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Review

The broadening spectrum of mitochondrial disease: Shifts in the diagnostic paradigm☆,☆☆

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ABSTRACT

Background: The diagnosis of mitochondrial disease requires a complex synthesis of clinical, biochemical, histological, and genetic investigations. An expanding number of mitochondrial diseases are being recognized, despite their phenotypic diversity, largely due to improvements in methods to detect mutations in affected individuals and the discovery of genes contributing to mitochondrial function. Improved understanding of the investigational pitfalls and the development of new laboratory methodologies that lead to a molecular diagnosis have necessitated the field to rapidly adopt changes to its diagnostic approach.
Scope of review: We review the clinical, investigational and genetic challenges that have resulted in shifts to the way we define and diagnose mitochondrial disease. Incorporation of changes, including the use of fibroblast growth factor 21 (FGF-21) and next generation sequencing techniques, may allow affected patients access to earlier molecular diagnosis and management.
Major conclusions: There have been important shifts in the diagnostic paradigm for mitochondrial disease. Diagnosis of mitochondrial disease is no longer reliant on muscle biopsy alone, but should include clinical assessment accompanied by the use of serological biomarkers and genetic analysis. Because affected patients will be defined on a molecular basis, oligosymptomatic mutation carriers should be included in the spectrum of mitochondrial disease. Use of new techniques such as the measurement of serum FGF-21 levels and next-generation-sequencing protocols should simplify the diagnosis of mitochondrial disease.
General significance: Improvements in the diagnostic pathway for mitochondrial disease will result in earlier, cheaper and more accurate methods to identify patients with mitochondrial disease. This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

1. Introduction

Mitochondrial disease represents a group of metabolic disorders with the common link of impaired mitochondrial function. Because the mitochondria play a crucial role in providing the main energy source for the cell, body tissues that require the most energy are most commonly affected. Organs that are most frequently affected include the brain, muscle, heart, retina and cochlea. The clinical presentation of mitochondrial disorders can be extremely variable, although for many different tissues, there are specific 'red-flags' that can provide clues to the diagnosis of a mitochondrial disease. The age of onset of mitochondrial disease is also highly variable, with some disorders presenting from very early in the neonatal period and others not becoming manifest until late in adulthood. Further complexity arises as a result of the dual genomic expression of mitochondrial proteins from both nuclear (nDNA) and mitochondrial DNA (mtDNA).

Since Luft's first description of a rare syndrome in a young woman who had evidence of mitochondrial dysfunction and symptoms of hypermetabolism in the absence of thyroid disease [1], the number of mitochondrial diseases has expanded to include over 100 distinct disease entities. With the advent of molecular genetics, the discovery of improved biomarkers and new sequencing platforms to perform targeted exome and whole genome sequencing, traditional approaches to diagnosing mitochondrial disease are now being challenged. We will review these conventional approaches, focus on the pitfalls of these established techniques and draw attention to the new advances that may improve the diagnostic pathway for affected patients. Application of these methods will represent shifts in the diagnostic paradigm for mitochondrial disease and may lead to improved diagnostic accuracy and clinical management of patients.

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2. Diagnosis of mitochondrial disease based on clinical features

2.1. Clinical features suggestive of mitochondrial disease

The diagnosis of mitochondrial disease is initially raised by specific clinical manifestations that cluster with additional features such as lactic acidosis or other evidence of impaired mitochondrial respiratory chain function (see Fig. 1). Affected patients may present anytime from birth to late adulthood and the clinical manifestations can vary depending on the age of symptom onset. Notably, the severity of disease may range from life-threatening to asymptomatic or oligosymptomatic mutation carriers. Why some individuals develop a syndromic illness and others fail to develop the full spectrum of disease is currently unknown. Furthermore, factors that influence the evolution of relatively mild disease in oligosymptomatic subjects to one with a more severe disease course remain unclear.

Children often present with different clinical features when compared to adults. Common clinical presentations of children include failure to thrive, motor regression, metabolic encephalopathy, seizures, ptosis, external ophthalmoplegia and cardiomyopathy. Most frequent clinical manifestations associated with adult mitochondrial disease include exercise intolerance, sensorineural hearing loss, ophthalmological abnormalities (retinal pigmentary changes, ptosis, progressive external ophthalmoplegia, optic atrophy), muscle weakness (proximal limb weakness, dysphagia, dysarthria), central nervous system involvement (focal neurological deficits, migraine, seizures), cardiac manifestations (cardiac arrhythmia, hypertrophic cardiomyopathy, conduction block), gastrointestinal system abnormalities (pseudo-obstruction, constipation) and endocrine abnormalities (diabetes, short stature and rarely hypoparathyroidism and hypogonadism). Some of these manifestations carry a short differential diagnosis, but many are non-specific and common. It is then important to consider the abnormality in more detail, that is, the hearing loss of mitochondrial disorders maybe asymmetrical and can partially recover [2]; mitochondrial disease associated diabetes is seen in younger, non-obese patients [3,4], and may only manifest during times of physiological stress. Fig. 1 summarizes these clinical features that are more specific for mitochondrial disease.

Clinical features may also cluster into recognizable syndromes. There are several of these that are common enough to warrant clinician awareness. The MELAS syndrome (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes) is most commonly caused by a tRNA point mutation, the m.3243A>G mutation [5–7] but other mtDNA mutations may cause the same phenotype including m.3271T>C, m.3291T>C, or m.13513G>A. MERRF syndrome (myoclonic epilepsy with ragged red fibers) is also associated with a tRNA point mutation, usually m.8344A>G, but m.8356T>C and m.8363G>A are also causally related to this syndrome. A single deletion in mtDNA may result in Kearns-Sayre syndrome, chronic progressive external ophthalmoplegia (CPEO) or Pearson's syndrome. Leber's hereditary optic neuropathy (LHON) is commonly associated with one of three point mutations, m.11778G>A, m.3460G>A, and m.14484T>C. Other recognizable syndromes of mitochondrial disease include NARP (neuropathy, ataxia and retinitis pigmentosa), Leigh's disease and MNGIE (mitochondrial neurogastrointestinal encephalomyopathy). Table 1 details classical mitochondrial disease syndromes [7–11].

In addition to suggestive clinical features, the family history may provide important information when a diagnosis of mitochondrial disease is being considered. Maternal inheritance is highly suggestive of a defect in mtDNA. This is because at conception, all surviving mtDNA is derived from the mother's ovum. This leads to the situation where a pathogenic mutation in an affected mother is transmitted to all her children, but only her daughters will transmit the mutation to their progeny. Hence, a disease that is expressed in both sexes, but not transmitted from a male to his progeny is highly suggestive of a mtDNA defect.

Identical pathogenic mutations may cause disease at different ages and cause different clinical syndromes [12]. Why this occurs is

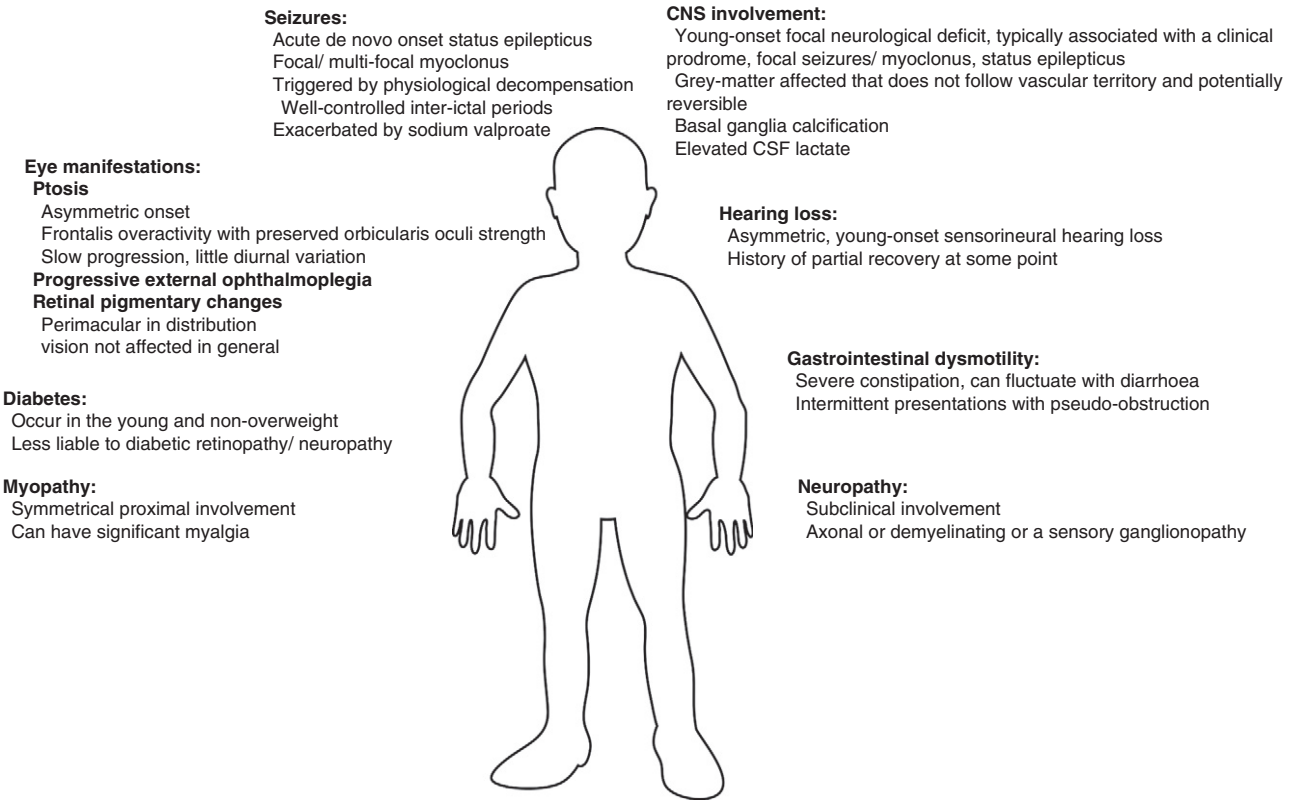

Fig. 1. Clinical features suggestive of a mitochondrial disorder.

Table 1
Summary of syndromic clinical phenotypes of mitochondrial disease (modified from C. Liang, C. M. Sue, How to Treat: Mitochondrial disease, Australian Doctor Mar 25 (2011) 27–34).

Clinical phenotype	Type of mutation typically associated with phenotype	Common clinical features
MELAS syndrome [4]	tRNA point mutations: m.3243A>G, m.3271T>C, m. 3291T>C, m.13513G>A	Cardinal — stroke-like episodes with clinical prodrome, areas of high signal on T2 weighted MRI that do not conform to vascular territories, lactic acidosis. Other — hearing loss, diabetes mellitus, gastrointestinal, short stature.
MERRF syndrome [5]	tRNA Point mutation: m.8344A>G, m. 8356T>C m.8363G>A	Cardinal — weakness, myoclonus and epilepsy. Other — multiple lipomata in 'collar' distribution, hearing loss, dementia, neuropathy.
Kearns-Sayre syndrome/CPEO [6]	Single deletion Deletion/duplications (rare)	Ptosis, PEO, retinal pigmentary abnormalities, short stature, ataxia, cardiac conduction abnormalities, proximal and bulbar weakness.
Leigh's disease [7]	Point mutation in protein subunit	Bilateral characteristic basal ganglia lesions, psychomotor retardation, seizures, movement disorders, lactic acidosis.
Leber's hereditary optic neuropathy [8]	Point mutation in protein subunit: m.11778G>A, m.3460G>A, m.14484T>C	Rapidly progressive, usually sequential visual loss commonly in young males. Characteristic fundal appearance.

unknown, but presumably may involve mechanisms such as tissue distribution of mtDNA mutations (that is, heteroplasmy, where mutant mtDNA coexists with the wild type mtDNA in varying proportions; threshold effects of the tissue at the time; environmental stressors and the underlying nuclear genetic backgrounds.)

As nuclear DNA mutations may also cause mitochondrial disease (nuclear genes are required to encode the majority of respiratory chain subunits, for the assembly of the respiratory chain, intergenomic signaling, protein import and mitochondrial motility), there may be a family history more suggestive of an autosomal dominant or recessive disease. In adults, mutations in POLG, thymidine phosphorylase, ANT1 and TWINKLE are the commonest nuclear encoded mitochondrial disease causing genes.

2.2. Problems with clinical diagnosis

Although mitochondrial disease may be suspected clinically, the diagnosis often remains uncertain until investigations such as a muscle biopsy or genetic tests confirm an abnormality. There have been several published diagnostic criteria which purport to assist in the recognition and diagnosis of mitochondrial disorders. Walker et al. were the first to propose a set of major and minor criteria for the identification of mitochondrial disorders [13]. The Walker criteria were then modified by Bernier et al. in 2002 and are now known as 'the modified Walker criteria' [14] to better serve the pediatric presentations. Other attempts to define diagnostic criteria include the Nijmegen Center for Mitochondrial Disorders scoring system [15] and the Mitochondrial Disease Criteria [16]. All of these diagnostic systems are based on a combination of clinical, laboratory, pathologic, biochemical, and genetic findings. They are useful for defining patients in research studies, but may not always be practical in day to day practice, where expensive investigations may be limited and where patients may be better served by a conclusive answer rather than a more ambiguous likelihood rating (e.g. 'possible' or 'probable' disease). Finally, poor phenotype–genotype correlations and the presence of oligosymptomatic and asymptomatic mutation carriers that obscure a positive family history can all affect the accuracy of clinical diagnosis in mitochondrial disease.

2.2.1. Phenotypic variability

Even within families carrying the same mutation, there is extreme phenotypic variability [12]. The m.3243A>G mutation, one of the most common mtDNA point mutations, provides a good example of this. The best known and severest clinical phenotype associated with the m.3243A>G point mutation is the MELAS syndrome. Potentially, this syndrome can be lethal in childhood. In addition to the MELAS syndrome, there are patients with milder phenotypes such as MIDD (maternally inherited diabetes and deafness), CPEO, non-syndromic symptom clusters, oligo/asymptomatic forms and a variety of other occasionally described phenotypes. We have found that only 25% of m.3243A>G mutation carriers suffer a stroke-like episode [17] and a UK study of 129 patients with the m.3243A>G mutation found that only about half of the patients had symptoms which befit a classical syndrome. 10% had features consistent with MELAS; 30% had MIDD, 6% had a MELAS/MIDD overlap, 7% had a CPEO overlap syndrome, 28% had a wide range of symptom clusters that did not conform to any syndromic diagnosis and 9% were asymptomatic [18]. It should be noted that as these patients were drawn from a mitochondrial database, it is possible that symptomatic disease may have been over-represented.

Another recent study investigating the clinical features of the MERRF syndrome associated mutation, m.8344A>G reported that 4 of the 39 patients within their database were asymptomatic and 4 were oligosymptomatic (isolated ptosis, hypogonadism and lipomatosis). The more severely affected patients had an array of both central nervous system and neuromuscular signs, in addition to other organ involvement in the form of diabetes, heart disease and multiple lipomata. The mean age of death was 37.5 years ($n = 4$) [19]. This again demonstrates the extreme phenotypic variability from asymptomatic to lethal multi-organ disease. This group also failed to find correlation between muscle mutation load and myopathic symptoms, and between the level of heteroplasmy and clinical severity or age of onset. There appeared to be few clues as to which patients were at risk of developing severe disease.

As there is extreme variability even within families, having an oligo/asymptomatic phenotype does not accurately predict the risk for the offspring [20]. Indeed, it is not uncommon for the minimally affected mother to be diagnosed after the assessment of a more severely affected child [21]. Asymptomatic at-risk family members cannot rely on a paucity of clinical symptoms and signs to determine their mutation status. It then becomes potentially important to pursue a genetic diagnosis even in patients with very mild disease.

Another problem with the classification of asymptomatic or oligosymptomatic mutation carriers is that the phenotype of individual patients may change throughout life. Although an affected patient is born with the genetic defect, it is very difficult to pinpoint when mitochondrial disease 'starts'. Patients may be well during early life and only develop symptoms as they age. Thus, a mutation carrier has the potential to develop symptoms anywhere along the full spectrum of mitochondrial disease. These patients carry a lifelong, relatively unpredictable risk of developing symptoms, and their offsprings are potentially at risk of severe disease (depending on specific genetic defect and mode of transmission). A Finnish study examined 33 adult patients with the m.3243A>G mutation. These patients were followed for 3 years and the progression of their symptoms assessed. Seven of the patients died during the follow-up period. Of the remaining patients, it was noted that sensorineural hearing loss progressed, echocardiographic changes progressed considerably, EEG findings changed and modified Rankin score became worse [22]. It is clear that even with this relatively short follow-up, an individual's phenotype and severity may change significantly over a lifetime.

2.2.2. Genotypic variability

Recognized mitochondrial disease phenotypes may be the result of different genotypes. A single syndromic phenotype may be the result

of different point mutations in mtDNA, mtDNA deletions or nDNA mutations. The MELAS phenotype is caused by the m.3243A>G point mutation in 80% of cases, but a similar phenotype is seen with m.3271T>C, m.3291T>C, or m.13513G>A mutations. Other MELAS causing mtDNA point mutations are increasingly reported in the medical literature. The combination of stroke-like episodes and lactic acidosis may also be seen as part of other mitochondrial syndromes. What may initially appear as a MELAS phenotype clinically may evolve into something different. Stroke-like episodes have been reported in Kearns-Sayre syndrome, Saguenay-Lac cytochrome oxidase deficiency and Leigh syndrome [23], further complicating the potential genotypic diagnosis of a patient who phenotypically has MELAS. A MELAS-like phenotype may also be the result of mutations in the POLG1 gene. A recent literature review identified 22 reported patients with stroke-like episodes as a consequence of POLG1 mutations, some of whom were initially misdiagnosed as mtDNA mutation related MELAS [24]. Differentiating between mtDNA and nDNA mutations is of particular importance given the differing mode of inheritance and implications for family members.

CPEO, using the term as a syndromic diagnosis, may be the result of mutations in mtDNA or nDNA and may be sporadic or follow mitochondrial or Mendelian inheritance patterns. Assigning inheritance pattern based on clinical information reduces the genotypic variability, but does not eliminate it, and this information is not always available or accurate. Sporadic CPEO is usually the result of a single large scale rearrangement of mtDNA. Maternally inherited CPEO is most commonly associated with the m.3243A>G mutation, but seventeen point mutations in six of the 22 mitochondrial tRNA genes have been identified as causing a CPEO phenotype. CPEO may also be inherited as a result of nDNA mutations in either an autosomal dominant or recessive pattern. These mutations usually cause multiple different large scale rearrangements of mitochondrial DNA. Six genes are associated with CPEO: ANT1, TYMP, Twinkle, POLG1, POLG2 and OPA1 [25]. Given this very large genotypic variability, the clinical examination and family history must be used to narrow down the possibilities, and guide the order of investigation. Most mitochondrial syndromes carry a genotypic differential and it is only the genotype that can accurately predict the mode of genetic transmission.

2.2.3. Oligosymptomatic and asymptomatic mutation carriers

Finally, a positive family history may be overlooked due to mild disease, variability in clinical manifestations or because many relatives may be asymptomatic carriers. Of note, in the m.8344A>G cohort mentioned above, 17 of 39 patients had no relatives with molecularly confirmed disease, and only 5 of these 17 had a maternal family history indicative of mitochondrial disease [19].

While only population screening will identify all asymptomatic carriers, genetic screening for asymptomatic at-risk family members may be more appropriate and of greater yield. Asymptomatic carriers may become symptomatic later in life and thus benefit from screening investigations, knowledge and avoidance of life-style factors which may exacerbate mitochondrial disease, and genetic counseling, should they wish to have children.

3. Diagnostic investigations for mitochondrial disease

Unless a classical mitochondrial disease phenotype is obvious, according to the currently proposed diagnostic criteria [13,14], steps to arrive at a more definitive diagnosis after clinically assessing a patient require identification of either indicative findings on a muscle biopsy, or the detection of a known pathogenic mutation for mitochondrial disease. Laboratory evidence suggesting abnormal oxidative metabolism, or abnormal respiratory chain enzymatic activities, may add to the strength of the diagnosis, but these often have poor sensitivity, and the methods used are not consistent between different laboratories [15]. Auxiliary investigations, such as serum lactate and pyruvate levels, imaging studies, and neurophysiological studies may similarly help to define the patient's disease syndrome, but are not definitive.

3.1. Diagnosis by muscle biopsy

Muscle biopsies are often necessary to better define the diagnosis for patients with suggestive symptoms who do not fit into a recognized clinical syndrome, and in whom initial common mutation screening is negative. Muscle samples may be used for morphological, biochemical and genetic analyses. When the muscle biopsy is taken, it is important that it is divided and stored differently for the various investigational processes. Portions of muscle should be frozen in liquid nitrogen cooled isopentane for morphological and histochemistrical studies, but freshly frozen without isopentane for biochemical studies. Other portions should be fixed in paraffin for histological examination or stored in glutaraldehyde for electron microscopy as necessary [26]. Mitochondrial genetic analysis for mtDNA mutations, including mtDNA rearrangements, deletion and depletion can be best identified using frozen (not fixed) muscle tissue.

Specific to mitochondrial disease, histological evaluation traditionally includes preparation of muscle sections with the modified Gomori trichrome stain, which identifies the historical “gold-standard” pathological hallmark referred to as the “ragged red fiber”. Ragged red fibers (RRFs) are myofibrils with excessive proliferation of mitochondria that appear red from the sphingomyelin content in the mitochondrial membrane [26]. Identification of more than 2% RRFs in a muscle biopsy sample qualifies the patient for a major diagnostic Walker criterion, whereas 1–2% RRFs in a muscle sample from a patient, 30–50 years of age (Fig. 2), or any amount found in those <30 years of age, qualifies the patient for a minor diagnostic Walker criterion [13,14].

Similarly, muscle sections can be stained with cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) stains. The presence of more than 2% COX-negative fibers in patients <50 years of age, or >5% in those over 50 years of age constitute a major diagnostic Walker criterion [13,14].

Demonstration of the ultrastructural changes of mitochondrial myopathy on electron microscopy complements light microscopy findings. The typical changes include: an excessive proliferation of mitochondria with increased number of abnormally shaped and enlarged mitochondria, abnormal architecture of the cristae in the mitochondria, and the identification of small intra-mitochondrial electron dense spherical granules or globoid inclusions, and paracrystalline inclusions (which are compressed crystallized cristae) [26,27]. Examination on electron microscopy can be more sensitive than histological findings.

3.2. Problems with diagnosis by muscle biopsy

Muscle biopsy is an invasive procedure that may require a general anesthetic, presenting an additional risk to the patient. There are issues with specimen handling and transport, particularly if the muscle is obtained from a site remote to the diagnostic laboratory. Biochemical studies need to be performed using muscle samples that are not frozen with isopentane; while electron microscopy requires a muscle biopsy preserved in glutaraldehyde to avoid artifacts. Such specific sample testing is best achieved when an institution has an established protocol for preparation, delivery and analysis of the muscle sample. Access to centers that have the appropriate level of expertise to perform biochemical and electron microscopic examination of muscle also represents further complexity to the diagnostic pathway, in addition to the further expense that these investigations and protocols attract.

Muscle biopsy results may have high false positive and false negative rates. In order to qualify for the major diagnostic criterion, the patients' biopsy needs to have a defined percentage of RRF or COX-negative fibers. This can make a definitive diagnosis difficult, as it is not uncommon for patients with mitochondrial diseases to have no RRF and/or a lower percentage of COX-negative fiber identified on their muscle

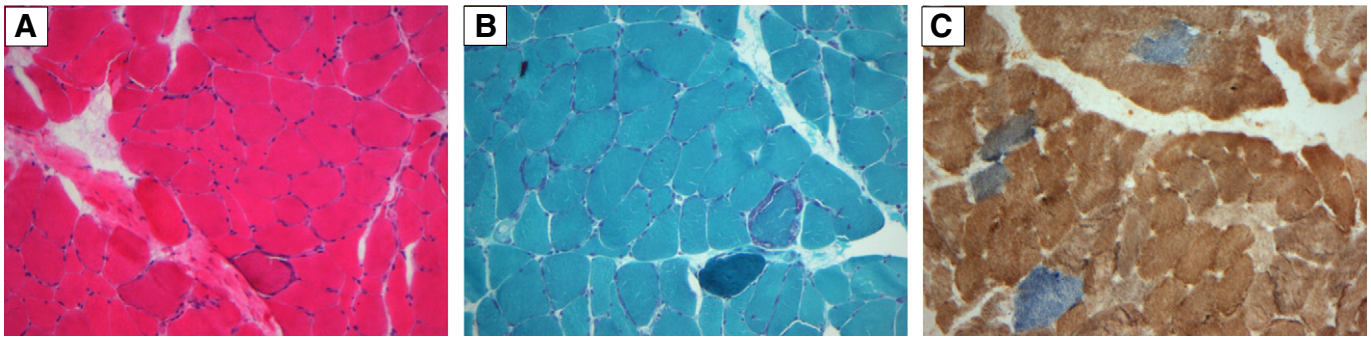

Fig. 2. A) Hemotoxylin & eosin stain: showing basophilic change to a myofiber, and a number of others with a basophilic rim. B) Modified Gomori trichrome stain: A ragged red fiber that stains darker is noted, and several others with a hint of red subsarcolemmal rim are seen. C) Cytochrome-c oxidase (COX) combined with succinate dehydrogenase (SDH) stain: the COX negative fibers (n = 4) clearly stand out after counter-staining with SDH. (Photos courtesy of Dr Janice Brewer)

biopsies. Moreover, the finding of RRF is dependent on age. RRFs are not generally found in biopsies of children <5 years [26], and may not be found in young adults with mitochondrial disease. Thus, there is poor sensitivity for younger patients. On the other hand, in one study, 85% of muscle specimens from older subjects showed >1 RRF per low power field [28]. RRFs can also be found in other conditions such as inflammatory myopathies such as inclusion body myositis [28], hereditary myopathies such as desminopathy, toxic myopathies (including in the use of statin and zidovudine) and metabolic myopathy with acid maltase deficiency, the latter condition in which the ultrastructural finding of paracrystalline inclusions in mitochondria can also be found [26].

Thus, muscle biopsy findings are not definitive in its diagnosis and need to be put into the context of the patient's clinical presentation, and the rest of other auxiliary investigations.

4. Diagnosis by genetics

4.1. The traditional approach to genetic diagnosis

Since the first report of mutations in mitochondrial DNA being associated with human disease in 1988, the finding of an established pathogenic mutation in a patient with clinical features of mitochondrial disease has proven to be the most definitive way to diagnose a patient with mitochondrial disease. Mitochondrial disease can be caused by mutations in either the mitochondrial or the nuclear genome. Hundreds of pathogenic mtDNA mutations have been reported in the literature, such that a database of reported mtDNA mutations can be found at www.mitomap.org. Mutations in the nuclear genome (nDNA) have also been reported to cause mitochondrial disease. Nuclear gene mutations associated with a primary mitochondrial disorder can cause defects in the respiratory chain subunits and ancillary proteins, impair intergenomic communication influencing mtDNA maintenance or expression, impair biosynthetic enzymes for lipids or cofactors, alter mitochondrial trafficking or biogenesis, or lead to defective apoptosis. Affected adults are more likely to have mutations in mtDNA, whereas affected children may have mutations in either genome.

Largely because of the expense and limited availability, genetic testing has traditionally been performed in a step-wise and selective manner, based on the patient's clinical phenotype and family history. For example, a patient with CPEO but no family history is suggestive of a sporadic mtDNA deletion whereas, if the same patient had a maternally transmitted pattern of inheritance, then consideration to common causative mutations in mtDNA (e.g. m.3243A>G) may be pursued. In contrast, a patient with CPEO and a Mendelian pattern of inheritance (in either an autosomal dominant or autosomal recessive manner) would justify sequencing of nuclear genes that are known to cause this phenotype (e.g. *POLG*, *ANT1*, *TWINKLE*) [29]. The results of other auxiliary tests can also direct targeted genetic testing. For example, if serum or urinary thymidine is elevated in a patient with ophthalmoplegia and gastrointestinal dysmotility, then the *TYMP* gene may be tested to confirm mitochondrial neurogastrointestinal encephalopathy (MNGIE) or; if mtDNA depletion is identified on Southern-blot, and the patient has elevated creatinine kinase, then testing for *RRM2B* or *TK2* genes might be considered.

4.2. Problem with diagnosis by genetics

The main problem with diagnosing mitochondrial disease with genetic testing is the vast number of causative genes that have been associated with mitochondrial disease. First of all, the number of candidate genes for testing amongst an ever-growing list of potential causative genes [30] can be difficult to rationalize, particularly given the variability in phenotype–genotype correlations.

For mitochondrial diseases, genetic testing needs to be performed in the correct clinical context and on the appropriately selected tissue. With regard to mtDNA mutations, selection of an appropriate tissue is important to optimize the chance of identifying a disease-causing mutation and may differ to that required for the identification of mutations in nuclear DNA. While some mtDNA mutations are homoplasmic and affect a single organ (such as for Leber's hereditary optic neuropathy), the majority of mitochondrial disorders caused by mutant mtDNA are heteroplasmic and may be selected out of various tissues (such as blood) over time [31–34], while being retained in others (such as muscles, hair follicles, and urine epithelial cells) [29]. For example, muscle is the tissue of choice for the identification of mtDNA rearrangements, deletions or depletion using Southern blot or quantitative real time PCR methodologies. In Kearns-Sayre syndrome (KSS) and chronic progressive external ophthalmoplegia (CPEO), mtDNA deletions are best detected in muscles, whereas in Pearson's syndrome, the mtDNA deletions may be found only in white blood cells [29].

4.3. Defining if mutational-carriers have the disease

Genetic testing of families with mitochondrial disease (e.g. in MELAS and MERRF) has revealed that the majority of family members are oligosymptomatic, but some have more severe disease, with the full clinical syndrome. Inclusion of oligosymptomatic mutation carriers under the umbrella of mitochondrial disease is warranted, given that these individuals may also develop a similar phenotype to the index case at a later point in time. In this sense, it is difficult to define when mitochondrial disease begins clinically. Probands often have clinical manifestations of mitochondrial disease heralding an acute presentation or the full syndromic illness. Factors that lead to the development of severe diseases are still largely unknown. Oligosymptomatic mutation carriers still require treatment and management of their clinical symptoms, and may transmit the pathogenic genetic mutation that can cause syndromic mitochondrial disease to their offspring.

5. Prevalence of mitochondrial DNA mutations in the community

Early epidemiologic studies estimated a minimum population prevalence of mitochondrial disease as between 9.2 and 16.5 in 100,000 [35–37]. These early studies aimed to determine the minimum birth prevalence of mitochondrial disease and may have underestimated the true prevalence of these disorders as they relied on *already diagnosed* case referrals and census data to calculate their estimates and did not account for the fact that many patients with mitochondrial disease in the community remain undiagnosed or misdiagnosed [38].

Another example is illustrated by prevalence studies on MELAS syndrome (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes), first described 1984 [7], which was most commonly associated with the m.3243A>G mutation in 1990 [6,39]. A Finnish study originally calculated a disease prevalence of 10.2/100 000, and a minimal m.3243A>G mutational prevalence of over 16.3/100,000 [40]. The mutational prevalence was subsequently revised to be much higher at 236/100,000 [41], amongst an Australian community-based cohort aged 50 years or older, indicating that undiagnosed, oligosymptomatic individuals are common in the population at large.

We continued our first series of community-based study to determine the prevalence of mitochondrial disease in the Australian population and found that 1 in 250 Australians had one of two common mtDNA mutations [41–43] and were thus at-risk of developing or had undiagnosed mitochondrial disease. Other prevalence studies have now also confirmed our findings and thus it has been established that the frequency of mtDNA mutations amongst 'healthy' or oligosymptomatic subjects is between 1 in 200–250 persons [43,44]. It is likely, however, that these studies, which are limited to testing only the common mutations, are still underestimating the true prevalence of all types of mitochondrial disease.

Thus, the problem arises as to whom we should define as having a mitochondrial disease. If we consider that only patients who manifest classical phenotypes as being affected, large numbers of oligosymptomatic affected individuals would be excluded and remain at risk of developing more severe disease. Lifestyle changes such as exercise programs, and supportive treatments can be helpful to minimize disease progression and ideally, disease monitoring should be offered to mutational carriers to minimize the risk of the disease in future [45]. If it is defined as all individuals carrying a known pathological mutation, then, the potential population needing medical attention could be very large. We propose that those individuals with known pathogenic mutations and at least one clinical manifestation be considered as having a mitochondrial disease, while unaffected mutations carriers should be classified as 'at-risk'. This highlights the need to better stratify the risks of these individuals in developing disease or future morbidity, in order to better assess the cost-effectiveness of screening and monitoring for the mutational carriers of each type of mitochondrial disease.

6. New advances in the diagnosis of mitochondrial disease

6.1. Next generation sequencing protocols

Because of the rapidly increasing number of mutated genes that are associated with mitochondrial disease, and the poor genotypic–phenotypic correlation in these patients, sequential sequencing of possible disease causing mitochondrial and nuclear genes can be prohibitively expensive and extensive. The utility of targeted exome sequencing and whole genome sequencing is currently being evaluated. New methods using massively parallel sequencing allows rapid, accurate sequencing of large amounts of DNA at lower costs [46]. First attempts to identify pathogenic mutations with next generation sequencing (NGS) protocols in a cohort of patients with severe infantile cases of mitochondrial disease defined on strict biochemical grounds and proven respiratory chain enzyme deficiency found that 26% of patients were identified as having pathogenic mutations causative of their mitochondrial disease [47]. Interestingly, only one child had an mtDNA mutation (deletion) and the other disease causing mutations were within nuclear encoded genes. Another study showed that their NGS protocol was able to reliably identify 17 of the 18 patients with known mtDNA mutations and all of the known nDNA mutations, demonstrating the diagnostic accuracy of this technique and underpinning the importance of correct tissue sampling, given that the mtDNA mutation 'missed' was only present at a low level of heteroplasmy (3%) in the tissue studied [48]. This latter study also demonstrated that when diagnostic criteria were made on less stringent clinical grounds, only 6% of cryptogenic patients were identified to have a disease causing pathogenic mutation. In both studies, additional patients were identified with sequence variants for which further studies were required to determine pathogenicity, emphasizing the need for further functional assessment of sequence variants identified by NGS protocols. NGS protocols and other genetic analyses that include long-range PCR analysis to detect multiple mtDNA deletions or depletion may also be helpful to identify the broad range of genetic defects and mutations associated with mitochondrial disease.

6.2. Fibroblast growth factor-21

Given that patient selection on clinical grounds may not be the best way to stratify DNA samples for NGS, we have proposed that the use of a biomarker such as serum of Fibroblast Growth Factor 21 (FGF-21) levels may be helpful in aiding the diagnostic pathway for patients with mitochondrial disease [49]. Serum levels of FGF-21 were first shown to be elevated in patients with mitochondrial disease by Suomalainen and colleagues, particularly if there was muscle involvement [50]. FGF-21 is a metabolic hormone and master regulator of energy balance and is particularly involved in lipid mobilization and the starvation response [51,52]. Mouse studies have shown that induction of FGF-21 is *specifically* due to respiratory chain deficiency [53]. We and others have found that serum FGF-21 levels display greater sensitivity than other classical indicators, such as serum lactate, pyruvate and creatine kinase levels and can distinguish mitochondrial disease from other neuromuscular disease and control subjects [49,50]. FGF-21 may be elevated in both mtDNA and nDNA encoded mitochondrial diseases [49] as well as adults and children with mitochondrial disease [49,50]. We propose to use this marker as a screening tool for patients suspected of having mitochondrial disease, so that those with elevated FGF-21 levels, and thus more

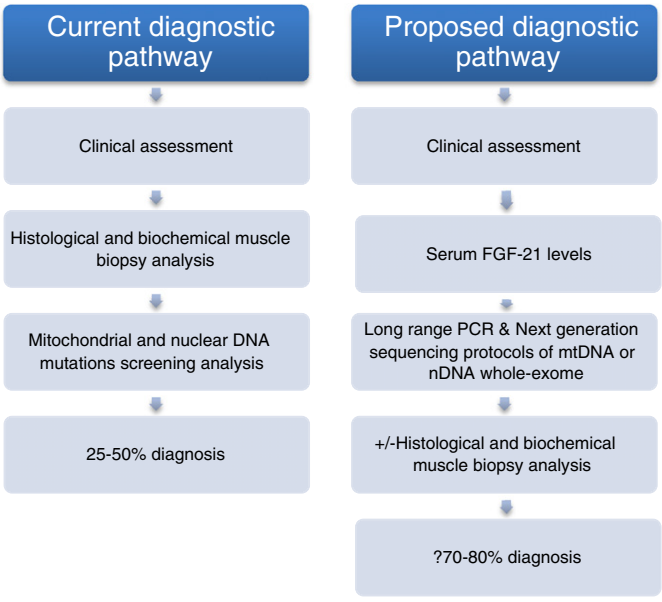

Fig. 3. Proposed changes to the diagnostic pathway for mitochondrial disease. Modified from C. M. Sue, The Mitochondrial Disease Report: Progress Towards Overcoming Life's Energy Crisis (2013).

likely to have the disease, can be prioritized for diagnostic genetic screening. However, FGF-21 levels may also be elevated in other conditions, including obesity and fasting. Thus, the clinical application of this biomarker needs to be further evaluated in larger well-characterized cohorts, with standardized protocols set up to ensure the clinical reliability of this test. We hypothesize that the diagnostic pathway for patients with mitochondrial disease may change as in Fig. 3.

7. Conclusions

The diagnosis of mitochondrial disease is complex and problematic. Traditional approaches to diagnose this group of disorders rely on clinical, auxiliary investigations and genetic analyses. There are problems and pitfalls with each of these approaches. New sequencing techniques such as next generation sequencing of targeted exomes have increased the diagnostic yield of older techniques. The use of serological biomarkers such as serum FGF-21 levels also offer promise to improve the diagnosis of such disorders. We conclude that new methodological advances in the genetic assessment of mitochondrial disease in combination with the discovery of new biomarkers such as serum FGF-21 levels will become established as a new diagnostic pathway for patients with mitochondrial disease. Identification of individuals with pathogenic mutations associated with disease symptoms indicative of mitochondrial disease will confirm that mitochondrial disease is no longer a rare group of fatal infantile or childhood-onset disorders but rather a vast group of disorders that can present at any age and potentially affect 1 in 200–250 people. Pathogenic mtDNA and nDNA defects are associated with a spectrum of clinical disease, inclusive of oligosymptomatic mutation carriers as well as recognized clinical syndromes. Muscle biopsy will no longer remain as the gold standard to diagnose mitochondrial disease as it will become ancillary to alternative diagnostic methods such as new NGS techniques in concert with the use of new biomarkers of mitochondrial disease. Improvements in the diagnostic pathway will establish a shift in the diagnostic paradigm for patients with mitochondrial disease, constituting further progress in the field of mitochondrial medicine.

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