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Review Article

Mitochondrial disorder diagnosis and management– what the pediatric neurologist wants to know

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ABSTRACT

Childhood-onset mitochondrial disorders are rare genetic diseases that often manifest with neurological impairment due to altered mitochondrial structure or function. To date, pathogenic variants in 373 genes across the nuclear and mitochondrial genomes have been linked to mitochondrial disease, but the ensuing genetic and clinical complexity of these disorders poses considerable challenges to their diagnosis and management. Nevertheless, despite the current lack of curative treatment, recent advances in next generation sequencing and -omics technologies have laid the foundation for precision mitochondrial medicine through enhanced diagnostic accuracy and greater insight into pathomechanisms. This holds promise for the development of targeted treatments in this group of patients. Against a backdrop of inherent challenges and recent technological advances in mitochondrial medicine, this review discusses the current diagnostic approach to a child with suspected mitochondrial disease and outlines management considerations of particular relevance to paediatric neurologists. We highlight the importance of mitochondrial expertise centres in providing the laboratory infrastructure needed to supplement uninformative first line genomic testing with focused and/or further unbiased investigations where needed, as well as coordinating an integrated multidisciplinary model of care that is paramount to the management of patients affected by these conditions.

1. Introduction

Mitochondria are dynamic organelles commonly known for their essential role in energy metabolism. Additionally, they play important roles in various other cellular processes including calcium homeostasis, heme and iron-sulphur biosynthesis, apoptosis, and the cellular stress response [1,2]. Here, we use the term mitochondrial disease (MD) to describe diseases with a primary, genetic disorder in the entire route of the pyruvate oxidation process (OXPHOS), including the pyruvate dehydrogenase complex (PDHc), the citrate cycle and the respiratory chain including adenosine triphosphate synthase (complex I–V) [3]. In this regard, the requisite "support machinery" of mitochondrial DNA (mtDNA)-related protein synthesis (including mtDNA replication, RNA metabolism, and translation), mitochondrial cofactors and their metabolism, and finally mitochondrial homeostasis (including protein import into mitochondria, lipid metabolism, fusion, and fission, quality control, etc.) are accordingly considered [3].

Together, the group of MDs account for the most common cause of neurological disease presenting in children (<18 years of age), often manifesting with clinical findings like developmental delay (DD), intellectual disability (ID), muscle weakness, epilepsy, or movement disorders [4–10]. By definition, these disorders are progressive and often lead to death before reaching adulthood [4]. Pathogenic variants in 373 genes across the nuclear and mitochondrial genomes have been associated with MD (Fig. 1, updated from Ref. [3]). Point-prevalence estimations based on clinical suspicion and genetic confirmation have been

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reported to be around 5/100,000 in children [11,12], while a population-based genotype study estimated the lifetime risk of nuclear-encoded recessive MDs to be closer to 50/100,000 [13]. One could therefore extrapolate the number of affected children living in the European Union to be around 40,000 [14]. Of note β-Oxidation defects are MDs but form a separate disease group and will not be covered in this review.

Both genetic and clinical heterogeneity pose significant challenges to the timely diagnosis of these disorders. The *diagnostic odyssey* metaphor aptly describes the often long and burdensome journey that parents face in seeking an explanation for their child's medical condition. Even in an era of genetic diagnostics, exome sequencing (ES) still leaves 50 % of individuals with suspected MD without a (genetic) diagnosis [15–17]. Once a diagnosis is established, a new *therapeutic odyssey* emerges, one that is equally daunting if not disheartening in the absence of effective disease-modifying therapies or management guidelines [18].

Against a backdrop of inherent challenges and recent technological advances in mitochondrial medicine, this review discusses the diagnostic approach to a child with suspected MD and outlines management considerations of particular relevance to paediatric neurologists.

2. Overview of challenges associated with diagnosing mitochondrial disease

2.1. The need for a (genetic) diagnosis

Reaching a molecular diagnosis has important implications for families, affecting financial decisions and access to relevant therapies, school support, and contact with peer support groups [18]. The precise genotype is equally informative for the treating team as well, offering

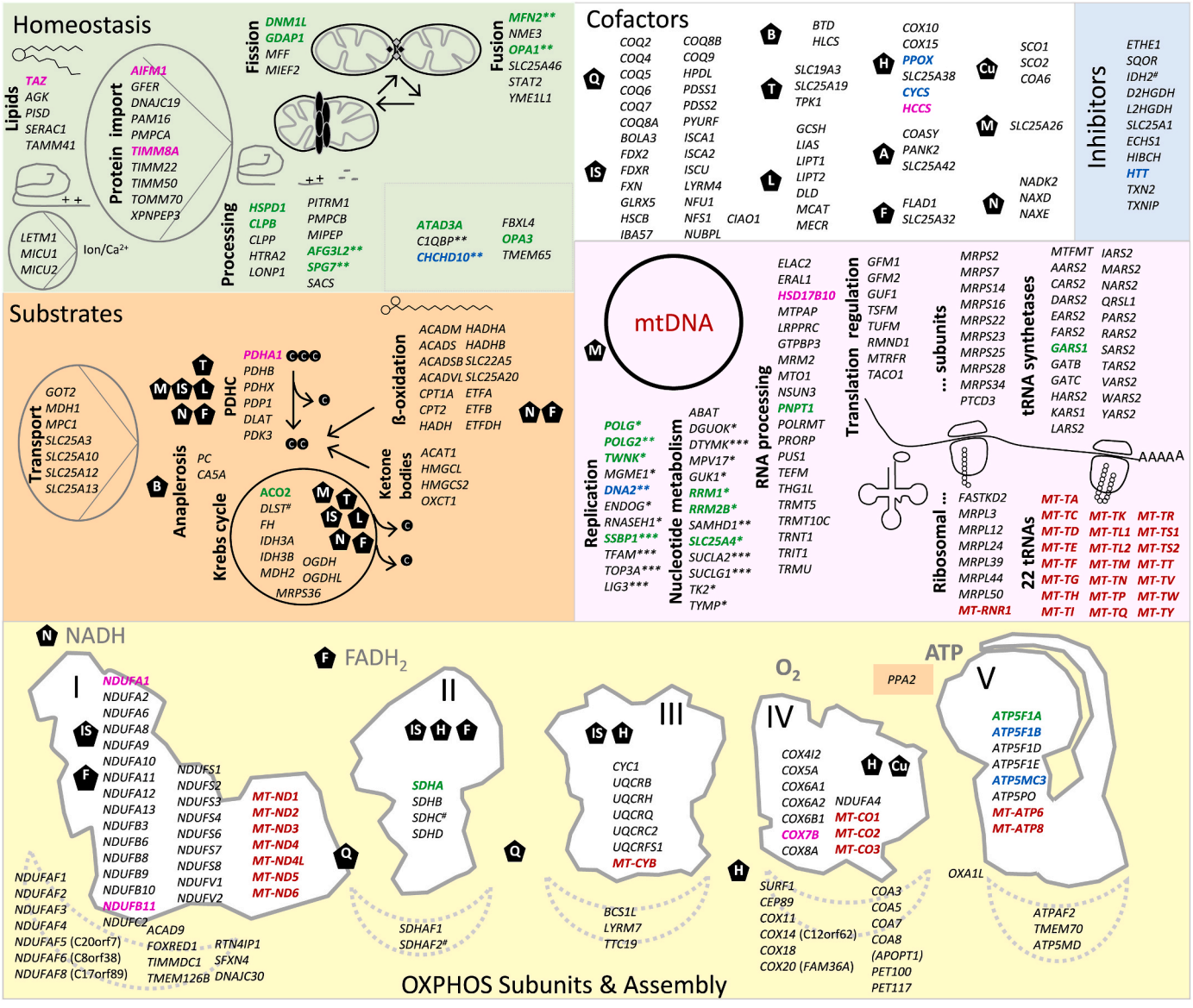

Fig. 1. Disease-relevant genes (n=373) in mitochondrial energy metabolism.
Genes in red are encoded on the mitochondrial DNA (mtDNA), genes in pink are X-linked, genes in blue result in either dominant or *de novo* defects, genes in green show either a dominant or recessive inheritance, and all other genes have an autosomal recessive inheritance mode. Pathogenic variants in these genes are associated with *mtDNA depletion and multiple mtDNA deletions, **multiple mtDNA deletions, ***mtDNA depletion. # *IDH2* gene defects have only been reported in cancer patients so far. Abbreviations: coenzyme Q10 (Q), iron-sulphur clusters (IS), biotin (B), thiamine pyrophosphate (T), lipoic acid (L), heme (H), coenzyme A (A), riboflavin/FMN/FAD (F), iron (Fe), copper (Cu), S-adenosyl-methionine (M), NAD(P)H (N). Modified from Ref. [3]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

insights into prognosis or likely complications, which can guide disease-specific counselling and inform individualised surveillance measures or palliative care planning where needed. The genotype can help identify tailored therapeutic intervention. For family planning, a molecular diagnosis can inform the decision-making process regarding prenatal or pre-implantation genetic diagnosis [19].

2.2. *"Any symptom, in any organ or tissue, at any age, and with any mode of inheritance"*

This adage [20] aptly reflects the unique and numerous challenges of MDs. Such challenges arise from the significant, clinical heterogeneity of these disorders, phenotypic overlap with non-MDs, the bigenomic sources of genetic variation underpinning them, and tissue specific variation (Fig. 2).

2.3. *Clinical heterogeneity – it's a spectrum!*

Traditionally, MDs have been clinically categorized into syndromes often eponymously named (e.g. Pearson [21] or Alpers-Huttenlocher syndrome (AHS) [22,23]) or assigned acronyms according to the constellation of symptoms observed, like mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome [24].

Furthermore, while syndromal names can be helpful in highlighting the multiple systems involved in MD in general, they are not reflective of paediatric presentations that often have less, more, and/or different signs and symptoms – a phenotypic spectrum due to incomplete penetrance and variable expressivity. For example, children affected by the m.3243A > G variant - the mtDNA variant most commonly associated with MELAS - often present with DD and/or ataxia but are much less likely experience a stroke like episode (SLE) during childhood [25]. However, labelling such a child as having MELAS can put the family into a state of anticipatory dread, waiting for the SLE, and impede disease processing and management of other signs and symptoms.

In *POLG*-related MD, another common inherited MD, the old nomenclature with many syndromal names (e.g. AHS, Sensory ataxia, neuropathy, dysarthria, and ophthalmoplegia (SANDO), Myoclonus, epilepsy, myopathy, and sensory ataxia (MEMSA)) was equally confusing and overly complicated in light of the continuum of clinical features that characterize a condition which manifests differently over the course of a lifetime [26]. The simplified classification of *POLG*-related MD according to age of onset (detailed below) has proven useful for guiding diagnostic workup and prognostication [5,26].

2.4. *One genotype - many phenotypes*

Pathogenic variants in the same gene can give rise to different phenotypes, an overlapping spectrum of disease with the predominant clinical findings often differing according to age, and where early onset is most often associated with the most severe presentation and course of disease. This is illustrated well in *POLG*-related MD [26], with early-onset disease (age of onset <12 years of age) being the most severe and having the worst overall prognosis, characterised by seizures, liver impairment, muscular hypotonia, feeding difficulties, and anaemia. In the juvenile/adult-onset form (12–40 years of age), ataxia, peripheral neuropathy and seizures predominate. Finally, late-onset disease (>40 years of age) is typically milder and with best prognosis, mainly characterised by ptosis and progressive external ophthalmoplegia (PEO), although peripheral neuropathy and ataxia are also common [26]. Another illustrative example is the MD caused by a single large-scale (5 kb) mtDNA deletion [27,28]. If symptoms present in the neonatal or early infantile period, bone marrow failure and exocrine pancreatic dysfunction predominate and are referred to as Pearson syndrome [21,29]. While many of these patients die early, some experience a phenotypic change to what is referred to as Kearns Sayre Syndrome (KSS) [30,

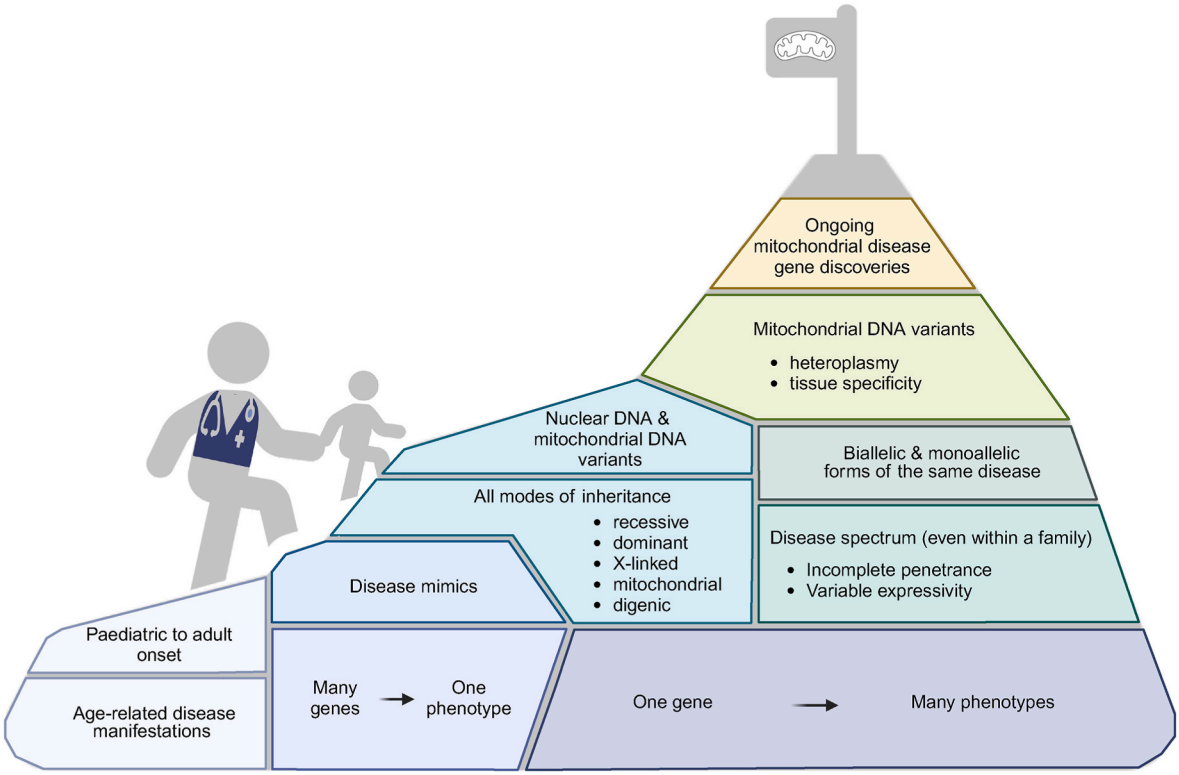

Fig. 2. Diagnostic challenges in mitochondrial medicine.
Establishing a molecular diagnosis in mitochondrial disease is fraught with challenges related to the clinical and genetic heterogeneity of these disorders. Created with BioRender.com.

31]. KSS summarizes many clinical findings (DD, developmental regression, ID, dementia, myopathy, ptosis, chronic progressive ophthalmoplegia, hearing loss, heart conduction defects, gastrointestinal issues, faltering growth, disturbed glucose homeostasis etc.) and different constellations of these clinical features, of varying severity, are seen in patients presenting at different ages [27]. The signs and symptoms often expand and progress over time, and in general, patients with haematological involvement have worse survival [27].

2.4.1. Phenocopies: many genotypes - one phenotype

Conversely, each clinical presentation can result from a defect in one of multiple genes (phenocopies), as best exemplified by Leigh syndrome spectrum (LSS), for which 113 gene-disease-associations (97 nuclear, 16 mtDNA) have been established to date [32]. Leigh syndrome is the most common MD in children and is diagnosed based on 1) the presence of bilateral, typically symmetric, T2 hyperintensities in brainstem and/or basal ganglia seen on magnetic resonance imaging (MRI); 2) at least one of the following: DD, developmental regression, encephalopathy, or psychiatric symptoms; and 3) one biochemical abnormality (e.g. elevated lactate in blood or cerebrospinal fluid, or OXPHOS enzyme activity deficiency) [32]. Consensus now favours using the term LSS instead, as cases that do not fulfil all criteria naturally exist.

2.4.2. Mitochondrial disease mimics - a special type of phenocopies

Phenotypic complexity and multisystem involvement should raise suspicion for a possible underlying MD but are also seen in other monogenic non-MDs. Pathogenic variants in one of the 373 mitochondrial disease genes are only found in half of the patients with suspected MD in whom a genetic diagnosis is reached with ES [16,33]. Examples of MD mimics include *RANBP2*-associated infection-triggered recurrent necrotizing encephalopathy, *SCN1A*-related epilepsy (Dravet syndrome) or *VPS13D*-related ataxia and spasticity [16,34,35].

2.5. What is in the name? - A proposal for a precise nomenclature

The complexity underpinning genotype-phenotype associations highlights the need for a precise and descriptive nomenclature, forgoing the use of acronyms and eponymous names in favour of a more systematic description of the gene involved, nuclear variant zygosity (i.e. monoallelic or biallelic) or mtDNA variant heteroplasmy, and the main phenotypic features. Illustrative examples are provided below.

- 8-month-old male with biallelic *POLG*-related MD with DD and recurrent status epilepticus and recurrent acute liver failure
- 16-year-old female with m.3243A > G-related MD (81 % heteroplasmy in blood) with ID, hearing impairment, and ataxia, without SLE
- 5-year-old male with *NDUFA2*-related MD with LSS characterised by mild DD, recurrent infection-triggered deterioration with loss of skills and slow recovery, generalized muscular hypotonia, intention tremor and ataxia, and symmetric brainstem T2 hyperintensities on MRI

2.6. Genetic underpinning of mitochondrial disease

2.6.1. Mitochondria are under bigenomic control – nuclear DNA and mitochondrial DNA

Childhood-onset MDs are, in about 80 % of cases, caused by pathogenic variants in nuclear genes, and conversely, variants in mtDNA account for 65–80 % of MDs with adult presentation (own unpublished data [36,37]). With only 13 proteins synthesized within the mitochondria itself, healthy mitochondrial function heavily relies on the contribution of over 1100 proteins encoded in the nuclear genome that are translocated to the mitochondria by sophisticated import systems [38]. To date 335 nuclear and 37 mitochondrial encoded genes have been associated with MD (Fig. 1).

2.6.1.1. Nuclear DNA. The group of MDs with complex I (CI) deficiency, affecting the first enzyme complex of the mitochondrial respiratory chain, illustrates the complexity of the genetics underlying MDs. CI deficiency, which often leads to LSS, was already the most common MD diagnosis of childhood in the “muscle biopsy era” and remains so in the current genomic era (Fig. 3). CI is one of the largest known human protein complexes. It is composed of 45 subunits, only 7 of which are encoded by mtDNA genes, and its correct assembly requires at least another 15 nuclear-encoded proteins [39,40]. Of note, isolated CI deficiency in muscle or fibroblasts is commonly observed in many cases of suspected MD that remain undiagnosed [41]. This likely points to secondary damage affecting this large enzyme complex (e.g. as a sign of severe disease in general or critical illness immobility), as well as vulnerability to pre-analytical problems affecting measurement of CI’s activity (e.g. repeated freezing and thawing), and presumably to a lesser extent, the influence of novel disease genes that remain to be discovered.

2.6.1.2. Mitochondrial DNA. Human mitochondria harbour their own genome, mtDNA, consisting of a small circular double-stranded DNA molecule [1]. Unlike the nuclear genome, which is only present in two copies in a post-mitotic cell, each cell contains hundreds to thousands mtDNA copies [1]. Human mtDNA is maternally inherited. Intriguingly, reports of biparental inheritance challenge the notion of strict maternal transmission of mtDNA to the offspring, although these exceedingly rare findings remain the exception and not the rule [42–44].

Pathogenic variants in mtDNA include single nucleotide variants as well as single large-scale deletions/rearrangements. The integrity of mtDNA can also be affected by multiple deletions arising in the context of primary defects in one of over 30 nuclear genes that control mtDNA maintenance. This can also result in reduced mtDNA content (mtDNA depletion). For example, pathogenic variants in the nuclear gene *POLG* account for the most prevalent mtDNA maintenance defect in childhood [5,45].

2.6.1.3. Heteroplasmy of mitochondrial DNA variants. The coexistence of mutant and wild-type mtDNA molecules within a cell, a situation termed heteroplasmy, further complicates interpretation of mtDNA variants. Although patients with severe disease tend to have a high heteroplasmy up to homoplasmy, the relationship between heteroplasmy and clinical phenotype is not straightforward, and often a certain “threshold” has to be exceeded for the disease to manifest [46]. The mtDNA m.3243A > G variant is the most common heteroplasmic mtDNA disease genotype, and broad intra-familial phenotypic heterogeneity is reported [47] Detecting low-level heteroplasmy in a sample that is easily accessible (blood, urine, fibroblasts, skeletal muscle) might not always reflect the true burden of disease, especially if higher proportions of the mtDNA variant are present in clinically relevant but less accessible tissues (brain, liver or cardiac muscle) [48]. Moreover, the tissue-specific heteroplasmy levels can change over time, with blood heteroplasmy levels of the m.3243A > G variant decreasing exponentially over the lifespan of an individual [47]. This is contrast to e.g. the m.8993T > G/C variant with nearly no tissue and age-related variation [49].

2.6.2. Various modes of inheritance

All modes of inheritance have been reported in MD (maternal, X-linked, autosomal recessive, autosomal dominant (Fig. 1) and variants regularly arise *de novo* even at recurrent positions [20,50]. For 23 nuclear genes, both monoallelic and biallelic forms of the associated disease occur [17] (Fig. 1), as is the case, for example, in *CLPB*-related MD resulting in overlapping phenotypes centred on a spectrum of neurological symptoms (e.g. DD/ID, epilepsy, brain atrophy), in addition to neutropenia and 3-methylglutaconic aciduria. Cataracts also feature in the phenotype of affected patients but, to date, have only been described in those with biallelic variants [51,52].

Although the phenotypic severity of monoallelic and biallelic forms

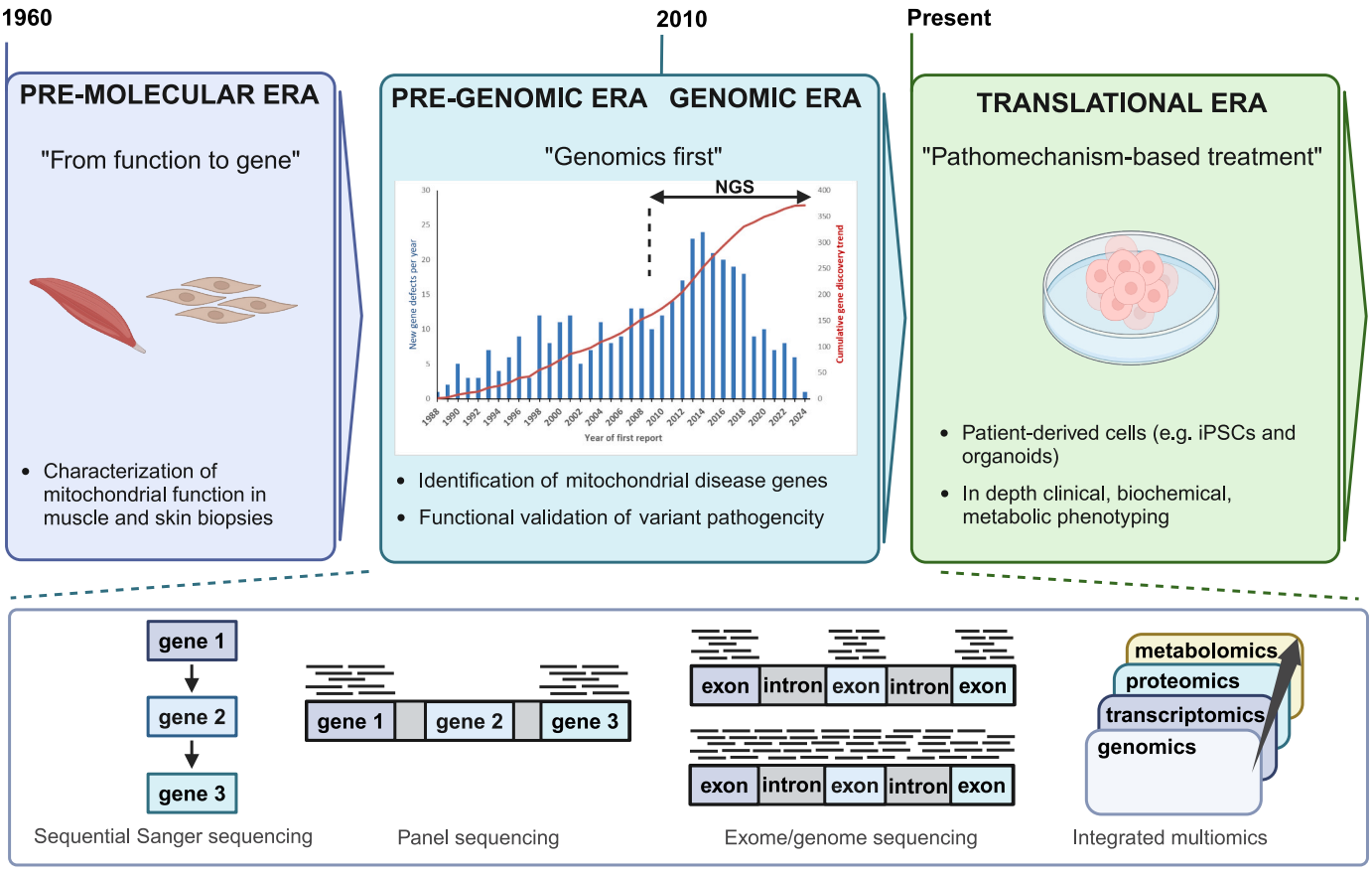

Fig. 3. Eras of mitochondrial medicine.
Schematic timeline of predominant eras of research focus in mitochondrial medicine. Created with BioRender.com.

of a disease can vary, the same organ systems are typically affected [52–54]. Yet, variants causing monoallelic disease are not seen in relation with the biallelic forms [52–54]. Interestingly, most of the proteins encoded by these genes participate in oligomeric structures. Thus, depending on the nature and location of the pathogenic variant, some monoallelic variants may exert a dominant negative effect, while other biallelic variants may have ensuing dosage reduction effects.

3. Diagnostic approach for suspected childhood-onset MD

The most important consideration when discussing the optimal diagnostic approach for suspected MD is to consider the diagnostic yield, the available infrastructure and experience, turn-around-times, as well the cost-effectiveness. In general, discussing or referring a patient with suspected MD to the nearest MECs can facilitate timely diagnosis.

3.1. The changing landscape of MD diagnostics - from muscle biopsy via next generation sequencing to pathomechanism-based treatment

Mitochondrial disease diagnostics have traditionally been laborious and predicated on immunohistochemical and/or biochemical investigations of mitochondrial function in biopsied tissues (e.g. muscle or skin) as the gold standard [3]. Again, CI-related MD can serve as a good example. Traditionally, biochemical confirmation of CI deficiency guided targeted molecular genetic investigations of known CI subunit-encoding genes, which for practical reasons usually started with mtDNA analysis before sequencing individual nuclear genes one by one. This sequential approach was invasive, time consuming and expensive, resulting in protracted and often inconclusive diagnostic odysseys with a diagnostic yield of only 10 % [55]. Indeed, as outlined earlier, CI deficiency can well be unrelated to MD. Furthermore, muscle biochemistry and histology may be normal in the absence of significant OXPHOS enzyme deficiencies, as can occur with defects in mitochondrial maintenance, fusion/fission, translation, transcription or abnormalities in membrane integrity or transport.

Thanks to technological and bioinformatics advances over the past decade, next generation sequencing (NGS) techniques have become established into routine clinical practice, and in present-day practice, the diagnostic paradigm for MD has shifted to a first-line genomic approach [3] (Fig. 3). With this diagnostic approach, muscle and/or other tissue biopsies are only considered secondarily if targeted functional testing is needed to help substantiate a variant of unknown significance (VUS), or if further untargeted investigations are warranted when a diagnosis remains to be identified after NGS [3] (Fig. 4).

3.2. The importance of phenotyping

3.2.1. Clinical phenotyping - any symptom in any organ or tissue

Clinical phenotyping remains indispensable to suspecting a possible underlying MD and is a skill that cannot simply be replaced by a genomic test. Evaluating the variety of symptoms experienced, symptom onset, evolution over time, and association to potential triggers (e.g., febrile infections) is key, as is family history.

Common non-specific neurological presentations include DD, ID, muscular hypotonia/generalized weakness, exercise intolerance, seizures, and encephalopathy. Myopathy is rarely found in isolation in childhood-onset MD, but is often part of a multisystem disorder, with the important exception of the early infantile reversible, purely myopathic m.14674T > C/G-related MD that should not be missed [56,57]. Listing the range of possible clinical findings is of little utility and beyond the scope of this review. Suffice to say, every single cell-type (with exception of mature red blood cells [58]), every tissue, and every organ depends

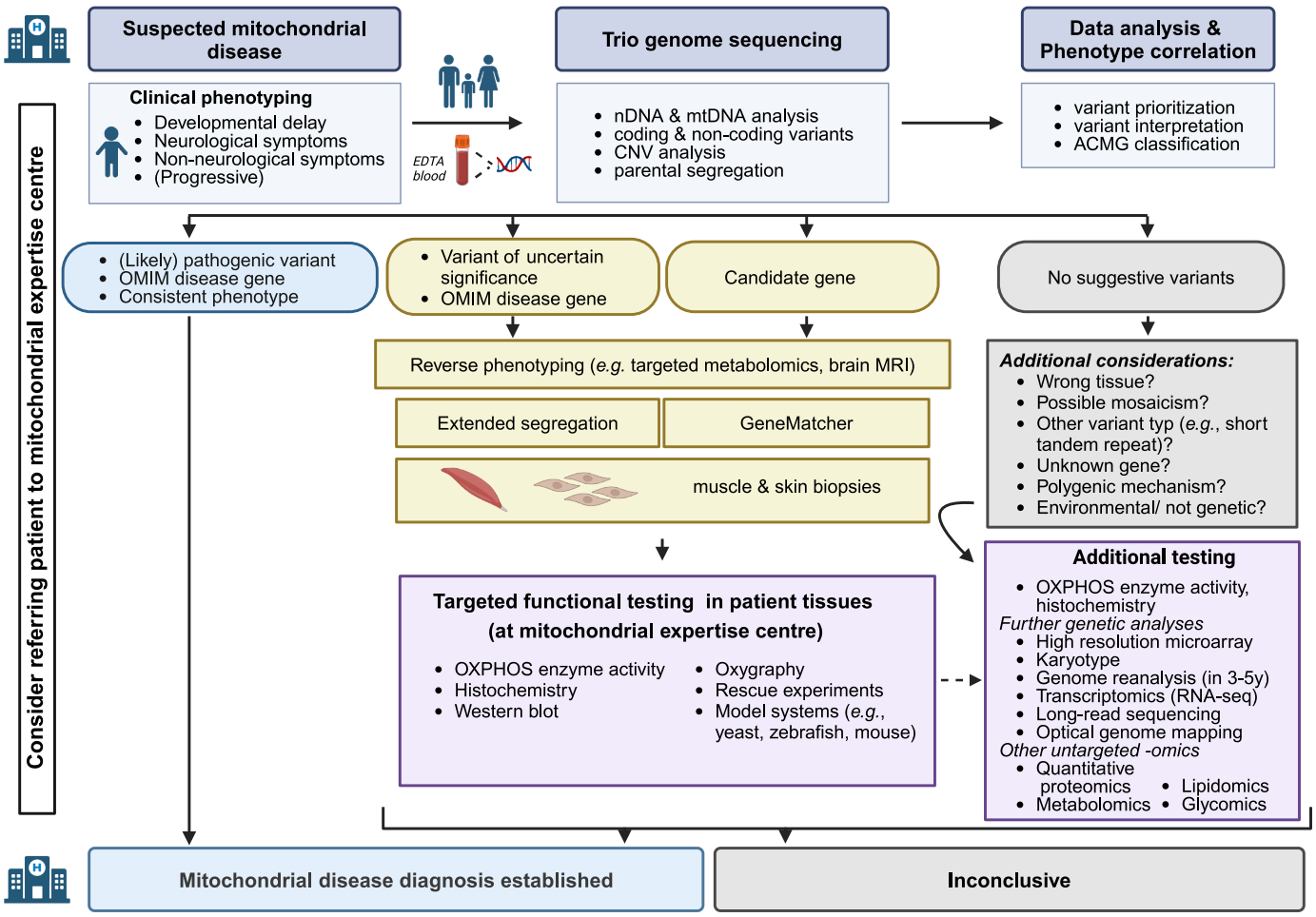

Fig. 4. Diagnostic flowchart for suspected paediatric mitochondrial disorders.
Schematic showing first-line genomic testing approach. Referring patients to MECs for assessment should be considered at any stage of this process. Created with BioRender.com.

on mitochondrial energy metabolism and can thus be affected, resulting in variable clinical phenotypes. Signs and symptoms related to the central nervous system (CNS) predominate (DD/ID, tone abnormalities, movement disorder, neuropathies, epilepsy/SLE, optic atrophy, retinitis pigmentosa, hearing loss etc.), with those related to skeletal muscle and, to a lesser extent, cardiac muscle also accounting for the most energy consuming organs in the body. Of note, unlike erythrocytes, erythroblasts still possess a nucleus and intact mitochondria [59]. Pathogenic variants in nuclear or mitochondrial DNA affecting these immature cell types can thus result in sideroblastic anaemia, a feature of large-scale mtDNA deletion-related MD (this phenotype characteristic of Pearson syndrome [21]) or *PUS1*-related MD [60], for example.

Consensus mitochondrial disease criteria (MDC) have been proven to facilitate mitochondrial diagnostics in children and provide a useful screening tool based on clinical features (muscular, neurological and multisystem involvement), metabolic abnormalities (e.g. high lactate) and findings on neuroimaging [33,61]. The composite score (out of 8) can thus help stratify patients according to their likelihood of having a MD, defined as possible (2–4), probable (5–7), or definite (8) [61]. Some versions of the MDC also account for the presence of histological anomalies (for a maximum total score of 12), although the simplified version is more useful at the bedside and can also guide the decision to carry out a muscle biopsy [61]. Even in the current NGS era, this scoring system still offers a reliable tool to predict disease [33]. A high MDC score increases the odds of finding a genetic diagnosis by ES, and conversely, if in a patient with a high MDC score ES analysis reveals a compatible variant in a mitochondrial disease gene, then this is likely to be a pathogenic finding [33].

3.2.2. *The role of biomarkers*

Depending on the phenotypic presentation, acylcarnitine profiles (e.g. in *SUCLA2*- [62] or *ECHS1*- [63] related MD) or urine organic acids (e.g. 3-methylglutaconic aciduria in *CLPB*- or *SERAC1*-related MD [64]) may help modify the index of suspicion, but their diagnostic value is limited. Repeatedly elevated lactate is certainly one of the better markers for MD in children, but one has to be aware that it is sensitive to pre-analytical issues (e.g. tourniquet effect), can be absent, and also does not correlate with disease severity [65]. Other markers like Fibroblast growth factor-21 (FGF-21) or neurofilament light-chain (NF-L) have currently very limited place in clinical routine diagnostics or follow-up.

3.2.3. *The role of brain MRI, MRS*

MRI and MR spectroscopy (MRS) are valuable tools in the work-up of suspected MD, and imaging-based genotype-phenotype correlations exist. Bilateral symmetric T2-weighted hyperintensities of the basal ganglia and brain stem are a diagnostic prerequisite of LSS, but leukoencephalopathy is also seen in MD [66,67]. Both can evolve over time, but, importantly, patterns on brain MRI do not correlate with MD severity, and MRI pattern seen in MDs may be observed in MD mimics (e.g. hypoxic ischaemic encephalopathy, *RANBP2* and *VPS13D* related disorders) [68]. Ultimately, MRI pattern recognition requires highly specialized expertise and NGS will still be required to unravel the exact

genotype. Hence, the role of neuroimaging in MD diagnostics has shifted away from a first-line diagnostic test to a supportive role that can aid variant interpretation and reverse-phenotyping. An important exception, of course, would be in the investigation of paediatric acute encephalopathy as occurs in *SLC19A3*-related MD (Biotin-thiamine-responsive basal ganglia disease [69,70]), in which case early cranial neuroimaging has a more prominent role in the initial diagnostic workup and should be performed in accordance with local evidence-based guidelines even when MDs are suspected [71,72].

3.3. Genomic sequencing approaches in MD

3.3.1. Advantage of exome and genome sequencing over gene panel sequencing, multiple molecular diagnoses, and digenic disease

The unbiased approaches of ES and GS in children with suspected genetic disorders have superseded the use of conventional testing with single gene or targeted panel sequencing, both in term of diagnostic yield and cost-effectiveness [73]. Targeted panel sequencing has the inherent inability to account for variants in novel disease genes or in genes responsible for MD mimics that are not included in the panel, as opposed to ES and GS, which can even facilitate detection of multiple molecular diagnoses in a single individual, thought to underlie complex phenotypes in about 5 % of cases [74]. Many genomic sequencing laboratories now apply 'virtual panels' to the ES/GS backbone to assist with variant prioritisation.

Another interesting aspect is the increasing knowledge about polygenic factors influencing the MD phenotype. Digenic inheritance is the simplest instance of a non-Mendelian disorder, characterized by the functional interplay of variants in two disease-contributing genes, and was recently reported for MDs. In patients with *DNAJC30*-related MD, the first form of nuclear-encoded Leber hereditary optic atrophy (LHON) to ever be described [75], a subgroup of patients presented with LSS and were found to harbour a pathogenic monoallelic variant in a CI-related gene in addition to the biallelic *DNAJC30* variants. The same was recently reported in patients with the m.11178G > A LHON variant who presented with LSS [76].

3.3.2. Diagnostic yield of exome and genome sequencing in suspected MD

Over the past decade, studies evaluating the utility of ES in cohorts of children with suspected MD, have reported diagnostic yields ranging from 35 % to 70 % [77]. Comparing these studies remains challenging, because of differences in study design, cohort size, case ascertainment, genetic testing done prior to ES, knowledge of MD genes, availability of population databases like the Genome Aggregation Database (gnomAD [78]), and the variability of genetic testing strategies used (singleton or trio ES, with/without mtDNA analysis and/or copy number variant (CNV) analysis).

In general, higher diagnostic yields are reported in highly selected cohorts in whom careful clinical and biochemical characterization has been undertaken beforehand (reviewed in Ref. [77]).

Although the mtDNA can be analysed from ES data [79], the utility of ES for mtDNA diagnostics is limited due to a failure to achieve very deep coverage, thus potentially negating the identification of low-level heteroplasmic variants. This perhaps is of less concern in affected children who are generally more likely to harbour causative nuclear DNA variants with higher levels of heteroplasmic mtDNA in blood compared to adult patients [47]. Another disadvantage is that ES is not suited to reliably detect single or multiple mtDNA deletions.

GS is not often performed as a first line test and is thought to contribute a diagnostic uplift of ~5 % after ES [80]. In mixed cohorts of children and adults with suspected MD who were evaluated with first-line GS *before* any other testing, the reported diagnostic yield was 35–55 % [37,81]. It is unknown how many and which MD genes are more often affected by intronic variants, one example being *TIMMDC1*-related MD where several cases with recurrent intronic variants have been reported [82]. It is also unclear how often CNV are seen in relation to MD, but they are certainly frequent in MD mimics.

Taken together, GS will likely replace ES in the near future, given the more rapid turn-around-time as no enrichment is needed, the greater coverage, and the first studies showing cost-effectiveness for this approach [83].

3.3.3. Limitations of current genomic diagnostics for MD and how to overcome them

3.3.3.1. The VUS pandemic, and how to flatten the curve with functional testing. The advent of NGS has exposed clinical diagnostic laboratories with the daunting task of having to evaluate variants as pathogenic or benign across the genome. This task can be supported by guidelines set by the American College of Medical Genetics and Genomics (ACMG [84]) These guidelines are necessarily conservative to minimize false positive identification of a benign variant as pathogenic. An unfortunate outcome of this stringency is that many variants are categorized as VUS in the ClinVar database [85], and thus remain clinically non-actionable. The VUS annotations that also include conflicted annotations have recently grown nearly 11 times faster than the (likely) benign or (likely) pathogenic. Both computational methods that help predict variant severity (considered 'supporting' evidence) and functional studies that test the effect of a variant in the laboratory (providing 'strong' evidence) can aid in 'de-VUSing' (reviewed in Refs. [86,87]). This highlights a clear need for tests to delineate rapidly, inexpensively, and accurately pathogenic from non-pathogenic variants. Functional testing using traditional focused biochemical assays to characterize mitochondrial function in tissue biopsy samples (Fig. 3) are key for this - now and in the future, with an ever-increasing number of VUS likely to be detected with GS. It is therefore of utmost importance that these techniques remain available in the laboratories at the MECs and be offered not only within a research context but as accredited tests with defined turn-around-times that are covered by the health care system.

3.3.3.2. How to proceed after negative exome. A negative genomic result in blood does not necessarily rule out the possibility of an underlying MD, and in contrast to other monogenic diseases (e.g. those causing isolated DD), functional testing in biopsied tissue can still be carried out if a MD is still suspected (Fig. 4). Although the rate of novel gene discoveries for MD has considerably slowed over the past 5 years, new gene-disease associations undoubtedly remain to be discovered considering the discrepancy between the >1100 nuclear-encoded proteins known to be involved in mitochondrial function and the currently known 373 MD genes. For this reason, periodic reanalysis of genomic data in unsolved cases is recommended every 3–5 years [88]. A negative blood-based ES or GS test result in an individual with suspected MD might also warrant further investigation in more appropriate tissues. Some pathogenic variants such as large-scale mtDNA deletions, for example, can be restricted to skeletal muscle in rare cases [89,90]. Finally, long-read sequencing will offer a promising alternative in the near future. It has the advantage of being able to measure repeat expansions, resolve structural variants often clustering around repetitive areas of the genome, and can detect epigenetic DNA and RNA modifications [91].

3.3.4. Integrative multi-omics

Advances in bioinformatics and high-throughput technologies have led to a surge in the development of unbiased –omics platforms that can offer deeper insight into underlying pathomechanisms. While (un)targeted **metabolomics** is replacing traditional "metabolic screening" that consists of separate tests, other techniques like **lipidomics** have mainly proven value for the functional validation of VUS [92].

RNA-sequencing is particularly useful to investigate the tissue-specific impact of splice variants in DNA and can also provide relative quantification of altered transcript levels (reviewed in Ref. [93]). For

MD, RNA-sequencing carried out in fibroblasts of ES-unsolved patients provided a diagnosis for 10 % of undiagnosed cases, including the discovery of a new MD gene, *TIMMDC1*, which encodes a complex I assembly factor [82]. Here, fibroblasts of two unrelated affected individuals showed aberrant expression and splicing leading to a 'poisonous exon' in the *TIMMDC1* transcript caused by a deep-intronic variant that was not detected by ES [82].

Quantitative **proteomics** offers functional insight into the consequence of genomic variants that result in degradation of the defective protein. This aided the discovery of the LSS disease gene *PTCD3*, where quantitative proteomics confirmed PTCD3 depletion and revealed the importance of this protein for mitochondrial translation and respiratory chain function [94].

As each of the individual -omics technologies continue to develop and eventually transition from research-based to clinical settings, integrated multi-omics analyses have the potential to improve our understanding of disease. This will also help stratify patients more objectively based on their multi-omics signatures and holds promise for identifying therapeutic targets in disease-relevant pathways.

4. Management

Management of childhood-onset MD is challenging. Reasons for this are multifactorial but ultimately come down to the fact that only small numbers of patients with the same genotype-phenotype-association are known, often with fluctuating but seldom comparable courses of disease. This explains why the natural histories of the different MDs are poorly defined. Consequently, validated outcome measures that are responsive to change are unavailable. Nevertheless, several general and more targeted approaches are of particular relevance for neuropaediatricians caring for this patient group. In general, many treatments used for patients with MD are no different from those used for other neurological diseases, and we therefore do not detail the management of constipation or sleeping disturbances, for example. For information on the general use and safety of medications in patients with MD we refer to Ref. [95]. As general anaesthesia poses a potential risk for decompensation in children with MD, assiduous perioperative management to avoid additional metabolic stress is essential. Both inhalational and intravenous GA agents have been used with success in this population [96]. Propofol can also be used with caution during induction anaesthesia (and even as

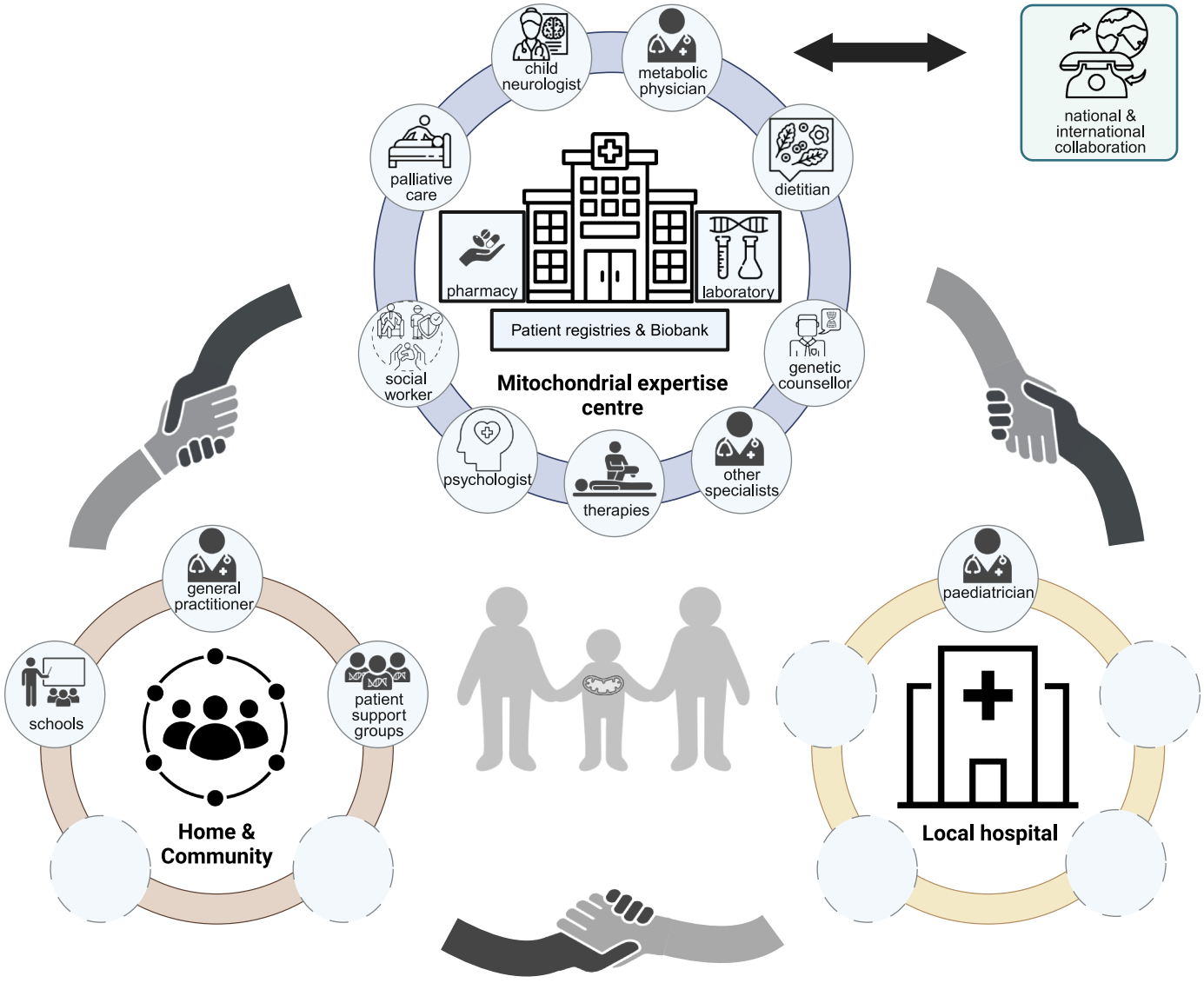

Fig. 5. Mitochondrial expertise centres and integrated care coordination.
Schematic highlighting the patient-centric approach to multi-disciplinary care coordination within mitochondrial expertise centres (MECs), with local hospital and community supports. Dotted circles reflect variation in the services available at local hospitals and within the community. Created with BioRender.com.

infusions of duration up to 48 h in refractory status epilepticus), being aware that prolonged infusions can cause severe lactic acidosis, rhabdomyolysis and cardiovascular collapse (propofol infusion syndrome) [95].

4.1. General considerations

4.1.1. Importance of mitochondrial expertise centres (MECs)

In the absence of a cure, multidisciplinary supportive care is all the more important to reduce morbidity and mortality, and to improve quality of life for patients and their families. Such models of complex care can be efficiently coordinated within specialized MECs (Fig. 5), as similar models of care used for other groups of disorders have been associated with improved patient outcomes [97]. In Europe, the European Reference Network for Hereditary Metabolic Disorders (MetabERN) ensures that these centres meet specific standards required to provide a high-quality service. This involves availability and good communication between experienced medical specialists as well as psychologists, pharmacists, dietitians, physiotherapists, occupational therapists, speech therapists, social workers, and a dedicated palliative/comfort care team. Standardized pathways for liaising with community-based services is also essential for empowering patients and their families, as is updating them with relevant current knowledge of advances in the field. Dedicated biochemical genetic laboratories are also embedded within these MECs, offering state of the art diagnostics including genetic testing and functional investigations. This not only allows for timely and reliable diagnostics, but also enables access to research avenues for a better understanding of the underlying pathomechanisms and development of targeted treatments. Of note, given the genetic heterogeneity of MDs and the multiple inheritance patterns, precise genetic counselling is of utmost importance. Another important duty of MECs is to curate a biobank where patient samples can be securely stored for use in future investigations. These centres also play an important role in establishing patient registries, allowing highly sought-after natural history studies to be carried out, and positioning themselves to be "trial ready" for upcoming therapies. Finally, a willingness to pool resources and to collaborate with other (inter)national centres, is also key.

4.1.2. Nutrition including ketogenic diet therapy

Children with MD regularly show feeding problems, failure to thrive, faltering growth, and/or gastrointestinal dysmotility [98]. Furthermore, mitochondrial energy production was shown to correlate with the age-related BMI [99]. Several small studies investigating adult and paediatric MD patients reported inadequate nutritional intake [100,101] establishing an association between body composition, physical functioning and protein intake. Finally, adults with the m.3243A > G variant [102], as well as children and adults with different MDs, were shown to profit from individually tailored dietary intervention especially with regard to body composition, handgrip strength, gastrointestinal complaints, quality of life and fatigue [103].

The ketogenic diet therapy (KDT) is a high fat, low-carbohydrate, and balanced protein diet that stimulates mitochondrial beta-oxidation and ketone body production for use as an alternative source of energy mainly by the central nervous system. It is the pathomechanism-based treatment for PDHc deficiency, bypassing the enzymatic defect [104]. The long-term efficacy and safety of the KDT in children with PDHc deficiency has been highlighted in a longitudinal study [105]. The greatest efficacy was observed in those with infantile or childhood presentation of disease, and when ketosis was sustained. Improvements in seizure frequency, ataxia and sleep were noted, together with increased alertness and improved behavioural outcomes [105]. This argues for the earliest possible introduction of KDT following the diagnosis of PDHC deficiency. Of note, pyruvate carboxylase deficiency (or *PC*-related MD) needs to be ruled prior to commencement of KDT, as gluconeogenesis in this condition is impaired and affected individuals depend on nutritional glucose [1]. Another contraindication for KDT are myopathies with multiple mtDNA deletions and ragged-red-fibres (e.g. *TWNK*-related MD) where patients can experience progressive muscle pain and rhabdomyolysis due to ragged-red-fibre damage. Interestingly an improvement in muscle strength was noted after two years of follow-up, suggesting activation of muscle regeneration [106]. For other MDs, a systematic review reported that data on the safety and efficacy of the KDT remains too limited to draw general recommendations [107]. Nevertheless, KDT was shown to be highly effective for seizure control and also reversed other clinical phenotypes like cardiomyopathy or movement disorder, as well as improved muscular symptoms [107]. Furthermore, a recent prospective, open-label, controlled study in children with MD and epilepsy found KDT to be effective for seizure control, particularly for patients with pathogenic mtDNA variants [108]. Adverse effects of KDT mainly relate to gastrointestinal complaints, but rhabdomyolysis can also occur in patients with mitochondrial DNA deletion(s) as a serious adverse event. Accordingly, KDT is a highly individualised management option in this fragile patient group and requires an experienced team at a specialized centre [107].

4.1.3. Exercise

Exercise intolerance is common in patients with MD and it is important to encourage physical activity nonetheless. A randomized controlled trial assessing the impact of aerobic endurance training in adults with mitochondrial myopathies, showed that such rehabilitation programs have a beneficial effect on exercise capacity (e.g. endurance performance, walking distance and muscle strength) and clinical symptoms [109], with similar findings in several other smaller studies [110–112]. In children with mitochondrial myopathies, the success of physical training schedules is less obvious, partly due to the challenges of maintaining motivation, particularly in those of young age and/or with behavioural problems [113]. Nonetheless, a small pilot study conducted in five children (>4 years old) with MD who participated in aerobic exercise training over 6–18 months, reported no significant disease progression in any of the participants, with improvements in exercise tolerance noted in one motivated individual [113].

4.1.4. Immunizations

The potential harmful impact of vaccine-preventable infections in medically vulnerable children is well known and can reasonably be extrapolated to children with MDs. While immunizations may cause similar, typically mild, inflammatory and metabolic responses, there is no evidence that immunizations exacerbate MD manifestations. However, there is increasing parental concern about vaccines in general and covid-19 vaccines in particular [114,115]. Nevertheless, a survey of parents of 95 children with MD found that adverse events following immunization were as frequent and benign as in the general population, and importantly no metabolic deteriorations or epileptic seizures were observed [116].

4.2. Disease-specific considerations (with importance for the paediatric neurologist)

4.2.1. The mitochondrial cocktail

Treatment of MD has traditionally involved prescribing a regimen of enzymatic co-factors (e.g. thiamine, riboflavin), antioxidants (e.g. coenzyme Q10) and other food supplements (e.g. carnitine), in variable combinations and dosages often based on the physician's gut feeling. These aim to improve enzyme function, reduce oxidative stress, support alternative energy production pathways, and remove toxic metabolites, to support cellular function during times of metabolic stress and prevent clinical decompensation. A Cochrane review of these therapies found no evidence supporting the use of any vitamin or cofactor intervention and there is hence no evidence backing the use of such "mitochondrial cocktails" [117]. Here again, one needs to realize that one single approach is unlikely to treat all the different types of MD. Rather,

knowing the causative genotype in many MD patients allows for targeted pathomechanism-based treatments. Examples of such are shown in Table 1, along with references for dosing guidance. Ideally, any intervention should be evaluated by means of a double-blinded n-of-1-trial to reach the highest evidence for or against treatment. In this regard, MECs have an important role in maintaining an updated knowledge base and providing these compounds in sufficient quality and quantity via their pharmacy (Fig. 3).

4.2.2. *Treatment of epilepsy including stroke-like episodes and a note on neuroinflammation*

Epilepsy may be the presenting feature of MD, with seizures occurring in 20–60 % of affected children depending on the underlying aetiology. All seizure semiologies may occur in children from focal to generalized. Seizures can be difficult to treat and may develop into drug-resistant epilepsy [130]. Evidence-based guidelines based on a recent systematic review and Delphi consensus expert opinion endorsed the adoption of the National Institute for Health and Care Excellence (NICE [131]) for the management of seizures and status epilepticus in MD [130]. Accordingly, levetiracetam may be trialled as first-line monotherapy for focal, generalized or myoclonic seizures in children (>28 days). In general, most of the anti-seizure medications that were reviewed for use in MD patients were considered not to be contraindicated. The notable exception relates to patients with *POLG* variants and/or individuals with liver impairment, in whom sodium valproate is absolutely contraindicated due to ensuing hepatotoxicity [95,130]. Importantly, this contraindication of valproate holds true only for *POLG*-related MD but not for all other MDs [132].

Stroke-like episode is a paroxysmal neurological manifestation thought to be neuronal hyper-excitability that affects a specific group of patients with MD, mostly adults, and children only in rare cases. The management of SLEs should focus on aggressive seizure management [133]. A systematic review found no robust evidence to prove the efficacy of l-arginine for both acute and prophylactic settings [134].

Mitochondrial diseases can also mimic neuroimmunological diseases like autoimmune encephalitis or acquired demyelinating syndrome (at first presentation) and may even respond to intravenous methylprednisolone and/or intravenous immunoglobulin (initially) [135–137]. Guidance in this regard from an experienced paediatric neurologist or epileptologist is highly recommended. Of note, there is increasing evidence that neuroinflammation and mitochondrial dysfunction are linked, and that immunotherapies targeting this pro-inflammatory response may have a beneficial role [138,139].

5. Conclusion and perspectives

Despite the current lack of curative treatment for MDs, it is encouraging nonetheless, to reflect on the historical arc of mitochondrial medicine for a greater appreciation of how far this field has come (Fig. 3). The first reports linking mitochondrial dysfunction to disease emerged in 1959–62, when the association between abnormal mitochondrial morphology in muscle, a biochemical uncoupling of mitochondrial respiration from ATP synthesis, and a euthyroid hypermetabolic state was first described in Luft disease [140]. This spawned the pre-molecular era of mitochondrial medicine, with a predominant focus on the use of histochemical and biochemical assays in muscle tissue to characterize abnormal mitochondrial morphology and function [141,142]. The molecular era started thirty years later, from 1988, when distinct types of pathogenic variants in mitochondrial DNA were first linked to some of the now widely recognized MD syndromes, including LHON [143] or KSS [30,31]. This opened the floodgates for discovery of new gene-disease associations, initially through sequential gene sequencing and mtDNA sequencing, and even more so over the past 15 years with the advent of NGS (as reviewed in Ref. [77]). Indeed, the yearly rate of new gene disease discovery increased five-fold in the NGS era, peaking at ~25 new disease genes in 2014 (Fig. 3). However, with most of the "low-hanging fruit" now picked, the rate of new gene defect characterization has markedly fallen over the past decade. The

Table 1
Examples of mitochondrial disease and pathomechanism based treatment.

Disease name (Mendelian Inheritance in Man (MIM) number)	Affected gene(s)	Suggestive clinical features	treatment option	Reference
ACAD9-related MD, ACAD9 deficiency (#611126)	*ACAD9*	Hypertrophic cardiomyopathy, myopathy	Riboflavin	[118]
Primary coenzyme Q10 deficiency (#607426, #616276, #614650, #616733, #612016, #615573, #614654, #614651, #614652)	*COQ2/4/6/7/8A/8B/9, PDSS1, PDSS2*	Spectrum encompassing neonatal encephalopathy, nephrotic syndrome, adult-onset myopathy	Ubiquinone (response highly variable, phenotypes with adults myopathic presentation seem to respond while no convincing response is seen in pediatric onset disease)	[119]
FARS2-related MD, Mitochondrial phenylalanyl tRNA synthetase deficiency (#614946)	*FARS2*	Spectrum encompassing developmental delay, ataxia, epilepsy	L-phenylalanine (one case report)	[120]
Mitochondrial malate-aspartate shuttle-related encephalopathies including Citrin deficiency (# 618721, #617339, #612949)	*GOT2, MDH2, SLC25A12*	Developmental and epileptic encephalopathy	Ketogenic diet, pyridoxine, serine	[121, 122]
NAXE-, NAXD-related MD, Early-progressive encephalopathy with brain oedema and/or leukoencephalopathy 1 & 2 (#617186, #618321)	*NAXE, NAXD*	Episodic neurologic deterioration and developmental regression, often associated with acute illness, fever, or mild trauma	Niacin	[123, 124]
Pyruvate dehydrogenase complex deficiency (#312170)	*PDHA1, PDHB, PDHX, PDP1*	Spectrum encompassing developmental delay, movement disorder, seizures, polyneuropathy lactic acidosis	Ketogenic diet, Thiamine	[125]
SLC19A3-related MD, Biotin-thiamine responsive basal ganglia disease (#607483)	*SLC19A3*	Spectrum encompassing early infantile lethal encephalopathy, acute onset encephalopathy with dystonia, seizures	Thiamine, Biotin (response well documented in infantile/childhood onset, questionable in neonatal onset disease)	[126]
TK2-related MD, Tk2-related mtDNA depletion syndrome 2 (#609560)	*TK2*	Myopathy	Thymidine, deoxycytidine	[127]
TPK1-related MD, Thiamine pyrophosphokinase deficiency (#614458)	*TPK1*	Acute onset encephalopathy, hypotonia, ataxia, developmental regression triggered by febrile infections	Thiamine	[128]
SLC52A3, SLC52A2-related MD, Brown-Vialetto-Van Laere syndrome-1 (#211530) & −2 (#614707)/Fazio-Londe disease (# 211500)	*SLC52A3, SLC52A2*	Rapidly progressive peripheral and cranial neuropathy, muscle weakness, vision loss, deafness, sensory ataxia, and respiratory compromise	Riboflavin	[129]

increasing use of GS together with the introduction of multi-omics methodologies will likely contribute to an increased diagnostic yield, but the uplift to date has been marginal [82,144]. Nevertheless, with recent bioinformatics advances heralding the era of big data, the adoption of a multi-omics approach in MDs together with the use of patient-derived cellular models (e.g. induced pluripotent stem cells and organoids) are already deepening our understanding of underlying pathomechanisms. Against this backdrop, this field stands to benefit from a translational mindset that seeks to develop targeted and personalised pathomechanism-based treatments. In this regard, the prospect of precision medicine for MD has never seemed closer.

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Not applicable.

Consent for publication

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Availability of data and materials

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