

Review

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# New diagnostic pathways for mitochondrial disease

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## Abstract

Mitochondrial diseases collectively represent the most common cause of inherited metabolic disease. They are estimated to affect at least 1 in 8,000 adults and at least 1 in 250 adults carry a disease-causing genetic mutation. They comprise a heterogeneous group of disorders caused by mutations in either the nuclear or mitochondrial genome, which ultimately result in dysfunction of the critical cellular energy producing mitochondrial respiratory chain. Owing to the key role of mitochondria in energy production, mitochondrial disorders predominantly manifest in tissues with high metabolic demand. However, they demonstrate significant phenotypic and genotypic variability, often rendering the diagnostic process protracted and challenging. Since Luft's first description of mitochondrial disease nearly 60 years ago, substantial evolution in diagnostic techniques have simultaneously improved the diagnosis and understanding of mitochondrial disease and biology, but the standard diagnostic approach has failed to evolve at the same pace. Although sequencing technologies and analysis for the diagnosis of mitochondrial disease continue to evolve, advances to date, our expanding understanding of mitochondrial diseases and the increasing affordability of these new technologies justify a paradigm shift in the diagnostic approach. We review the progression, impact and challenges of diagnosing mitochondrial diseases and propose a minimally invasive "genetics first" approach incorporating stratification using non-invasive biomarkers, followed by non-targeted next-generation sequencing, such as whole genome sequencing. Such an approach may improve diagnostic yield and streamline diagnosis, leaving invasive investigations to address diagnostic challenges and functional validation of novel variants.



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**Keywords:** Mitochondrial disease, diagnosis, next-generation sequencing, genetics

## INTRODUCTION

Mitochondrial diseases comprise a diverse group of genetic disorders characterised by disrupted cellular energy metabolism, which may arise due to mutations in either the mitochondrial (mtDNA) or nuclear (nDNA) genome<sup>[1-4]</sup>. Collectively, mitochondrial diseases represent the most common cause of inherited metabolic disease<sup>[1]</sup>, estimated to affect at least 1 in 8000 adults<sup>[5]</sup>. However, population-based studies indicate that the prevalence of mitochondrial disease may be as high as 1 in 250 adults, with the majority of cases being under-recognised<sup>[6-8]</sup>.

Present in all nucleated cells of the body, mitochondria are dynamic intracellular organelles that are central to cellular homeostasis and metabolism. They host a variety of biochemical pathways and play a primary role in energy generation<sup>[9]</sup>. Consequently, mitochondrial diseases frequently manifest in tissues with high energy requirements<sup>[10]</sup>. Although mitochondrial diseases may present with one of many well-defined clinical syndromes, clinical manifestations are protean, ranging from single organ, mild or oligo-asymptomatic disease to severe or life-threatening multi-organ dysfunction. Moreover, symptoms and signs may overlap with more common conditions and evolve throughout an individual's lifespan<sup>[4,11-13]</sup>. Even for experienced clinicians, the vast clinical and genetic variability can render specific genetic diagnoses challenging, and the process may become a protracted “odyssey”, taking years before achieving molecular diagnosis<sup>[14]</sup>.

Mitochondrial medicine has seen substantial advances in diagnostic technologies over the last 50 years, from the pre-molecular era of histological analysis of muscle to rapidly accelerating identification of the molecular aetiologies of disease using next-generation sequencing (NGS) technologies. The notoriously heterogeneous nature of mitochondrial diseases, their individual rarity, genotypic and phenotypic variability and overlapping presentations with other genetic disorders, make them an ideal candidate group for a non-targeted approach to genetic diagnosis. Although there remain important challenges to such an approach, including optimising bioinformatic pipelines, classification and functional validation of variants and cost, early studies support their utility<sup>[3,4,15-23]</sup>. Whole exome sequencing (WES) approaches have markedly improved diagnostic yield, highlighted the genetic variability of diseases, facilitated the diagnosis of monogenic mitochondrial mimics and advanced the understanding of mitochondrial biology, opening up potential therapeutic avenues<sup>[3,4,15-23]</sup>. Whole genome sequencing (WGS) offers further potential, through unbiased, simultaneous bigenomic sequencing with improved coverage, incorporation of non-coding regions and excellent mtDNA coverage depth<sup>[24-26]</sup>.

The traditional and prevailing diagnostic approach, however, recapitulates the technological evolution in mitochondrial disease diagnosis, moving from clinical evaluation to invasive biopsy and subsequently, targeted sequencing, reserving WES or WGS for consideration in undiagnosed cases<sup>[4]</sup>. In this article, we briefly review the history of mitochondrial disease diagnosis, its evolution, impact and outstanding challenges, and propose an alternative, minimally invasive “genetics first” approach, which complements clinical evaluation with serum biomarkers for stratification, followed by exploratory bigenomic NGS. Recourse to more invasive techniques, including muscle biopsy, is reserved for aetiological uncertainty, identification of tissue-specific variants and functional validation of novel variants. Such an approach has the potential to streamline diagnosis and limit invasive investigations, without increasing costs, whilst optimising reciprocal gains in the understanding of mitochondrial biology and potential therapeutic avenues.

## AN HISTORICAL PERSPECTIVE ON THE APPROACH TO DIAGNOSIS

Techniques for diagnosing mitochondrial disease have significantly advanced since Ernster and colleagues described the enzyme activity of skeletal muscle mitochondria in 1959<sup>[27]</sup>, paving the way for their identification of Luft's first reported case of mitochondrial disease<sup>[28]</sup>. Subsequent development of the modified Gomori trichrome stain<sup>[29]</sup> allowed rapid identification of "ragged red" fibres on muscle biopsy, the first pathologic hallmark of mitochondrial disease. Clinical-histological descriptions of mitochondrial diseases ensued, and early diagnostic criteria were based on recognising a constellation of features comprising a clinical syndrome - such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) - combined with biochemical and/or histopathological evidence from muscle tissues<sup>[30-36]</sup>. This approach resulted in biases towards identified disease syndromes, and led to underdiagnosis of those with non-classical symptoms<sup>[37]</sup>.

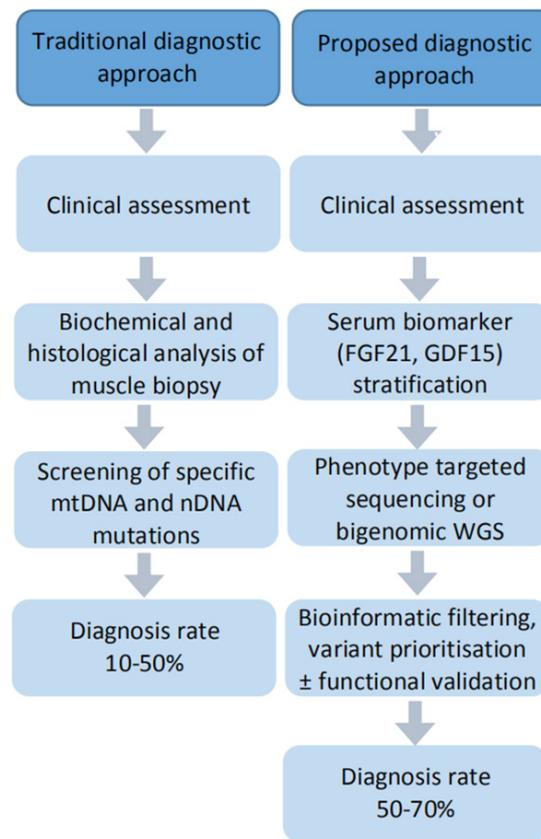
The mitochondrial genome was sequenced in its entirety in 1981<sup>[38]</sup> and the first two reports of genetic causes of mitochondrial diseases were published in 1988<sup>[39,40]</sup>. Shortly afterward, the m.3243A>G mutation was identified as the (most common) cause for the MELAS syndrome<sup>[41]</sup>. Since this discovery, more than 300 pathogenic mtDNA point mutations, deletions and rearrangements have been reported, involving almost all 37 mtDNA-encoded genes<sup>[1,42]</sup>. Although the nuclear genome encodes a vastly greater proportion of the mitochondrial proteome (~1200 genes)<sup>[43]</sup>, including over 320 genes implicated in disease to date<sup>[1,3,4,44-46]</sup>, causative nuclear gene involvement was only definitively established some years after the first mtDNA mutations were identified<sup>[47,48]</sup>. Nuclear disease plays a significantly greater role than initially appreciated, accounting for the majority of childhood-onset disease<sup>[16]</sup> and a substantial proportion of adult-onset mitochondrial disease<sup>[5]</sup>. Early tools for genetic diagnosis were limited, testing one or a small panel of common mtDNA point mutations, with poor sensitivity for heteroplasmy below ~30%-50% by Sanger sequencing<sup>[42]</sup>. Despite limitations, the advent of genetic diagnosis permitted greater appreciation of the broad spectrum and phenotypic variability associated with specific mutations and mitochondrial diseases in general<sup>[49-52]</sup>, which has continued to expand alongside improving sequencing techniques.

Technology to facilitate routine clinical genetic diagnosis did not become readily available until the mid-2000s<sup>[42]</sup> and, historically, relied on sequential Sanger sequencing of clinically prioritised individual genes - a costly, laborious and limited approach, which necessarily biased towards known genotype-phenotype correlations. Consequently, definitive genetic diagnosis was difficult to achieve and molecular diagnosis rates remained low<sup>[53]</sup>, with clinical and biochemical characterisation of greatest utility (albeit imperfect) in confirming or excluding mitochondrial disease. This embedded a "biopsy first" approach, with the clinical and biochemical phenotype guiding targeted genetic testing<sup>[4,54]</sup>. The advent of powerful, high-throughput, NGS technologies enabling simultaneous interrogation of many, or all genes<sup>[55]</sup>, has transformed genetic diagnosis. Consequently, the genetic landscape of mitochondrial diseases - and understanding of mitochondrial biology - has expanded rapidly over the last two decades, challenging established aetiological concepts of disease and clinical diagnostic approaches.

## LIMITATIONS OF THE TRADITIONAL FUNCTION TO GENE APPROACH

Numerous iterations of a diagnostic algorithm for mitochondrial diseases have been proposed. Although the complexity of the disease has precluded consensus, common is a "function to gene" approach centred on muscle biopsy: combining clinical features with biochemical and enzymatic characterisation from muscle biopsy to guide targeted genetic testing. However, there are significant limitations of this approach, prompting calls for a paradigm shift to a "genetics first" approach followed by functional validation.<sup>[54,56,57]</sup> [Figure 1](#) provides a comparative summary of these approaches.

Muscle biopsy can be a helpful diagnostic tool, demonstrating histological and - often more sensitive - ultrastructural changes, as well as providing biochemical and enzymatic information. Muscle tissue may



**Figure 1.** Comparative summary of traditional and proposed diagnostic approaches to mitochondrial diseases. Adapted from Liang *et al.*<sup>[13]</sup>

also be utilised for genetic analysis, particularly mtDNA rearrangements, deletion and depletion studies where sensitivity in blood is limited due to heteroplasmy<sup>[13,58,59]</sup>. However, technological improvements have increased the sensitivity for detecting point mutations and certain deletions in blood or urine<sup>[60]</sup>.

Biopsy is an invasive procedure, which often requires general anaesthetic, presenting significant risk to those with mitochondrial disease, and adding significant cost to the diagnostic process. Furthermore, appropriate preparation, analysis and interpretation of muscle biopsy presents many technical challenges that can impact upon results and in turn, diagnosis and care<sup>[61]</sup>. Specimen handling, transport and the varied preparation requirements are therefore critical for reliable and reproducible findings, as is interpretation by a clinician with appropriate expertise<sup>[13,37,58,62,63]</sup>. Biochemical and enzymatic analysis are best performed at an experienced laboratory with established normal criteria. As assays can vary between laboratories, inter-laboratory comparison is challenging<sup>[58,62,64,65]</sup>. Both false positive and false negative results occur; light microscopy can be normal in up to half of affected patients and findings may evolve over time<sup>[37]</sup>. Sensitivity is limited, especially for ragged red fibres, which are age-dependent, and in young children and young adults with mitochondrial disease. Specificity is also limited, with mitochondrial changes occurring in a variety of other myopathies including toxic exposures<sup>[13,63,66,67]</sup>. Ultrastructural changes identified on electron microscopy, although present in up to a third of patients in whom light microscopy is normal, may also be absent in affected patients, and can similarly be seen in various other conditions<sup>[37,63]</sup>, whilst normal respiratory chain biochemical activity in muscle does not exclude mitochondrial disease<sup>[68,69]</sup>. Although microscopic and biochemical findings from biopsy may provide strong evidence for mitochondrial disease when present, they cannot always differentiate between primary mitochondrial disease and secondary mitochondrial dysfunction, have limited utility to guide specific management and prognosis, and cannot inform genetic counselling<sup>[54,58,63]</sup>.

Clinical characterisation remains imperative - not least to identify and proactively manage organ involvement - with certain phenotypes indicative of a specific or restricted genotype, particularly amongst “classical” mtDNA-based syndromes, such as MELAS. However, even where a defined syndrome is present, genetic heterogeneity is common (e.g., Leigh Syndrome<sup>[52]</sup>). More often, clinical features do not neatly fit a specific clinical syndrome and presentations can be heterogeneous, with poor phenotype-genotype correlation and therefore, low predictive value for specific genetic diagnosis<sup>[61,70]</sup>. Muscle biopsy findings do not reliably predict specific genetic aetiologies either<sup>[71]</sup>. Accordingly, the traditional biopsy-first approach, followed by Sanger sequencing of clinically prioritised individual genes has been estimated to achieve genetic diagnosis in only approximately 11% of patients overall<sup>[53]</sup>. Further, incremental sequencing costs can exceed the costs of WES with targeted panel analysis<sup>[53]</sup> whilst the iterative process can prolong the diagnostic odyssey for individuals and reinforce diagnostic bias<sup>[14,37]</sup>.

## THE IMPACT AND CHALLENGES OF EVOLVING NGS TECHNOLOGIES

Targeted nuclear gene panels incrementally improve diagnosis compared to traditional single-gene Sanger sequencing, with rates reported between 6%-37% after mtDNA sequencing and dependent on the selected gene set and patient group, as summarised in Table 1<sup>[22,23,53,61,71-74]</sup>. However, the vast majority of patients remain undiagnosed. This approach focuses on commonly known disease genes and mutations, contributing less to the collective understanding of mitochondrial biology and disease. Unsurprisingly then, approaches utilising WES combined with mtDNA sequencing (either in advance, or incorporated into WES<sup>[75-77]</sup>) have further improved genetic diagnosis rates to between 35%-68%, depending on the selected patient group, as summarised in Table 2<sup>[15-21,23]</sup>. In one study, 31% of cases resolved through WES would have been missed using contemporaneous MitoCarta-based panels<sup>[15]</sup>. These results have included many novel disease genes and mutations, (43%-51% of cases in two paediatric studies<sup>[16,17]</sup>), thus expanding genotypic heterogeneity, whilst demonstrating greater phenotypic heterogeneity of known disease genes<sup>[3,23,78]</sup>, highlighting the shortcomings of candidate gene approaches.

NGS technologies have dramatically accelerated the identification of novel mitochondrial disease genes and mutations, with around 15-20 new genes discovered annually over the past decade and more than 350 genes across the nuclear and mitochondrial genome implicated in disease<sup>[3,4,46]</sup>. Identification and functional validation of novel gene and mutation candidates have in turn provided novel insights into mitochondrial structure, function, dynamics, and mechanisms of disease<sup>[23,79]</sup>. Even early studies evaluating NGS technologies recognised their potential to revolutionise the diagnostic process for heterogeneous disorders, such as mitochondrial disease<sup>[42]</sup>. WES or WGS are already resolving many outstanding challenges associated with mitochondrial disease genetics, in turn improving patient care<sup>[21]</sup>. However, a distinction must be drawn between routine clinical genetic testing and the ongoing interchange between research and genetic diagnosis. The latter is critical for expanding the list of known pathogenic variants, improving the understanding of mitochondrial biology and disease, enabling refinement of the diagnostic pipeline and enhancing the routine interpretation of genetic variants. This is necessary to positively impact the evolution of genetic diagnosis from here, as well as inform clinical management, family planning and potential therapeutic avenues.

A further important benefit of comprehensive, non-targeted sequencing is the identification of pathogenic non-mitochondrial disease variants: mimics and phenocopies, especially neurological disorders and neuromuscular diseases, amongst other monogenic disorders, providing definitive genetic diagnosis, and, at times, important therapeutic options<sup>[56]</sup>. Depending on the cohort selection criteria for WES studies, proportions of (solved) cases attributable to mitochondrial diseases range from 25%-89%<sup>[15,17,19-21]</sup> underscoring the clinically important overlap with other monogenic disorders. Given the broad range of overlapping disorders to be considered, necessitating multiple sequential panels, the additional cost of exome sequencing is rapidly negated - and costs continue to decrease. In contrast to targeted gene panels,

**Table 1. Diagnostic yield from NGS panel studies in mitochondrial disease**

Authors	Patients (n)	Age	Disease characteristics	Prior sequencing	Sequencing approach mtDNA (n); nDNA (n); %	Nuclear genes	Yield: overall mtDNA % (n) [of solved] nDNA % (n) [of solved]	MD % (n) of solved cases	Novel % (n) of solved cases
Schoonen et al. <sup>[52]</sup> 2019	127	Paed all Onset < 20 yo Most < 1 yo	All MD Bioc RCD	Nil	mtDNA, nDNA panel ± WES NGS mtDNA (M) (all) Panel (68%) WES (6%)	136 genes (3 custom panels)	6% (8/127); 75% (6/8) of WES 0% (0/127) 6% (8/127) [all solved]	63% (5/8 pts) 62% (8/13 var)	62% (8/13 var)
Plutino et al. <sup>[72]</sup> 2018	80	Adult predom 70% adult 30% paed	All MD Clin/bioc/histology	NS	2 step NGS NGS mtDNA screen (all) Panel: targeted (all)	281 genes (custom)	29% (23/80) 19% (15/80) [65% (15/23)] 10% (8/80) [35% (8/23)]	N/A (Only analysed MD genes)	40% (4/10 nDNA var)
Legati et al. <sup>[23]</sup> 2016	125	Paed predom 62% < 1yo	All MD Clin/bioc	mtDNA analysis Single n genes	2 step: panel ± WES N/A Panel (all); WES (8%)	132 genes (custom)	20% (25/125); 15% panel; 5% WES N/A 20% (25/125) 19/125 panel; 6/10 WES	84% (21/25)	NS
Lieber et al. <sup>[73]</sup> 2013	102	Mixed Range: 0-64 Mean: 27 yo	Def/HS 80% IS/LS 20% + control 18%	Varied mtDNA Single n genes	mtDNA + exome panel MitoExome mtDNA (all) MitoExome panel (all)	1598 genes (MitoExome, expanded)	23% (23/102); 17/18 control, 6/84 new 12% (12/102) [52% (12/23)] 11% (11/102) [48% (11/23)]	50% (3/6 new diagnoses)	17% (1/6 new diagnoses)
DaRe et al. <sup>[61]</sup> 2013	148	Paed predom 83% < 18 yo Range 0-83	Mixed Def/HS-MD 36% bioc RCD	Varied mtDNA Single n genes	Targeted exome panel N/A Panel: targeted (all)	447 genes (custom)	9% (13/148) N/A 9% (13/148) [all solved]	31% (4/13 pts)	48% (10/21 var)
Neveling et al. <sup>[53]</sup> 2013	44	Mixed Mean 11.4 Range 2-30	All MD Bioc (M/Fib)	mtDNA, POLG, ≤ 10 n genes	Targeted exome panel N/A Panel: targeted (all)	211 genes (custom)	16% (7/44) N/A 16% (7/44) [all solved]	100% (7/7 pts)	NS
Calvo et al. <sup>[74]</sup> 2012	42	Paed All NN/1	All MD Clin/Bioc RCD	NS	mtDNA + exome panel MitoExome mtDNA (all) MitoExome panel (all)	1034 genes (MitoExome)	31% (13/42) 2% (1/42) [8% (1/13)] 29% (12/42) [92% 12/13]	100% (13/13)	23% (3/13 pts)
Vasta et al. <sup>[71]</sup> 2012	26	Paed all Onset < 1 yo 88%	Mixed: Def, HS and LS-MD 2 + control	10/26 mtDNA seq	Targeted NGS panel N/A NGS panel (all)	908 genes	27% (7/26); 2/2 control, 5/24 new N/A 27% (7/26) [all solved]	71% (5/7 pts)	62% (8/13 var)

Bioc: biochemical; Clin: clinical; Def: definite; Fib: fibroblast; HS-MD: high suspicion MD; IS-MD: intermediate suspicion MD; LS-MD: low suspicion MD; LR-PCR: long range PCR; M: muscle; MD: mitochondrial disease; mo: months; RCD: respiratory chain deficiencies; mtDNA: mitochondrial DNA; n: nuclear; nDNA: not applicable; NGS: next generation sequencing; NN: neonatal; NN/I: neonatal/infant; NS: not stated; Paed: paediatric; + control: positive control; predom: predominant; pts: patients; var: variants; WES: whole exome sequencing; yo: years old

WES or WGS is applicable for most genetic disorders<sup>[60]</sup>, can incorporate a virtual gene panel initially if desired, and expand if inconclusive, improving cost-effectiveness, and can be readily re-investigated<sup>[19]</sup>.

Whilst the substantial benefits are evident, there are evolving challenges too. Vast amounts of data are generated by exome and genome sequencing. This presents important practical challenges for both sufficient, secure data storage and comprehensive and accurate analysis - particularly of the many variants of uncertain significance and novel variants requiring functional validation - as well as ethical questions pertaining to the identification and reporting

**Table 2. Diagnostic yield from WES studies in mitochondrial disease**

Authors	Patients n	Age	Disease characteristics	Prior sequencing	Sequencing approach mtDNA (r; %) nDNA (r; %)	Nuclear genes	Yield: overall mtDNA % (n) [of solved] nDNA % (n) [of solved]	MD % (n) of solved cases	Novel % (n) of solved cases
Theunissen et al. <sup>[15]</sup> 2018	117	Paed predom Onset < 18 yo 77%	MD 74% (clin/ bioc) NM 26%	Nil	2 step NGS: NGS whole mtDNA (B ± U:M) (all) WES (94/117; 80%)	Unfiltered	68% (80/117) 20% (23/117) [29% (23/80)] 49% (57/117) [71% (57/80)]	73% (58/80) 23 mtDNA   35 nDNA	NS
Puusepp et al. <sup>[20]</sup> 2018	28	Paed all Onset < 7 yo NN/I predom	Def MD 14% HS-MD 57% I/LS-MD 29%	Targeted mtDNA Single n genes	WES + mtDNA Off-target (all) WES (all)	Unfiltered	57% (16/28) 0% (0/28) [0% (0/16)] 57% (16/28) [100% (16/16)]	25% (4/16) 75% Def MD 25% HS-MD 0% I/LS-MD	70% (19/27 var)
Kohda et al. <sup>[21]</sup> 2016	142	Paed all Onset < 15 yo 46% < 1 mo 42% NN	All MD Bioc RCD	NS	mtDNA, WES, CGH LR-PCR mtDNA (all) WES (all)	Unfiltered	35% (49/142) 7% (10/142) [20% (10/49)] 25% (35/142) [71% (35/49)] 59% (67/113) (36% LS, 90% HS)	86% (42/49) 67% (40/60 var)	61% (30/49 pts) 67% (40/60 var)
Pronicka et al. <sup>[17]</sup> 2016	113	Paed all All < 27 yo	HS-MD 35% IS-MD 27% LS-MD 37%	Routine (NS)	WES (after routine) NS WES (all)	Unfiltered	59% (67/113) (36% LS, 90% HS) 5% (6/113) [9% (6/67)] 54% (61/113) [91% (61/67)] LS)	70% (46/67 pts) 20% LS-MD 97% HS-MD	51% (50/99 var)
Wortmann et al. <sup>[19]</sup> 2015	109	Paed Young adult All < 27 yo	HS-MD 39% IS-MD 40% LS-MD 21%	mtDNA analysis CGH	2 step: VP ± WES N/A WES (all)	VP (custom) 238 genes Unfiltered	38% (42/109) (57% HS, 35% LS) N/A	62% (26/42)	NS
Ohtake et al. <sup>[16]</sup> 2014	104	NS	All MD Bioc RCD	mtDNA analysis	WES (after mtDNA) N/A WES (all)	Unfiltered	38% (42/109) [all solved] 43% (45/104) N/A	100% (45/45) 17 variants, 27 genes	98% (44/45 pts)
Taylor et al. <sup>[18]</sup> 2014	53	Paed all 96% < 15 yo 66% < 1 yo	All MD Bioc MRCD	mtDNA analysis CGH	WES (after mtDNA) N/A WES (all)	Unfiltered	60% (32/53) N/A 60% (32/53) [all solved]	100% (32/32) NS (12.5% novel genes)	NS

B: Blood; Bioc: biochemical; Clin: clinical; CGH: comparative genomic hybridization; Def: definite; HS-MD: high suspicion MD; IS-MD: intermediate suspicion MD; LS-MD: low suspicion MD; LR-PCR: long range PCR; M: muscle; MD: mitochondrial disease; mo: months; (M)RCD: (mixed) respiratory chain deficiencies; mtDNA: mitochondrial DNA; n: nuclear; n: number; N/A: not applicable; NGS: next generation sequencing; NN: neonatal; NN/I: neonatal/infant; NS: not stated; Paed: paediatric; predom: predominant; pts: patients; U: urine; var: variants; VP: virtual panel; WES: whole exome sequencing; yo: years old

of incidental findings. In 2015, the American College of Medical Genetics and Genomics and the Association for Molecular Pathology released revised guidelines informing sequence variant interpretation, incorporating limited guidelines for mitochondrial variant interpretation and noting specific associated challenges<sup>[81]</sup>. Despite this framework, interpretation remains challenging and inherently subjective, particularly for mtDNA variants<sup>[82]</sup>. Therefore, clinical and biochemical phenotyping remain important for successful utilisation and accurate interpretation of evolving sequencing techniques<sup>[19]</sup>, with family trio sequencing incorporated where feasible, especially for paediatric cases and segregation studies in adults, to rapidly prioritise de novo variants. Increasing use of WES and WGS combined with evolving “omics” techniques, including metabolomics, proteomics and transcriptomics, are enabling further interrogation and evaluation of variants, generating data to inform variant prioritisation and assignment, pathophysiological insights and therapeutic options<sup>[83]</sup>.

## A PROPOSED NEW APPROACH

We suggest a genetics first diagnostic approach given the technical suitability of NGS for mitochondrial disease genetics and the expanding capability to reliably identify and call mitochondrial disease variants<sup>[26]</sup>. A genetics first diagnostic approach is also advocated for by others<sup>[56,57]</sup> and a proposed process is outlined in [Figure 2](#) (adapted from Davis *et al.*<sup>[1]</sup>).

The first stage aims to stratify the population for testing by answering two questions: (1) “is mitochondrial disease likely?” and, if so, (2) “is there a distinctive phenotype indicative of the genotype?” to inform the most appropriate genetic testing strategy. The next stage focuses on molecular diagnosis - either identifying a known pathogenic mutation, validating a novel mitochondrial-disease causing variant, or identifying a genetic phenocopy.

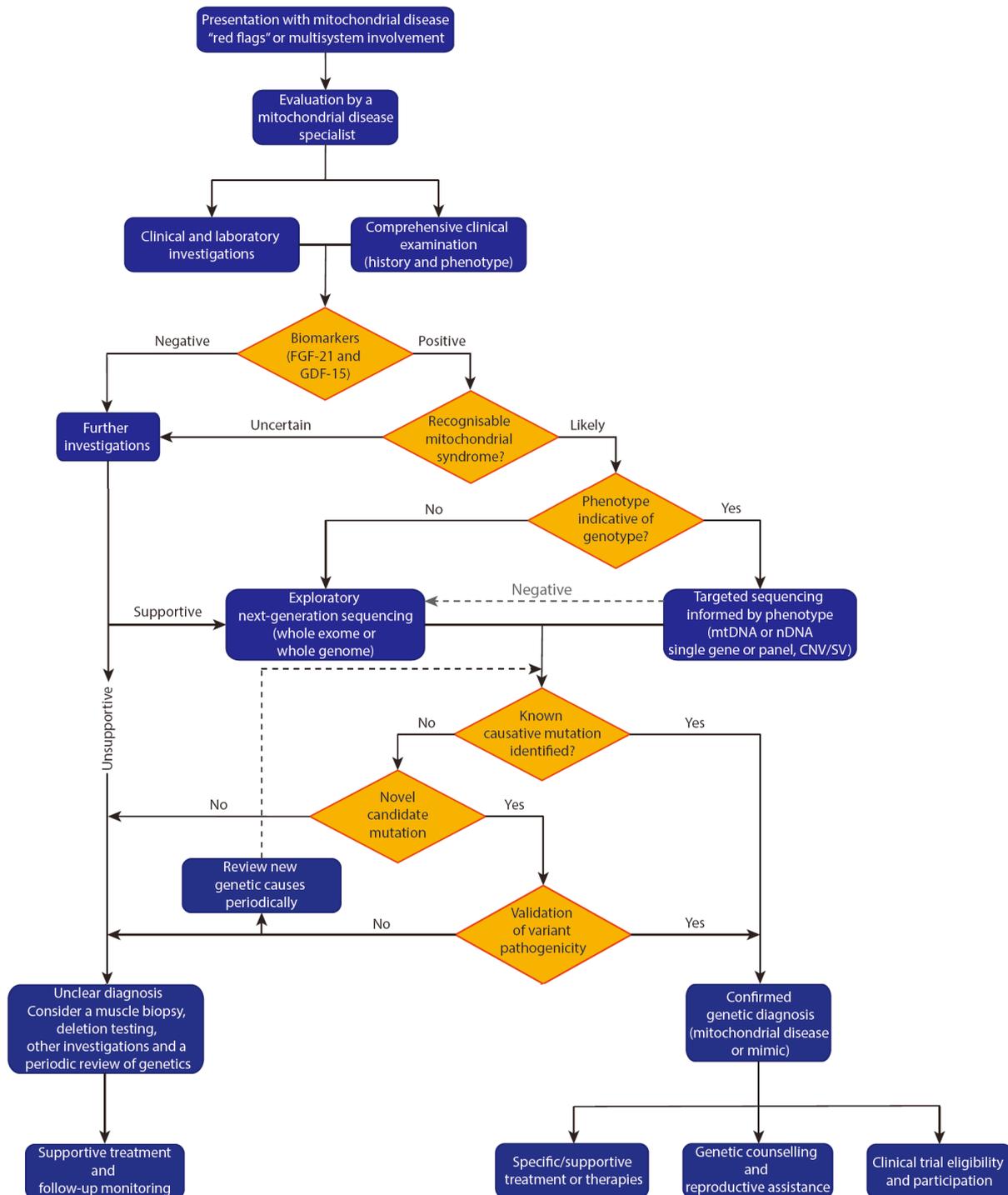
A careful and comprehensive history, including inheritance pattern where possible, together with comprehensive clinical examination, enables accurate clinical phenotyping and should be combined with tailored initial investigations to characterise organ involvement and form an initial clinical estimate of the likelihood of mitochondrial disease. Routine laboratory investigations, including those aimed at excluding infective or inflammatory processes and other mimics, should be undertaken alongside specific evaluation of serum lactate and pyruvate, creatine kinase (CK) and a urinary metabolic screen: indicators of disease but with limited sensitivity and specificity<sup>[54,66]</sup>. In adults, neuroimaging typically includes MRI of the brain (ideally with MR spectroscopy of CSF), and may demonstrate characteristic or non-specific patterns, or be normal<sup>[66,84-86]</sup>. Electroencephalogram, nerve conduction studies and electromyography may complement the initial clinical evaluation. Cardiac evaluation with electrocardiogram, 24-hour holter monitor and echocardiogram is critical to evaluate potentially life-threatening organ involvement and bedside ophthalmological examination may be augmented by retinal photography, or formal ophthalmological evaluation where appropriate.

The incorporation of biomarkers may aid clinical stratification (discussed below). If initial clinical evaluation and investigations are equivocal and/or biomarkers are negative, further supportive evidence for disease should be sought, prior to initiating comprehensive genetic testing. For example, the yield from detailed ophthalmological evaluation is high<sup>[87]</sup>, with findings often specific for mitochondrial disease, whereas other investigations, such as GI motility, although predictive of a positive genetic diagnosis when present, are less specific for mitochondrial diseases or a particular genetic culprit<sup>[88]</sup>.

The combination of suggestive clinical features, inheritance and initial investigations, together with positive biomarkers, should prompt the clinician to progress to genetic evaluation. Where a classical phenotype suggests a deletion syndrome, or one of a restricted group of causative genes or mutations, established targeted sequencing approaches in an appropriate tissue source (deletions often require uroepithelium or muscle) are readily available, rapid and cost-effective. If targeted sequencing returns negative, and in the many instances where a specific genetic cause or candidate is not able to be proposed, a comprehensive sequencing approach encompassing all potentially causative genes should be considered (discussed further below).

If, after comprehensive bigenomic sequencing, a genetic diagnosis still cannot be established, a review of the clinical presentation, consideration for further investigations - including muscle biopsy for biochemical and enzymatic studies, and genetics (in post-mitotic tissue) - and a periodic review of genetic data should be undertaken, as bioinformatics pipelines, variant analysis and the catalogue of known disease genes and pathogenic mutations are rapidly evolving.

With this proposed approach, muscle biopsy is not omitted entirely. Rather, it is selectively utilised to achieve specific end-points. Scenarios where early incorporation of muscle biopsy may be relevant include



**Figure 2.** A proposed diagnostic approach for mitochondrial disease

the genetic diagnosis of mtDNA deletion syndromes, where less invasive sources (blood leukocytes/uroepithelium) have been unrevealing, and for consideration where there is substantial uncertainty regarding the underlying aetiology that warrants further evaluation before proceeding further toward definitive diagnosis. However, it should be noted that in the latter context, WES has demonstrated utility in patients with a lower pre-sequencing likelihood of mitochondrial disease - the “possible” rather than “probable” group - as it can identify coding variants causing mitochondrial disease and monogenic

disease mimics<sup>[15,19,20]</sup>. Therefore, careful consideration should be given to whether invasive investigation is justified at this stage. Later incorporation of muscle biopsy may be relevant for evaluation and functional validation of identified novel variants<sup>[4]</sup>, in cases where definitive genetic diagnosis is not forthcoming, for investigation using more disease-relevant post-mitotic tissues, including to interrogate mtDNA deletions<sup>[89]</sup> and/or histological and biochemical evidence of mitochondrial disease in the absence of genetic diagnosis.

## THE ROLE OF SERUM BIOMARKERS

The addition of sensitive and specific serum biomarkers to the initial evaluation may aid stratification of genetic testing. Traditional and commonly tested serum biomarkers of mitochondrial disease include lactate, pyruvate, their ratio, and CK. However, results may vary substantially, depending on factors including activity, diet and sample handling<sup>[90]</sup> and they lack sufficient sensitivity and specificity for clinical utility in mitochondrial disease<sup>[91]</sup>. Recently, more sensitive and specific serum biomarkers have been identified, although there remains scope for improvement.

Elevated levels of fibroblast growth factor-21 (FGF-21) have been demonstrated in people with muscle-manifesting mitochondrial diseases, compared to non-mitochondrial disease and healthy controls<sup>[91-93]</sup>. Further research indicates FGF-21 levels best correlate with defects of mitochondrial translation and may be normal in defects of respiratory chain complexes or their assembly factors<sup>[93]</sup>. More recent functional studies of mitochondrial myopathy in human and mouse models demonstrate the crucial role of FGF-21 in the integrated mitochondrial stress response (ISR<sup>mt</sup>), activating the systemic stress response and inducing systemic metabolic consequences<sup>[94]</sup>. However, FGF-21 levels can also be elevated in non-mitochondrial diseases, including some non-mitochondrial myopathies, cancer, obesity, renal disease, diabetes and liver disease<sup>[90]</sup>, limiting diagnostic utility independent of clinical context.

The elevation of growth differentiation factor 15 (GDF-15) was identified in Thymidine Kinase (TK2)-related mitochondrial disease<sup>[95]</sup>. It was further evaluated in patient cohorts with mitochondrial and non-mitochondrial diseases<sup>[96-100]</sup>, with some suggestion that GDF-15 levels may correlate with disease severity<sup>[97]</sup>. Davis and colleagues demonstrated improved diagnostic sensitivity and a higher diagnostic odds ratio for GDF-15 compared to FGF-21, noting that GDF-15 was potentially more broadly applicable than FGF-21<sup>[96]</sup>. This was followed by suggestion of better correlation with mitochondrial translation and mtDNA maintenance defects<sup>[90]</sup>. GDF-15 may also be elevated, albeit to a lesser degree, in non-mitochondrial muscle and metabolic diseases, pregnancy, diabetes, cancer, liver fibrosis and cardiovascular disease<sup>[90,101]</sup>, and may reflect oxidative stress<sup>[101]</sup>.

Both FGF-21 and GDF-15 are non-invasive serum assays, and although not independently diagnostic<sup>[101]</sup>, offer superior utility to classical biomarkers<sup>[99]</sup>. They therefore complement clinical evaluation and can better inform decision making on subsequent costly tests such as NGS, whilst noting clinically relevant limitations.

## WHICH SEQUENCING APPROACH?

Although targeted NGS panels achieved early advances in genetic diagnosis, there are clear benefits of WES or WGS approaches. Both generate vastly more data and demand upfront resources for analysis, although costs are rapidly decreasing, and can simultaneously analyse mtDNA, identify novel disease genes and variants, as well as monogenic phenocopies.

WES has demonstrated increased diagnostic yield in mitochondrial disease studies as outlined above<sup>[15-23]</sup>, although it has frequently been utilised only for nuclear genome analysis following dedicated mtDNA genome sequencing. Off-target WES reads sufficiently capture mtDNA to assemble a mitochondrial genome<sup>[102]</sup> and analyse mtDNA variants with reasonable precision<sup>[75]</sup>, owing to the abundance of mtDNA

relative to nDNA. However, greater depth of coverage is required for reliable detection of low-heteroplasmy variants<sup>[76]</sup>. Dedicated mtDNA enrichment enables simultaneous analysis of mtDNA, with enhanced detection of low heteroplasmy variants, down to 8%<sup>[76]</sup>. Despite vast progress, however, a substantial proportion (30%-70%) of patients remain undiagnosed following WES<sup>[15-21]</sup>. Whilst this may reflect bioinformatic prioritisation or evolving analytic pipelines, there remain a number of insufficiencies in WES: coverage may be non-uniform and importantly limited in certain regions (especially G-C rich)<sup>[103]</sup> and indels and copy number variations may not be reliably identified<sup>[104]</sup>. Furthermore, PCR and mtDNA enrichment also introduce sequencing error and bias, the nature and extent of which depend on the selected kit and methods<sup>[103,105]</sup>. By definition, causative variants in non-coding regions are also omitted by WES. WGS can overcome all of these limitations to offer further utility, with promising early data in rare diseases<sup>[104]</sup> that may justify the modest additional cost.

PCR-free whole genome sequencing avoids sequencing error and biases introduced by library amplification, offering more consistent breadth and depth of coverage of coding regions<sup>[24]</sup> as well as covering the extensive non-coding regions. WGS can detect small and large chromosomal copy number variants<sup>[82]</sup>, an increased proportion of single nucleotide variants and structural variants<sup>[24,25,104]</sup>. It also offers superior mtDNA coverage (1200-4000× with acceptable coverage depths of the nuclear genome, between 14-30×), allowing reliable detection of low-heteroplasmy variants, down to 2% or less<sup>[26,57]</sup>. Whilst analysis of mitochondrial variants presents unique challenges compared to interpretation of nuclear variants<sup>[106]</sup> which have more established bioinformatics pipelines, we have developed a novel dedicated tool, *mity*<sup>TM</sup> to offer automated, integrated mtDNA variant calling from WGS data<sup>[26]</sup>. Nuclear and mtDNA bioinformatics pipelines may be linked, facilitating simultaneous analysis of both nuclear and mitochondrial genomes from a single, minimally-invasive sample<sup>[26]</sup>. WGS therefore offers comprehensive, simultaneous bigenomic sequencing with superior mtDNA coverage depth and heteroplasmy sensitivity, whilst reducing introduced sequencing error and bias, and should therefore be the preferred sequencing option. Early WGS results from mitochondrial disease studies indicate the yield is at least equivalent for known variants, with potential for improved yield with novel variant identification and as analysis - especially of non-coding regions - evolves.

## **CONCLUSION: A MINIMALLY INVASIVE, STREAMLINED APPROACH TO MITOCHONDRIAL DISEASE GENETIC DIAGNOSIS**

Despite significant advances in technology and understanding of mitochondrial biology over recent decades, the diagnosis of mitochondrial disease continues to present a challenge to the clinician and a large proportion of cases remain undiagnosed. Whilst the prevailing diagnostic paradigm advocates a “function-to-gene” approach centred on muscle biopsy, the substantial benefits of a “genetics-first” approach justify a paradigm shift. Such an approach, as proposed here, incorporating clinical evaluation, serum biomarker stratification and early bigenomic WGS, offers the potential to streamline a less invasive diagnostic process for patients, improve diagnostic yield, inform individual prognosis and the collective understanding of mitochondrial biology and ultimately pave the way for substantial therapeutic advances.

## **DECLARATIONS**

### **Authors' contributions**

Made substantial contributions to data interpretation, conception and design of the work, revision of the manuscript: Watson E, Davis R, Sue CM

Drafting: Watson E

Made technical support: Davis R, Sue CM

### **Availability of data and materials**

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declare that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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