Table 2. Diagnostic yield from WES 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
Theunissen *et al.*[15] 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.*[20] 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.*[21] 2016	142	Paed all Onset < 15 yo 46% < 1 mo	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)]	86% (42/49)	61% (30/49 pts) 67% (40/60 var)
Pronicka *et al.*[17] 2016	113	Paed all 42% NN	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)]	70% (46/67 pts) 20% LS-MD 97% HS-MD	51% (50/99 var)
Wortmann *et al.*[19] 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 38% (42/109) [all solved]	62% (26/42)	NS
Ohtake *et al.*[16] 2014	104	NS	All MD Bioc RCD	mtDNA analysis	WES (after mtDNA) N/A WES (all)	Unfiltered	43% (45/104) N/A 43% (45/104) [all solved]	100% (45/45)	98% (44/45 pts) 17 variants, 27 genes
Taylor *et al.*[18] 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)

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[81]. Despite this framework, interpretation remains challenging and inherently subjective, particularly for mtDNA variants[82]. Therefore, clinical and biochemical phenotyping remain important for successful utilisation and accurate interpretation of evolving sequencing techniques[19], 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[83].

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[26]. A genetics first diagnostic approach is also advocated for by others[56,57] and a proposed process is outlined in Figure 2 (adapted from Davis *et al.*[1]).

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[54,66]. 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[66,84-86]. 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[87], 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[88].

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

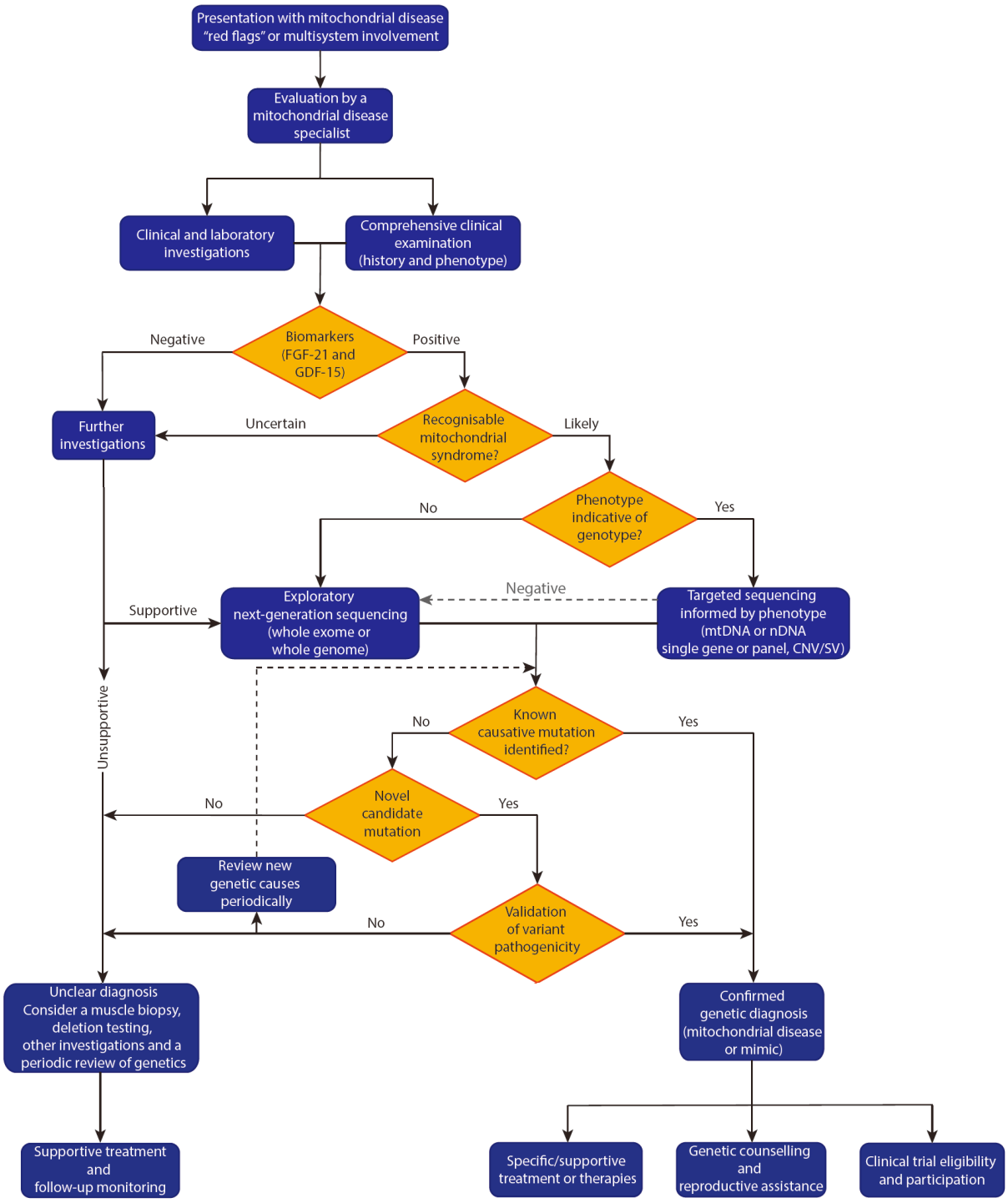

Figure 2. A proposed diagnostic approach for mitochondrial disease

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[15,19,20]. 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[4], in cases where definitive genetic diagnosis is not forthcoming, for investigation using more disease-relevant post-mitotic tissues, including to interrogate mtDNA deletions[89] 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[90] and they lack sufficient sensitivity and specificity for clinical utility in mitochondrial disease[91]. 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[91-93]. 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[93]. 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^{mt}), activating the systemic stress response and inducing systemic metabolic consequences[94]. 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[90], 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[95]. It was further evaluated in patient cohorts with mitochondrial and non-mitochondrial diseases[96-100], with some suggestion that GDF-15 levels may correlate with disease severity[97]. 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[96]. This was followed by suggestion of better correlation with mitochondrial translation and mtDNA maintenance defects[90]. 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[90,101], and may reflect oxidative stress[101].

Both FGF-21 and GDF-15 are non-invasive serum assays, and although not independently diagnostic[101], offer superior utility to classical biomarkers[99]. 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[15-23], 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[102] and analyse mtDNA variants with reasonable precision[75], owing to the abundance of mtDNA

relative to nDNA. However, greater depth of coverage is required for reliable detection of low-heteroplasmy variants[76]. Dedicated mtDNA enrichment enables simultaneous analysis of mtDNA, with enhanced detection of low heteroplasmy variants, down to 8%[76]. Despite vast progress, however, a substantial proportion (30%-70%) of patients remain undiagnosed following WES[15-21]. 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)[103] and indels and copy number variations may not be reliably identified[104]. Furthermore, PCR and mtDNA enrichment also introduce sequencing error and bias, the nature and extent of which depend on the selected kit and methods[103,105]. 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[104] 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[24] as well as covering the extensive non-coding regions. WGS can detect small and large chromosomal copy number variants[82], an increased proportion of single nucleotide variants and structural variants[24,25,104]. 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[26,57]. Whilst analysis of mitochondrial variants presents unique challenges compared to interpretation of nuclear variants[106] which have more established bioinformatics pipelines, we have developed a novel dedicated tool, *mity*™ to offer automated, integrated mtDNA variant calling from WGS data[26]. Nuclear and mtDNA bioinformatics pipelines may be linked, facilitating simultaneous analysis of both nuclear and mitochondrial genomes from a single, minimally-invasive sample[26]. 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

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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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