²¹.

5.2.2 Thiamine transporter disorder (biotin-responsive basal ganglia disease - BBGD)

Thiamine-pyrophosphate is the active form of thiamine. Usually, three phenotypes are recognized under this defect: a precocious LSS; a milder LSS and Wernicke encephalopathy in adulthood^{122,123}. Biochemical studies show low to very low free thiamine in CSF and indicate the *SLC19A3* gene sequencing. The supplementation of high doses of biotin and thiamine has good clinical response and positive impact in preventing deleterious outcomes¹²³.

5.2.3 Lipoyltransferase 1 deficiency (LIPT1)

Lipoic acid is fundamental in fatty acid synthesis and mitochondrial assembly factors functioning. *LIPT1* is thiamine dependent and involved in other pathways such as PDHc, glycine cleavage system, α -ketoglutarate dehydrogenase (KGDH), branched chain α -ketoacid dehydrogenase complex (BCKDH) and iron metabolism¹²⁴. Its deficiency manifests as early onset encephalopathy, pulmonary hypertension and hyperlactacidemia^{125,126}. Biochemically normal to elevated branched-chain amino acids and glycine levels in serum and/or CSF¹²⁷. So far, no effective treatment is proven but studies report a cocktail of several co-factors (pantothenate, nicotinamide, vitamin E, thiamine, biotin, and α -lipoic acid) rescued LIPT1 activity in fibroblasts and reduced the iron accumulation¹²⁸.

6. Prenatal diagnosis and genetic counseling

A diagnosis of mitochondrial disorder is demanding in investigation and in communicating the diagnosis to the patient and the family. In a family with already characterized index-case, relatives are also important to be screened for clear and assertive prenatal counseling. In this subject, a careful detailed familial history must be provided, since identifying the inheritance mode could shorten the diagnosis pathway enabling a more precise the genetic counselling more precise^{129,130}.

6.1 Mitochondrial inheritance

MtDNA has some particularities implied to itself. The segregation pattern is mitotic and beholds an unpredictable expression threshold, which directly affects the mutation performance in human organs and the genetic counseling¹¹. Additionally, during pregnancy these thresholds are variable. However, the

m.8993T>G/C is exception remaining in stable ratings throughout all the pregnancy in all tissues¹³¹. For the remaining mutations, it is safe to offer genetic counseling in heteroplasmy above 95%. The risk for the proband's family members basically depend on the mutation and its heteroplasmy. The correspondent evaluation is accessed through urine and blood from the mother and chorionic villus biopsy (CVB) for the fetus. MtDNA rearrangements are mostly sporadic and transmission to offspring is very rare. On the contrary, point mutations are passed to offspring in variable heteroplasmic amounts. Therefore, the same woman may have normal to differently affected children¹³².

White *et al* confirmed that the maternal heteroplasmy in m.8993T>G required to offsprings severe outcomes was smaller than T>C. Besides, these specific mutation loads remain stable during pregnancy. Heteroplasmy rates under 60% drastically reduce the incidence of severe presentations. For the other mutations, as m.9176T>C⁷⁵⁸⁸, a safer counseling relies extreme mutation loads are retrieved during investigation¹³³.

6.2 Nuclear inheritance

The inheritance may be autosomal recessive, dominant or X-linked. As in mtDNA inheritance, the family pedigree is extremely useful. Therefore, using this information, genetic counseling must be offered to any couple welcoming the idea of a pregnancy. The conduction and decision are under medical group and the family's choice by fully addressing concernments of a new offspring⁶².

7. Disease models for LSS

Given the precocious fatal outcomes, several studies and interventions are being accessed in MDs¹³⁴. Unfortunately, LSS does not have curative treatment so far and simulations through experimental models of the mitochondrial disorders are still ongoing, especially to mtDNA mutations due to heterogeneous outcomes¹³⁵. Hence, a deeper understanding of physiopathological elements is key to providing answers to MDs. So far, the therapeutic options are limited.

7.1 Yeast

S.cerevisae and *Y. lipolytica* were models to replicate MD and pursue answers. On one hand, *S. cerevisiae* provided insights for mt-tRNA disorders such as MELAS, MERRF, CPEO and related myopathies since surviving without a

functional mtDNA¹¹⁰. On the other hand, the evolutionary gap between species and the lack of CI turned out to be obstacles in using *S. cerevisiae*, demanding a proper aerobic model¹³⁶. Another yeast, *Y. lipolytica* was used to fulfill CI studies doubts, but still lacked homology and evolutionary aspects.

7.2 Worm model

C. elegans is a nematode and relevant model for sharing a consistent part of its genome with the human genome, presenting transparent small size, easing the manipulation, clear observation of results, and the extensively known neuronal system which is convenient when investigating neurodegenerative disorders¹³⁷. However, the mild phenotypical expression in *C. elegans* lacked resemblance to humans, demanding further investigation into another model¹³⁸.

7.3 Fruit fly

D. melanogaster shared the same genomic resemblance and specific conserved genes related to neurodegeneration pathways, suitable for LSS¹³⁹. Interestingly, tests in *Drosophyla* homologue *SURF1* knockout produced a COX deficiency resulting in the same effects and interference in other complexes. Despite addressing mtDNA heteroplasmic mutations, the lack of homology with humans still prevented robust conclusions¹¹⁰.

7.4 Zebrafish

D. rerio or zebrafish is a vertebrate to model for neurodegenerative disorders since their CNS resembles the human CNS and genome are 70% like ours¹⁴⁰. Besides, zebrafish display valuable advantages like high reproduction rate with fertilized eggs, transparent evolutionary phases with easy visual perception of effects, fast developmental phases enabling studies to begin in early developmental stages and benefited from simple genetic manipulation to gain and loss of function mutations^{141,142}. Furthermore, visual aspect is key to access deficits and effects as well as interventions results. Currently, it is one of the preferred options to model for MDs and neurodegenerative disorders.

7.5 Mouse

A mammalian model was still required to conduct a full experiment, guarantee reproducible outcomes and more precise results especially *ndufs4*^{-/-}

model to CI deficiency¹⁴³. However, mouse model has mtDNA segregation pattern and random drift unlike humans, demonstrating *in vivo* tissue/organ preferences and age-related pattern of manifestation within different genotypes. Nevertheless, the inconvenience relied on high lethality rate in neonatal period and variable phenotypic resemblance of mutant mice to the targeted phenotype still leaving some gaps to be filled in¹³⁵.

7.6 Transmitochondrial cybrids

After mitochondrial genome was identified, laboratorial testing reproduced mtDNA genomic molecules depleted and deleted when exposed to ethidium bromide, which was later successfully achieved in chicken embryo model¹⁴⁴. However, generating the cell lines in exact phase of cellular division cycle demand a long-term exposure to substances which affect survival gene expression, disorganize organelles and membrane patterns and outcomes in oncological cell lines are variable and unstable^{145,146}.

7.7 Induced pluripotent stem cells (iPSC) models

The revolutionary breakthrough published by Yamanaka *et al* using iPSC opened a plethora of possibilities in the research field. Beyond cellular reprogramming, iPSC allows simultaneously using the patient's own tissue for personalized interventions and accessing results¹⁴⁷. Yet, the heteroplasmy rate is unpredictable in resultant cells and inducing strictly the desirable cellular type^{148,149}. Since reproducing features of the patients, this is an alternative to test individualized treatment choices. The high demand for resources is still an impediment for its wide use in modelling for LSS¹⁴⁸.

7.8 Organoids

The tridimensional cellular cluster model to more complex organizations are the organoids, well indicated to structural assessment. They are originally from somatic cells or iPSCs derived and model the more complex environment in macroscale¹⁵⁰. Especially for neurological tissue, the bidimensional cultures failed to demonstrate more detailed developmental disturbances, mitochondrial network disruption and relevant metabolic changes illustrated by the organoids¹⁵¹. Although a major complexity assessment is achieved by this model, some lacks are recognizable such as additional supportive tissues and cellular

types to the principal tissue in focus, which leaves more cellular interactions to be clarified¹⁵².

8. Therapeutic strategies

As in MDs, LSS comprise wide phenotypic performance. It is laborious to identify single strategies to address these cases. Therefore, no curative treatment is yet defined given the vast genotypic background causing LSS but particular interventions have been tested focusing on the specific molecular defect. The options could be addressed as supportive or general.

8.1 General treatment

General treatment aims to give support to major treatable conditions and symptoms displayed by the patients. Co-factors, antiepileptic drugs, dietary management and the new studied therapeutic interventions are summarized below.

8.2 Precision medicine

The lack of robust evidence in methodology such as double-blind randomized trials are prejudicial in establishing evidence-based guidelines. There is no effective treatment to LSS so far¹⁵³. The available data in unrelated studies published so far directs the treatment according to the metabolic defect and molecular background.

8.2.1 Thiamine and biotine

In BBGD, thiamine and biotin replacement are meant to be done as soon as possible for having the best responses in early onset cases¹⁵⁴. The late onset patients did not benefit from therapeutical intervention possibly due to irreversible neuronal damage. Moreover, early treatment is recommended by substantially changing the outcomes. The recommended dosage of thiamine is 20mg/kg/day in primary defects of thiamine and 30-40mg/kg/day in secondary defects such as PDHc, where biotin prescription is 5-10 mg/kg/day⁶². Alongside, the follow up of these patients in long-term supplementation is crucial to determine the real effect and possible toxicity of these compounds⁶¹.

8.2.2 Coenzyme Q10 (CoQ10)

CoQ10 is an auxiliary factor in ETC performs antioxidant role in cells. In patients with LSS and CoQ10 deficiency, the supplementation had a dramatic improvement in symptoms¹⁵⁵. The tolerated dose is up to 3000mg/day but again, long term follow up is required to check on possible toxic effects¹⁵⁶.

8.2.3 Idebenone

This CoQ10 derivative compound is an attempt to overcome the limitations of CoQ10 in mitochondrial uptake and utilization by crossing blood-brain-barrier (BBB) enhancing ATP production¹⁵⁷. Long symptoms stabilization was reported in a LS patient for a few years¹⁵⁸ and it's approved to Leber's Hereditary Optic Neuropathy (LHON)¹⁵⁹.

8.2.4 Vatiquinone (EPI-743)

Vatiquinone (EPI-743) is a synthetic quinone oxidoreductase that selectively inhibits 15-lipoxygenase (15-LO), an enzyme involved in pathways related to inflammation, oxidative stress, and ferroptosis. It has been under clinical investigation for the genetic diagnosis and treatment of mitochondrial disorders, including Leigh syndrome (LSS), mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), POLG-related disorders, and Friedreich's ataxia. LSS patients had less frequent hospitalization in the first 6 months of therapy¹⁶⁰. When stopping medication, they experienced relapsed symptoms. The mechanism of functioning is through replenishing the glutathione cellular pool, enhancing mitochondrial ETC. There is still ongoing study phase III and the efficacy in long term follow up is needed to be checked¹⁶¹. Reports claim that Vatiquinone is effective in prevent cellular death in the culture treated with BSO/Fe(III)Citrate but no effect was seen in paraquat cultured cells¹⁶².

8.2.5 Pyruvate

Pyruvate has been applied to LSS patients with different molecular backgrounds by changing NADH/NAD ratio by stimulating glycolytic pathway and removing the accumulations of hydrogen peroxide¹⁶³. The patients in general had positive impact on lactate levels and symptomatology, except for a COX deficiency patient¹⁶⁴. In one case of treatment suspension, the patient displayed relapsed symptoms but recovered after resuming treatment¹⁶⁵.

8.2.5 Rapamycin

A selective inhibitor of mTOR which induces amino acid metabolism reducing the intermediary compounds of glycolysis mitigating MDs symptoms^{166,167}. It was tested in LSS knock-out mice model *NDUFS4* which had a longer life span and didn't manifest neurological symptoms, while the untreated mice had high levels of lactate and pyruvate which were recovered by rapamycin.

8.2.6 Bezafibrate, nicotinamide riboside, resveratrol, AICAR

Enhancing mitochondrial biogenesis is an alternative to replenish the lacking ATP by offsetting the metabolic defect if the defect partially blocking the MRC. Compounds such as bezafibrate, nicotinamide riboside, resveratrol are being studied as alternatives to address the ATP deficit¹⁶⁸. Bezafibrate, a PGC-1 α activator, presented conflictive results in mouse models¹⁰⁸ while AICAR, also PGC-1 α activator, has shown to recover cytochrome oxidase c activity in SCO2 deficiency mice¹⁶⁹. The nicotinamide riboside, an activator of *Sirtuin1* and precursor of NAD⁺, demonstrated positive effects in muscle tissues but not in CNS¹⁶⁸. On the other hand, resveratrol, another *Sirtuin 1* activator, enriched MRC in complexes I and IV, enhancing ATP production and diminishing lactate levels¹⁷⁰. It is reported a Complex I activity rescue *C.elegans* using resveratrol. By balancing the NAD⁺/NAD ratio lifespan was expanded, suggesting the resveratrol could transpose the blood-brain barrier¹⁷¹.

8.2.7 Ketogenic diet

A less invasive intervention in a chronic progressive disorder could address the major contribution where treatment meets the dietary diary needs. The Ketogenic diet (KD) is a more convenient choice according to a metabolic shift simulating an intermittent fasting permanent state. The shift in glycolytic metabolism provides viable support without glucose as main fuel. In metabolic disorders such as PDHc, KD alleviated symptoms in significant matter also reverting the neuroimaging lesions in some cases¹²¹.

9. Gene therapy

The latest advances have been made regarding gene therapy for CIV deficiency. Reports were made using knockout mice through adenovirus in favor of gene therapy, but they failed to determine the phenotype related to *SURF1* deficiency highlighting the importance in having a well-defined phenotype and

checking the reproduction of these characteristics in the simulation model¹⁰⁵
Studies for *SURF1* deficiency (SURF1 Gene Therapy Program) have finished phase II.

10. Prognosis

Apart from treatable cases, the prognosis remains obscure due to the unavailability of optimal therapeutic options in such heterogeneous and variable phenotypes. The neonatal neurological presentations are closer to more relapses and poorer outcomes. Acute compensation was more frequent in epileptic patients and acute infections were the main cause of relapses also increasing hospitalization. The early onset before six-month-old, brainstem abnormalities, increased lactate levels on (CSF), hospitalization in Intensive Unit Care and cardiomyopathy show a worse prognosis¹⁷².

Chapter II. Aims and objectives

To genetically identify the underlying genes associated to LS and LLS, the variants implicated in clinical symptoms and access the results in highly suspicious patients, we aim to:

1) Perform mitochondrial genome sequencing, already developed and standardized at Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA) and predict impact on protein production/function by bioinformatic tools if a suspiciously variant is found. Leigh and Leigh-like syndrome are basically secondary to an impairment in mitochondrial energy generation. The mtDNA genome is composed of 37 genes that control mitochondrial metabolism and functioning, easily accessed in a short period of time and giving a valuable possibility for finding disease-associated variants. In this case, we will verify if it is an already described mutation or a variant of uncertain significance (VUS) according to American College of Genetics and Genomic Medicine (ACMG).

2) Design a custom-made targeted nuclear gene panel using next generation sequencing (NGS) to screen disease-associated mutations for LS and LLS to characterize genetically suspicious patients and proceed to bioinformatic analysis. This gene targeted panel focuses on nuclear genes implied in controlling and assuring mitochondrial metabolism. If the first step fails to determine the variant in mitochondrial genome, a panel covering nuclear genes involved in mitochondrial energy generation will be executed.

3) Screen nuclear genes by clinical exome sequencing (CES) for the identification of variants in nuclear genes and proceed to bioinformatic evaluation. CES covers 5.000 genes across various samples and assures the timely completion of this task.

4) Perform biochemical studies (MRC, UOA) if any metabolite is related to a specific biomarker as first approach to functional determination. Considering previous steps 1, 2, or 3 pointing to any potentially causative variant, the presence of a pathognomonic biomarker will be useful biochemical functional studies allow accessing the protein repercussion under the identified variant. Some alterations produce a specific abnormal UOA profile or some reduced activity in MRC, easily accessed by biochemical studies available at INSA.

Chapter III. Patients and methods

1. Patients

Since the 1990s, the Newborn Screening and Metabolic Disorders Unit of the Human Genetics Department at the National Institute of Health Doutor Ricardo Jorge (INSA) Porto has received patient samples from the five main Portuguese hospital centers. This project aimed to genetically characterize patients suspected of having Leigh Syndrome (LSS). Sample selection was primarily based on our database, which included uncharacterized patients with NARP/MILS mutations. No additional studies were performed until 2018; thereafter, new uninvestigated cases from 2019 to 2023 were incorporated (first phase). Clinical information was obtained from referring physicians, and patients meeting the criteria for Leigh Syndrome (LS) and Leigh-like Syndrome (LLS) were selected.

Subsequently, we revisited a previously characterized cohort for LSS (comprising LS and LLS) (second phase), as well as an undiagnosed group investigated for mitochondrial disorders (MDs) whose clinical features were compatible with LSS (third phase). Among the diagnosed group, 11 patients had been previously reported by our group, while the remaining 29 were molecularly characterized and reported here for the first time.

In total, 82 patients were included (see Figure 6), consisting of 42 males and 40 females, with ages ranging from 0 to 36 years. These patients exhibited neurological, radiological, and metabolic features consistent with LSS. Additionally, the undiagnosed patients were studied through population background analyses to identify relevant correlations between haplogroups and phenotypes.

The study was approved by the local Ethics Committees of INSA and informed consent for genetic studies was obtained from all participants or their relatives, in accordance with the Declaration of Helsinki (Supplementary Material 1).

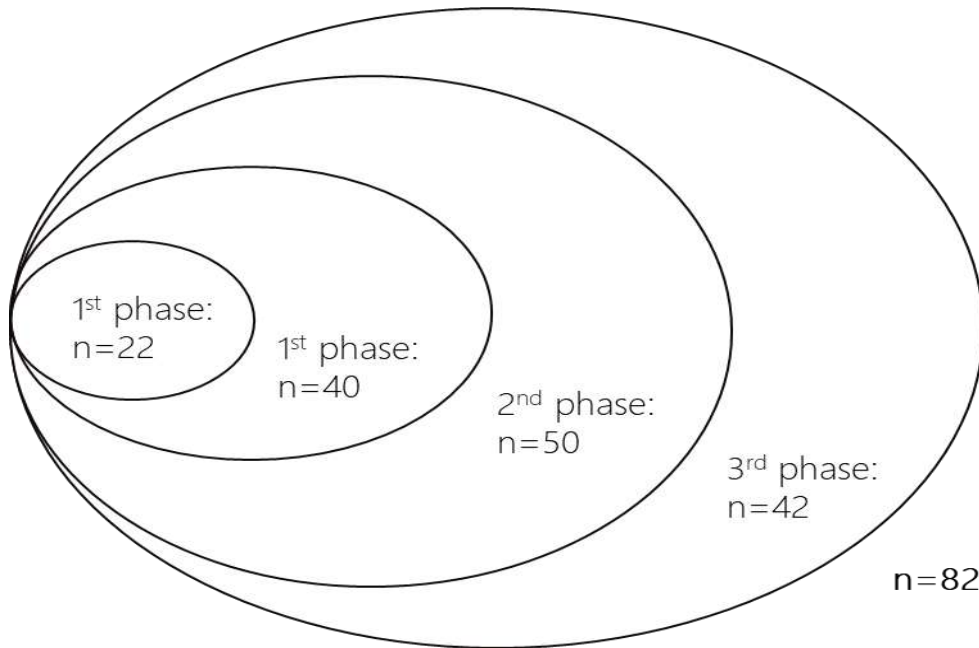


Figure 6. Sample selection methodology during the project phases, with a total of 82 patients included in the study. The first phase equals 40 patients (12 newly diagnosed and 28 revisited); the second phase equals 50 patients (22 newly studied and 28 revisited); the third phase equals 42 uncharacterized patients (ten from previous phases and 32 new), totalizing 82 studied patients.

2. Methods

2.1 DNA extraction, sequencing, and variant analysis

High purity DNA was extracted from peripheral blood leucocytes and/or muscle biopsy material according to standard procedures. A custom-made targeted nuclear gene panel was designed using Suredesign software (Agilent Technologies) and includes 209 nuclear genes, from which 188 were known to be involved in MDs and 21 were candidates based on their role or participation in different pathways that include genes responsible for clinical presentations mimicking MD (Supplementary material 2). Genes of interest were captured with SureSelect QXT kit (Agilent Technologies), followed by sequencing on the Illumina MiSeq platform. Variant calling and annotation were performed using available commercial programs, such as Surecall (Agilent Technologies) and wAnnoVar (wannovar.wglab.org) with mean coverage depth of 50x, and heterozygous/homozygous variants were checked and confirmed.

Nuclear variants were filtered taking into account: *i*) the type of pathogenic variant (missense, frameshift, stop-gain or stop-loss, and splice-site variants), *ii*) *in silico* predictors (SIFT, PolyPhen-2, MutationTaster) and presence in databases (dbSNP, 1000 Genomes, HGMD professional, ClinVar, ExAC, OMIM, gnomAD¹⁷³),

and *iii*) 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. Furthermore, we searched for copy number variations through the analysis of coverage data, using cnMOPS20¹⁷⁴.

The entire human mtDNA was enriched by a single amplicon, using back-to-back primers, by long-range PCR. Indexed paired-end DNA libraries were prepared and an equimolar pool was sequenced, according to the manufacturer instructions. FASTQ files were aligned to the mtDNA reference sequence with SeqMan NGen (DNASTar). Variant calling and annotation were performed using the following commercial programs: SeqMan NGen and SeqMan Pro (DNASTAR).

Mitochondrial variants were filtered considering: *i*) the type of pathogenic variant, *ii*) the population frequency, *iii*) in silico predictors and presence in available databases (Mitomap, MitImpact2, HmtVar), and *iv*) heteroplasmy>5%. All variants detected that had the potential to be disease-causing were confirmed by Sanger sequencing using the BigDye Terminator Cycle Sequencing Version 3.1 (Applied Biosystems) and analyzed on an ABI 3130XL DNA Analyzer¹⁷⁵.

When DNA from additional family members were available, co-segregation studies were also performed.

Chapter IV. Results

Scientific material was published according to the aims of this thesis after proceeding with patients' investigation. The first step was a general review of the theme as our first publication: **Molecular basis of Leigh Syndrome Spectrum: a current look.** *Orphanet J Rare Dis.* 2020;15(1):31. doi: 10.1186/s13023-020-1297-9, which is the most relevant features from clinical suspicion to molecular basis, counseling and treatment updates relatively to LS diagnosis.

The results of molecular investigations are detailed in our second publication **Leigh Syndrome Spectrum: A Portuguese Population Cohort in an Evolutionary Genetic Era.** *Genes.* 2023;14(8):1536. doi: 10.3390/genes14081536, where a major part of our cohort was characterized, and the heterogeneous background was revealed. The percentage of positive identification is expressive and encouraged us to look carefully at our results and our practical approach. Since results were unexpected, giving the positive rates confirmation in MDs screening is between 30-40% of closure. Therefore, the motivation to produce a general assessment to clinicians based on a merge of clinical and laboratorial experiences over the last decades, a practical guideline for patients' investigation encompassing clinical suspicion criteria until proper molecular choice of confirmation is explained in our third publication **A comprehensive approach to the diagnosis of Leigh Syndrome Spectrum.** *Diagnostics.* 2024;14(19). doi: 10.3390/diagnostics14192133.

We also decided to look deeper into the uncharacterized clinically suspected LSS patients who underwent biochemical evaluation and molecular studies to reassess their investigation, which contributed to our cohort. It is known that the genetic background is associated with a particular feature in protective or disruptive way. Therefore, uncharacterized patients were additionally studied in populational genetic background to check whether the population background informed by the haplogroups could eventually correlate with the phenotype.

IV.1 Molecular basis of Leigh Syndrome Spectrum: a current look

REVIEW

Open Access



Molecular basis of Leigh syndrome: a current look

Manuela Schubert Baldo* and Laura Vilarinho

Abstract

Leigh Syndrome (OMIM 256000) is a heterogeneous neurologic disorder due to damage in mitochondrial energy production that usually starts in early childhood. The first description given by Leigh pointed out neurological symptoms in children under 2 years and premature death. Following cases brought some hypothesis to explain the cause due to similarity to other neurological diseases and led to further investigation for metabolic diseases. Biochemical evaluation and specific metabolic profile suggested impairment in energy production (OXPHOS) in mitochondria. As direct approach to involved tissues is not always possible or safe, molecular analysis is a great cost-effective option and, besides biochemical results, is required to confirm the underlying cause of this syndrome face to clinical suspicion. The Next Generation Sequencing (NGS) advance represented a breakthrough in molecular biology allowing simultaneous gene analysis giving short-time results and increasing the variants underlying this syndrome, counting over 75 monogenic causes related so far. NGS provided confirmation of emerging cases and brought up diagnosis in atypical presentations as late-onset cases, which turned Leigh into a heterogeneous syndrome with variable outcomes. This review highlights clinical presentation in both classic and atypical phenotypes, the investigation pathway throughout confirmation emphasizing the underlying genetic heterogeneity and increasing number of genes assigned to this syndrome as well as available treatment.

Keywords: Leigh syndrome, Leigh-like syndrome, NARP, MILS, OXPHOS, Review

Introduction

Mitochondria are a very particular multitask organelle with their own functioning. Composed by two membranes and a circular DNA (mtDNA), they command cellular energy production [1]. The metabolism and bio-cycle obey a self-signaling instead exclusive nuclear control. Mitochondria are capable of dividing themselves and producing their own material, including mtDNA products, to support their and major cell issues, requiring a refined and highly specialized arsenal to achieve optimal balance between replication and proteomic machinery in DNA synthesis [2]. They are responsible for energy production through tricarboxylic acid cycle (TCA) and electron transport chain (ETC), that is the main feature to generate ATP in the aerobic metabolism through an oxidative phosphorylation (OXPHOS), depending mainly on their inner membrane integrity to accomplish the entire process [3] (Fig. 1). ETC occurs with

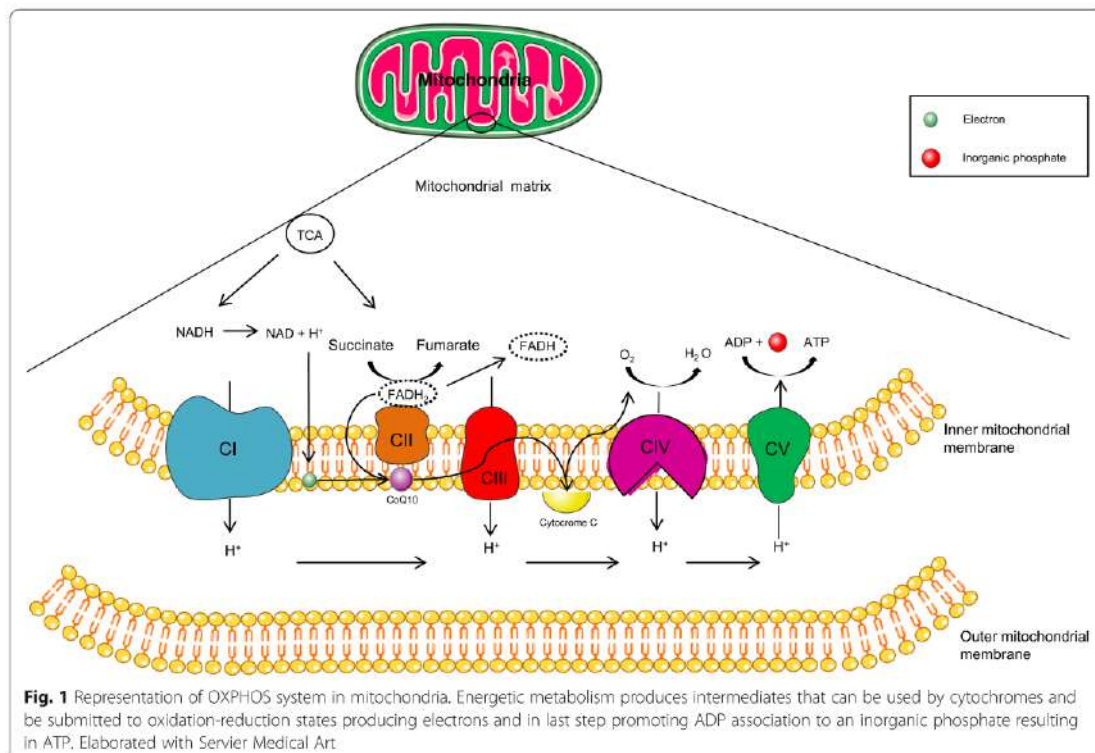
electron transport in changing membrane potentials, ion flux associated with cofactors, as riboflavin and coenzyme Q10, and generate ATP to sustain cellular demands. Some organs, like muscles and brain, require more energy to maintain their homeostatic functioning. Any damage in OXPHOS will diminish considerably energy supplies in high energy demand organs and it might produce signs and symptoms. Replication and homeostasis are a dynamic bioprocess and may lead to accumulation of pathogenic variants. These organelles have a peculiar condition to display heterogeneous presentation of DNA. There might be normal (wild-type) mtDNA along with mutated mtDNA with different rates between organs, even in the same mitochondria, in the same organism: this condition is defined as heteroplasmy [4]. Mitochondrial genome is inherited down maternal line, meaning that the offspring is identical in mtDNA. Although natural precautions are provided, such as bottleneck phenomenon, mtDNA is vulnerable to accumulation of novel mutations and it might enhance along time [5, 6]. When counting these quantities they

* Correspondence: manuela.baldo@insa.min-saude.pt

Newborn screening, metabolism and genetics unit - human genetics department, Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA), Porto, Portugal



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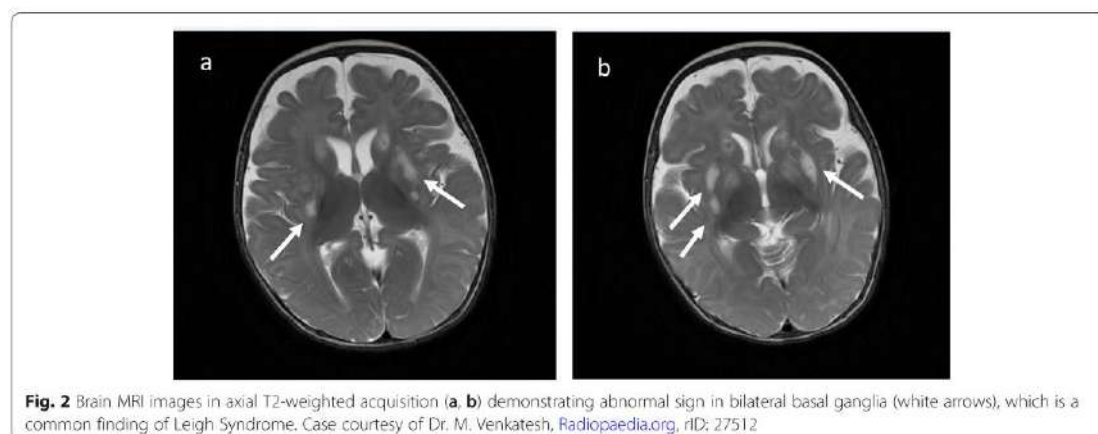


may vary between descendents and organ to organ in the same organism, producing different percentage of energy and possibly clinical expression. Regarding their flaws they are classified in point mutations or defects of maintenance which may be depletion or multiple DNA deletion types, linked to variants that interfere quantitatively or qualitatively in mtDNA synthesis. The DNA maintenance defects are linked to nuclear DNA (nDNA) variants inherited in autosomal dominant or recessive manner [7]. In this context, there are authors that defend their similarity in physiopathology discouraging group separation but one *continuum* of the same [8]. Therefore, mitochondrial diseases may be due to any interference in ATP metabolism somehow affecting the OXPHOS process.

Clinical presentation

Mitochondrial disorders of energy generation are the most frequent group of inborn errors of metabolism due to a dysfunction in OXPHOS (Fig. 2). They comprise distinct types of transmission (X-linked, autosomal, mitochondrial/maternal) due to nuclear, mitochondrial genome or in the interplay between two genomes resulting in great heterogeneity and wide range of presentation [9], from prenatal to adulthood. In childhood the

most common presentation is Leigh syndrome (LS) or subacute necrotizing encephalopathy. It was first described by Denis Archibald Leigh in 1951 through *post-mortem* investigation of a 7-month-old boy with progressive neurologic symptoms [10, 11]. Since first description based on histopathological findings, many tests tried to support the diagnosis revealing a damage in mitochondrial metabolism [12]. Currently, over 75 nuclear genes are related to this syndrome and still in expanding scenery [13]. Its variable onset brought up the diagnosis to be present in more age groups. The classical form usually starts before 2 years of age, even manifesting in neonatal period, and presents with hypotonia, epilepsy, respiratory stress, neurodevelopmental delay, ataxia and lactic acidosis. Typical neuroimaging reveals symmetrical hyperintensity in T2-weighted images in magnetic resonance imaging (MRI) in basal ganglia and/or brainstem with a lactate peak in affected areas in spectroscopy (Fig. 2). In addition cerebral white matter, thalamus, spinal cord and *cerebellum* may be affected as well [14]. Such identifiable imaging findings are a response to OXPHOS dysfunction and lower ATP generation. The neurons of higher energy demand in dysfunctional stock of ATP trigger a stress cascade culminating in gliosis and vacuolization of neuronal



tissue over time [15]. The late-form presents in more heterogeneous way, with behavioural/psychiatric findings, intellectual decline, movement disorders, headaches, memory loss or even mimicking a multiple sclerosis-phenotype [16]. Although highly specific to central nervous system there may be non-neurological symptoms. This condition is classified as Leigh-like syndrome (LLS) and comprises cardiac, hepatologic, gastrointestinal, hematological abnormalities and dysmorphic features [17]. As the main basis is impairment in mitochondrial ATP production, stress situations (infection, prolonged fasting, vaccine, anesthesia, surgery) may trigger an acute decompensation due to increased energy demand and produce symptoms. These patients are vulnerable and must undergo to careful investigation. Neuroimaging can provide valuable information and help medical group making decisions, except it requires in certain cases the patient to go through an anesthetic procedure and it might be deleterious even if successfully accomplished. Anesthetic components, as well as some antiepileptic drugs, may reduce even more the metabolism failing in producing ATP [18]. Relatively to imaging studies and considering the genetic heterogeneity involved, this pathology may display different findings. Complex I deficiency usually presents basal ganglia involvement, stroke-like lesions (mtDNA) and leucoencephalopathy (nDNA). Complex IV – cytochrome *c* oxidase – deficiency resembles complex I imaging findings. Complex V deficiency, mainly ATP6 and ATP8 related genes, exhibits basal ganglia and brainstem involvement with cerebellar atrophy but no stroke-like lesions, which may shorten the investigation. The translation defects might exhibit other findings such as: calcification in basal ganglia, cortical and cerebellar atrophy and stroke-like lesions in Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis and Stroke-like episodes (MELAS). Pontocerebellar hypoplasia is seen in *RARS2*

and pyramidal tract and spinal cord involvement associated with a lactate peak in spectroscopy are well associated to *DARS2*. A specific and occasionally reversible white matter dystrophy sparing the periventricular rim is suggestive of *EARS2* involvement and severe cortical atrophy with putaminal hyperintensity is relevant for *FARS2* involvement [19–21]. The maintenance defects display cortical and cerebellar atrophy with stroke-like lesions in *POLG* association [22], as *SUCLA2/SUCLG1* display basal ganglia involvement and cerebral atrophy [23]. In case of having such suggestive characteristics, molecular diagnosis may be easier. As far as the overall investigation it includes clinical records, family history, physical examination, biochemical evaluation in body fluid samples (lactate, pyruvate, glucose, blood gas profile, amino acids profile in blood/ spinal fluid and organic acids in urine), specific muscular tissue analysis (assessment of mitochondrial respiratory chain complexes) and ideally genetic identification of the responsible variant. As biochemical approach is not always elucidative or safe, genetic evaluation has become an eligible initial step in investigation especially if a pattern of signs and/or symptoms is found suggesting a specific condition or complex/complexes deficiency. Despite ongoing trials and progress, there is no curative treatment available, only towards supporting symptoms and avoiding metabolic crisis.

OXPHOS defects

Complex I (ubiquinone oxidoreductase - NADH): the first mitochondrial complex is known to be the most associated to mutations in mitochondrial diseases, with several LS related genes described so far, being the largest mitochondrial complex involved in ETC [24]. The LS spectrum of presentation is wide regarding the underlying variant, presenting with ataxia, oculomotor apraxia, seizures, neurodevelopmental delay, dystonia, failure to

thrive, cardiomyopathy and apnea. Leukoencephalopathy is basically a signature feature of complex I deficiency [11]. There are some mainly related genes to complex I assembly factors and subunits with LS phenotype previously described, new related-genes with variable phenotypes and new mutations in previously related genes, confirmed through assertive functional methods (Tables 1 and 2).

Baertling F. et al described a patient with *NDUFA4* missense variants c.194T>C (p.Leu65Pro) displaying an early-onset with neurodevelopmental regression, hypotonia, failure to thrive and irritability [25]. Biochemical profile showed hyperlactacidemia in plasma and cerebral spinal fluid (CSF). Brain imaging suggested LS, respiratory chain complexes analysis in fibroblasts demonstrated reduced complex I activity confirming WES results. Incecik F. et al confirmed a consanguineous late-onset LS presented with progressive ataxia and dysarthria with normal serum biochemical analysis but abnormal spectroscopy, in which a lactate peak was apparent in both *putamen* and right caudate topography [24]. *NDUFV1* homozygous variants c.1268C>T (p.Thr423Met) were identified pointing to a complex I deficiency. Previous descriptions for complex I include early-onset presentation displaying muscle hypotonia, spastic diplegia,

Table 1 Nuclear genes involved in OXPHOS causing Leigh and Leigh-like syndrome

Biochemical defect	Associated nuclear genes
Complex I Deficiency	<i>NDUFA1, NDUFA2, NDUFA9, NDUFA10, NDUFA12, NDUFS1, NDUFS2, NDUFS3, NDUFB8, NDUFS4, NDUFS7, NDUFS8, NDUFV1, NDUFV2</i>
Complex II Deficiency	<i>SDHA</i>
Complex III Deficiency	<i>UQCRCQ</i>
Complex IV Deficiency	<i>NDUFA4, COX8A</i>
Assembly factors deficiency	
Complex I	<i>NDUFAF2, NDUFAF4, NDUFAF5, NDUFAF6, C17ORF89, FOXRED1, NUBPL</i>
Complex II	<i>SDHAF1</i>
Complex III	<i>BCS1L, TTC19</i>
Complex IV	<i>SURF1, COX10, COX15, SCO2, PET100</i>
Cofactors and biosynthesis	
Pyruvate Dehydrogenase Complex	<i>PDHA1, PDHX, PDHB, DLAT, DLD</i>
Biotinidase Deficiency	<i>BTD</i>
Thiamine Deficiency	<i>TPK1, SLC19A3</i>
Lipoic acid	<i>LIPT1, LIAS, BOLA3</i>
Amino acid	<i>HIBCH, ECHS1</i>
Coenzyme Q10 Deficiency	<i>PDSS2, COQ9</i>

Table 2 Nuclear genes involved in other mitochondrial functions causing Leigh and Leigh-like syndrome

Mitochondrial function	Associated nuclear genes
Mitochondrial DNA maintenance	<i>FBXL4, POLG, POLG2, SUCLA2, SUCLG1, TWNK, SLC25A4, MPV17</i>
Mitochondrial translation	<i>GTPBP3, TRMU, EARS2, FARS2, IARS2, GFM1, GFM2, LRPPRC, TACO1, MTFMT, C12orf65, MRPS34, MRPS39 (PTCD3), NARS2, TSFM</i>
Mitochondrial dynamics	<i>SLC25A46, DNMTL, MFN2, RRM2B</i>
Mitochondrial material import/export	<i>SLC25A19</i>
Membrane phosphocomponents	<i>SERAC1</i>
Sulfur dioxygenase	<i>ETHE1</i>
AAA oligomeric +/- ATPase	<i>CLPB, SPG7</i>
RNA import	<i>PNPT1</i>
RNA-specific adenosine deaminase	<i>ADAR, RNASEH1</i>
Nuclear translocation system	<i>RANBP2</i>
Nuclear pore complex	<i>NUP62</i>
Manganese transportation	<i>SLC39A8</i>

oculomotor dysfunction, ataxia, lethargy, without leukodystrophy in brain imaging. Lou X. et al presented an early-onset case in a 7-month-old boy with torticollis, brain MRI compatible with LS, who evolved to metabolic crisis and progressive lesions in basal ganglia in imaging studies [26]. Whole Exome Sequencing (WES) identified missense variants c.418 C>T (p.Arg140Try) – new variant – and c.595 C>T (p.Arg199Try) in *NDUFS3* confirmed by Sanger sequencing and tested in B-lymphocytes of the *trio*. Confirmed segregation pattern, both variants were characterized implicated in reducing complex I activity. Beyond nuclear control, there are also mtDNA variants that reduces complex I functioning. The co-difying mtDNA genes as mitochondrial encoded ND genes *MTND* (1–6) can interfere in complex I electron pumping and therefore produce a compatible phenotype. *MTND1* m.3697G>A previously reported in MELAS is suggested to be implied as well [27]. Ma YY. et al screened Chinese patients with LS and identified previously reported variants as m.10191 T>G in *MTND3* causing the phenotype in their group and suggested to be the most frequent variant in their population [28]. Another variant reported by Ruiter EM. et al to be frequent is m.13513 G>A in *MTND5*. A biochemical proven complex I deficiency in association with optic atrophy and/or a cardiac conduction defect syndrome was found and described in their cohort [29]. Despite its occurrence in MELAS, Leber's Hereditary Optic Neuropathy (LHON) and overlap of these two, Sudo A. et al found 7% of this variant's

prevalence in their study [30]. The same situation occur in m.14487 T>C in *MTND6* broad phenotype possibilities, related to severe LS in homoplasmic rates and in lower rates to progressive myoclonic epilepsy in adulthood [31].

Complex II, III and Coenzyme Q10: far less common than complex I (around 8%). Complex II - SDH (succinate dehydrogenase) has four subunits, the most important codified by *SDHA* and *SDHB* genes, being *SDHA* the most implicated. Patients may present with early onset and classic rapid progression or late form involving Kearn-Sayres, dementia, myoclonic epilepsy and surviving to late childhood [32]. Complex III (ubiquinol:cytochrome c oxidoreductase) is rare to be implicated to mitochondrial diseases in general and three nuclear genes mainly involved in LS - *UQCRCQ*, an assembly protein, *TTC19*, an assembly factor and *BCSIL* [33]. *BCSIL* is the most frequent out of three, a complex chaperone that encodes ATPases necessary for complex assembly. *BCSIL* deficiency often manifests as GRACILE-like/ LLS combining low weight at birth, renal and hepatic dysfunction, hypotonia and developmental delay. The previous described are encompassed in an intermediate dysfunction phenotype, also named "myopathy *BCSIL* phenotype" [34]. *UQCRCQ* codifies a subunit of complex III, and is related to early-onset severe LLS identified in a consanguineous Israeli Beduin kindred family. Descriptions of *TTC19* range from psychiatric symptoms and ataxia [35] to global neurodevelopmental delay and language regression in early childhood [36]. In a report, it is described that the majority of patients with complex III deficiency related to *TTC19* have consistent neuroradiological findings to LS [14]. Coenzyme Q10, or ubiquinone, is a vital ETC component that promotes electron transport via flavoproteins from complex I and II to III [37]. This deficiency might manifest with ataxia, migraine, ptosis, deafness, lactic acidemia and display neuroradiological findings of LS. Signs recognition must be carefully taken under consideration when supplementing this cofactor. Van Maldergem L. et al compared two sisters with confirmed deficiency of Coenzyme Q10 (CoQ10) in muscle tissue, with important recovery of abilities, behaviour and growth parameters with 300 mg/day [38]. Glover EI. et al tried a randomized trial with CoQ10 to mitochondrial disorders but no meaningful results were observed especially in higher doses, leaving questions around supplementation dose when no proven deficiency [39]. The ideal is testing CoQ10 in muscle tissue directly measuring ETC functioning and quantifying the cofactor or through molecular diagnosis related to this cofactor [37].

Complex IV (Cytochrome c oxidase - COX): The *SURF1* variants are the most common playing part in Complex IV assembly, counting majority of cases

described so far. Despite relatively homogeneous clinical findings like hypertrichosis even in presymptomatic state [40], neuroimaging may display uncommon features such as leukodystrophy and atrophy [14]. They appear to have better prognosis than other syndromes caused by genes implied in complex IV metabolism, such as *ETHE1* and *LRPPRC*. The *ETHE1* is related to ethylmalonic encephalopathy (OMIM #602473) and may manifest as LS due to accumulation of sulfide, which reduces complex IV functioning and justifies the resemblance. *LRPPRC* variants are related to first description in French-Canadian population and present a characteristic phenotype, including facial dysmorphic features, liver disease and recurrent lactic acidosis [41]. In addition to interfering in cytochrome c oxidase function, *LRPPRC* interposes in global mitochondrial translation and mitochondrial RNA (mRNA) maturation, in ATP synthase complex and lipid metabolism in peroxisomes, playing more roles but presenting mainly as complex IV deficiency. Such findings may highlight the phenotype difference from other LS causes [42]. Previously related to complex I, *NDUFA4* is recently proven through functional studies to codify a complex IV subunit [43], presents as late-onset form with learning disabilities, epilepsy and survival into adulthood [14].

Complex V (ATP synthase): Unlike other complexes, complex V is mainly under mtDNA control and represents the last OXPHOS step - generating ATP. The two subunits (F0 and F1) produce a conformational change when receiving ADP, coupling to inorganic phosphate (Pi) and converting ADP into ATP. Some variants are well related to mitochondrial diseases and even LS. The *MTATP6* m.8993 T>G/C variant, that damages the F0 portion of ATPase [44], is associated to Maternally Inherited Leigh Syndrome (MILS) and to Neuropathy, Ataxia and Retinitis Pigmentosa (NARP) syndrome with basically the heteroplasmic rates defining the phenotype. Considering rates over 60% and lower than 85% it is expected a NARP syndrome phenotype; 85% or more it is expected a MILS phenotype, although variations in these values and phenotypes have been reported. Besides heteroplasmic rates a common finding related to this variant is hypocitrullinemia in amino acids evaluation, which may shorten the investigation as Vilarinho L. et al demonstrated in a report that two out of three families had low levels of citrulline [45] and Lopes T. et al reported in a patient [46]. In addition to *MTATP6* are m.9176 T>G and m.9185 T>C, described to be associated with late-onset cases [47]. In a Portuguese study of m.9176 T>C, Carrozzo R. et al investigated two sisters with LS where one of them had low levels of this metabolite, displaying a new possibility to LS with hypocitrullinemia as well [48].

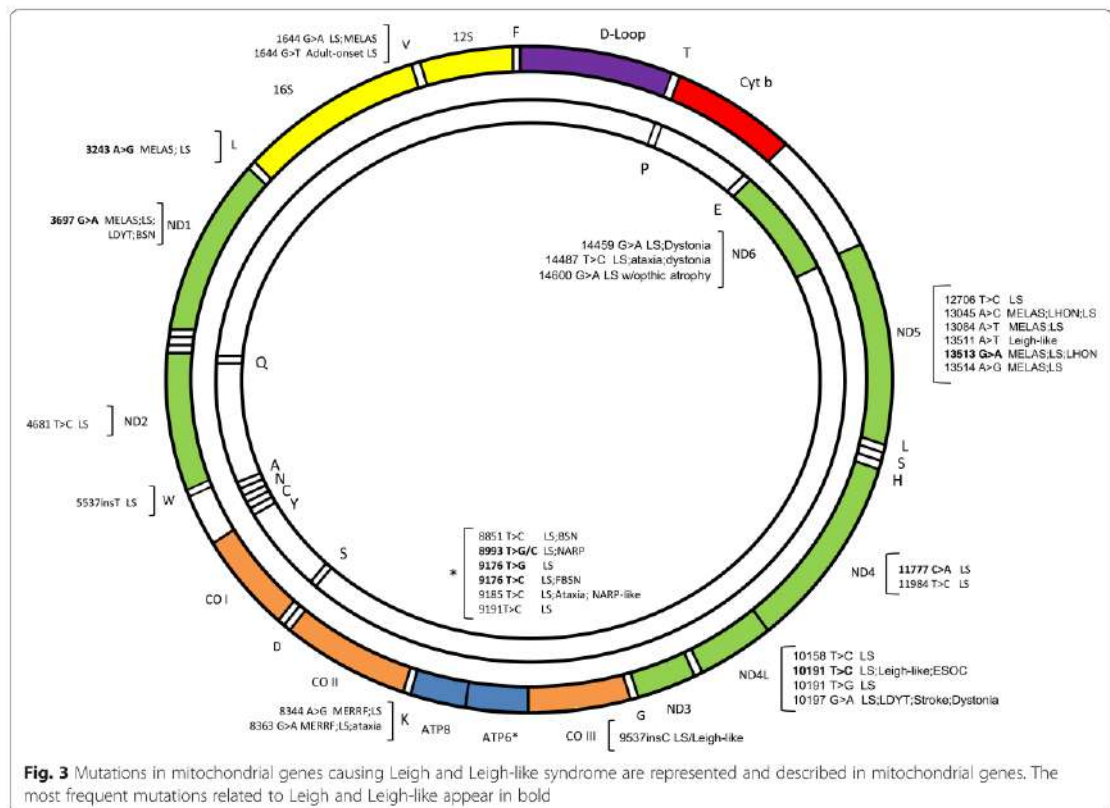
Combined deficiencies: Since mtDNA encodes components of complexes I, III, IV and V, a pathogenic variant that affects mtDNA maintenance can result in combined OXPHOS impairment. Whereas deletion syndromes are related to rearrangements in DNA, depletion syndromes produce reduction in DNA copy numbers [49]. New reports include LS to the nomenclature due to overlapping phenotypes as genes associated with mtDNA deletion or depletion syndromes were identified in these patients [50]. The most frequent causes of mtDNA depletions are related to *SUCLA2* and *SUCLG1*. By affecting succinyl-CoA synthetase, an important step of TCA, they manifest mild to elevated levels in methylmalonic acid in organic acids profile and reports suggest that this metabolite is good in surveillance and investigating the disease [14]. *SUCLA2* tends to present as early-onset LLS syndrome with muscular atrophy and sensorineural hearing impairment, while *SUCLG1* can express in a severe and fatal phenotype including harsh hyperlactacidemia and pronounced liver dysfunction. However, descriptions of indistinguishable phenotype between them have reported, adding a confusing factor to investigation [51]. *SERAC1* is responsible for cholesterol trafficking and remodeling phosphatidylglycerol 34:1 to 36:1, a structural cell membrane component cardiolipin. *SERAC1* is related to MEGDEL syndrome, a progressive syndrome defined by 3-Methylglutaconic aciduria, Deafness, Encephalopathy and LLS [52, 53]. Other important function in mitochondria is DNA translation, resulting in mitochondrial nucleic acids production. It is performed by modifying proteins (mt-tRNA) and constitutes another group implied in combined deficiencies. *MTFMT* was the first mt-tRNA reported to be responsible for LS, being c.626C>T a frequently identified variant in this group [14]. Subsequently, came over others like *MTTL1* and *MTTK* to be linked to LS: m.3243A>G *MTTL1* responsible for MELAS and m.8344A>G *MTTK* related to Myoclonic Epilepsy with Ragged Red Fibers (MERRF). After production of nucleic acids, mtDNA must undergo through proteins synthesis to be polymerized, an event under *POLG* control [54]. Despite directly involved in mitochondrial bioprocesses, *POLG* is not maternally inherited but in an autosomal dominant or recessive manner. *POLG* is related to Alpers-Huttenlocher Syndrome (severe encephalopathy, seizures, and liver failure) but accordingly to the variant might produce various phenotypes, like Alpers, Progressive External Ophthalmoplegia (PEO) and LS. Its deficiency produces a LLS phenotype, which includes hepatic dysfunction as Alpers [8] and may include hypertrophic olivary degeneration as in *SURF1* deficiency [55]. As *POLG*, other rare genes related to mtDNA maintenance but non-related to LS are currently proven to express the phenotype. Souza, PVS.

et al published in a recent study in a heterogeneous age group (0-69y) uncommon responsible genes and new variants in formerly related to LS/LLS genes: *SPG7*, a mitochondrial protease, was related to basal ganglia abnormalities never referenced before; *RRM2B*, a nucleic acid supplier to mitochondrial machinery, evidenced parkinsonism and LS features in imaging in an unusual phenotype. *MFN2*, a protein related to mitochondrial dynamic bioprocesses, displayed basal ganglia lesions with movement disorders and deafness. *TWNK*, a helicase involved in replicating mitochondrial genome, presented with stroke-like episodes and never before confirmed LS phenotype. *MPV17*, an essential protein to keep nucleic acids pool, is related to Navajo neurohepatopathy not comprising LS features previously. *SLC25A4*, an inner transmembrane mitochondrial channel to ADP-ATP, displayed unusual central nervous system abnormalities consistent with LS. *RNASEH1*, an endonuclease to degrade RNA, manifested late-onset LS with progressive parkinsonism, *pes cavus* and cerebellar ataxia [56]. Even though the most common causes in mtDNA LS/LLS are point mutations or small deletions [7], DNA rearrangements (deletion) and depletion syndromes are rising in number accounts to mitochondrial disorders due to new genetic tools. Hence, latest reports phrase that NGS have enabled diagnosis and expanded the previously known LS/LLS phenotypes (Fig. 3).

Other defects non-OXPHOS

Biotine-responsive basal ganglia disease (BBGD)/*SLC19A3* deficiency: thiamine pyrophosphate (TPP) is an important cofactor of more steps in energy production playing main role in several pathways in neurometabolism and *SLC19A3* is a specific biotin-dependent thiamine transporter in basal ganglia system [57–59]. The lack of thiamine might produce three phenotypes along life: early-onset LS, the BBGD in childhood and Wernicke Encephalopathy in adulthood [60]. BBGD mainly presents with hyperlactacidemia, vomiting, seizures and encephalopathy. The CNS free-thiamine dosage is frequently in low quantities nearly to absence, which can enhance diagnosis suspicion [61]. The neuroimaging is consistent with LS except cortical and subcortical abnormalities that are unusual for classic LS presentation. In *SLC19A3* deficiency thiamine and biotin supplementation might improve symptoms and have promising outcomes [57].

Pyruvate Dehydrogenase Complex deficiency (PDHC): pyruvate dehydrogenase complex that is composed by units E1, E2 and E3 - the E1-alpha and E1-beta require active thiamine (TPP) which interferes in converting pyruvate in acetyl-CoA in TCA, the main substrate to ETC. In a deficiency state, the exceeding pyruvate is transformed in lactate forbidding the ETC to move



forward with glycolysis [62, 63]. The X-linked PDHc deficiency (PDHA1) is related to *PDHA1* and it is the most frequent PDHc deficiency. Variants in *PDHA1* produce high lactate, pyruvate and alanine levels, failure to thrive, seizures. Basal ganglia and *corpus callosum* abnormalities are common feature. Treatment is based in dietary modification by enhancing calories through lipids, thiamine supplementation in high quantities and controlling lactate/pyruvate levels.

Lipoyltransferase 1 deficiency (*LIPT1*): lipoic acid is necessary to many assembly complexes and intermediate mitochondrial metabolism in ETC to generate ATP [64]. The metabolism to its active form requires active thiamine as a cofactor, implying integrity in related pathways to succeed and its deficiency interferes in other pathways like glycine cleavage system, PDHc, α -ketoglutarate dehydrogenase (KGDH) and branched-chain α -ketoacid dehydrogenase complex BCKDH - E3 form. Its deficiency produces metabolic acidosis due to hyperlactacidemia, normal/elevated branched-chain amino acids and glycine levels in serum and/or CSF. Reposition of lipoic acid is not effective, restricting specific treatment options [65].

Biochemical investigation

As first approach to a suspicion of LS it is reasonable to evaluate the possible affected pathways, considering energy supplies and metabolic intermediates generated in response. As so, blood gas analysis, lactate and pyruvate, glucose and electrolyte profile are the main metabolic basis. LS include normal/raised lactate/pyruvate levels in plasma and/or cerebral spinal fluid (CSF) and may present with metabolic acidosis and hypoglycemia in decompensation status. Specific profiles as amino acids and organic acids are helpful and may give hints of the underlying cause. Amino acids profile in plasma may show alanine or citrulline alterations, compatible as well to the syndrome. Alanine is produced in response to high lactate levels and citrulline is diminished in a specific mtDNA variant – m.8993 T>G, shortening investigation [66, 67]. A specific metabolite search in plasma, such as thiamine pyrophosphate (TPP), could allow a thiamine transporter deficiency diagnosis due to *TPK1*, a treatable cause of LS [68]. Organic acids profile may reveal specific pattern of acids in urine suggesting the blocked pathway

(methylmalonic, 3-methylglutaconic) or even intermediates of TCA, often present in mitochondrial defects. In CSF evaluation a free-thiamine dosage would help the investigation of thiamine transporter deficiencies, such as *SLC19A3*, another treatable cause of LS related to thiamine transporter deficiency [61]. Lactate levels represent the local metabolism status being a promising finding [11]. As it relates sensitively to central nervous system (CNS) metabolism it is more specific than peripheral lactate levels that are under many confusing factors, such as sample collection manner, material stocking and others. Raised levels may also be identified in spectroscopy but it is not understood yet the minimum quantity to be present to be identified [69] even in a more accurate investigation with proton acquired imaging. However, in many asymptomatic or undiagnosed cases spectroscopy may show precocious local metabolism impairment and lead the investigation towards metabolic etiology [70]. Some findings in neuroimaging are found to be suggestive of mitochondrial disease as stroke-like lesions, *striatum* commitment and brainstem alterations associated with lactate peak in spectroscopy [71]. Furthermore, as not always neuroradiologic features or biochemical approach are positive, especially for mitochondrial disorders, new biomarkers are been studied in order to better help in a less invasive manner such as tissue biopsy. FGF-21 (fibroblast growth factor 21) and GDF-15 (growth and differentiation factor 15) are serum cytokines produced in liver and muscle tissue that were found to be raised in mitochondrial diseases, particularly in muscle manifesting mitochondrial diseases, when compared to previously used biomarkers (lactate, pyruvate, lactate to pyruvate ratio and creatine kinase). In order to correlate circulating levels to disease and prove specificity and sensitivity parameters, some authors published data comparing them with each other and each of them to the standardized serum biomarkers. It was proposed that FGF-21 correlates well with disease severity and has sufficiently specific and sensitive to mitochondrial myopathies but few years later it was verified that nonmitochondrial heterogeneous groups also displayed raised levels of this component. Shortly after GDF-15 was suggested to have more sensitivity and specificity rates than FGF-21, a result showed especially in children, and associating their measurements would be better than isolated [72]. Yatsuga A. et al even described better statistic profile of GDF-15 over FGF-21 for severity and mitochondrial basis except for non efficient discriminative profile in cardiac muscular involvement etiology [73]. Lehtonen JM. et al reported raised levels

of FGF-21 in mitochondrial translation defects and mtDNA deletions and correlated positively with disease severity being a possible biomarker to follow up [74]. Morovat A. et al demonstrated that FGF-21 displays considerable overlapping values between mitochondrial and nonmitochondrial diseases, increased levels in other conditions yet stability in repeated measurements to follow up procedures and important positive correlation in ophthalmoplegia in adults even suggesting to dismiss muscle assessment to produce a definite diagnosis [75]. Given the disagreement, a Russian group recently published results comparing mitochondrial disease groups with nonmitochondrial controls for each biomarker and had no significant results in their cohort studies pointing raised levels in metabolic nonmitochondrial group, though mitochondrial group had higher values, which require further investigation of these biomarkers [76].

A more invasive and harmful possibility for investigation and diagnosis is tissue biopsy (skin, muscle). Although quite criterious in acquiring samples due to invasive collection procedure, histopathology for muscle sample and OXPHOS analysis for both muscle and skin fibroblasts are still an alternative to a biochemical assessment and are profitable in providing a diagnosis. Muscle samples should be processed right away for entire OXPHOS assessment or if not must be frozen and then complexes must be evaluated individually [77]. Despite precautions with technique and procedure (local anesthesia), OXPHOS assessment in muscle biopsy is preferable under general anesthesia. However, regarding clinical phenotype may add little contribution depending on mainly affected organ. To perform muscle biopsy in parallel to genetic evaluation is a practice that is required in cases of great clinical instability or rapidly progressive symptoms, such as classical Leigh syndrome presentation, in isolated muscle involvement disease and in cases that muscle biopsy could provide diagnosis and change or start specific treatment. The negative genetic evaluation or only confirmation of already disease causing genetic findings does not require simultaneously or prompt muscle evaluation [78]. Skin fibroblasts are an alternative to muscle in small children, less invasive, but in a heteroplasmy possibility they may result normal or inconclusive as well [11].

Genetic evaluation

When it comes to DNA analysis there are alternatives to address the respective content. Mitochondrial DNA is a circular DNA, double-stranded, easier to access and sequence than nuclear DNA. The investigation decision relies in having a specific suspicion or not. Whenever identified a novel variant

there has to be a prediction of its behavior *in vivo*. The bioinformatic tools are helpful in estimating these results *in silico* predictors as MutationTaster, PolyPhen-2, SIFT by performing an analysis and, therefore, providing an estimate result [79–81]. In children with early presentation the investigation in molecular matters should start in mitochondrial DNA. The most frequent mutation related to this phenotype in early childhood is m.8993 T>G/C related to maternally-inherited Leigh syndrome (MILS) and NARP syndrome [82]. The more time passes by, the more mitochondria are vulnerable to nDNA command, which may point first molecular investigation in late-onset groups to nDNA. In deletion/depletion syndromes, this rule is not valid. They depend on accumulation of abnormal variants in mtDNA, enhanced by time, to produce symptoms. It is known that the bottleneck phenomenon narrows the mutation loads by producing lower mitochondrial replication whenever higher mutant loads are present and the opposite as well, but it may be influenced at every cycle [2]. The particular relevance of onset is due to literature results demonstrating that in many suspicious or unclear cases the first genetic approach is NGS – gene panels, clinical exome sequencing, whole exome/genome sequencing. The difference is the coverage of genes in an individual analysis: 5000 genes in clinical exome and 20,000 in whole exome but still representing 2% of the DNA – the exomic (coding) regions, where genome includes all coding and non-coding sequences [83]. In nDNA defects, it's valuable to search or recognize signals and symptoms that strengthen possibilities, leading to one probable disease-causing gene. In case of specific phenotypic findings the search might be direct, shortening time for diagnosis [11]. When the phenotype is not well defined, a wide investigation is required. Nogueira C. et al published a report in use of NGS techniques for mitochondrial diseases in adults and children, displaying favorable results in gene-panel NGS assessment to provide molecular diagnosis to this selected group and suggesting the complementary investigation through exome in order to expand candidate genes to evaluation giving the panel limitation design and gene targets [84]. A recent report of a Brazilian group with variable age presenting diverse neurological features was assessed by magnetic resonance of the brain and spinal cord, biochemical profile, muscle biopsy and further genetic characterization by WES. Surprisingly the results came suggestive of LS/LLS by mtDNA-maintenance defects. The variants were not previously related to them, something a gene panel would have missed. A

Polish group investigated pediatric patients that were selected to WES evaluation in a Health Center with mitochondrial diseases suspicion based on the Mitochondrial Disease Criteria. They reported 90% of positive results in their cohort especially in neonatal patients, a highly unstable age range and susceptible to severe and fatal clinical presentations [85]. In contrast, Taylor RW. et al analyzed 53 patients of two centers from distinct countries who had respiratory chain assessment in muscle biopsy with evidence of multiple respiratory chain complex deficiency but no pathogenic variant identified in mtDNA. WES was performed and 32 patients had results involving 18 candidate genes, including recurrent pathogenic variants and novel variants in mitochondrial genes that could possibly be disease-causing. The group described the negative identification of candidate variants and the difficulties of validating results of non-reported variants in literature until then leaving 21 patients undiagnosed [86]. In pediatric or rapidly progressive ill patients a correct diagnosis in fast performance is still challenging, even more in hospitalized ones. The need has stimulated new modalities of genetic evaluation such as the rapid Whole-Exome/Genome Sequencing (rWES/rWGS). Saunders CJ. et al explained the use of rWGS in Neonatal Intensive Care Unit patients using a rapid genome analysis promising less than 3 days to results. Crossing clinical information and candidate genes identified in rWGS the diagnosis was directed and time-shortened [87]. This technique might be promising as in hospitalized children demonstrating enhancing diagnosis, readjustment in treatment manners, saving costs of unnecessary hospitalization as Farnaes L. et al described, and even in approach to fetal hydrops in a Niemann Pick type C fetus [88, 89]. Although proven to be effective, rapid approaches are even rarer than the classic WES or WGS and might be questionable particularly in non mendelian inherited disorders identification [87]. The unsatisfactory or inconclusive outcomes like Taylor RW. et al described motivate emerging fields as the “omics” evaluation, namely transcriptomics, is one of them. The analysis of transcriptome, or the group of DNA transcripts, could explain the non mendelian inherited diseases and other modalities of expression (aberrant expression, aberrant splicing and mono-allelic expression) that exome and genome themselves would miss. Furthermore, WGS would detect more variants by analyzing the genome material but interpreting coding and non-coding new information enhance the challenge and require validation as well. Analyzing the RNA-sequencing (RNA-seq) in clinical practice would provide stronger

evidence for WES and WGS results [83]. Despite robust findings in bioinformatic techniques the functional analysis must be taken in order to prove themselves or not, where more “omics” (ex: proteomics, metabolomics) tasks are welcome to take place and be a positive approach to effective diagnosis. Indeed, it requires a more accurate investigation to better comprehension and improve diagnosis effectiveness but still is an open door to be explored [90].

Genetic counseling and prenatal diagnosis

LS has mitochondrial and nuclear DNA as possible causes of disease. Hence, it is important to define whether autosomal, X-linked or maternally inherited to genetic counseling and future decisions. If the mother has already lost a child to the disease or is known to harbour mtDNA mutation, it is well expected to undergo through testing. Prenatal diagnosis is feasible when there is possibly known causative mutations with well established physiopathology, segregation pattern and linkage studies [91, 92]. The objective is to provide an assessment of the risk to the fetus in developing disease antenatal or in childhood. When a mtDNA variant is involved there has to be a proper determination of heteroplasmy percentage in the mother by testing blood and urine sample and the fetus by chorionic villus biopsy testing (CVB). Even though assuming pathogenic rates above 60%, in many cases, it is not possible to assure that offspring will be affected considering possible changes in these numbers until birth [92, 93]. One variant that has stable mutation load levels throughout the gestation period and in most affected tissues including blood is m.8993 T > G/C, favoring proper diagnosis and counseling during pregnancy. Some articles reviewed different mtDNA variants, as the m.9176 T > C, and adopted more directive counseling in extreme mutation loads [94]. Considering that the mutant load may change during pregnancy and vary from tissue to tissue, careful advice is recommended. If negative there is no indication of a pregnancy termination. If below 10% or above 90% genetic counseling may be straightforward. Between these values it's unsafe to be determinative of the future. Relatively to autosomal recessive trait an evaluation is required when previous diagnosis in offspring or positive family history, recurring to amniocentesis to test the fetus. If the fetus has mutated DNA in both alleles certainly will be affected, leaving the geneticist and the family the final decision.

Treatment

Specific mitochondrial support: currently there is no sustained evidence of effective interventions in mitochondrial diseases [17], except few are “treatable” mitochondrial diseases [95]. In patients with *SLC19A3*

mutations some improvement is seen, especially in early presentation, in thiamine (10–20 mg/kg/day) and biotin (10–15 mg/kg/day) oral supplementation [96]. They seem to enhance some transporters modulated by *SCL19A3* [50]. The same is observed in high doses supplementation of thiamine (30–40 mg/kg/day) in patients with *PDHA1* deficiency by stabilizing the PDHc [96, 97]. Primary thiamine defects, such as *TPK1* deficiency, are also under treatment option in thiamine reposition in 20 mg/kg/day [96]. CoQ10 is another that is reasonably effective in mitochondrial disorders, besides its primary deficiency, providing recovery of neurologic symptoms. Although acting in electron transfer from complexes in ETC and playing an antioxidant part in many cellular processes [98] and even higher dose are well tolerated even benefits are discussed. The recommended dose of CoQ10 is 10–30 mg/kg/day via oral supplementation [96].

General treatment: some general treatments have been studied along with specific therapies in order to ameliorate symptoms, avoid disease progression, clinical decompensation and enhance quality of life. A derivative from CoQ10, idebenone, was introduced due to easier tolerance profile and mitochondrial uptake, allowing results by a better improvement of ATP generation [99]. Recently, an alternative drug - EPI-743, a Coenzyme Q10 analog - has been developed and proved to have a better therapeutic profile. The first trial reported a promising improvement in patients with genetically confirmed mitochondrial disease, leading to a second trial. The patients who remained until the study was completed evolved with improvement and the one that discontinued medication fell back to symptoms [100]. EPI-743 increases intracellular glutathione levels by enhancing reduced intracellular glutathione stock [101]. Epilepsy is a common feature of LS demanding antiepileptic drugs to be considered to control the episodes. The choice of an antiepileptic drug must be taken considering the collateral profile and the benefits associated with the medication. It is already known that valproic acid, phenobarbital and phenytoin have deleterious effects in ETC, yet in case of uncontrolled prolonged crises (epileptic status) the option is based on risk - benefit of the emergency or medication availability [102]. Along with medications, some alternatives in dietary modification might be helpful. In some inhibitors of mitochondrial metabolism defects (*HIBCH*, *ECHS1*) a valine-restricted diet is an option, even with unclear responses so far [96]. Another option is the ketogenic diet is a dietary modality that avoids carbohydrates and focuses on lipids and proteins, producing ketone bodies instead of glucose as a cellular fuel [103]. Despite trials, ketogenic diet is still controversial to mitochondrial disease [95]. In *ETHE1* deficiency metronidazole associated to N-acetyl-cysteine

might reduce sulfides and improve clinical and biochemical status [14]. As until now there is no curative treatment the available choices must be optimized in order to best benefit the patients in providing care and leaves an open door to new studies in finding new and, hopefully, definitive therapeutic option.

New therapies - as reproductive assistance and gene therapy are emerging and slowly expanding the resources field availability. Zhang J. et al described a live born baby resultant of an oocyte spindle transfer. The mother carried homoplasmy m.8993 T > G in germline cells, had multiple pregnancy losses, positive family history of LS, and decided to conceive again mitigating risks of a new unhealthy baby. Tests in donor material and in blastocyst for mutation load were made and came in safe range percentage. After birth, new samples were collected and preserved in safe levels [104]. This is an alternative to mothers who are known to harbor a pathogenic mitochondrial mutation. As far as direct intervention, gene therapy is being developed for LS. Allotopic gene expression was reported by Manfredi G. et al in mammalian nucleus to rescue the *MTATP6* function in producing ATP successfully achieved [105]. Tanaka M. et al reported the use of a restriction endonuclease to mitochondria managing to eliminate only in mutated mtDNA and preserving wild type in cultured cells [106]. New techniques like TALENS (Transcription activator-like effector nucleases) to mitochondria (mito-TALEN's) were also reported to be quite selective in patient-derived cells harboring m.14459 G > A [107]. Di Meo I. et al seek to repair neurologic affected areas by LS in animal model and reported only partial improvement in neurologic symptoms due to a selective distribution of the vector to certain brain areas requiring further studies to have better results [108].

Prognosis

Even with diagnosis and management improvements, the survival remains poor. As a heterogeneous disease, it takes time for diagnosis and little chances of having specific interventions as they are needed. Sofou K. et al presented a cohort of 130 patients with early presentation, described their natural history and identified possible factors of impact on survival as disease severity. In their cohort premature births, interventions in immediate neonatal period were present in little frequency yet pointing that prenatal presentation is still true. Pathological findings at neonatal period and epilepsy were described to be related to more frequent relapses as the age of onset, brainstem abnormalities in neuroimaging studies and hospitalization in Intensive Unit Care due to acute exacerbations were identified as poor prognosis predictors. Some groups published surviving rates that rarely encompass the third decade of life. The mortality

is precocious not only in LS, but in mitochondrial diseases in general, due to complications and deterioration of organs and systems. Delayed diagnosis compromising optimal care also contributes to poor prognosis [109].

Conclusion

LS is a neurodegenerative disease with broad phenotype and genotype presentations, in expanding picture. Besides diagnosis tools progresses, like NGS, new serum biomarkers and functional studies, treatment options are still restricted. It is still a challenge to make diagnosis and offer an optimal treatment due to its heterogeneous causes, therefore enhancing the need of new trials and further research to enhance quality of life and better providing care to the patients.

Abbreviations

ADAR: Adenosine deaminase RNA Specific; ADP: Adenosine Diphosphate; ATP synthase: Complex V; *MTATP6*: ATP synthase 6; ATP: Adenosine Triphosphate; *BBGD*: Biotin-responsive basal ganglia disease; BCKDH: Branched-chain α -ketoacid dehydrogenase complex; *BCSL1*: Ubiquinol-cytochrome c reductase complex chaperone; *BOLA3*: BOLA family member 3; *BTBD*: Biotinidase; *C12Orf5*: Chromosome 12 open reading frame 65; *C12ORF89*: NADH:ubiquinone oxidoreductase complex I, assembly factor 8 (NDUFAF8); *CLPB*: Caseinolytic peptidase B protein homolog; CNS: Central nervous system; Complex I: NADH (Nicotinamide Adenine Dinucleotide Dehydrogenase)/ubiquinone oxidoreductase; Complex III: Ubiquinol:cytochrome c oxidoreductase; CoQ10: Coenzyme Q10; *COQ9*: Coenzyme Q9 homolog; *COX*: Cytochrome c oxidase (complex IV); *COX10*: Cytochrome c oxidase assembly homolog COX10; *COX15*: Cytochrome c oxidase assembly homolog COX15; *COX8A*: Cytochrome c oxidase subunit 8a; CSF: Cerebral spinal fluid; CVB: Chorionic villus biopsy; *DLAT*: Dihydrolipoamide S-acetyltransferase; *DLD*: Dihydrolipoamide dehydrogenase; DNA: Deoxyribonucleic acid; *DNM1L*: Dynamin 1 like protein; *EARS2*: Glutamyl-tRNA synthetase 2; EPI-743: *Vatiquone*; ETC: Electrons Transport Chain; *ETHE1*: Persulfide dioxygenase; FADH/FADH₂: Reduced forms of Flavin Adenine Dinucleotide (FAD); *FARS2*: Phenylalanyl-tRNA synthetase; *FBLX4*: F-Box and leucine rich repeat protein 4; FGF-21: Fibroblast growth factor 21; *FOXRED1*: FAD-dependent oxidoreductase domain-containing protein 1; GDF-15: Growth and differentiation factor 15; *GFM1*: Mitochondrial elongation factor g 1; *GFM2*: Mitochondrial elongation factor g 2; GRACILE: Growth Retardation, Aminoaciduria, Cholestasis, Iron overload, Lactic acidosis, and Early death; *GTPBP3*: GTP binding protein 3; *HIBCH*: 3-hydroxyisobutyryl-CoA hydrolase; *IARS2*: Isoleucyl-tRNA synthetase 2; KGDH: α -ketoglutarate dehydrogenase; LHON: Leber's Hereditary Optic Neuropathy; *LIAS*: Lipoic acid synthetase; *LIPIT1*: Lipoyltransferase 1; *LIPIT2*: Lipoyltransferase 1 deficiency; LLS: Leigh-like Syndrome; *LRPPRC*: Leucine rich pentatricopeptide repeat containing; LS: Leigh Syndrome; MEGDEL: 3-Methylglutaconic aciduria, Deafness, Encephalopathy and Leigh-like syndrome; MELAS: Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes; MERRF: Myoclonic Epilepsy with Ragged Red Fibers; *MFN2*: Mitofusin-2; MILS: Maternally Inherited Leigh Syndrome; Mito-TALENS: mitochondrial transcription activator-like effector nucleases; *MPV17*: Mitochondrial inner membrane protein 17; MRI: Magnetic resonance imaging; mRNA: Mitochondrial RNA; *MRPS34*: Mitochondrial ribosomal protein s34; *MRPS39*: Mitochondrial ribosomal protein s39; mtDNA: Mitochondrial DNA; *MTFMT*: Mitochondrial methionyl-tRNA formyltransferase; *MTND 1-6*: Mitochondrial encoded ND genes 1 to 6; *MTND1*: Mitochondrial encoded ND gene 1; *MTND3*: Mitochondrial encoded ND gene 3; *MTND5*: Mitochondrial encoded ND gene 5; *MTND6*: Mitochondrial encoded ND gene 6; *MTTK*: Mitochondrial tRNA for lysine; *MTTL1*: Mitochondrial tRNA for leucine; mt-tRNA: Mitochondrial transfer RNA; NADH: Reduced Nicotinamide Adenine Dinucleotide (NAD); NARP: Neuropathy, Ataxia and Retinitis Pigmentosa; *NARS2*: Asparaginyl-tRNA synthetase 2; nDNA: Nuclear DNA; *NDUFA1*: NADH:ubiquinone oxidoreductase complex I alpha subcomplex subunit 1; *NDUFA10*: NADH:ubiquinone oxidoreductase complex

I alpha subcomplex subunit 10; *NDUFA12*: NADH:ubiquinone oxidoreductase complex I alpha subcomplex subunit 12; *NDUFA2*: NADH:ubiquinone oxidoreductase complex I alpha subcomplex subunit 2; *NDUFA4*: NADH:ubiquinone oxidoreductase complex I alpha subcomplex subunit 4; *NDUFA4*: NADH:ubiquinone oxidoreductase complex I alpha subcomplex subunit 4; *NDUFA9*: NADH:ubiquinone oxidoreductase complex I alpha subcomplex subunit 9; *NDUFA2*: NADH:ubiquinone oxidoreductase complex I, assembly factor 2; *NDUFA4*: NADH:ubiquinone oxidoreductase complex assembly factor 4; *NDUFA4*: NADH:ubiquinone oxidoreductase complex I, assembly factor 4; *NDUFA5*: NADH:ubiquinone oxidoreductase complex I, assembly factor 5; *NDUFA6*: NADH:ubiquinone oxidoreductase complex I, assembly factor 6; *NDUF8*: NADH:ubiquinone oxidoreductase 1 beta subcomplex subunit 8; *NDUF51*: NADH:ubiquinone oxidoreductase Fe-S protein 1; *NDUF52*: NADH:ubiquinone oxidoreductase Fe-S protein 2; *NDUF53*: NADH:ubiquinone oxidoreductase Fe-S protein 3; *NDUF54*: NADH:ubiquinone oxidoreductase Fe-S protein 4; *NDUF57*: NADH:ubiquinone oxidoreductase Fe-S protein 7; *NDUF8*: NADH:ubiquinone oxidoreductase Fe-S protein 8; *NDUFV1*: NADH:ubiquinone oxidoreductase core subunit v1; *NDUFV1*: NADH:ubiquinone oxidoreductase flavoprotein 1; *NDUFV2*: NADH:ubiquinone oxidoreductase flavoprotein 2; *NGS*: Next Generation Sequencing; *NUPB*: Nucleotide-binding protein-like; *NUP62*: Nucleoporin p62; *OXPHOS*: Oxidative Phosphorylation; *PDHA1*: Pyruvate dehydrogenase alpha-1; *PDHB*: Pyruvate dehydrogenase e1 beta subunit; *PDHC*: Pyruvate dehydrogenase complex; *PDHX*: Pyruvate dehydrogenase complex component x; *PDSS2*: Decaprenyl-diphosphate synthase subunit 2; *PEO*: Progressive External Ophthalmoplegia; *PET100*: Cytochrome c oxidase chaperone PET100; *PNPT1*: Polyribonucleotide nucleotidyltransferase 1; *POLG*: DNA polymerase gamma; *POLG2*: DNA polymerase gamma accessory subunit; *PolyPhen-2*: Polymorphism Phenotyping v2; *RANPB2*: RAN-binding protein 2; *RNASEH1*: Ribonuclease H1; *RNA-seq*: RNA sequencing; *RRM28*: Ribonucleotide reductase regulatory tp53 inducible subunit m2b; *rWES*: Rapid Whole Exome Sequencing; *rWGS*: Rapid Whole Genome Sequencing; *SCL25A4*: Solute carrier family 25 member 4; *SCO2*: SCO Cytochrome c oxidase assembly protein 2; *SDH*: Succinate dehydrogenase (complex II); *SDHA*: Succinate dehydrogenase complex subunit a; *SDHA1*: Succinate dehydrogenase flavoprotein subunit; *SDHB*: Succinate dehydrogenase complex subunit b; *SIFT*: Scale-Invariant Feature Transform; *SLC19A3*: Solute carrier family 19 member 3; *SLC25A4*: Solute carrier family 25 member 4; *SLC39A8*: Solute carrier family 39 member 8; *SPG7*: Matrix ATPases associated with diverse cellular activities (AAA) peptidase subunit, paraplegin; *SUCLA2*: Succinate-CoA ligase, ADP-forming, beta subunit; *SUCLG1*: Succinate-CoA ligase, alpha subunit; *SURF1*: Surf1 locus protein 1; *TACOT1*: Translational activator of cytochrome c oxidase I; *TALENS*: Transcription activator-like effector nucleases; *TCA*: Tricarboxylic Acid cycle; *TPK1*: Thiamine pyrophosphokinase 1; *TPP*: Thiamine pyrophosphate; *TRMU*: tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase; *TSM*: Translation elongation factor; *TTC19*: Tetrapeptide repeat domain/protein 19; *TWINK*: Twinkle mitochondrial DNA helicase; *UQCRCQ*: Ubiquinol-cytochrome c reductase complex III subunit VII; *WES*: Whole Exome Sequencing

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IV.2 Leigh Syndrome Spectrum: A Portuguese Population Cohort in an Evolutionary Genetic Era

Article

Leigh Syndrome Spectrum: A Portuguese Population Cohort in an Evolutionary Genetic Era

Manuela Schubert Baldo ¹, Célia Nogueira ^{1,2}, Cristina Pereira ^{1,2}, Patrícia Janeiro ³, Sara Ferreira ⁴, Charles M. Lourenço ⁵, Anabela Bandeira ⁶, Esmeralda Martins ^{6,7}, Marina Magalhães ⁸, Esmeralda Rodrigues ⁹, Helena Santos ¹⁰, Ana Cristina Ferreira ¹¹ and Laura Vilarinho ^{1,2,*}

- ¹ Research and Development Unit, Human Genetics Department, National Institute of Health Doutor Ricardo Jorge, 4000-055 Porto, Portugal; manuela.baldo@insa.min-saude.pt (M.S.B.)
- ² Neonatal Screening, Metabolism and Genetics Unit, Human Genetics Department, National Institute of Health Doutor Ricardo Jorge, 4000-055 Porto, Portugal
- ³ Inherited Metabolic Disease Reference Center, Lisbon North University Hospital Center (CHULN), EPE, 1649-028 Lisbon, Portugal
- ⁴ Inherited Metabolic Disease Reference Center, Pediatric Hospital, Hospital and University Center of Coimbra, 3004-561 Coimbra, Portugal
- ⁵ Neurogenetics Department, Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto 15090-000, Brazil
- ⁶ Oporto Hospital Centre, University of Porto, 4099-001 Porto, Portugal
- ⁷ Unit for Multidisciplinary Research in Biomedicine, Instituto de Ciências Biomédicas Abel Salazar, Porto University, 4050-313 Porto, Portugal
- ⁸ Department of Neurology Porto Hospital and University Centre, EPE, 4050-011 Porto, Portugal
- ⁹ Reference Center for Inherited Metabolic Disorders, University Hospital Centre S. João, 4200-319 Porto, Portugal
- ¹⁰ Department of Pediatrics, Hospital Centre, EPE, 4434-502 Vila Nova de Gaia, Portugal
- ¹¹ Department of Pediatrics, Hospital D. Estefânia, 1169-045 Lisbon, Portugal
- * Correspondence: laura.vilarinho@insa.min-saude.pt



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Abstract: Mitochondrial diseases are the most common inherited inborn error of metabolism resulting in deficient ATP generation, due to failure in homeostasis and proper bioenergetics. The most frequent mitochondrial disease manifestation in children is Leigh syndrome (LS), encompassing clinical, neuro-radiological, biochemical, and molecular features. It typically affects infants but occurs anytime in life. Considering recent updates, LS clinical presentation has been stretched, and is now named LS spectrum (LSS), including classical LS and Leigh-like presentations. Apart from clinical diagnosis challenges, the molecular characterization also progressed from Sanger techniques to NGS (next-generation sequencing), encompassing analysis of nuclear (nDNA) and mitochondrial DNA (mtDNA). This upgrade resumed steps and favored diagnosis. Hereby, our paper presents molecular and clinical data on a Portuguese cohort of 40 positive cases of LSS. A total of 28 patients presented mutation in mtDNA and 12 in nDNA, with novel mutations identified in a heterogeneous group of genes. The present results contribute to the better knowledge of the molecular basis of LS and expand the clinical spectrum associated with this syndrome.

Keywords: leigh syndrome; mitochondrial disorders; mutational spectrum; clinical spectrum; NGS

1. Introduction

Mitochondrial disorders (MD) are the most common group of inherited metabolic disorders. These disorders might result from mutations in mtDNA, nDNA, or even genome interplay malfunctioning. In pediatric populations, LS is the most frequent neurodegenerative MD, reaching up to 1:40,000 live births [1,2]. It encloses progressively fatal neurologic failure, high lactate levels, and characteristic symmetrical lesions in brain stem and basal ganglia [2,3]. The diagnostic criteria include clinical, neuro-radiological, biochemical, and molecular findings. The developments in molecular diagnosis enabled an easy recognizing of this pathology but

also expanded the phenotypical features, as well as the knowledge on the molecular basis of this syndrome. Since Holt et al. first reported m.8993T>G in 1990 [4], several mutations in *MT-ATP6* were subsequently published, also associated with LS, such as 9176T>C [5] and m.10191T>C [6]. The quick advance in nDNA sequencing provided helpful information since biochemical studies and mtDNA sequencing do not allow diagnosis confirmation in every cases. Fatal early-onset presentations, classically seen associated with complex I (CI) deficiency [7,8], were importantly explored through sequencing of structural mitochondrial respiratory chain (MRC) components coding genes. Additional clinical presentations that did not fulfill the clinical and/or radiological criteria for LS but strongly resembled the syndrome were named Leigh-like syndrome (LLS), considering that the evolution of symptoms could be sufficient to complete LS criteria in the future [1,2,9]. Since LLS is diagnosed in patients with nuclear-gene-related mutations, the condition is now denominated Leigh syndrome spectrum (LSS) [9], where LS and LLS are considered a probable continuum of the same disorder [9,10] (Table 1). The molecular advances through NGS and genomics made it possible to obtain huge amounts of information in a timely way, enabling an increase in diagnosis sensitivity, but bringing some uncertainties [11]. The plethora of information produced by these technologies demanded new data for comparison that is not widely available or extensively established. Furthermore, these techniques demand time and expertise to build robustness [12,13]. In mtDNA, for example, identifying a pathogenic variant may allow a condition to be named, but might fail to explain different phenotypes associated with a common underlying genetic lesion and intrafamilial phenotypic differences [14]. To address these questions, precision medicine was evolved through approaches known by “omics” (metabolomics, proteomics, and transcriptomics). The integrative medicine studies dive into deep microenvironments to solve these questions. Despite recent advances it is still too early to apply it in routine testing [15]. While precision medicine is not routine, molecular biology refinements are eventful in promoting faster diagnosis and supporting interventions and counseling to LSS patients.

Table 1. LSS clinical and radiological features [9].

Leigh Syndrome Spectrum (LSS)	
Clinical features *	Metabolic decompensation (elevated lactate levels in blood and/or cerebrospinal fluid) and/or characteristic symptoms during acute illness (episodic). Neurologic manifestations: hypotonia, spasticity, movement disorders (dystonia, chorea), cerebellar ataxia, and peripheral neuropathy. Other systems manifestations: hypertrophic cardiomyopathy, anemia, renal tubulopathy, liver involvement, ptosis, and muscle weakness.
Radiological features	Multiple symmetric bilateral lesions in the basal ganglia, thalamus, brain stem, dentate nuclei, and optic nerves **.

Legend: * Includes LLS features, such as: no evidence of abnormal energy metabolism, atypical neuropathology, and/or incomplete evaluation, to the classic LS criteria resulting in LSS. ** Unilateral lesions in brain stem, basal ganglia, spinal cord, cerebellum; variation in the distribution or character of lesions or with the additional presence of unusual features, such as extensive cortical destruction (neuronal loss).

Unfortunately, there are no curative options for MD yet. Supporting treatment is still the best and only option, and includes dietary management, cofactors administration, specific support of disabilities, and respiratory intervention. Clinical trials for treatments, such as mTOR inhibitors, EPI-743, are undergoing to evaluate its effectiveness in preventing mortality and morbidity, with promising results [16]. In this paper we present data from a cohort of 40 patients diagnosed with LSS highlighting the genetic heterogeneity identified in our cohort, contributing to expand the LSS.

2. Materials and Methods

Patients:

A cohort of 40 confirmed LSS individuals, from birth (0 months old (m.o.)) to 36 years, 20 males and 20 females, and respective positive family members, were followed in different clinical reference centers from Portugal. From this group, 11 had already

been reported by our group and the remaining 29 were molecularly characterized and reported here for the first time. This study was approved by the Ethics Committees of the National Institute of Health Doutor Ricardo Jorge, and informed consent for genetic studies was obtained from all investigated subjects, or their relatives, in agreement with the Declaration of Helsinki.

DNA extraction, sequencing, and variant analysis:

High-purity DNA was extracted from peripheral blood leucocytes and/or muscle biopsy material according to standard procedures. A custom-made targeted nuclear gene panel was designed using SureDesign software (Agilent 2023 7.10.1.1, Agilent Technologies, Santa Clara, CA, USA) and includes 209 nuclear genes, from which 188 were known to be involved in MD and 21 were candidates based on their role or participation in different pathways that include genes responsible for clinical presentations mimicking MD (Appendix A). Genes of interest were captured with a SureSelect QXT kit (Agilent Technologies), followed by sequencing on the Illumina MiSeq platform. Variant calling and annotation were performed using available commercial programs such as Surecall (Agilent Technologies) and wAnnoVar (wannovar.wglab.org/ (accessed on 23 January 2023)). Variants were filtered, taking into account (i) the type of pathogenic variant (missense, frameshift, stop-gain or stop-loss, and splice-site variants), (ii) in silico predictors (SIFT, PolyPhen-2, MutationTaster) [17–19] and presence in databases (dbSNP, 1000 Genomes, HGMD professional, ClinVar, ExAC, OMIM, gnomAD), and (iii) the population frequency (variants with a minor allele frequency (MAF) > 1% in the 1000 Genomes Project (<http://www.1000genomes.org> (accessed on 23 January 2023) and Exome Variant Server databases (<http://evs.gs.washington.edu> (accessed on 23 January 2023) were filtered out). Furthermore, we searched copy number variations through the analysis of coverage data, using cnMOPS [20]. The entire human mtDNA was enriched by a single amplicon, using back-to-back primers, by long-range PCR [21]. Indexed paired-end DNA libraries were prepared and an equimolar pool was sequenced, according to the manufacturer's instructions. FASTQ files were aligned to the mtDNA reference sequence with SeqMan NGen (DNASTar). Variant calling and annotation were performed using the following commercial programs: SeqMan NGen and SeqMan Pro (DNASTAR). Variants were filtered considering (i) the type of pathogenic variant, (ii) the population frequency, (iii) in silico predictors and presence in databases (Mitomap, MitImpact2, HmtVar), and (iv) heteroplasmy > 5%. All variants detected that had the potential to be disease-causing were confirmed by Sanger sequencing using the BigDye Terminator Cycle Sequencing Version 3.1 (Applied Biosystems, Foster City, CA, USA), and analyzed on an ABI 3130XL DNA Analyzer. When DNA from additional family members was available, co-segregation studies were also performed [22].

3. Results

3.1. Mitochondrial DNA Mutations

A total of 28 out of 40 patients, 14 males and 14 female patients, presented mutations in mtDNA. The *MT-ATP6* gene was the most frequent spot of mutations identified in our cohort, corresponding to 48% of the patients. The previously known m.8993T>G (p.Leu156Arg) and m.8993T>C (p.Leu156Pro) are present in 16 patients: 12 patients T>G and four patients T>C (P1 to P16). P7's mother presented the same mutation m.8993T>G and same heteroplasmy of her child, but exhibiting the neuropathy, ataxia, and retinitis pigmentosa (NARP) phenotype. Two other mutations were identified in the *MT-ATP6* gene: m.9185T>C (p.Leu220Pro) in two patients (P17 and P18) and the m.9176T>G (p.Leu217Arg) in P19, who had low citrulline levels identified on neonatal screening and confirmed on plasma amino acid analysis by ion exchange chromatography. Other, less frequent mutations were identified in our cohort in other mtDNA genes associated with LSS were m.6547T>C (p.Leu215Pro), m.4142G>T (p.Arg279Leu), m.3243A>G, m.10197G>A (p.Ala47Ile), m.10191T>C (p.Ser45Pro), m.13094T>C (p.Val253Ala), m.13513G>A (p.Asp393Asn), and a single-large-scale deletion of 4977bp (Table 2). In *MT-TL1*, the m.3243A>G mutation was identified in P20. The family, namely, mother and two siblings, underwent investigation and all of them harbored

the m.3243A>G mutation, but no symptoms were reported at the time. Two other patients had mutations in *MT-ND3*: P21 had m.10191T>C (p.Ser45Pro) mutation, and in P22 we found m.10197G>A (p.Ala47Pro) in nearly homoplasmic state. In *MT-ND5* we identified mutations in three patients: P23 was positive for m.13094T>C (p.Val253Ala), and P24 and P25 with m.13513G>A (p.Asp393Asn). In *MT-ND1*, we identified the mutation m.4142G>T (p.Arg279Leu) in P26 and *MT-CO1* P27 was found to have m.6547T>C (p.Leu215Pro). P28 was diagnosed in the first decade of life through a Southern-blot analysis attesting a single-large-scale deletion of 4977 bp (m.8470-m.13447) in 61% of heteroplasmy in muscle, the most common deletion in the mtDNA (Table 2).

Table 2. Results of investigation of LSS patients' mtDNA in this cohort.

Patient	Gender	Age of Diagnosis	Gene	Mutation Data			Reference	Symptoms
				Variant	Heteroplasmy	Status (Mitomap) [22]		
P1	F	7 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Muscle: 95%	P	Holt, 1990 [4]	Psychomotor regression, loss of contact, lethargy, generalized seizures, dystonia.
P2	F	2 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Muscle: homoplasmic	P	Holt, 1990 [4]	Hypotonia, progression to apnea after febrile illness, dystonia.
P3	F	8 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Blood: homoplasmic	P	Holt, 1990 [4]	Intellectual impairment, spastic paraparesis, hyperlactacidemia.
P4	F	9 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Blood: 95%	P	Holt, 1990 [4]	Mitochondrial encephalopathy.
P5	M	1 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Muscle: 95%	P	Holt, 1990 [4]	Hypotonia, myoclonic epilepsy, regression of milestones.
P6	F	29 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Blood and muscle: >95%	P	Holt, 1990 [4]	LSS
P7	F	16 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Muscle: 75%	P	Holt, 1990 [4]	LSS
P8	M	6 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Muscle: 85%	P	Holt, 1990 [4]	LSS
P9	M	1 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Blood and muscle: homoplasmic	P	Holt, 1990 [4]	LSS
P10	F	1 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Muscle: 90%	P	Holt, 1990 [4]	Neurodevelopmental delay, hypotonia, epilepsy.
P11	F	19 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Muscle: 95%	P	Holt, 1990 [4]	Deafness, peripheral neuropathy, ataxia.
P12	F	2 y	MT-ATP6	m.8993T>G (p.Leu156Arg)	Muscle: 98%	P	Holt, 1990 [4]	Failure to thrive, eosinophilic esophagitis.
P13	F	23 y	MT-ATP6	m.8993T>C (p.Leu156Pro)	Muscle: >95%	P	De Vries, 1993 [23]	LSS
P14	M	8 y	MT-ATP6	m.8993T>C (p.Leu156Pro)	Muscle: >95%	P	De Vries, 1993 [23]	LSS
P15	F	10 y	MT-ATP6	m.8993T>C (p.Leu156Pro)	Blood and muscle: >90%	P	De Vries, 1993 [23]	Numbness, ataxia.
P16	M	8 y	MT-ATP6	m.8993T>C (p.Leu156Pro)	Muscle: 98%	P	De Vries, 1993 [23]	Mitochondrial disorder.
P17	F	1 y	MT-ATP6	m.9185T>C (p.Leu220Pro)	Muscle: 70%	P	Moslemi, 2005 [24]	Neurodevelopmental delay, microcephaly and hypotonia.
P18	M	32 y	MT-ATP7	m.9185T>C (p.Leu220Pro)	Blood: homoplasmic	P	Moslemi, 2005 [24]	LSS
P19	M	0 m.o.	MT-ATP6	m.9176T>G (p.Leu217Arg)	Blood: homoplasmic	P	Thyagarajan, 1995 [25]	Still asymptomatic (studied to clarify newborn screening alterations).
P20	M	10 y	MT-TL1	m.3243A>G	Muscle: 73%	P	Poulton, 1988 [26]	Epilepsy, learning disability, ataxia, hyperlactacidemia.
P21	F	4 y	MT-ND3	m.10191T>C (p.Ser45Pro)	Blood: 80%, muscle: 90%	P	Taylor, 2001 [6]	Hypotonia, neurodevelopmental delay, recurrent respiratory infections in first months of life, epilepsy.

Table 2. Cont.

Patient	Gender	Age of Diagnosis	Gene	Mutation Data			Reference	Symptoms
				Variant	Heteroplasmy	Status (Mitomap) [22]		
P22	M	7 y	MT-ND3	m.10197G>A (p.Ala47Ile)	Muscle: 100%	P	Kirby, 2004 [27]	Epilepsy, failure to thrive, recurrent illness, hypotonia, high serum lactate levels.
P23	M	11 y	MT-ND5	m.13094T>C (p.Val253Ala)	Blood: 100%	P	Valente, 2009 [28]	Severe hypotonia.
P24	F	8 m.o.	MT-ND5	m.13513G>A (p.Asp393Asn)	Blood: 70%	P	Chol, 2003 [29]	Neurodevelopmental delay, cardiomyopathy, hypotonia.
P25	M	7 y	MT-ND5	m.13513G>A (p.Asp393Asn)	Blood: 58%, muscle: 88%	P	Chol, 2003 [29]	Ataxia and apnea.
P26	M	12 y	MT-ND1	m.4142G>T (p.Arg279Leu)	Muscle: 80%	R	Pereira, 2019 [30]	Spastic deambulation with cerebellar signs, hyperreflexia, clonus, dysarthria, dystonia, cognitive impairment.
P27	M	13 y	MT-CO1	m.6547T>C (p.Leu215Pro)	Muscle: 50%	R	Pereira, 2019 [30]	Hypotonia, hyperlactacidemia.
P28	M	7 y	MT-ATP6 to MT-ND5	4977bp-del (m.8470-m.13447)	Muscle: 61%	P	Vilarinho, 1997 [31]	Epilepsy, short stature, cerebellar ataxia, tremor.

Legend: LSS: Leigh syndrome spectrum; y: year; F: female; M: male P: confirmedly pathogenic; R: reported; m.o.: months old.

3.2. Nuclear DNA Mutations

A targeted 209 nuclear-gene panel allowed molecular characterization of 12 of the 40 patients, five males and seven females, which presented mutations in *SLC19A3*, *NDUFV2*, *NDUFS8*, *NDUFS1*, *POLG*, *SURF1*, *LRPPRC*, and *HIBCH* (Table 3). In nuclear genes associated with CI, we found mutations in four patients: P29 presented in compound heterozygosity in *NDUFS8*, the previously reported mutations c.196C>T (p.Arg66*) and c.287G>A (p.Arg96His); in P30 we identified a compound heterozygous for *NDUFS1* c.470A>T (p.Lys157Met) and c.1798G>C (p.Glu600Gln), which are not reported in databases. Segregations studies confirmed inheritance, and these variants are class IV according to the American College of Medical Genetics (ACMG) scoring system classification [32]. P31 was homozygous for *NDUFV2* splicing mutation c.120+5_120+8delGT, previously described in the literature, which was also identified by prenatal test in her sibling (P32) [22]. In nuclear genes of CIV, we identified mutations in four patients: P33, P34, P35, and P36. P33 and P34 are siblings with different clinical symptoms, and the investigation by NGS retrieved c.19_35dup17* (p.Ala13Cysfs*65)/c.845_846del (p.Ser282Cysfs*9) mutations in *SURF1*, while P35 had isolated psychiatric symptoms and also presented the mutation c.19_35dup17* (p.Ala13Cysfs*65), in homozygous condition, in *SURF1*. P36 was also studied by NGS and was revealed to be homozygous c.74G>A (p.Arg25His) in *LRPPRC*, previously unreported in the literature, which is a class III according to ACMG scoring system classification [32]. Despite fitting into the phenotype and confirmative segregations studies, it remains as VUS, as we will discuss. Co-factors such as thiamine are also related to LSS. We had two patients investigated in our cohort manifesting symptoms of biotin-responsive basal ganglia disease (BBGD), P37, and P38, which presented homozygous mutations c.74dupT (p.Ser26Leufs*19) and c.980-14A>G, respectively, in *SLC19A3* [33]. The patients P39 and P40 had abnormal metabolic work-up with increased dried blood spot propionylcarnitine (C3) in the acylcarnitine profile (by tandem mass spectrometry MS/MS) and increased erythro-2-methyl-2,3-dihydroxybutyric acid and 3-methyl-glutaconic acid in urinary organic acids profile by gas chromatography/mass spectrometry (GC/MS). The NGS gene panel identified in P39 homozygous mutation c.488G>T (p.Cys 163Phe) and in P40 c.129dupA (p.Gly44Argfs*20)/c.910C>T (p.Pro304Ser) in *HIBCH*, coherent to an organic aciduria due to a disturbance in valine catabolic pathway to be further discussed [34,35]. All identified nuclear mutations in this cohort are shown in Figure 1.

Table 3. Results of investigation of LSS patients' nDNA in this cohort.

Patient	Gender	Age of Diagnosis	Gene	Mutation Data					Symptoms			
				Allele 1	Reference	ClinVar	ClinVar Phenotype	Allele 2		Reference	ClinVar	ClinVar Phenotype
P29	M	36 y	NDUF58	c.196C>T (p. Arg66*)	Nogueira, 2019 [22]	ND	ND	c.287G>A (p. Arg96 His)	Nogueira, 2019 [22]	ND	ND	Short stature, Leigh syndrome compatible neuroimaging.
P30	M	2 y	NDUF51	c.470A>T (p. Lys157 Met)	ND	ND	ND	c.1798G>C (p. Glu600 Gln)	ND	ND	ND	Axial hypotonia, failure to thrive, neurodevelopmental delay, nystagmus.
P31	F	2 y	NDUFV2	c.120+5_120+8 delGT	Bénit, 2003 [36]	P	Mitochondrial Complex I deficiency	c.120+5_120+8 delGT	Bénit, 2003 [36]	P	Mitochondrial Complex I deficiency	Severe eczema, epilepsy, axial hypotonia, neurodevelopmental delay, suspicion of Leigh syndrome.
P32	M	prenatal		c.120+5_120+8 delGT	Bénit, 2003 [36]	P	Mitochondrial Complex I deficiency	c.120+5_120+8 delGT	Bénit, 2003 [36]	P	Mitochondrial Complex I deficiency	Prenatal screening, P31 sibling.
P33	F	3 y	SURF1	c.19_35dup17* (p. Ala13 Cysfs*65)	Tiranti, 1998 [37]	P	LSS	c.845_846 del (p. Ser282 Cysfs*9)	Tiranti, 1998 [37]	P/LP	LSS	Ataxia, hypotonia, tremors, Tetralogy of Fallot.
P34	F	1 y		c.19_35dup17* (p. Ala13 Cysfs*65)	Tiranti, 1998 [37]	P	LSS	c.845_846 del (p. Ser282 Cysfs*9)	Tiranti, 1998 [37]	P/LP	LSS	Neurodevelopmental delay, hypotonia, bradycardia.
P35	M	15 y		c.19_35dup17* (p. Ala13 Cysfs*65)	Tiranti, 1998 [37]	P	LSS	c.19_35dup17* (p. Ala13 Cysfs*65)	Tiranti, 1998 [37]	P	LSS	Visual hallucinations.
P36	F	4 y	LRPPRC	c.74G>A (p. Arg25 His)	ND	VUS	Congenit lactic acidosis	c.74G>A (p. Arg25 His)	ND	VUS	Congenit lactic acidosis	Encephalopathy, brain atrophy.
P37	F	1y	SLC19A3	c.74dupT (p. Ser26 Leufs*19)	Debs, 2010 [33]	P	BBGD	c.74dupT (p. Ser26 Leufs*19)	Debs, 2010 [33]	P	BBGD	Neonatal epileptic encephalopathy responsive to biotin, thiamine, Coenzyme Q10 and riboflavin.
P38	F	5y		c.980-14A>G	Debs, 2010 [33]	LP	BBGD	c.177G>A (p. Trp59*)	Debs, 2010 [33]	ND	ND	Episodic ataxia responsive to biotin, thiamine, Coenzyme Q10 and riboflavin.
P39	M	3 m.o.	HIBCH	c.488G>T (p. Cys 163Phe)	Wojcik, 2019 [38]	LP	HIBCH deficiency	c.488G>T (p. Cys 163Phe)	Wojcik, 2019 [38]	LP	HIBCH deficiency	Stop of progression of the neurodevelopment and feeding difficulties.
P40	F	6 y		c.129dupA (p. Gly44 Argfs*20)	Peters, 2015 [35]	LP	HIBCH deficiency	c.910C>T (p. Pro304 Ser)	ND	ND	ND	Axial hypotonia, psychomotor regression after intercurrent illness, ataxia.

Legend: ND: not described; P: pathogenic; LP: likely pathogenic; m.o.: months old.

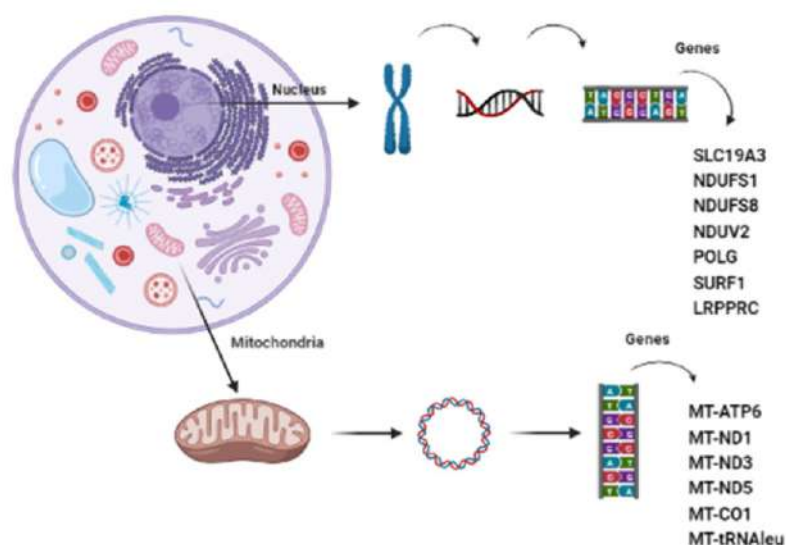


Figure 1. Mitochondrial and nuclear genes with mutations in our cohort, implicated in LSS.

4. Discussion

The first group of patients in our cohort were molecularly characterized using targeted molecular approaches, the only available in previous decades, as PCR/RFLP and sanger sequencing of selected gene regions. The complete mtDNA sequencing by NGS was an important achievement that enabled the analysis of the total mtDNA genes, increasing the detection power and allowing the identification of several other mutations already linked to other phenotypes that are now part of the LSS. A total of 48% of the patients from our cohort presented mutations in the *MT-ATP6* gene, which is higher than could be expected based on published results [39]. This result could be the reflection of a particular molecular background of Portuguese LSS patients, or a bias associated with the studied cohort. Most index cases had m.8993T>G/C with mutation loads over 95% in muscle and/or blood and recurrent symptoms such as hypotonia, weakness, neurodevelopmental delay or regression, epilepsy, movement disorders, and deafness. The discovery of variable phenotypes of m.8993T>G/C was important to distinguish LSS from and NARP [40], for which the heteroplasmic rates showed that LSS classically display higher than 85% and NARP up to 85%. An additional explanation for the differences in clinical expression is the highly conserved position in *MT-ATP6* resulting in less tolerance to T>G rather than T>C in 8993-point mutations due to the amino acid change, as T>C results in maintaining nonpolar amino acids while the T > G results in substitution of nonpolar amino acid for a charged one, which could produce an important destabilization in the catalytic site of the ATP synthase where this mutation occurs [4,16,41]. In family members of our cohort, we identified two mothers with 75% of heteroplasmy in m.8993T>G: one yet asymptomatic, the other exhibiting NARP syndrome. P7's mother with NARP displayed the same heteroplasmic rates, but this was calculated in DNA extracted from blood, while P7's heteroplasmy was accessed in muscle tissue, making the correlation difficult to perform. Our hypothesis for the observed clinical presentations is based not only on the overall different mutation load but also on the distribution of mutated mtDNA by the different tissues. Plasma amino acids profiles can exhibit changes that point to these mutations, namely, low citrulline levels [42,43]. Even at newborn screening (and after excluding urea cycle disorders), low citrulline is suggestive of *MT-ATP6* mutations 8993T>G/C and m.9176T>C/G [44]. A recent paper [45] tried to correlate these mutations to an

assertive metabolic profile phenotype, such as the one we found in P19, with low citrulline levels at newborn screening and m.9176T>G (p.Leu217Arg) in homoplasmies [45]. The study's results showed that reduced citrulline levels and increased propionylcarnitine (C3) and 3-hydroxy-isovaleryl/2-methyl-butyrylcarnitine (C5-OH) might be associated with *MT-ATP6* mutations [45,46]. P17 was investigated due to psychomotor delay, hypotonia, and microcephaly and harbors m.9185T>C (p.Leu220Pro) in 70% of mtDNA molecules while P18 had an LSS and harbors the same mutation in homoplasmies. This mutation is particularly relevant in LSS presenting with ataxia and late-onset cases where ataxia is the core symptom [47,48], albeit fulminant presentation being known as an early-onset LSS with infantile spasms with this mutation [49].

In the tRNA^{Leu} *MT-TL1*, the MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) mutation m.3243A>G has been also identified in different phenotypes such as LSS, maternally inherited deafness and diabetes (MIDD), and isolated myopathy [50–52]. In our cohort, P20 presented m.3243A>G and epilepsy, ataxia, hyperlactacidemia, and learning disabilities, with imaging studies characteristics of LSS, an expected phenotype to this mutation [31]. The m.3243A>G usually has a variable heteroplasmy percentage in different tissues and does not respect the usual threshold for MD, as mentioned by Chinnery et al. and by Pann et al. [50,53]. Another mutated gene associated with LSS is *MT-ND3*, where we also found mutations in our cohort. The m.10191T>C (p.Ser45Pro) may manifest as a remarkable CI deficiency, inducing an early neonatal fulminant phenotype [54] or milder cases of encephalopathy, such as P21 [55], and small fiber neuropathy [56], but configuring dystonia and dysarthria as main symptoms [57]. It can also be associated with a slowly clinical progression from infancy to adulthood [58], leading to the association of mtDNA mutations to “spectrum” nomenclature, which was only acknowledged to nDNA mutations [9]. P22 with the mutation m.10197G>A (p.Ala47Thr) in *MT-ND3* also presented a mild phenotype, similar to P21. Mutations in the *MT-ND5* gene were also identified in our cohort. We found m.13094T>C (p.Val253Ala) in homoplasmies in P23, a mutation previously described associated with cerebellar syndromes [59], CI deficiency, or combined complexes deficiencies [60], presenting our patient a marked hypotonia. In pediatric populations, LSS appears to represent a less favorable prognosis [61]. P24 and P25 presented m.13513G>A (p.Asp393Asn), a mutation that has already been outlined by Ruiter et al. in a patient with CI deficiency, cardiac rhythm perturbation, and optic atrophy [62], with our patients presenting cardiomyopathy and hypotonia, and ataxia and apnea, respectively. Our cohort also includes a patient with m.4142G>T (p.Arg279Leu) in *MT-ND1* (P26 [30]) and another one with m.6547T>C in *MT-CO1* (P27 [30]).

Another group of mutations in mtDNA are the rearrangements, such as single-large-scale mtDNA deletions (LMD), a genetic cause of MD expressing a wide range of clinical features. The rearrangements result into a spectrum of syndromes: Pearson, Kearns Sayre Syndrome (KSS), a subtype of chronic progressive external ophthalmoplegia (CPEO), and CPEO itself [63,64]. P28 was investigated due to epilepsy, short stature, cerebellar ataxia, and tremor, and Southern-blot analysis detected an LMD of 4977bp-del (m.8470-m.13447). This association is extremely rare because the early-onset spectrum of LMD, Pearson syndrome, is usually a potentially fatal disorder disabling the progression of the spectrum to other clinical entities, namely, LSS, which makes the association rare [65,66]. Despite the prognosis, the patients who survive may develop LSS or a multiorgan deficiency in the future [63].

The patients of our cohort identified with nDNA mutations were all screened by NGS, which represents a satisfactory middle-term cost-utility approach, providing a significant amount of data in a reasonable time, delivering a broader diagnostic performance when set side by side to conventional procedures. The complexes are the major structural components of the MRC resulting in mitochondrial optimized ATP generation [57]. As we observed in our cohort, the CI and CIV most frequently harbor pathogenic mutations, followed by non-OXPHOS defects. In total, in nDNA we identified ten pathogenic mutations, three likely-pathogenic variants, one reclassified in our perspective through ACMG,

symptomatology, and biochemical analysis, and one VUS that did not meet criteria to be reclassified.

The CI is the first and largest MRC complex [67] and is widely associated with LSS, with several genes involved [3,57], resulting in 33 subtypes of mitochondrial CI deficiency (MCID), from first year of life rapidly progressing to fatal course [68,69] to a wide range of presentations [70–72]. P30 presented the c.470A>T (p.Lys157Met)/c.1798G>C (p.Glu600Gln) variants in *NDUFS1*, which are not reported in databases so far. Standard classification scores by ACMG resulted in class IV, likely pathogenic, for both variants. In silico predictors such as Polyphen-2 and SIFT described them as deleterious, and Mutation Taster as probably damaging. Segregation studies showed heterozygous parents. The clinical manifestations are described in LSS and CI deficiency, altogether supporting a probable pathogenicity. P31 displayed, with homozygous mutation, c.120+5_120+8delGT in *NDUFV2*, a classic *NDUFV2* phenotype represented by hypertrophic cardiomyopathy and encephalopathy [73,74], alongside with a rare skin condition. In less frequent cases, *NDUFV2* mutations manifest as LSS, which was reported by Liu et al. in two families [75].

The CIV is also a critical part of the MRC as the terminal catalytic site to transfer electrons into oxygen [76]. *SURF1* is a gene that plays part in the assembly of CIV, while *LRPPRC* impacts the mitochondrial translation [77]. We identified P35 with *SURF1* mutations displaying atypical presentation with isolated psychiatric symptoms represented by visual hallucinations with suggestive LSS neuroimaging. Molecular assessment retrieved homozygous c.19_35dup17* (p.Ala13Cysfs*65) in *SURF1*, a gene typically related to a group of homogeneous symptoms (hypertrichosis [78] and peripheral neuropathy), specific imaging findings (leukodystrophy), and high mortality in childhood [79]. However, heterogeneous phenotypes linked to *SURF1* mutations have been reported [80]. P36 was investigated for encephalopathy and brain atrophy. NGS revealed the homozygous mutation c.74G>A (p.Arg25His) in *LRPPRC* gene, a rare variant in population, classified as ACMG class III. Segregation studies were performed, confirming inheritance in our patient. In silico predictors are controversial; Polyphen-2 and SIFT pointing to deleterious role, and Mutation Taster to benign role, while ClinVar classifies it as a VUS associated with congenital lactic acidosis Saguenay-Lac-Saint-Jean, a subtype of LSS [81]. This feature gives us a likely connection between the genotype and the patient's phenotype, with this variant having the potential to be, in the future, reclassified as likely pathogenic.

Thiamine pyrophosphate is an important cofactor of several steps in energy production and *SLC19A3*, a biotin-dependent thiamine transporter in the central nervous system, is fundamentally present in the basal ganglia system [82]. BBGD is a well-known cause of LSS [83] frequently manifesting in childhood with vomiting, seizures, encephalopathy, movement disorders, and free-thiamine dosage in cerebrospinal fluid typically low to absent [84,85]. P37 and P38 investigated in our cohort had mutations in *SLC19A3*. P37 developed neonatal epileptic encephalopathy with homozygous c.980-14A>G in *SLC19A3* and P38 displayed episodic ataxia with homozygous c.74dupT (p.Ser26Leufs*19). Both patients are under treatment with biotin and thiamine and had important recovery of symptoms, which demonstrates the importance of recognizing the phenotype and starting effective intervention [82,83].

Organic acidurias are also a proven cause of LSS. In the case of *ECHS1* and *HIBCH* genes, encoding a hydratase and a hydrolase enzyme, respectively, in the final valine metabolism, LSS is the most common clinical manifestation of these genetic deficiencies [86–88]. Frequent symptoms identified in these cases are developmental delay or regression, hypotonia, and epilepsy [89]. A recent Spanish study demonstrated that *HIBCH* deficiency was completely correlated to LSS, and *ECHS1* deficiency displayed LSS, but also fatal neonatal lactic acidosis and paroxysmal dystonia. The biochemical profile was divergent between them, which could guide the diagnosis [90]. P39 was confirmed with *HIBCH* deficiency, being homozygous for c.488G>T (p.Cys 163Phe). P40 is a compound heterozygous for a known pathogenic mutation in *HIBCH* c.129dupA (p.Gly44Argfs*20) alongside an unreported variant c.910C>T (p.Pro304Ser). The new variant meets ACMG criteria as class III. In silico estimates

in Polyphen-2, SIFT, Mutation Taster suggest a deleterious behavior. Also, in this case, the positive segregation pattern, clinical manifestations expressed as LSS and biochemical analysis of acylcarnitine profile displaying elevation of 3-hydroxyisobutyryl carnitine, and urinary organic acids showing intermediate metabolites of valine degradation were associated with a reported pathogenic variant we would consider to be a likely pathogenic variant, ACMG Class IV, under this information.

5. Conclusions

The present work presents data on a Portuguese cohort of 40 LS patients and allows the expansion of the molecular knowledge on this syndrome and the clinical spectrum associated with it. The present data stress the high clinical and genetic heterogeneity associated with this syndrome. The evolution of the molecular technics from PCR/RFLP to sanger sequencing and finally NGS was a path leading to an unprecedented increase in sensitivity in the diagnosis of LS. The adoption by our lab of NGS allowed the clarification of unsolved cases for many years, being useful in revealing the molecular etiology in children and adult patients by identifying new variants, characterizing more cases, and expanding the LSS scenery of mutations, since rare and undescribed variants were pivotal for our cases; albeit this is only the beginning.

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Appendix A

Nuclear Gene Panel for Mitochondrial Disorders

AARS2; ABCB7; ACAD9; ACO2; ADCK3; AGK; AIFM1; ALAS2; ALG13; APTX; ATP13A2; 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; CTSF; CWF19L1; CYC1; DARS2; DGUOK; DLAT; DLD; DNA2; DNAJC19; DNAJC5; DNM1L; DOLK; EARS2; ECHS1; ELAC2; ETF A; ETFB; ETFDH; ETHE1; FARS2; FASTKD2; FBXL4; FDX1L; FLAD1; FOXRED1; GDAP1; GFER; GFM1; GLRX5; GRN; 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; MPL; 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;

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IV.3 A comprehensive approach to the diagnosis of Leigh Syndrome Spectrum

Guidelines

A Comprehensive Approach to the Diagnosis of Leigh Syndrome Spectrum

Manuela Schubert Baldo ^{1,2,*}, Luísa Azevedo ^{3,4}, Margarida Paiva Coelho ^{2,3,5}, Esmeralda Martins ^{2,3,5} and Laura Vilarinho ^{1,6,*}

¹ Research and Development Unit, Human Genetics Department, National Institute of Health Doutor Ricardo Jorge, 4000-053 Porto, Portugal

² School of Medicine and Biomedical Sciences (ICBAS), University of Porto, 4050-313 Porto, Portugal; mmargaridacoelho.dca@chporto.min-saude.pt (M.P.C.); esmeralda.dia@chporto.min-saude.pt (E.M.)

³ UMIB—Unit for Multidisciplinary Research in Biomedicine, School of Medicine and Biomedical Sciences (ICBAS), University of Porto, 4050-346 Porto, Portugal; lazevedo@icbas.up.pt

⁴ ITR—Laboratory for Integrative and Translational Research in Population Health, 4050-600 Porto, Portugal

⁵ Pediatrics Department, Northern Mother and Child Centre, Reference Centre for Metabolic Disorders, Santo António Hospital University Centre, 4050-651 Porto, Portugal

⁶ Neonatal Screening, Metabolism and Genetics Unit, Human Genetics Department, National Institute of Health Doutor Ricardo Jorge, 4000-053 Porto, Portugal

* Correspondence: schubert.neuroped@gmail.com (M.S.B.); laura.vilarinho@insa.min-saude.pt (L.V.)

Abstract: Background: Leigh syndrome spectrum (LSS) is a novel nomenclature that encompasses both classical Leigh syndrome and Leigh-like phenotypes. Given the heterogeneity of disease presentation, a new consensus published recently addressed the main issues and proposed general guidelines towards diagnosis. Based on these recommendations, we developed a simple pipeline that can be useful in the diagnosis of LSS. Methods: We combined previously published criteria with our own experience to achieve a diagnostic framework that can provide faster satisfactory results with fewer resources. Results: We suggest adding basic biochemical tests for amino acids, acylcarnitine, and urinary organic acids as parallel investigations, as these results can be obtained in a short time. This approach characterized 80% of our cohort and promoted specific intervention in 10% of confirmed cases. Conclusions: Genetic studies are crucial in the diagnosis of LSS, but they are time-consuming and might delay tailored interventions. Therefore, we suggest adding more affordable and less complex biochemical studies as primary tests when investigating treatable causes of LSS.

Keywords: Leigh syndrome spectrum; diagnosis; mitochondrial disorder; neurodegeneration



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1. Introduction

Leigh syndrome (LS) is the most frequent mitochondrial neurodegenerative disorder in childhood, affecting approximately 1:40,000 live births [1–3] with predominantly neurologic involvement, such as neurodevelopmental delay, regression, or psychiatric symptoms [4–6]. The first case was described by Denis Archibald Leigh in 1951 and revealed a constellation of isolated neurological findings in the autopsy of a 7-month-old boy [7]. Currently, the term “spectrum” is a new designation merging Leigh and Leigh-like syndrome that highlights the disease as a continuum and includes suspicious cases that exhibit atypical presentations in the Leigh syndrome “universe” [5,8]. Symptoms primarily result from the dysfunction of the central nervous system and manifest as epilepsy, motor and/or speech delay, hypotonia, apnea, pyramidal signs, and movement disorders [4]. These clinical manifestations correspond to neuroimaging features of bilateral symmetric basal ganglia involvement as well as brainstem and spinal cord regions. Typically, the first symptoms begin in the first years of life, but there is an increasingly number of cases with adulthood

presentation [9]. Atypical and/or mild presentations have been observed in adulthood mimicking Parkinson disease, spinocerebellar ataxia, and multiple sclerosis [10].

An intact oxidative phosphorylation (OXPHOS) system is essential to the aerobic metabolism. When hampered, glycolytic products such as lactate (L) and/or pyruvate (P) are abnormally high, demonstrating an ineffective aerobic metabolism. Lactate is an intermediate compound of energy production, considered a noble part in the redox signaling state and a regulator of homeostasis, representing a relevant source of energy [11]. Lactate and pyruvate can accumulate in body fluids such as blood, urine, and cerebral spinal fluid (CSF). Double lactate peaks or a pyruvate peak in magnetic resonance spectroscopy (MRS) in correspondent topography for LSS is valued as biochemically supportive of the diagnosis [5]. Because pyruvate has a variable range of sensitivity and a strong specificity threshold, it is usually considered in association with lactate, using the ratio between them (an L/P ratio higher than 20) as a more specific biomarker for mitochondrial disorders (MDs) [12]. High alanine can also reflect a glycolytic metabolism through the conversion of lactate to alanine; therefore, it is an important marker in suspected MDs. OXPHOS functional studies (OXPHOS complex activities), usually performed in muscle biopsies, are closest to mitochondrial metabolism, as they are considered to “mirror” the patient’s metabolic condition [13]. Despite being highly recommended in clinical practice, deep tissue biopsy requires invasive procedures and anesthesia, adding risks for patients.

LSS may arise either from mitochondrial DNA (mtDNA) or nuclear DNA (nDNA) mutations via autosomal dominant, autosomal recessive, X-linked, or maternal inheritance. Identification of the inheritance mode is crucial for genetic counseling. *MT-ATPase6* m.8993T>G/C stands out as a frequent pathological variant in mtDNA marked by hypocitrullinemia in most cases, while in nuclear genome human surfeit 1 (*SURF1*), leucine rich pentatricopeptide repeat containing (*LRPPRC*) complex IV (COX) and complex I (CI) deficiencies are more frequently seen [3]. However, the absence of a straightforward genotype–phenotype relationship is a characteristic of many genetic diseases, such as LSS, and arises largely due to incomplete penetrance and variable expressivity [14,15]. The phenotype of mtDNA carriers linked to LSS is variable, which indicates that the associated genetic background and/or environmental variables can alter the expression and penetrance of disease-causing variants. Their interactions might result in a multitude of clinical presentations with varying degrees of severity, making it difficult to predict the outcome associated with a specific pathological variant [16–18].

LSS criteria were recently revisited by the Clinical Genome Resource (ClinGen) Mitochondrial Disease Gene Curation Expert Panel (Mito GCEP) [5]. A patient displaying a combination of neurologic symptoms and compatible neuroradiologic criteria supported by biochemical findings most likely has LSS. Neuroimaging criteria include bilateral, typically symmetric, T2-weighted hyperintensities on MRIs or hypodensities on CT scans of the brainstem and/or basal ganglia with or without bilateral T2 hyperintensity on MRIs or hypodensity on CT scans of the thalamus, cerebellum, subcortical white matter, and/or spinal cord [5]. The observation of neurological symptoms, such as delay, regression, or psychiatric symptoms, obtained with predefined biochemical tests and neuroimaging studies might not result in a definitive diagnosis. Current diagnostic strategies include mandatory genetic confirmation [6,8,19,20].

In high-income countries, whole exome sequencing (WES) is often a readily available first line test. However, in other economies, technical and financial constraints interfere with following such guidelines, turning the diagnostic process into an odyssey for patients and their families. Moreover, not all cases are solved through genetic studies, and inconclusive results are more frequent than desired.

We propose a cost- and time-effective pipeline based on Mito GCEP work [5] and our own experience, prioritizing frequent and treatable causes, which can be adjusted to clinical practices of different realities.

2. Materials and Methods

We revisited a cohort of 50 LSS-suspected individuals (23 males and 27 females) investigated at our center during the last three decades. These patients were studied through a sequential gene-targeted approach for the most frequent mtDNA and nDNA mutations in diagnosed LSS cases [21,22]. We identified practical cases in which a more comprehensive initial approach than that proposed by the Mito GCEP would benefit a timelier diagnosis and phenotype–genotype correlation, enabling adaption to each service. Other causes of LSS with easily obtainable biochemical markers identified through a literature review from other groups were also included. Neuroimaging patterns and criteria were previously defined [5] and are outside the focus of this work.

3. Results

Considering our expertise and the literature reviewed, we propose a diagnostic protocol for the diagnosis of LSS, focusing on treatable causes and shortening diagnostic time. Primarily based on the Mito GCEP’s recommendations, our approach can be adapted to distinct economies, as shown in Figure 1.

From a clinical perspective, the phenotype is still a relevant factor and can suggest a molecular diagnosis, such as *NDUFV2* mutations identified in two siblings of our cohort displaying encephalopathy and cardiomyopathy. However, MDs are such a heterogenic group of disorders that they require the more robust support given by the following steps of investigation [21,22]. To support our suggestions, we revisited data previously collected through phenotype characterization and basic biochemical studies leading to a genetic diagnosis in a few of our cases.

MT-ATPase6 mutations, especially m.8993T>G/C, were the most frequently found variants (16/50). *MT-ATPase6* m.8993T>G/C and m.9176T>G/C impact complex V (CV) and result in low to absent citrulline levels in approximately 80% of patients [23–26]. Amino acid studies are also helpful in distinguishing MDs from pyruvate dehydrogenase deficiency (PDHD), a known cause of LSS in which branched chain amino acids follow plasma alanine levels. According to the Nijmegen mitochondrial disease criteria scoring system, alanine levels above 450 µM are a highly sensitive parameter for MDs [27,28].

Organic acidurias are also a known cause of LSS commonly detected through a urinary organic acids profile. Mitochondrial enzyme defects HIBCHD and ECHS1D are well established causes of LSS resulting from an impairment in valine metabolism [29–31]. They differ from each other biochemically by urinary and acylcarnitine profile and represent a treatable cause of LSS [29].

In our experience, 10% of characterized cases (4/40) corresponded to treatable etiologies, with two cases bearing *SLC19A3* mutations and two with *HIBCH* mutations [21]. An *HIBCH* patient presented failure to thrive in early months of life. Urinary organic acids displayed valine pathway metabolites, which led us to first consider *ECHS1D*, another treatable cause of LSS. However, acylcarnitine profile showed high levels of 3-hydroxyisobutyryl carnitine, which is more frequently seen in *HIBCHD* [29]. In this case, two variants were identified in *HIBCH*: a definitively pathogenic variant associated with a variant of unknown significance (VUS) according to ACMG criteria [32]. Based on the distinctive biochemical profile, we proposed the reclassification of the latter variant. From our perspective, basic metabolic studies are mandatory for all patients, including an L/P ratio [33], plasma amino acids and acylcarnitine profiles, and urinary organic acids, which achieved up to 80% (40/50) of the diagnostic rate, as shown in Figure 2.

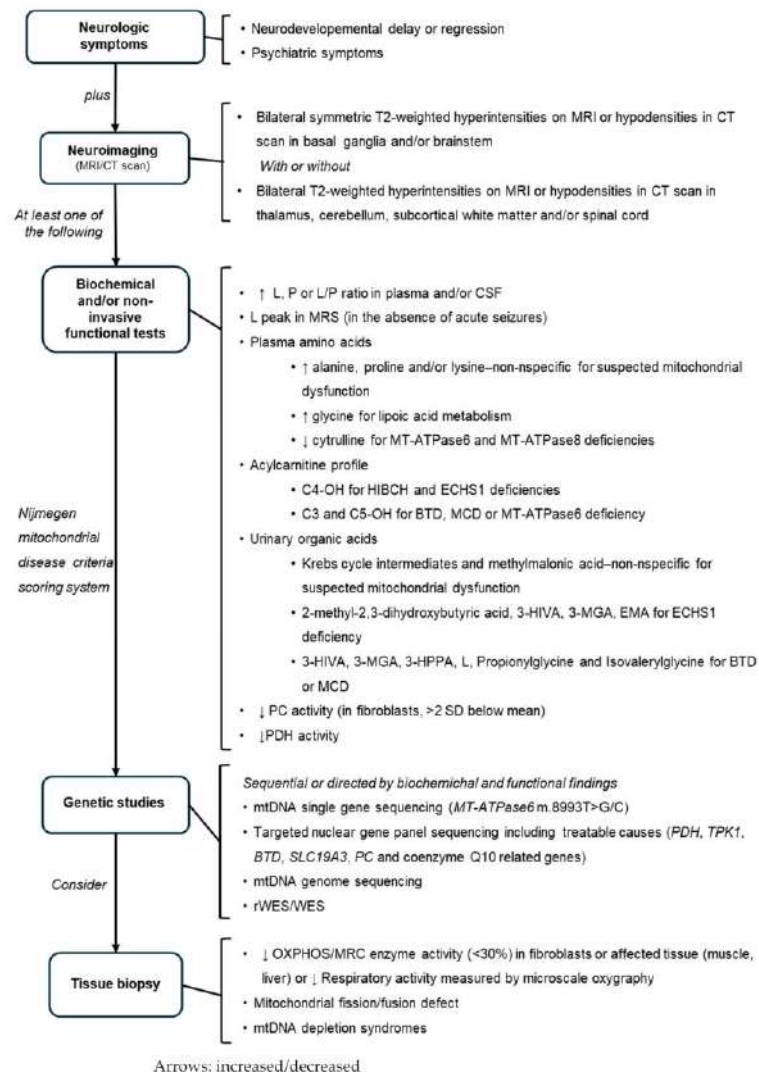


Figure 1. Proposed algorithm for the diagnosis of LSS. Abbreviations: L = lactate; P = pyruvate; CSF = cerebrospinal fluid; CT = computed tomography; LSS = Leigh syndrome spectrum; MRI = magnetic resonance imaging; MRS = magnetic resonance spectroscopy; C4-OH = 3-hydroxybutyrylcarnitine; C3 = propionylcarnitine; C5-OH = 3-hydroxyvaleryl carnitine; HIBCH = 3-hydroxyisobutyryl-CoA hydrolase; ECHS1 = short-chain enoyl-CoA hydratase deficiency; BTBD = biotinidase deficiency; MCD = multiple carboxylase deficiency; 3-HIVA = 3-hydroxyisovaleric acid; 3-MGA = methylglutacetic acid; EMA = ethylmalonic acid; 3-HPPA = 3-hydroxy propionic acid; PC = pyruvate carboxylase; SD = standard deviation; PDH = pyruvate dehydrogenase; SLC19A3 = solute carrier family 19 member 3; rWES = rapid whole exome sequencing; WES = whole exome sequencing; OXPHOS = oxidative phosphorylation; MRC = mitochondrial respiratory chain.

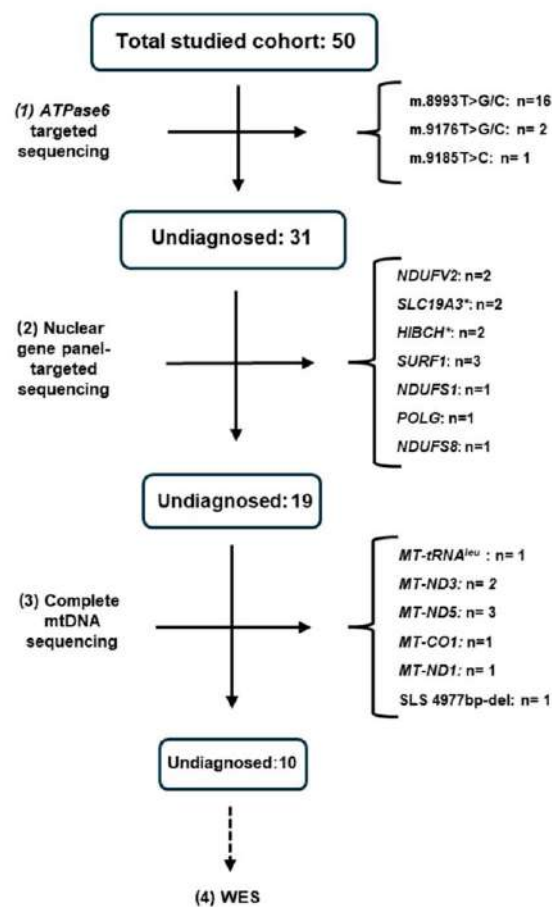


Figure 2. Results of molecular studies in our clinical cohort. * = treatable cases in our cohort; NDUFV2 = NADH:ubiquinone oxidoreductase core subunit v2; SLC19A3 = solute carrier family 19 member 3; HIBCH = 3-hydroxyisobutyryl-CoA hydrolase; SURF = human surfactant protein 1; NDUFS1 = NADH:ubiquinone oxidoreductase core subunit S1; POLG = DNA polymerase subunit gamma; NDUFS8 = NADH:ubiquinone dehydrogenase iron-sulfur protein 8; MT-tRNA^{leu} = mitochondrially encoded tRNA leucine 1; MT-ND3 = mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 3; MT-ND5 = mitochondrial genome coding for the NADH:ubiquinone oxidoreductase chain 5 protein; MT-CO1 = mitochondrially encoded cytochrome c oxidase 1; MT-ND1 = mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1; SLS 4977bp-del = single-large scale deletion of 4977 bp; WES = whole exome sequencing.

The *SLC19A3* patient displayed episodic ataxia and regression after intercurrent illness (previously diagnosed with two encephalitis episodes). Neuroimaging revealed cavitations in the basal ganglia area with brain atrophy, a mandatory differential diagnosis with CI deficiency by NADH:ubiquinone oxidoreductase core subunit S1 (*NDUFS1*) and NADH:ubiquinone oxidoreductase core subunit V1 (*NDUFV1*) mutations [34], as biotin responsive basal ganglia disorders (BBGDs) are treatable. *SLC19A3* mutations were identified through a targeted nuclear gene panel, allowing personalized treatment with higher doses of thiamine and biotin with full recovery.

In non-critically ill patients, a stepwise sequential and targeted approach can be cost-effective. Nevertheless, selected cases require invasive procedures. A deep tissue biopsy can be performed *ad initium* for specific organ manifestation (e.g., liver disease), rapidly progressive symptoms, critically ill patients, and in cases where access to a mitochondrial/rare disorders center is delayed [1,6,35]. In the remaining cases, tissue studies prove useful for heteroplasmy confirmation or clarifying biochemical and/or genetically unclear results as functional assay proof [6,35,36]. For biochemical assays, liver, muscle, and cultivated fibroblasts cells are the main sources selected to perform tests related to mitochondrial fusion/fission defects, and OXPHOS deficiency, and mitochondrial respiratory chain enzymatic activity tests.

4. Discussion

LSS is a common presentation of primary MDs with high clinical heterogeneity in clinical manifestation [6]. Several patients face multidisciplinary evaluations that delay diagnosis and appropriate treatment for years [5,6]. This work represents previously recommended criteria in clinical practice for diagnosing LSS to which we associated our experience accumulated over the years [21,22,25,37,38]. Although we acknowledge that our cohort of patients was not large, we also believe that the framework presented here facilitates the early diagnosis of treatable conditions.

4.1. Biochemical Features

Biochemical data are important in the process of LSS diagnosis. Pyruvate links glycolysis and the tricarboxylic acid cycle, presenting as acetyl-CoA, and should be included in the initial screening of suspected LSS patients. Moreover, lactate in CSF can be higher in MDs with neurological presentation [39], for which LSS is the most common form.

A previous study revealed that the L/P ratio in blood and CSF is useful for differential diagnosis between MDs and pyruvate dehydrogenase deficiency, being more accurate in patients with higher blood lactate levels [40]. In our experience, the presence of high lactate levels associated with an L/P ratio over 20 and a strong presence of ketone bodies in the urine are the first alert towards mitochondrial pathologies [41,42].

Mitochondria are also involved in amino acids synthesis and catabolism. Therefore, the evaluation of these cellular processes can provide important metabolic insights into multiple systems simultaneously. For example, alanine levels result from pyruvate transamination and reflect both lactate and pyruvate levels, and glycine is elevated in lipoic acid deficiency due to its pivotal role in the glycine cleavage system [43–45]. Hyperalaninemia can also be related to hyperammonemia frequently found in organic acidurias. Citrulline is another important marker, as its decreased levels are present in approximately 80% of patients carrying *MT-ATPase6* mutations [23,24]. This finding allows directed gene testing for m.8993T>G/C and m.9176T>G/C variants [25,26]. There are limitations to the usefulness of biochemical testing, and MDs diagnosis is not possible despite normal biochemical testing. OXPHOS assessment in fibroblasts is an alternative test before more tissue collection, and should be considered, especially in infants [1]. Nevertheless, it might not be informative [46,47].

Data on urinary organic acids and acylcarnitine profile are also important, as they can direct specific therapeutic interventions. High levels of methylmalonic acid (MMA) associated with tricarboxylic acids intermediates can be identified in mtDNA depletion syndromes secondary to succinate-CoA ligase ADP-forming subunit beta (*SUCLA2*) and succinate-CoA ligase GDP/ADP-forming subunit alpha (*SUCCL1*), as well as in methylmalonic acidurias [48,49]. In ethylmalonic encephalopathy, ethylmalonic acid (EMA) is found in the urinary profile associated with methylsuccinate, butyryl, isovalerylglycines, and lactic acidosis [48]. EMA may be elevated in short-chain acyl-CoA dehydrogenase deficiency (SCADD) and glutaric aciduria type II (MADD). 3-MGA is a compound found in patients with MDs such as 3-methylglutaconic aciduria and deafness, encephalopathy and “Leigh-like” (MEGDEL) syndrome, Barth syndrome, and mitochondrial CV deficiency nuclear type 2 (MC5DN2) syndromes [48,50,51]. Moreover, mitochondrial enzyme defects

(HIBCHD and ECHS1D) differ from each other by the presence of high levels of 2-methyl-2,3-dihydroxybutyric acid in urine in ECHS1D and the elevation of 3-hydroxyisobutyryl carnitine in plasma observed in HIBCHD [29–31].

An acylcarnitine profile can be useful in newborn screening to confirm fatty oxidation disturbances and organic acidurias. Multiple carboxylase deficiency (MCD) is a severe disorder that blocks the multiple carboxylase metabolism via interference in the biotin metabolism. Usually, it is indicated by abnormal levels of propionylcarnitine (C3) and/or hydroxyisovalerylcarnitine (C5-OH) in acylcarnitine studies and carboxylase intermediate compounds in urine workups. Nevertheless, without an MCD confirmation, a CV deficiency should be suspected in children with elevated C3 or C5-OH [48]. Additionally, a newborn screening series reported abnormal C3 and/or C5-OH levels, but enzymatic and molecular studies did not confirm MCD. Two babies in this cohort displayed neurological symptoms. The following molecular studies identified *MT-ATPase6* mutations [52].

4.2. Molecular Studies

In our center, we experienced an 80% diagnostic rate (40/50) using mtDNA single gene sequencing by Sanger followed by targeted nuclear gene panel and then mtDNA genome by next generation sequencing (NGS). As shown in Figure 2, steps 1 and 2 were sufficient to diagnose 62% of our cohort, and after the molecular approach (3 steps), 80% of cases were clarified. Undiagnosed cases (10/50) were directed to WES analysis. Molecular analysis is challenging in MDs cases, with variable results in biochemical screening. After inconclusive initial analysis, phenotype and data re-analysis are recommended [53–55]. This strategy can increase the diagnostic rate by up to 26% in WES [56]. A negative molecular result does not exclude the possibility of the patient carrying a deleterious rearrangement of DNA or replacements in functionally important non-coding regions [57,58]. Maximizing analytical efforts may enhance success in WES analysis, such as including a trio-analysis, in which familial variants and more complex inheritance patterns can be clarified [59]. mtDNA sequencing of specific affected tissue [60], methylation studies [61], and short-read sequencing (SRS), particularly in neurodegenerative disorders [62,63], are other important strategies that can yield a definite diagnosis.

Soon, rapid sequencing methods, such as long-read sequencing, can stand out as an alternative in molecular diagnosis [60]. Even though NGS enables several simultaneous analyses, results might take weeks or months to be officially reported [64]. In critical cases, we should prioritize target-gene sequencing if a suspected genotype is supported by biochemical and/or imaging methods, which may save time and avoid potentially fatal outcomes.

4.3. Tissue Biopsy

For many years, a specific tissue analysis was the first line approach to diagnosing MDs [6]. The evolution towards NGS performed in DNA extracted from leukocytes prevented a more invasive approach in many cases [65]. In fact, when evaluating patients suspected of having LSS, careful consideration should be given to how deep tissue fragments are obtained and the risks associated with this invasive process. Therefore, specific tissue collection should be reserved for severely ill patients who are unable to seek health support in a specialized center for rare/mitochondrial disorders, patients without alternative material sources for tests, rapidly progressive cases, and cases with specific organ involvement (e.g., the liver). For cases with conflicting genetic results, deep tissue biopsy can be necessary to confirm mitochondrial dysfunction through biochemical studies (assessing oxygen consumption via microscale oxygraphy, or OXPHOS enzyme deficiency), morphologic abnormalities (e.g., mitochondrial fission/fusion defects), confirming VUS, especially mitochondrial respiratory chain complexes, and assessing heteroplasmy levels [6,35].

5. Conclusions

The clinical presentation of LSS includes diverse neurological and radiological features. Since its first description in 1951, several questions related to the underlying causes of the disease remain without a definitive answer. The lack of detailed diagnostic criteria has delayed appropriate interventions and optimal prognosis. The incorporation of basic assays can enhance the number of diagnosed cases, as molecular assessment is unevenly accessible across diagnostic centers. As the diagnostic framework improves, we anticipate an adjustment in the pathway towards medical interventions in centers where NGS is not yet widely available to avoid preventable consequences and increase the quality of life of affected individuals.

6. Future Perspectives

Growth/differentiation factor 15 (GDF-15) and fibroblast growth factor 21 (FGF-21) are cytokine stress markers of mitochondrial dysfunction and have gained attention for their potential use in the diagnosis of MDs [11]. GDF-15 is produced in response to cellular stress, playing essential roles in both regulatory stress and inflammatory response. It was also identified in the hindbrain, where its receptor GFRAL is overtly expressed, although its exact role in MDs remains elusive [66]. Most reports claim statistical significance firmly correlating it to mitochondrial translation and deletions defects [11,67]. FGF-21 is a hormone-like cytokine linked to mitochondrial myopathies, disease severity, and respiratory chain deficient fibers [68,69]. Additionally, in genetically confirmed mitochondrial hepatopathies and translation defects, a stronger correlation is seen with GDF-15 levels rather than FGF-21 levels [70]. In vanishing white matter neurodegeneration, a non-mitochondrial early degenerative disease, GDF-15 is markedly elevated in CSF [66]. These biomarkers are also identified in multiple disorders, such as cardiovascular disorders [71], type 2 diabetes [72], and pediatric headaches [73]. Therefore, a more extensive work-up validation for adoption in routine screening for MDs is required.

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IV.4 Uncharacterized patients of a MDs suspected cohort – from clinical to populational perspective

1. Introduction

A database of the most frequent mtDNA variants in Portuguese population was built, based on the analysis of the complete mtDNA over 42 samples studied at INSA laboratory. The database includes all the mtDNA variants corresponding haplogroups as well as the clinical features associated with the individuals. It will allow not only to trace the variation of mtDNA (based on the analysis of the whole molecule, rather than isolated fragments) in the Portuguese population, as well as identify, or not, associations with neurological clinical features of the individuals.

2. Patients and Methods

2.1 Patients

A group of 42 unrelated Portuguese individuals (29 male and 13 female) were selected from an in-house database. A part of this cohort was reanalyzed from previous work by Nogueira *et al*¹⁷⁴. The individuals were selected based on clinical suspicion of mitochondrial disorders considering personal information (age, personal history remarks, family history/familial occurrence), presence of specific symptoms by systems (neurological, muscular, hepatological, cardiac, renal and others), laboratorial parameters (amino acids profile, organic acids profile, lactate and pyruvate), histological parameters (in case of a previous muscle biopsy), neuroimaging available findings and other molecular or specific assessments previously tested. All patients included in this study gave their informed consent and the study was previously approved by the local Ethics Committee (Supplementary material S1).

2.2 Methods

The DNA extraction for NGS was performed from peripheral blood leukocytes and muscle biopsy homogenate according to standardized protocol procedures. The mitochondrial DNA whole genome sequencing for blood extraction was enriched by a single amplicon, using back-to-back primers, by long-range PCR. Indexed paired-end DNA libraries were prepared and an equimolar pool was sequenced, according to the manufacturer instructions. FASTQ files were aligned to the mtDNA reference sequence with SeqMan Ngen (DNASTar). Variant calling and annotation were performed using the following

commercial programs: SeqMan NGen and SeqMan Pro (DNASTAR). Variants were filtered considering: the type of pathogenic variant, the population frequency, in silico predictors and presence in databases (Mitomap, MitImpact2, HmtVar), and heteroplasmy $> 5\%$ ¹⁷⁴. For muscle samples, the entire mtDNA was amplified using primers from Applied Biosystems. Sanger sequencing of PCR products was carried out with the BigDye v3.1 terminator cycle sequencing methodology on Applied Biosystems. The sequences obtained were edited and aligned with the Cambridge Reference Sequence and the consensus was made using the SeqScape software v2.5 (Applied Biosystems). All the variations along with their position were noted and searched in the human mitochondrial genome databases such as Mitomap (<http://www.mitomap.org>), mtDB (<http://www.genpat.uu.se/mtDB>) and HmtDB (<http://www.hmtdb.uniba.it:8080/hmdb>) for their significance. The mutations were submitted to MToolBox, APOGEE, Mitoclass.1 and HmtVar for a prediction of pathogenicity. These recent tools were developed for mtDNA using machine learning based approaches. The HmtVar explore human mitochondrial variability data and their pathological correlations. The phylogenetic analysis and the mammalian conservation index were also evaluated. The material was not tested to single/multiple mtDNA deletions or insertions¹⁷⁵.

2.3 Analysis of mitochondrial variants

We started by revisiting all variants previously detected in patients to be further analyzed in MITOMAP (Kogelnik 1996, 1997, 1998) for haplogroup classification, MitImpact prediction tools as APOGEE, that is a consensus classifier which combines predictors directed to non-synonymous coding mutations, selected by the database as the main predictor in “allele search”, and MitoTIP for the analysis of tRNA variants. The APOGEE scores from 0 to 1.0, from possibly benign to likely pathogenic, with the following intervals: 0-0.25: neutral/likely benign (LB); >0.25-0.50: neutral/possibly benign; >0.5-0.75: possibly pathogenic and >0.75: likely pathogenic. MitoTIP scores from 0 to 100% with the following intervals: 0-25%: likely benign; >25-50%: possibly benign; >50-75%: possibly pathogenic and >75%: likely pathogenic. MitoTIP also has the confirmed pathogenic status to the classification. The variants were also submitted to MitImpact predictors for more accurate prediction. The pathogenicity predictors available in MitImpact are PolyPhen2, FatHMMW, EFIN

SP, PANTHER, CADD, SNPDryad, SIFT, PROVEAN, EFIN HD, PhD-SNP, SNAP, FatHmm, Mutation Assessor, VEST, MutationTaster, MitoClass1. The pathogenicity metapredictors available on MitImpact are APOGEE, COVEC WMV, DEOGEN2, CAROL, MetaSNP, Condel and MtoolBox. Allele frequencies were obtained from Mitomap and GnomAD¹⁷³. Alongside with APOGEE, the results were also checked in for Mitoclass.1 and MtoolBox predictors in MitImpact compilation as suggested by Bris¹⁷⁶.

3. Results

3.1 Distribution of clinical features

We studied a total of 42 individuals for which the presence of symptomatology was compatible with a mitochondrial disease but with no known disease-associated variant either in the mtDNA or in nDNA. We started by grouping by symptoms and found that neurologic and muscular symptoms are very frequent in our cohort (Figure 6). 41 patients (95.3%) showed neurologic symptoms, muscular or both, while one (2.3%) displayed the neuromuscular and non-neuromuscular symptoms, and one (2.3%) showed isolated non-neuromuscular symptoms. The most frequent neurological features were neurodevelopmental delay (NDVD), epilepsy, ataxia, and intellectual disability. The most frequent muscular features were myopathy, myalgia, and weakness. There is a group of patients that present both muscular and neurologic symptoms (figure 6 – group NN+MM). These symptoms are frequently found in individuals with diagnosis of MDs such as LSS, MERRF, MELAS and ataxic syndromes^{2,177,178}. These affected individuals may display diverse clinical pictures. These findings are compatible with the scenario expected in the clinical presentation of MDs and reinforce the fact that these diseases show a great heterogeneity of symptoms making the establishment genotype-phenotype correlations difficult¹⁷⁹⁻¹⁸².

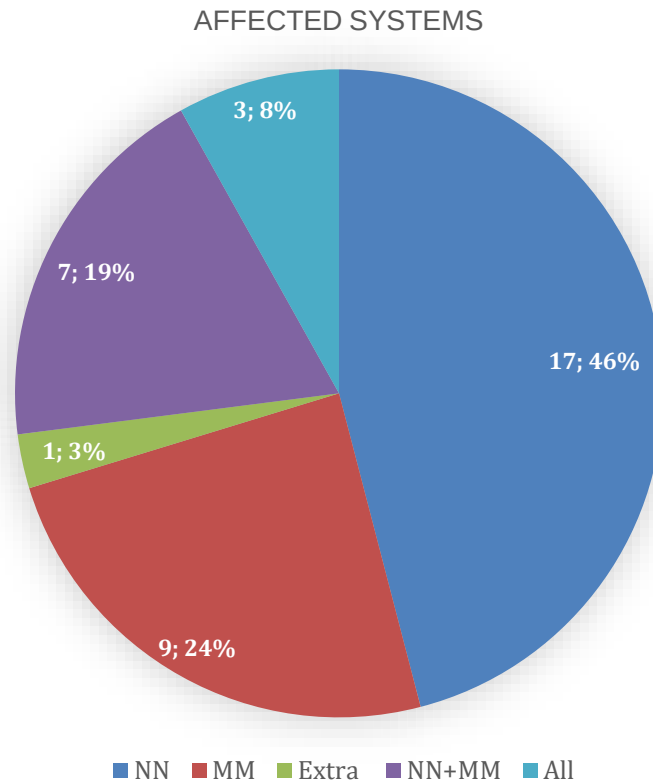


Figure 6. Symptoms' frequency distribution in our cohort. Legend: NN= neurological symptoms; MM= muscular symptoms; Extra= other systems involvement (hepatic, thyroid); NN+MM= neuromuscular symptoms; All= the three categories simultaneously present.

3.2 Haplogroup analysis

To assess the haplotypic structure of our cohort, the haplogroup profile was set in accordance with MITOMAP database (www.mitomap.org) using complete mtDNA information as shown in Figure 7. Our cohort represent a Portuguese population and, therefore, the results replicated the profile and frequencies displayed in a previous work in this population¹⁸³, indicating that the studied cohort is representative of the general Portuguese population and no significant deviation has been found.

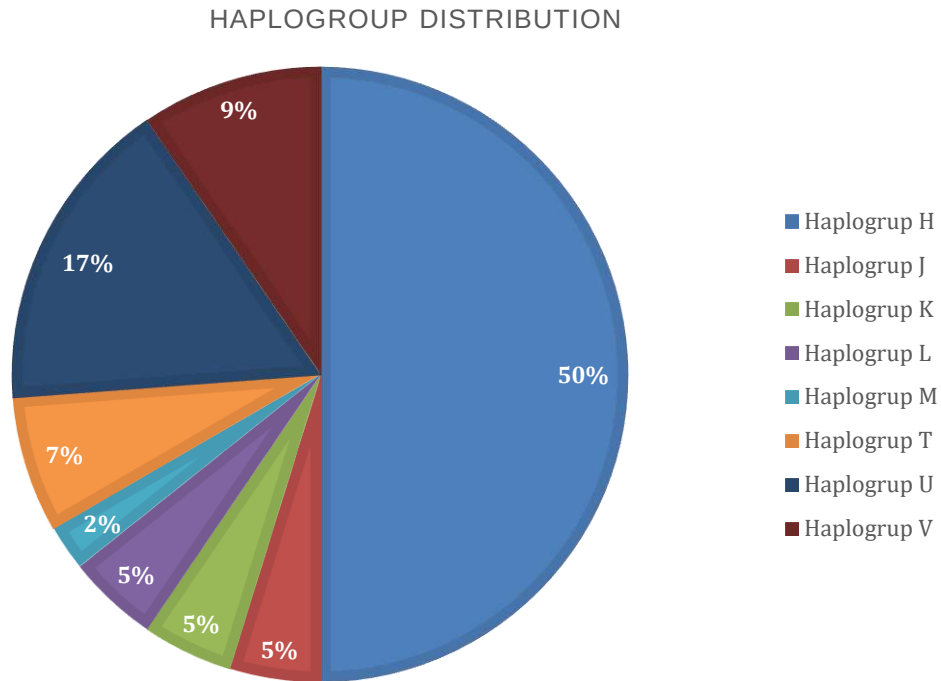


Figure 7. The haplogroups distribution found in our cohort.

3.3 Analysis of genetic variability

As stated above, the whole mtDNA genome was sequenced in all individuals here analyzed and no disease-causing variant has been identified. Therefore, we revisited the data to explore the variants previously detected but considered VUS to predict their putative biological effect and, ultimately, their possible association to the symptoms presented by the individuals.

The analysis started with the observation of the frequency of all the VUS previously found in our study. A frequency higher than 1% was observed in our data in over 140 variants, indicating that they are most likely to be benign polymorphic alleles, although reports in literature suggest an increased susceptibility to other conditions such as Parkinson disease, Alzheimer disease, cancer and bipolar disorder, and these cases were overlooked^{184–186}.

We also looked for variants whose frequencies are under 1% in general population which were selected for further evaluation in relation to location, amino acid substitution, frequency, site conservation among vertebrate species, reference literature/publications and APOGEE scoring for non-synonymous variants¹⁸⁷ and MitoTIP for tRNA variants¹⁸⁸. We also submitted to Mitomap *in silico* tools prediction contained in MitImpact. The variants identified in our

cohort that are rare also belong to haplogroup U defining variants, therefore, unremarkable for pathological performance.

4. Discussion

For the 42 samples studied, no definite molecular diagnosis was established. Therefore, we reanalyzed the data focusing on the VUS detected in the samples. Most of them are present in frequencies higher than 1% in the population which indicates an unlikely association to the pathological scenario manifested by our patients, although some of these variants were reported to be related to other mitochondrial conditions or haplogroup definers^{189,190}.

The haplogroup analysis revealed that samples pertain to a typical European profile, with predominance of Haplogroup H, J, L, K, T, V and U. The haplogroup H is the most frequent in our cohort and the most frequent in Iberic Peninsula¹⁹¹ since it emerged in the Near East and spread to European territory¹⁹² in post-glacial period. Haplogroup U is the second most frequent among our samples, comprising 17% of the total. The haplogroup J, another European group, present in Galicia territory and other western European population^{193,194}, was identified in 9% of individuals. The haplogroup L, the African root of modern anatomic human's haplogroups, was found in 9.4% of individuals, a frequency that is higher than the data for the Portuguese population studied by Pereira *et al.*¹⁹⁵

Contrasting to our results, Marques *et al.*¹⁸³ found a frequency of 12% of a Haplogroup R, although in our analysis only one individual was classified as belonging to this Haplogroup. This may be explained by the fact that the previous work focused on hypervariable regions and included specific SNPs to differentiate branches derived from the same ancestral haplogroup, particularly in case of R and H haplogroups, whereas our analysis was based on the complete mtDNA molecule.

We further investigated whether a possible link between specific haplogroups and the most frequent phenotypes could exist, but no link was found to the observed phenotypes.

Furthermore, the expected symptoms were accordingly to the most ATP dependent organs and systems and the haplogroup pattern was irrelevant in correlating to any particularity of our investigated cohort which fails to contribute to determining a specific diagnosis for them.

Chapter V. General discussion and future perspectives

1. General overview

LSS is classically considered an MD and follows the characteristic clinical and molecular heterogeneity. Therefore, LSS possess the same diagnostic, prognostic and genetic counselling challenges as any other MD. Nevertheless, there are some known causes of LSS, such as biotin or thiamine deficiency, that are not considered MD, even though they are known to have a direct impact in mitochondrial function.

With the growing number of reports of Leigh-like syndrome, experts felt the need to uniformize nomenclature and case definition, as previously stated in Chapter 1.

Until recently, diagnosis relied strictly on specific muscle tissue studies where DNA sources were closely related to the exhibited phenotype. Unfortunately, obtaining deep tissue samples involves invasive procedures and submits the patients under several risks, demanding alternative techniques to achieve diagnosis.

DNA sequencing evolved from RFLP and long-range PCR to complete sequencing of genomes allowing faster results. The first-generation sequencing entered the first results, yet it took a long time to merge preparation, technique and results. The long-time impediment for urgent cases demanded faster results and the ability to address more than one gene at a time. Additionally, the simultaneous sequencing of genes could save efforts through optimizing resources¹⁹⁶. Therefore, NGS was a breakthrough in MDs diagnosis maximizing interventions, costs and available results versus time spent in the process. In the last decade, the number of related genes to LSS enhanced approximately 30%, currently counting more than 100 genes responsible for the LSS⁵⁴. Therefore, the contribution that molecular advances brought to LSS is invaluable.

Nonetheless, pathophysiological mechanisms are still eloping and need to be further explored, for example thiamine metabolism was also confirmed to be involved in LSS. The genetic defect may help to elucidate pathophysiological mechanisms that are common and identify pathways amenable to therapeutic intervention.

Our work allowed us to systematically perform genetic studies in a cohort of LSS patients and contributed to a significant number of diagnosed cases.

The combination of this systematic approach with previous laboratory experience allowed us to create a pipeline designed to diagnose a higher number of patients by associating basic (metabolic) biochemical data as an initial approach.

The proposed pipeline is applicable to low to middle-income countries for which broad genetic studies are not always feasible. Priority must be given to treatable causes and by addressing phenotypical and biochemical studies prior to NGS, we could facilitate early intervention in such treatable cases as BBGD.

2. Detailed results

By reviewing the available information in a general review (see Chapter IV.1), we studied a particularly suspicious LSS cohort (see Chapter IV.2). Using the molecular techniques associated with our expertise, we applied them to this cohort and, furthermore, using phenotypic observations, biochemical and molecular studies we created a practical pipeline for conducting suspected LSS patients to a diagnosis (see Chapter IV.3).

Phenotypic traits are part of the official criteria for MDs in general as a valuable step in the search for diagnosis^{197,198}. In LSS, some additional clinical manifestations can aid phenotype-genotypes correlation and fasten definitive results. This can be illustrated by the case we reported in siblings with *NDUFV2* mutations where cardiomyopathy associated with neurological impairment directed molecular studies.

Psychiatric symptoms were the latest criteria associated with LSS. In our cohort we have diagnosed a patient presenting with visual hallucinations and neuroimaging supportive of LSS due to COX deficiency and biallelic *SURF1* mutations.

Organic acidurias are acute intoxicating metabolic disorders known to cause LSS. By using biochemical approach as the first step, these disorders are accessed, and prompt intervention can be done until molecular studies are concluded. The results should be interpreted according to the phenotype to avoid potential conflicts and mistakes. In the case of well determined metabolic pathways, such as valine degradation, the presence of its metabolites is confirmatory of a defect in its metabolism. We reported some patients of valine degradation impairment that were diagnosed due to biochemical support albeit presenting VUS in molecular studies for which we considered likely pathogenic according to ACMG¹⁹⁹ in face of a strong biochemical support^{200,201}.

Characteristically, the patients with MILS exhibited an amino acid hallmark, a very low to absent citrulline level in plasma^{65,202}. The finding was consistently identified in patients with m.8993T>G/C mutations, the most common cause of mtDNA-related LSS, as supported by our published cohort. This suggests that it can be pertinent to start genetic studies with the *MT-ATPase6* gene. Additionally, we identified VUS in some molecular studies that would possibly be considered by biochemical/neuroimaging studies support a reclassification from VUS to likely pathogenic (see Chapter IV.2). The remaining cases were studied by a custom made targeted nuclear gene panel and the whole mtDNA sequencing accomplishing another 21 diagnoses. By achieving this diagnostic rate of 49%, we put together a pipeline published (see Chapter IV.3), where we highlighted simple biochemical analysis and clinical information, especially in treatable cases where time is crucial. Our approach seems effective since published diagnostic rates are less than 40%²⁰³. Going back to basics, reviewing diagnostic criteria and studies step-by-step, could save us from inappropriate or more invasive interventions.

The main bias we have identified in our work was in sample selection, as it depended on the clinical information provided. This clinical information is typically a summary, and we assume that some data is missing, including in phenotype data, biochemical, neuroradiological or even familial occurrence. In selected cases, the medical team was contacted to further discuss relevant information to phenotype-genotype correlation.

Although our work did not focus on brain MRI description, considering that in LSS patients, the neuroradiological features are obligatory criteria, systematic neuroradiological review should be preferred. Also, periodical MRI could add relevant and valuable information for definitive genetic diagnosis.

For biochemical description, we should note that biochemical studies were conducted by different Centers which limited our access to a more detailed work-up.

The undiagnosed patients (42/82) were analyzed against a populational background since haplogroups might be involved from both as protective factors or risk factors affecting disease onset and/or outcome. The populational profile reflects the European background, and no correlation was seen between the haplogroups, and the non-haplogroup definer variants present in our cohort. Still, these unclarified cases were directed to be further explored in the future.

The number of associated genes with LSS grew expressively requiring routine updates in molecular pipelines. Since we used our in-house genetic panel and CES considering our population background at the time of the study, it is acknowledgeable that some diagnosis might have been missed.

Our patients were studied by our step-by-step proposal (see Chapters II and III). Our nuclear targeted gene panel (and later CES) was elaborated at the beginning of the project. Since then, several other genes were identified to cause LSS (e.g. *ABAT*^{204,205}). The rapidly expanding molecular background must be considered to regularly update the targeted genes. Re-evaluation of genetic data is universally suggested and can provide further diagnosis without the need for new sample collection²⁰⁶.

Data reanalysis of our cohort is scheduled for the near future to include novel variants and possible reclassification of previously uncertain or likely pathogenic variants. Also, proceeding to a Trio-analysis is helpful in segregation pattern or *de novo* mutations. Nevertheless, other complex genetic causes should be studied by proper techniques²⁰⁷.

3. Future perspectives

3.1 Biochemical features

Biochemical studies are part of the screening for MDs. As discussed before, the intermediate compounds may indicate specific metabolic impairments shortening the clinical investigation pathway. Proteins are break down to amino acids and some, such as alanine and citrulline, have direct relevance. Alanine has a direct correlation to lactate levels, which can be revealing for MDs and LSS^{198,208}. On the other hand, citrulline has a direct relation with ATPase complex function. Mutations coding for units and subunits of this complex block the proton pumping and change the membrane electro gradient. Citrulline sources are basically dietary needing to be absorbed by enterocytes in small intestine, which are more sensitive to ATPase activity⁶⁵. Therefore, the inefficiency of ATPase complex has a direct impact on this amino acid metabolism. Extremely low values (<5µmol/L) are highly suggestive of ATPase deficiency, after excluding organic causes such as short bowel syndrome.

Additionally, persisting in investigating and refining the pre-existent available biomarkers for screening could potentially be a successful measure. Still, lactate and pyruvate are the main easily available biochemical compounds

when investigating a possible MD⁷⁴. Moreover, the ratio between them shows better correlation when lactate is elevated in venous blood sample²⁰⁹. The sensitivity and specificity concerning these biomarkers are variable and vulnerable to external interferences in the results. Yet, they are valuable biochemical screening and could be the best choices in the future⁷⁴.

The cytokines GDF-15 and FGF-21 have been proposed as alternatives to lactate and pyruvate because they are related to mitochondrial dysfunction, especially in the mtDNA rearrangement defects⁷³. Yet, they are not available in rare diseases Centers nor validated as a method because they are elevated in several distinct non-mitochondrial disorders and neurodegenerative conditions preventing their inclusion as screening biomarkers^{210,211}.

3.2 Molecular studies

In the uncharacterized cohort, a total of 42 patients will undergo re-evaluation encompassing clinical, radiological, and metabolic assessments. Upon the completion of these evaluations, cases that remain undiagnosed will be referred to WES.

3.3 Disease models

Diagnosis is important from an individual perspective but also fundamental for the understanding of neurodegenerative disorders. The lack of a consensus model to simulate neurodegenerative disorders has been the major challenge in building knowledge in LSS and planning more effective measures for the patients.

Non-mammalian organisms such as yeasts, fruit-fly and several simple models were ineffective due to evolutionary differences, but zebrafish proved useful to simulate *SURF1* deficiency and convenient for having a short life cycle, a recognizable stage of development, similarity to the human nervous system plus enabling interventions according to each developmental phase. Zebrafish *surf1*^{-/-} developed LSS major features and allowed to test therapeutical interventions²¹².

Therefore, non-mammalian models are straightforward options in simulating lifespan, neurological impairment and therapeutical intervention testing.

Mice are evolutionary closer to humans, being more accessible and completing a full life cycle in a relatively short period of time. For *ndufs4 ko/-*, the mouse model for mtDNA based mutations showed deleterious effect on the eggs leading to premature death of the offspring¹³⁵ but at the same time revealing in showing affected brain regions, metabolic studies, and pharmacological toxicity of the performed interventions²¹³.

In vitro primary cellular models, such as fibroblasts, are suitable and advantageous since they are easily obtained, carrying all genetic information for both genomes and cultivated in controlled conditions. Additionally, they are relevant in demonstrating pathophysiology and molecular mechanisms aspects but failing to be reproducible when the majority of symptoms are in muscle tissues or CNS based²¹³. Transmitochondrial cybrids could illustrate impairments from both genomes, but mtDNA mutations are best explored by this model for studying biochemical features and delimitating the genetic location²¹⁴.

On the other hand, advanced/pluripotent cell lines are versatile but extremely dispendious since the reprogramming process is quite disturbing for the cellular environment which may result in genomic responses to the stress changing the material information²¹⁵. iPSCs must be differentiated into the specific cell type and assured to reproduce the aimed genetic background.

Organoid models illustrate human organs and more complex tissues¹⁵⁰ since bidimensional cultures failed to detail events neurological tissue¹⁵¹. Still, the lack of demonstration of additional neuronal structures, for example, means further detailing is required to deepen the understanding of organs and tissues developments for neurodegenerative disorders²¹⁶.

There is not a model that addresses all aspects of neurodegenerative disorders, from physiological aspects to interventional strategies. The model must be chosen to best fit and reproduce the targeted questions. From our perspective, we plan to develop a zebrafish model, which is being implemented at INSA, to study LSS features of neurodegeneration and phenotypical expression.

Chapter VI. Conclusion

LS and LLS present a heterogenous phenotype which is currently denominated as spectrum (LSS) to accommodate the multiplicity of phenotypic and genotypic expression. After improvement in molecular studies techniques, the pivotal outcomes took place and helped clarify underlying uncertainties. Their progression continues to expand the knowledge of the heterogeneity of the disease spectrum counting over 100 genes so far. Yet, there are shortcomings along the way to be addressed. The disease models are attempts to simulate *in vitro* the *in vivo* condition. Despite the promising results and several models available, there is not a universal eligible model for simulating for LSS since every one of them displays particularities that are suitable for specific traits of the studied profile. Additionally, some models allow interventions during the experimental phases, a valuable characteristic to explore treatment options.

Nevertheless, diagnosing LSS is still a challenge to overcome. Based on our results, we vouch for the relevance of basic biochemical steps associated with clinical phenotypic traits to be valid in highlighting and supporting molecular results, as well as screening already known etiologies of LSS. Despite molecular breakthroughs are key for confirming diagnosis, they are still not sufficient to being the final word in all cases.

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Supplemental material

S1. Ethics Committee approval



centro hospitalar
do Porto

Hospital Santo António | Hospital Maria Pia | Maternidade Júlio Dinis | Hospital Joaquin Urbano

Largo Prof. Abel Salazar
4099-001 PORTO
www.hgsa.pt

Exma. Sra. Prof.ª Doutora Laura Vilarinho
Instituto Nacional Ricardo Jorge
Rua Alexandre Herculano, 321
4000-053 Porto

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	Dr.ª ELIA GOMES
Presidente	Vogal Executiva
Prof. Doutor JOSE BARRIOS	Dr.ª EUI PEDROSO
Diretor Clínico	Vogal Executivo
Enf.ª EDUARDO ALVES	
1.º Vice-Presidente	

* Em todas as eventuais comunicações posteriores sobre este estudo é indispensável indicar a nossa ref.ª.

APRECIACÃO E PARECER PARA A REALIZAÇÃO DE PROJETO DE INVESTIGAÇÃO

Título: "Defeitos genéticos das doenças mitocondriais: abordagem por sequenciação de nova geração"		Ref.º: 2016.194(164-DEFI/153-CES)
		Investigador: Prof.ª Doutora Laura Vilarinho Instituto Nacional Ricardo Jorge

DIREÇÃO DE ENFERMAGEM: ☒ NÃO SE APLICA ☐ PARECER FAVORÁVEL ☐ PARECER NÃO FAVORÁVEL Data: _____	PRESIDENTE DO CONSELHO DE ADMINISTRAÇÃO: ☒ PARECER FAVORÁVEL ☐ PARECER NÃO FAVORÁVEL Data: 18/4/17 DR. PAULO BARBOSA Presidente do Conselho de Administração do CHP
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Em conformidade. Pode ser autorizado


 Diretora do DEFI
 26/4/2017
 Prof.ª Doutora Luísa Lobato
 Diretora do DEFI

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.: 2016.194(164-DEFI/153-CES)
Protocolo/Versão: PI	Promotor: o(a) próprio(a)	Investigador: 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

S2. List of genes included the NGS gene panel in this study

AARS2; ABCB7; ACAD9; ACO2; ADCK3; AGK; AIFM1; ALAS2; ALG13; APTX; ATP13A2; 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; CTSF; 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; GRN; 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; NDUF51; NDUF52; NDUF53; NDUF54; NDUF56; NDUF57; NDUF58; NDUFV1; NDUFV2; NFU1; NOTCH3; NUBPL; OPA1; OPA3; PARS2; PC; PCK2; PDHA1; PDHB; PDHX; PDP1; PDSS1; PDSS2; PLD1; PMM2; PNPT1; POLG; POLG2; PPT1; PPT2; 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; UQCRCQ; VARS2; WARS2; WDR45; WFS1; YARS2.

INSTITUTO de Ciências BIOMÉDICAS ABEL SALAZAR