A Diagnosis of "Possible" Mitochondrial Disease: An Existential Crisis

Sumit Parikh*, MD, Mitochondrial Medicine Center, Neurologic Institute, Cleveland Clinic, Cleveland, OH

Amel Karaa*, MD, Mitochondrial Disease Program, Genetics Unit, Massachusetts General Hospital, Boston, MA.

Amy Goldstein, MD, Mitochondrial Medicine Frontier Program, Division of Human Genetics, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA; University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA

Enrico Bertini, Unit of Neuromuscular and Neurodegenerative Disorders, Bambino Gesu' Children's Research Hospital, Rome, Italy

Patrick F. Chinnery, MRC Mitochondrial Biology Unit & Department of Clinical Neurosciences, University of Cambridge, Cambridge Biomedical Campus, Cambridge, CB2 0QQ, UK

John Christodoulou, Neurodevelopmental Genomics Research Group, Murdoch Children's Research Institute, Melbourne and Department of Paediatrics, Melbourne Medical School, University of Melbourne, Melbourne, Victoria, Australia

Bruce H. Cohen, Department of Pediatrics and Rebecca D. Considine Research Institute, Akron Children's Hospital, Akron, OH, USA and Northeast Ohio Medical University, Rootstown, OH, USA

Ryan L. Davis, University of Sydney, Faculty of Medicine and Health, Northern Clinical School, Sydney, New South Wales, Australia; Department of Neurogenetics, Kolling Institute, University of Sydney and Royal North Shore Hospital, Sydney, New South Wales, Australia

Marni J. Falk, Mitochondrial Medicine Frontier Program, Division of Human Genetics, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA; University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA

Carl Fratter, NHS Specialized Services for Rare Mitochondrial Disorders of Adults and Children UK, Oxford, UK and Oxford Medical Genetics Laboratories, Oxford University Hospitals, Oxford, UK

Rita Horvath, Wellcome Centre for Mitochondrial Research, Institute of Genetic Medicine, Newcastle University, Central Parkway, Newcastle upon Tyne, NE1 3BZ UK; Department of Clinical Neurosciences at the University of Cambridge Box 165, Addenbrooke's Hospital, Cambridge Biomedical Campus, Cambridge CB2 0QQ

Mary Kay Koenig, Department of Pediatrics, Mitochondrial Center, University of Texas McGovern Medical School, Houston, Texas, USA

Michelangelo Mancuso, Department of Experimental and Clinical Medicine, Neurological Institute, University of Pisa, Italy

Shana McCormack, Mitochondrial Medicine Frontier Program, Division of Human Genetics, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA; University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA

Elizabeth M. McCormick, Mitochondrial Medicine Frontier Program, Division of Human Genetics, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA

Robert McFarland, Wellcome Centre for Mitochondrial Research, Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, NE 2 4HH UK

Victoria Nesbitt, NHS Highly Specialised Services for Rare Mitochondrial Disorders, Oxford University Hospitals, Oxford, OX3 9DU, UK; Wellcome Centre for Mitochondrial Research, Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK

Manuel Schiff, Reference Center for Inborn Errors of Metabolism, Robert-Debré University Hospital, APHP, UMR1141, PROTECT, INSERM, Université Paris-Diderot, Sorbonne Paris Cité, Paris, France

Hannah Steele, Department of Neurology, Sunderland Royal Hospital, Sunderland, SR4 7TP, UK. Previously at Wellcome Centre for Mitochondrial Research, Institute of Genetic Medicine, Newcastle University, Central Parkway, Newcastle upon Tyne, NE1 3BZ UK

Sylvia Stockler, Department of Pediatrics, Division of Biochemical Diseases, University of British Columbia

Carolyn Sue, University of Sydney, Faculty of Medicine and Health, Northern Clinical School, Sydney, New South Wales, Australia; Department of Neurogenetics, Kolling Institute, University of Sydney and Royal North Shore Hospital, Sydney, New South Wales, Australia; Department of Neurology, Royal North Shore Hospital, Northern Sydney Local Health District, Sydney, New South Wales, Australia

Mark Tarnopolsky, Department of Pediatrics, Neuromuscular and Neurometabolic Clinic, McMaster University, Hamilton, Canada

David Thorburn, Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne, Victoria, Australia; Victorian Clinical Genetics Services, Royal Children's Hospital, Melbourne,

Victoria, Australia; Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia

Jerry Vockley, Department of Pediatrics, University of Pittsburgh School of Medicine; Center for Rare Disease Therapy, Children's Hospital of Pittsburgh, Pittsburgh, PA 15224

Shamima Rahman, Mitochondrial Research Group, UCL Great Ormond Street Institute of Child Health, London, UK; Metabolic Unit, Great Ormond Street Hospital NHS Foundation Trust, London, UK

**These authors contributed equally to this manuscript.*

Corresponding Author

Sumit Parikh, MD

parikhs@ccf.org

Cleveland Clinic, 9500 Euclid Ave, S60, Cleveland, OH 44195.

Word Count (text) 2997

Number of Figures: 0

Number of Tables: 5

Abstract

Primary genetic mitochondrial diseases are often difficult to diagnose, and the term “possible” mitochondrial disease is used frequently by clinicians when such a diagnosis is suspected. There are now many known phenocopies of mitochondrial disease. Advances in genomic testing have shown that some patients with a clinical phenotype and biochemical abnormalities suggesting mitochondrial disease may have other genetic disorders. In instances when a genetic diagnosis cannot be confirmed, a diagnosis of “possible” mitochondrial disease may result in harm to patients and their families, creating anxiety, delaying appropriate diagnosis and leading to inappropriate management or care. A categorization of *“diagnosis uncertain”*, together with a specific description of the metabolic or genetic abnormalities identified, is preferred when a mitochondrial disease cannot be genetically confirmed.

Author contributions

SP, AK, AG conceived the presented idea and prepared a document outline including needed supplementary material.
SP, AK, AG, SR developed and supervised the manuscript.
SP, AK, AG, EB, PFC, JC, BHC, RLD, MJF, CF, RH, MKK, MM, SM, EMM, RM, VN, MS, HS, SS, CS, MT, DT, JV, SR commented on, approved and helped expand on the presented idea, drafted and revised portions of the manuscript including supplementary materials and commented on and approved the final draft.

Details of funding: This project did not receive direct funding.

Patient consent and IRB: IRB involvement was not required for this project

Key Words:
Mitochondrial Disease Diagnosis, Genetic Disorders, Patient Harm

Introduction

Primary mitochondrial disorders (PMD) are genetic metabolic disorders that directly impair normal mitochondrial structure or function including electron-transport chain (ETC) activity.[1] They are due to mutations in either maternally-inherited mitochondrial DNA (mtDNA) or one of hundreds of nuclear DNA (nDNA) genes that encode components involved in mitochondrial structure and function. PMDs can present at any age and be multisystemic or selectively involve only a single organ. They can present as a well-defined canonical syndrome or a constellation of phenotypes, although typically at least one "red-flag" symptom is usually present at disease onset.[2]

With advances in next-generation sequencing (NGS) and the discovery of a multitude of new disease genes, the ability to diagnose PMDs has improved enormously compared to just a few years ago. The diagnosis still remains challenging due to heterogeneous manifestations combined with limitations of currently available biochemical and genetic testing methods. Despite recent advances, many individuals with suspected mitochondrial disease may remain without a confirmed genetic diagnosis, presenting a challenge to the clinician in not only establishing a mitochondrial disease diagnosis but also in knowing how to categorize, counsel and manage the patient with a suspected PMD where a genetic diagnosis is not yet possible. [3–7]

These limitations contribute to continued variation in diagnostic categorization of patients depending on the opinion of the treating provider.[8] Diagnostic terms such as "unlikely," "possible" or "probable" mitochondrial disease, originally proposed as part of research diagnostic criteria[9–11] were developed prior to genetic advances and may end up being inaccurate and misleading to patients and care providers, impacting or limiting proper counseling and the pursuit of further diagnostic testing. The complex and variable clinical presentation of mitochondrial diseases means that many unexplained disorders could conceivably have a mitochondrial etiology, so if a concrete alternative diagnosis cannot be

made using conventional investigations, there is a tendency to use the label 'possible' mitochondrial as a working diagnosis until an alternative emerges. Patients and families may inadvertently be burdened by the fear of the progressive nature of PMDs, the potential complications and early demise, as PMDs have no known cure. Thus, a diagnosis of "possible" mitochondrial disease may do more harm than good and consequently a new categorization for these patients is necessary.

Mitochondrial Disease can no longer be diagnosed on the basis of phenotypic features alone

A high index of suspicion for the possibility of a mitochondrial disease is appropriate when there is multi-system involvement or the presence of so-called "red-flags" such as stroke-like episodes in a non-vascular distribution (seen in Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke [MELAS] syndrome), bilateral symmetrical T2-weighted hyperintense MRI lesions in the basal ganglia and/or brainstem (Leigh syndrome), or chronic progressive external ophthalmoplegia (CPEO) and myopathy. Some of these "red-flag" symptoms were the subject of a previous review.[2] However, the growing list of genetically confirmed mitochondrial diseases has also led to an expanding list of variable phenotypes that should be suspected in the differential diagnosis of PMD, some of which are outlined in Table 1. [12–14]

Table 1 Differential diagnosis of selected phenotypes commonly associated with mitochondrial disease

Phenotype	Mitochondrial cause	Limited differential diagnosis
Dystonia	Leigh syndrome, deafness-dystonia syndrome, other mitochondrial encephalomyopathies	Biotinidase deficiency, thiamine transporter deficiency 2, *ADAR* mutations (Aicardi- Goutières syndrome 6), organic acidaemias (especially glutaric aciduria type I), NBIA, acute (viral) necrotising encephalopathy, mutations in *NUP62*, *RANBP2* and *PDE8B*, primary genetic dystonias
Epileptic encephalopathy	Alpers-Huttenlocher syndrome, many other	Many genetic epileptic encephalopathies, including Dravet syndrome and *KCNQ2* mutations, Pyridoxine dependent

	mitochondrial disorders	epilepsies (Antiquitin deficiency, PNPO deficiency), viral encephalitis
Progressive myoclonic epilepsy	MERRF	Ramsay Hunt syndrome, Unverricht-Lundborg disease, Lafora body disease, sialidosis, *PRICKLE1* mutations
Leukoencephalpathy	Complex I deficiency, Complex II deficiency, SURF1 deficiency (rarely), disorders of mitochondrial translation and Fe-S cluster assembly	Vanishing white matter disease, lysosomal storage disorders, Canavan disease, Alexander disease, Pelizaeus-Merzbacher(-like), hypo/dysmyelination
Ataxia	*ADCK3* mutations, ataxia-neuropathy syndromes e.g. SCAE, MIRAS, MERRF, NARP, disorders of coenzyme Q_{10} biosynthesis	Spinocerebellar ataxias, CAPOS syndrome
Demyelination	MNGIE	ADEM, multiple sclerosis
Peripheral neuropathy	Mutations in *POLG, MPV17, KARS* and *SURF1*; part of multisystem disease in many mitochondrial disorders, e.g. MNGIE	Other non-mitochondrial genetic causes of Charcot-Marie-Tooth syndromes, riboflavin transporter deficiency, toxic neuropathies, critical illness
Ptosis and ophthalmoplegia	PEO, KSS, MNGIE, MELAS	Some congenital myopathies, pseudo upgaze impairment in *OPMD*, horizontal gaze palsy and scoliosis (*ROBO3* mutation)
Optic neuropathy	LHON, ADOA, Leigh syndrome	Toxic optic neuropathy (e.g. methanol, cyanide, tobacco)
Hypertrophic cardiomyopathy with lactic acidosis	Complex I deficiency, *TMEM70* mutations, Sengers syndrome (AGK deficiency), disorders of mitochondrial translation	Viral infection
Dilated cardiomyopathy with lactic acidosis	Barth syndrome, disorders of mitochondrial phospholipid remodelling, other	Viral infection

	mitochondrial cardiomyopathies	
Exocrine pancreatic insufficiency	Pearson syndrome	Cystic fibrosis
Diabetes and deafness	MIDD, other mtDNA mutations	Type II diabetes mellitus with incidental nonsyndromic deafness
Sideroblastic anaemia	Pearson syndrome, MLASA, TRNT1 deficiency, *PUS1* or *YARS2* mutations	Blackfan-Diamond syndrome, Schwachman-Diamond syndrome, X-linked sideroblastic anaemia
B cell immune deficiency	TRNT1 deficiency	Primary immunodeficiency disorder
Liver failure	Mitochondrial DNA (mtDNA) depletion syndromes,	NBAS, LARS and IARS deficiencies, viral infection, lysosomal storage disorders, other syndromic genetic conditions
Renal tubulopathy/failure	Pearson and Kearns-Sayre syndromes, *RMND1*-related disease	Gitelman syndrome, Fanconi Bickel (*SLC2A2* mutations) syndrome, other syndromic genetic conditions
Myopathy	Part of multisystem disease in many mitochondrial disorders especially mtDNA depletion syndromes	Congenital muscular dystrophies, myositis, many other disorders
Rhabdomyolysis	Mitochondrial myopathies (e.g. *MTCO1*, *MTCO2*, *MTCO3* and *MTCYB* mutations)	*LPIN1* mutations, fatty acid oxidation defects (VLCAD, LCHAD), TANGO deficiency, glycolytic defects, toxic, post-exercise
Low copper	Cytochrome oxidase deficiency	Menkes, *SLC33A1* mutations
Complex multisystem disorders	Many mitochondrial disorders, particularly in childhood	Congenital disorders of glycosylation, peroxisomal disorders, lysosomal storage disorders, other syndromic genetic conditions

Key:
ADEM acute disseminated encephalomyelitis
ADOA autosomal dominant optic atrophy
CAPOS cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss
Fe-S iron-sulphur
KSS Kearns-Sayre syndrome

LHON Leber hereditary optic neuropathy
MERRF myoclonic epilepsy with ragged red fibers
MIDD maternally inherited diabetes and deafness
MIRAS mitochondrial recessive ataxia syndrome
MLASA myopathy, lactic acidosis, sideroblastic anemia
MNGIE mitochondrial neurogastrointestinal encephalomyopathy
NBIA neurodegeneration with brain iron accumulation
PEO progressive external ophthalmoplegia
SCAE spinocerebellar ataxia with epilepsy

In contrast, genetic testing has revealed that non-mitochondrial disorders may present with symptoms suggestive of mitochondrial disease. Without confirmatory genetic evidence, an erroneous diagnosis of a PMD may be made. For example, myopathy with ophthalmoplegia may also be seen in some cases of congenital myopathies with mutations in *MYH2,*[15] *MTM1* in male patients and female carriers,[16] *DNM2,*[17] and recessive *RYR1* mutations,[18–22] as well as in late-onset nemaline myopathy[23] and in congenital myasthenia caused by mutations in *CHAT* encoding the choline acetyltransferase.[24] Similarly, patients with branched-chain organic acidurias can manifest with non-hemodynamic strokes,[25] as can patients with congenital disorders of glycosylation.[26] Bilateral striatal necrosis (with MRI lesions resembling those observed in Leigh syndrome) has been reported with genetic mutations in the nuclear pore proteins *NUP62*[27] and *RANBP2*[28] or in the cyclic nucleotide phosphodiesterase *PDE8B*.[29] Many of these disorders may also be associated with secondary mitochondrial dysfunction on biochemical testing as discussed later and illustrated in Table 2. Clinicians clearly need to exercise caution when considering mitochondrial disease, in order not to narrow the differential too quickly simply based on aspects of the clinical phenotype and astutely ensure that even "red flag" features of a PMD are placed in the correct context of the patient's comorbid symptoms, family history and course of disease.

Table 2 Mitochondrial Dysfunction Identified in Select Other Genetic Disorders

Disorder	Mitochondrial defect	Reference by PubMed ID number
AOA1 (*APTX* mutations)	Coenzyme Q_{10}	15699391
Desminopathy	CS, mtDNA depletion (35%)	26097489

Dravet syndrome (*SCN1A* mutations)	Variable OXPHOS deficiencies	20392657; 21906962
EXOSC3 and *EXOSC8* related diseases	Low Complex I and pyruvate dehydrogenase activities, low mtDNA copy number, increased expression of mitochondrial genes	28687512; 24989451
GLUT1 deficiency	Complex I	22156785
GM3 synthase deficiency	Respiratory chain dysfunction in fibroblasts and liver	22990144
LCHADD	Complex III, COX	16417669
Limb immobilization	COX and CS	19654872
Lysosomal diseases: GM1-gangliosidosis, mucopolysaccharidosis IIIC, multiple sulfatase deficiency, Krabbe disease, Gaucher disease, Niemann Pick disease type C	Multiple OXPHOS deficiencies attributed to excessive production of mitochondrial reactive oxygen species and dysregulated calcium homeostasis with mitochondria-induced apoptosis and neurodegeneration	28132808
MADD (*ETFDH, ETFA* or *ETFB* mutations)	Complex I and II deficiencies; Riboflavin and Coenzyme Q_{10} responsive	17412732
Molybdenum co-factor deficiency	COX	16417669
MTHFR mutations	Complex I deficiency	21131308
Multiple carboxylase deficiency	Complex III	16417669
NBIA (PKAN)	Complex III	16417669
Neonatal haemochromatosis	Complex III (liver)	16417669
Neuroferritinopathy (*FTL1)*	Complex I or multiple Complex deficiency	17142829
NPHS3 (PLCE1 deficiency)	COX	21365190
Neuronal Ceroid Lipofuscinosis (*CLN2* and *CLN3*-related)	Partial deficiency in fatty acid oxidation enzymes and the storage of subunit c of mitochondrial ATP synthase in fibroblasts	8971698
ORAI1 related disease	Impaired lipid metabolism and fatty acid oxidation in skeletal muscle, heart and liver due to abnormal store-operated Ca^{2+} entry	28132808
Organic acidemias	Coenzyme Q_{10}, multiple OXPHOS deficiencies and free radical induced oxidative damage	21329767; 28753922; 28753922
Ras/MAPK pathway mutations	Variable OXPHOS deficiencies	26097489
Rett syndrome (*MECP2* mutations)	Variable OXPHOS deficiencies	26741492
SCAR10	Coenzyme Q_{10}	25182700
Spinal muscular atrophy	Complexes I-IV, mtDNA depletion	12557011; 25844556
STXBP1 mutation (*de novo*)	Complex I	25418441
Zellweger syndrome	Complexes II+III, COX	25287621; 28753922

Biochemical diagnostic tests remain imperfect

Consensus criteria to help standardize the evaluation of patients with potential PMD, outlining a streamlined approach and reviewing the strengths and limitations of many of the current testing modalities were suggested in 2015 by the Mitochondrial Medicine Society (MMS), an international group of clinicians specializing in mitochondrial disease.[30] This exercise aimed to decrease the variability that exists in approaches used by clinicians to diagnose PMDs.[8]

When a mitochondrial disorder is suspected, biochemical screening in blood, urine and cerebrospinal fluid (CSF) remain the initial tests of choice quickly followed by next-generation sequencing (NGS) of mtDNA and nDNA from white blood cells, with additional genetic studies in muscle when needed, particularly in adult-onset cases. Whole exome sequencing (WES) is useful, and along with whole genome sequencing (WGS) is quickly becoming the first or second line genetic test in patients with suspected mitochondrial disease.[1, 5]

Histopathological, biochemical and genetic analysis of tissue including muscle remain important tools to further delineate the phenotype and ascertain the relevance of any genetic variants identified in blood, but should no longer be considered first or second line tests when suspicion of a PMD is high and appropriate genetic testing is available.[30] Select disorders, such as CPEO, may warrant the need for further diagnostic testing in muscle. Additional considerations regarding these tests have been reviewed previously[30] and are summarized below and in Table 3 and discussed in detail in the supplementary material (Supplement – Testing).

Table 3 Limitations of Testing

Current Limitations of Biochemical Testing
Imperfect sensitivity and specificity
Secondary mitochondrial dysfunction leading to abnormal results

Inter-lab variability of methods and reference ranges
Challenges with tissue processing

Current Limitations of Genetic Testing
Incomplete understanding of the role of the entire genome in mitochondrial function
Novel genes still being identified
Interpretation of nuclear and mtDNA variants of uncertain significance
Lack of understanding of tissue-specificity of mtDNA mutations
Unclear relevance of low heteroplasmy levels of pathogenic mtDNA mutations
mtDNA deletions and depletion may be observed in non-mitochondrial disease

Challenges with biochemical testing

Biochemical studies in blood and urine such as lactate, amino acids, organic acids, and including the recently identified biomarkers growth differentiation factor 15 (GDF15) and fibroblast growth factor 21 (FGF21), along with functional assays in various tissues such as ETC enzyme analysis, all have less than optimal sensitivity and specificity, especially when interpreted in isolation from the clinical context.[30–35]

Abnormalities on ETC enzyme analysis may occur for a multitude of reasons outside of PMD including secondary mitochondrial dysfunction from other causes such as other genetic diseases, limb immobilization,[36] and in liver failure from non-mitochondrial causes.[37, 38] The list of other genetic disorders where some degree of secondary mitochondrial dysfunction in various tissues is seen seems ever-growing (Table 2) and includes spinal muscular atrophy (SMA),[39] X-linked adrenoleukodystrophy (ALD),[40] Phelan-McDermid syndrome, Down syndrome, Zellweger syndrome, the “rasopathies” (disorders caused by mutations in the Ras-MAPK pathway) and a variety of other conditions.[41–46] Causes of this secondary dysfunction have been discerned for very few of these disorders and the extent of mitochondrial dysfunction is variable and may not meet the diagnostic criteria threshold for ‘definite’ mitochondrial disease.[47] Therefore, evidence of biochemical dysfunction on functional testing alone, especially when mild or moderate, should not lead to a conclusive diagnosis of PMD.[42, 45, 48][49][47] When used with rigor, mitochondrial disease criteria may help the

clinician selectively better stratify truly high-risk patients.[50] However, mitochondrial disease diagnostic criteria were all developed at a time prior to the advent of NGS, when only limited genetic testing was available, and strongly emphasized the importance of abnormal biochemical findings in tissue.[10, 50, 51] This inevitably led to many patients being diagnosed with 'possible' mitochondrial disease.

Challenges with genetic testing

The advent of rapid, relatively low cost, NGS technologies has allowed for a genetic diagnosis to be made in many more patients with PMD. A growing number of nuclear genes has been associated with mitochondrial function (1500 to-date) [52, 53] although only around 350 or so have firmly been linked to causing human mitochondrial disease.[1, 54, 55] With more routine use of WES, new nuclear genes impacting mitochondrial function continue to be discovered. In some patients with a prior suspected but unconfirmed mitochondrial disease diagnosis, WES has also identified non-mitochondrial diseases.[56] In other cases, variant and milder phenotypes of PMD have been identified.[57] The ability to detect clearly pathogenic mutations in suspected PMD via genetic studies remains imperfect, with a reported diagnostic yield ranging from 25-75%.[3–7] The lack of understanding of the entire genome beyond the exome and increasing findings of variants of unknown significance (VUS) add to the diagnostic complexity.

MtDNA can now be accurately sequenced in its entirety for a relatively low cost and it is possible to detect levels of heteroplasmy of less than 5% in tissue, including blood. Genetic testing of mtDNA continues to be impacted by aspects of tissue specificity of mutations in mtDNA and varying degrees of heteroplasmy in easily attainable tissue. With newer testing methods able to detect low levels of heteroplasmy, common pathogenic mtDNA mutations (such as m.3243A>G) at low mutation load may mistakenly be attributed to cause a patient's phenotype.[58] These issues and others are discussed in further detail in the supplementary material (Supplement – Testing) but lead to the clear concern that simply testing the mitochondrial genome in leukocytes is not always adequate, and that mtDNA testing including

quantification and deletion analysis in other tissues (skeletal muscle, liver, buccal, urine sediment) may be needed. Furthermore, even though many defects in mtDNA maintenance may be diagnosed by WES, there remains a significant number in which the causative genes remain unknown. Muscle or liver biopsy (depending on the phenotype), along with reliable assessment of mtDNA copy number compared to age specific control ranges and/or long PCR for multiple deletions, are needed to diagnose these patients.

Despite the current limitations of genetic testing, the need for genetic confirmation of a PMD diagnosis is becoming a necessity. The number of phenocopies identified together with the less than perfect specificity of biochemical studies raises the concern of a mistaken diagnosis and the potential of missing a separate treatable disease. Accurate genetic diagnosis of a PMD allows care providers and affected families to better understand the condition, for the provision of appropriate genetic counseling, and for the development of targeted therapies. For some PMDs where the natural history is better known, clinicians and families can more accurately predict the disease course and provide appropriate clinical management and preventative care.[59] The need for a genetic diagnosis in PMD is now essential for eligibility in clinical trials. Pre-implantation genetic diagnosis for nuclear and mtDNA disorders and mitochondrial donation techniques also requires a prior confirmed genetic diagnosis.

Ending a "Possible" Diagnosis of Mitochondrial Disease

Previously established diagnostic criteria,[9–11] developed prior to advances in genetic testing, relied heavily on biochemical functional tests. They were intended to serve as research categorization tools in the era of only a basic understanding of mtDNA as it relates to mitochondrial illness and prior to our knowledge of any but a handful of the hundreds of nuclear genes that are now known to cause mitochondrial disease. In addition, they were often not adhered to in the strictest fashion by clinicians. These diagnostic categorizations subsequently infiltrated the clinic and many more patients began to be labelled as having

"possible" mitochondrial disease. Others have received the diagnosis of "mitochondrial myopathy" because of abnormalities seen in muscle histology or microscopy alone, even though this finding may exist due to other genetic, metabolic or neurodegenerative diseases.

While genetic testing has improved, it is not currently possible to confirm the diagnosis at a genomic level in every case. Some patients may have a coincidentally identified pathogenic mtDNA mutation with low levels of heteroplasmy or a VUS in a nuclear gene bioinformatically predicted to impact mitochondrial function that may make a clinician consider a "possible" mitochondrial disease diagnosis.

Given that patients with symptoms suggestive of mitochondrial disease may or may not ultimately have a PMD, it is increasingly important to establish better diagnostic criteria, or at least a unified approach to categorizing these patients, to avoid significant variability in diagnostic labelling, genetic counseling and management. With the growing number of clinical, biochemical and genetic phenocopies of PMD being identified, it has become prudent that a definitive diagnosis of mitochondrial disease should only be provided when a confirmed pathogenic genetic defect has been identified. Utmost caution must be used when providing a diagnosis based on biochemical abnormalities in tissue alone and the strictest application of biochemical diagnostic criteria is needed. Patients with strong biochemical and clinical evidence for a PMD should be periodically re-evaluated as diagnostic testing advances.

There is a clear concern that a diagnosis of "possible" mitochondrial disease may result in harm. First and foremost, some patients who receive a diagnosis of a "possible" or "suspected" mitochondrial disease may not recognize the impermanence of such a diagnosis and remain carrying this label for many years without having their symptoms periodically reassessed and a more specific diagnosis investigated as knowledge and diagnostic tools improve. Over time, the categorization of "possible" is often dropped by some providers and non-mitochondrial specialists providing routine care for the patient. Some families may cling to the diagnosis even after having had a different genetic disease confirmed, as it is the diagnosis they have become

most familiar with over time. Testing for another disorder may be delayed from the clinician's side if they are not aware of this diagnostic uncertainty. Other treatable disorders may not be diagnosed, or diagnosis may be delayed.

A diagnosis of "possible" mitochondrial disease may also create an unfounded fear of worsening morbidity and mortality. Certain families of patients given a diagnosis of "possible" mitochondrial disease often overlook the uncertainty of the diagnosis and become overly concerned that they or a family member may manifest all of the symptoms a patient with a PMD may develop, including neurodegeneration or early death, even in instances where their symptoms are relatively mild.

Lastly, patients with a diagnosis of "possible" mitochondrial disease may receive inappropriate care or be over-medicalized. Counseling of disease expectations and management may vary based on how patients are categorized.[60] Unnecessary medical interventions may be offered to some during times of catabolic stress. Some medications may not be used due to a concern of potential mitochondrial toxicity. New symptoms that a patient may manifest may inappropriately be explained away by the underlying diagnostic label rather than looking for other potentially treatable causes. These and other concerns are summarized in Table 4.

Table 4 Potential Harms Arising from a Diagnosis of "Possible" Mitochondrial Disease

Ending diagnostic odyssey prematurely
Missing potentially treatable disorders
Psychological burden of mitochondrial disease diagnosis: parent/patient fear of progressive or degenerative disorder
Inaccurate recurrence risk counseling
Inappropriate preventative care
Unnecessary medical interventions at times of catabolic stress
Avoidance of needed medications owing to fear of mitochondrial toxicity
Inappropriate reproductive decisions taken

Some of these very issues and challenges are outlined in example cases provided in the supplementary material (Supplement – Cases). In addition to the disorders outlined in Table 2, the supplementary cases illustrate instances where a patient may have symptoms suggesting the possibility of mitochondrial disease, often with biochemical abnormalities suggesting mitochondrial dysfunction, but the final diagnosis is not a PMD. Diagnosis is often delayed due to the mistaken diagnosis. Examples include a manganese transporter disorder with bilateral basal ganglia hyperintensities and elevated FGF21 levels (Case 1), oculopharyngeal muscular dystrophy with ragged red and cytochrome *c* oxidase (COX)-negative fibers (Case 2), Lesch-Nyhan syndrome with putaminal and thalamic abnormalities, lactic acidosis and reduced Complex I enzymatic activity in muscle (Case 3) and Niemann-Pick Type C with Complex I deficiency leading to a delay in being prescribed Miglustat (Case 4). In some of these instances, mitochondrial functional testing was notably abnormal, meeting biochemical diagnostic criteria for a mitochondrial disease. In contrast, select other cases (Cases 5-8) illustrate a delayed PMD diagnosis due to limitations of genetic testing in blood, findings of low levels of heteroplasmy or findings of a VUS. Case 5 illustrates an instance of a female with MELAS-like symptoms. Other cases (Cases 6-8) show the challenges in interpreting nuclear and mtDNA VUS.

Recommendations

In patients without a confirmed genetic diagnosis, there is a need for clinicians and the mitochondrial disease community to utilize diagnostic labels that clearly state that the diagnosis is uncertain even when mitochondrial dysfunction has been identified. A category of *"genetic diagnosis uncertain; mitochondrial biochemical dysfunction or mitochondrial genetic variant of unknown significance identified"* is preferable to a diagnosis of "possible" or "probable" or "suspected" mitochondrial disease. Other terminology that should be avoided is listed in Table 5. Depending on the clinical situation, patients may be further stratified into a *"high risk"* for a PMD to guide management.

Table 5 Terminology to avoid when a mitochondrial diagnosis is uncertain

"Possible," "Probable" or "Suspected" Mitochondrial Disease
Mitochondrial Myopathy

Mitochondrial Cytopathy
Mitochondrial Metabolism Disorder
Defect of Mitochondrial Metabolism

Our proposal to utilize a diagnostic label of *"genetic diagnosis uncertain"* for all such cases would allow clinicians and patients to remain actively engaged in the diagnostic odyssey, review the prior data periodically and take advantage of technological advances in genetic testing and new disease descriptions. Conducting relevant screening of other systems and monitoring for other organ involvement would allow better definition of the phenotype and not overlook disease progression. The clarity of the diagnostic label may prevent inappropriate or unnecessary care and allay fears of a progressive or degenerative disease.

Further categorization of selected patients as possible "high risk" for a PMD would allow for closer monitoring for mitochondrial disease related systemic comorbidities or extra cautions during times they are at risk of metabolic decompensations. If the phenotype is especially suggestive of a PMD, it may be appropriate to manage such a patient as if they have a genetically confirmed PMD for the time being – especially if they have previously experienced metabolic decompensation during times of illness or medical stress. Unexpected, acute changes in clinical status warrant thorough medical evaluation including laboratory testing to investigate potential mitochondrial dysfunction. However, the *"diagnosis uncertain"* designation would prevent any misunderstanding among medical teams. If the phenotype is not as suggestive of a PMD, it may be prudent to avoid over-medicalization of the patient and simply continue more routine monitoring.

As diagnostic standards for mitochondrial disease continue to evolve, these patients should remain under the care of a clinician who can assist in providing up-to-date recommendations regarding further testing. The MMS has such recommendations available online at www.mitosoc.org.

Conclusion

Despite advances in diagnostic techniques and molecular genetics, a subset of patients with suspected mitochondrial disease remains without a confirmed genetic diagnosis. The path these patients take to receiving a diagnosis is arduous and, at times, circuitous. Newer NGS based genetic studies offer the ability to streamline the approach to diagnosis for some patients. Others remain with a constellation of symptoms, findings of mitochondrial dysfunction on functional testing, and no clear pathogenic genetic mutation. Patients diagnosed with a "possible" mitochondrial disease might be found to have a non-mitochondrial genetic disorder once new testing modalities are utilized. A mistaken diagnosis of mitochondrial disease may prematurely end their diagnostic journey, over-medicalize their care, and potentially limit access to appropriate treatments for the actual underlying condition. To alleviate this dilemma, such patients would be better served by clinicians avoiding the diagnostic term "possible" mitochondrial disease.

Acknowledgements

RH is a Wellcome Trust Investigator (109915/Z/15/Z), who receives support from the Wellcome Centre for Mitochondrial Research (203105/Z/16/Z), Medical Research Council (UK) (MR/N025431/1), the European Research Council (309548), the Wellcome Trust Pathfinder Scheme (201064/Z/16/Z) and the Newton Fund (UK/Turkey, MR/N027302/1). PFC is a Wellcome Trust Senior Fellow in Clinical Science (101876/Z/13/Z) and a UK NIHR Senior Investigator, who receives support from the Medical Research Council Mitochondrial Biology Unit (MC_UP_1501/2), the Medical Research Council (UK) Centre for Translational Muscle Disease (G0601943), EU FP7 TIRCON, and the National Institute for Health Research (NIHR) Biomedical Research Centre based at Cambridge University Hospitals NHS Foundation Trust and the University of Cambridge. SR is supported by a Great Ormond Street Hospital Children's Charity Research Leadership Award (V1260) and by research grant funding from the NIHR Great Ormond Street Hospital Biomedical Research Centre and the Lily Foundation.

EB, SR and MR are members of the European Reference Network for Rare Hereditary Metabolic Disorders (METABERN) - Project ID No 739543. MetabERN is partly co-funded by the European Union in the framework of the Third Health Programme "ERN-2016 - Framework Partnership Agreement 2017-2021.

MM is supported by research grants from Telethon and MITOCON Italian patients' association (grants GSP09004 and GSP16001)

MT would like to thank Dan Wright and family for a kind donation for research in mitochondrial disease and to Kathy Corkins and Warren Lammert and family for a kind donation to establish the Corkins-Lammert Mitochondrial Disease Center at McMaster University.

REFERENCES

1 Rahman J, Rahman S. Mitochondrial medicine in the omics era. *Lancet (London, England)* 2018;**391**:2560–74.

2 Haas RH, Parikh S, Falk MJ, Saneto RP, Wolf NI, Darin N, Cohen BH. Mitochondrial Disease: A Practical Approach for Primary Care Physicians. *Pediatrics* 2007;**120**:1326–33.

3 Calvo SE, Compton AG, Hershman SG, Lim SC, Lieber DS, Tucker EJ, Laskowski A, Garone C, Liu S, Jaffe DB, Christodoulou J, Fletcher JM, Bruno DL, Goldblatt J, Dimauro S, Thorburn DR, Mootha VK. Molecular diagnosis of infantile mitochondrial disease with targeted next-generation sequencing. *Sci Transl Med* 2012;**4**:118ra10.

4 Wortmann SB, Koolen DA, Smeitink JA, van den Heuvel L, Rodenburg RJ. Whole exome sequencing of suspected mitochondrial patients in clinical practice. *J Inherit Metab Dis* 2015;**38**:437–43.

5 Taylor RW, Pyle A, Griffin H, Blakely EL, Duff J, He L, Smertenko T, Alston CL, Neeve VC, Best A, Yarham JW, Kirschner J, Schara U, Talim B, Topaloglu H, Baric I, Holinski-Feder E, Abicht A, Czermin B, Kleinle S, Morris AAM, Vassallo G, Gorman GS, Ramesh V, Turnbull DM, Santibanez-Koref M, McFarland R, Horvath R, Chinnery PF. Use of whole-exome sequencing to determine the genetic basis of multiple mitochondrial respiratory chain complex deficiencies. *Jama* 2014;**312**:68–77.

6 Lieber DS, Calvo SE, Shanahan K, Slate NG, Liu S, Hershman SG, Gold NB, Chapman BA, Thorburn DR, Berry GT, Schmahmann JD, Borowsky ML, Mueller DM, Sims KB, Mootha VK. Targeted exome sequencing of suspected mitochondrial disorders. *Neurology* 2013;**80**:1762–70.

7 Kohda M, Tokuzawa Y, Kishita Y, Nyuzuki H, Moriyama Y, Mizuno Y, Hirata T, Yatsuka Y, Yamashita-Sugahara Y, Nakachi Y, Kato H, Okuda A, Tamaru S, Borna NN, Banshoya K, Aigaki T, Sato-Miyata Y, Ohnuma K, Suzuki T, Nagao A, Maehata H, Matsuda F, Higasa K, Nagasaki M, Yasuda J, Yamamoto M, Fushimi T, Shimura M, Kaiho-Ichimoto K, Harashima H, Yamazaki T, Mori M, Murayama K, Ohtake A, Okazaki Y. A Comprehensive Genomic Analysis Reveals the Genetic Landscape of Mitochondrial Respiratory Chain Complex Deficiencies. *PLoS Genet* 2016;**12**. doi:10.1371/journal.pgen.1005679

8 Parikh S, Goldstein A, Koenig MK, Scaglia F, Enns GM, Saneto R, Anselm I, Collins A, Cohen BH, DeBrosse SD, Dimmock D, Falk MJ, Ganesh J, Greene C, Gropman AL, Haas R, Kahler SG, Kamholz J, Kendall F, Korson MS, Mattman A, Milone M, Niyazov D, Pearl PL, Reimschisel T, Salvarinova-Zivkovic R, Sims K, Tarnopolsky M, Tsao CY, van Hove J, Walsh L, Wolfe LA. Practice patterns of mitochondrial disease physicians in North America. Part 1: Diagnostic and clinical challenges. *Mitochondrion* 2014;**14**:26–33.

9 Morava E, van den Heuvel L, Hol F, de Vries MC, Hogeveen M, Rodenburg RJ, Smeitink JA. Mitochondrial disease criteria: diagnostic applications in children . *Neurology* 2006;**67**:1823–6.

10 Bernier FP, Boneh A, Dennett X, Chow CW, Cleary MA, Thorburn DR. Diagnostic criteria for respiratory chain disorders in adults and children. *Neurology* 2002;**59**:1406–11.

11 Thorburn DR, Sugiana C, Salemi R, Kirby DM, Worgan L, Ohtake A, Ryan MT. Biochemical and molecular diagnosis of mitochondrial respiratory chain disorders. *Biochim Biophys Acta* 2004;**1659**:121–8.

12 Martikainen MH, Chinnery PF. Mitochondrial disease: mimics and chameleons. *Pract Neurol* 2015;**15**:424–35.

13 Needham M, Duley J, Hammond S, Herkes GK, Hirano M, Sue CM. Mitochondrial disease mimicking Charcot-Marie tooth disease. *BMJ Case Rep* Published Online First: 2009. doi:10.1136/bcr.06.2009.2001

14 Gardeitchik T, Wyckmans J, Morava E. Complex Phenotypes in Inborn Errors of Metabolism: Overlapping Presentations in Congenital Disorders of Glycosylation and Mitochondrial Disorders. Pediatr. Clin. North Am. 2018;**65**:375–88.

15 D'Amico A, Fattori F, Bellacchio E, Catteruccia M, Servidei S, Bertini E. A new de novo missense mutation in MYH2 expands clinical and genetic findings in hereditary myosin myopathies. *Neuromuscul Disord* 2013;**23**:437–40.

16 Biancalana V, Scheidecker S, Miguet M, Laquerrière A, Romero NB, Stojkovic T, Abath Neto O, Mercier S, Voermans N, Tanner L, Rogers C, Ollagnon-Roman E, Roper H, Boutte C, Ben-Shachar S, Lornage X, Vasli N, Schaefer E, Laforet P, Pouget J, Moerman A, Pasquier L, Marcorelle P, Magot A, Küsters B, Streichenberger N, Tranchant C, Dondaine

N, Schneider R, Gasnier C, Calmels N, Kremer V, Nguyen K, Perrier J, Kamsteeg EJ, Carlier P, Carlier RY, Thompson J, Boland A, Deleuze JF, Fardeau M, Zanoteli E, Eymard B, Laporte J. Affected female carriers of MTM1 mutations display a wide spectrum of clinical and pathological involvement: delineating diagnostic clues. *Acta Neuropathol* 2017;**134**:889–904.

17 Catteruccia M, Fattori F, Codemo V, Ruggiero L, Maggi L, Tasca G, Fiorillo C, Pane M, Berardinelli A, Verardo M, Bragato C, Mora M, Morandi L, Bruno C, Santoro L, Pegoraro E, Mercuri E, Bertini E, D'Amico A. Centronuclear myopathy related to dynamin 2 mutations: Clinical, morphological, muscle imaging and genetic features of an Italian cohort. *Neuromuscul Disord* 2013;**23**:229–38.

18 Bevilacqua JA, Monnier N, Bitoun M, Eymard B, Ferreiro A, Monges S, Lubieniecki F, Taratuto AL, Laquerrière A, Claeys KG, Marty I, Fardeau M, Guicheney P, Lunardi J, Romero NB. Recessive RYR1 mutations cause unusual congenital myopathy with prominent nuclear internalization and large areas of myofibrillar disorganization. *Neuropathol Appl Neurobiol* 2011;**37**:271–84.

19 Wilmshurst JM, Lillis S, Zhou H, Pillay K, Henderson H, Kress W, Müller CR, Ndondo A, Cloke V, Cullup T, Bertini E, Boennemann C, Straub V, Quinlivan R, Dowling JJ, Al-Sarraj S, Treves S, Abbs S, Manzur AY, Sewry CA, Muntoni F, Jungbluth H. RYR1 mutations are a common cause of congenital myopathies with central nuclei. *Ann Neurol* 2010;**68**:717–26.

20 Clarke NF, Waddell LB, Cooper ST, Perry M, Smith RLL, Kornberg AJ, Muntoni F, Lillis S, Straub V, Bushby K, Guglieri M, King MD, Farrell MA, Marty I, Lunardi J, Monnier N, North KN. Recessive mutations in RYR1 are a common cause of congenital fiber type disproportion. *Hum Mutat* 2010;**31**. doi:10.1002/humu.21278

21 Kondo E, Nishimura T, Kosho T, Inaba Y, Mitsuhashi S, Ishida T, Baba A, Koike K, Nishino I, Nonaka I, Furukawa T, Saito K. Recessive RYR1 mutations in a patient with severe congenital nemaline myopathy with ophthalomoplegia identified through massively parallel sequencing. *Am J Med Genet A* 2012;**158A**:772–8.

22 Das S, Dowling J, Pierson CR. *X-Linked Centronuclear Myopathy*. 1993.

http://www.ncbi.nlm.nih.gov/pubmed/20301605 (accessed 27 Apr2018).

23 Wengert O, Meisel A, Kress W, Dekomien G, Angstwurm K, Heppner FL, Goebel HH, Stenzel W. Progressive external ophthalmoplegia as initial manifestation of sporadic late-onset nemaline myopathy. J. Neurol. 2011;**258**:915–7.

24 Mullaney P, Vajsar J, Smith R, Buncic JR. The natural history and ophthalmic involvement in childhood myasthenia gravis at the hospital for sick children. *Ophthalmology* 2000;**107**:504–10.

25 Testai FD, Gorelick PB. Inherited metabolic disorders and stroke part 2: Homocystinuria, organic acidurias, and urea cycle disorders. Arch. Neurol. 2010;**67**:148–53.

26 Briones P, Vilaseca MA, García-Silva MT, Pineda M, Colomer J, Ferrer I, Artigas J, Jaeken J, Chabás A. Congenital disorders of glycosylation (CDG) may be underdiagnosed when mimicking mitochondrial disease. *Eur J Paediatr Neurol* 2001;**5**:127–31.

27 Basel-Vanagaite L, Muncher L, Straussberg R, Pasmanik-Chor M, Yahav M, Rainshtein L, Walsh CA, Magal N, Taub E, Drasinover V, Shalev H, Attia R, Rechavi G, Simon AJ, Shohat M. Mutated nup62 causes autosomal recessive infantile bilateral striatal necrosis. *Ann Neurol* 2006;**60**:214–22.

28 Neilson DE, Adams MD, Orr CMD, Schelling DK, Eiben RM, Kerr DS, Anderson J, Bassuk AG, Bye AM, Childs A-MM, Clarke A, Crow YJ, Di Rocco M, Dohna-Schwake C, Dueckers G, Fasano AE, Gika AD, Gionnis D, Gorman MP, Grattan-Smith PJ, Hackenberg A, Kuster A, Lentschig MG, Lopez-Laso E, Marco EJ, Mastroyianni S, Perrier J, Schmitt-Mechelke T, Servidei S, Skardoutsou A, Uldall P, van der Knaap MS, Goglin KC, Tefft DL, Aubin C, de Jager P, Hafler D, Warman ML. Infection-triggered familial or recurrent cases of acute necrotizing encephalopathy caused by mutations in a component of the nuclear pore, RANBP2. *Am J Hum Genet* 2009;**84**:44–51.

29 Appenzeller S, Schirmacher A, Halfter H, Bäumer S, Pendziwiat M, Timmerman V, De Jonghe P, Fekete K, Stögbauer F, Lüdemann P, Hund M, Quabius ES, Ringelstein EB, Kuhlenbäumer G. Autosomal-dominant striatal degeneration is caused by a mutation in the phosphodiesterase 8B gene. *Am J Hum Genet* 2010;**86**:83–7.

30 Parikh S, Goldstein A, Koenig MK, Scaglia F, Enns GM, Saneto R, Anselm I, Cohen BH, Falk

MJ, Greene C, Gropman AL, Haas R, Hirano M, Morgan P, Sims K, Tarnopolsky M, Van Hove JLK, Wolfe L, DiMauro S. Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet Med* 2015;**17**:689–701.

31 Lehtonen JM, Forsström S, Bottani E, Viscomi C, Baris OR, Isoniemi H, Höckerstedt K, Österlund P, Hurme M, Jylhävä J, Leppä S, Markkula R, Heliö T, Mombelli G, Uusimaa J, Laaksonen R, Laaksovirta H, Auranen M, Zeviani M, Smeitink J, Wiesner RJ, Nakada K, Isohanni P, Suomalainen A. FGF21 is a biomarker for mitochondrial translation and mtDNA maintenance disorders. *Neurology* 2016;**87**:2290–9.

32 Montero R, Yubero D, Villarroya J, Henares D, Jou C, Rodríguez MA, Ramos F, Nascimento A, Ortez CI, Campistol J, Perez-Dueñas B, O'Callaghan M, Pineda M, Garcia-Cazorla A, Oferil JC, Montoya J, Ruiz-Pesini E, Emperador S, Meznaric M, Campderros L, Kalko SG, Villarroya F, Artuch R, Jimenez-Mallebrera C. GDF-15 is elevated in children with mitochondrial diseases and is induced by mitochondrial dysfunction. *PLoS One* 2016;**11**:1–15.

33 Yatsuga S, Fujita Y, Ishii A, Fukumoto Y, Arahata H, Kakuma T, Kojima T, Ito M, Tanaka M, Saiki R, Koga Y. Growth differentiation factor 15 as a useful biomarker for mitochondrial disorders. *Ann Neurol* 2015;**78**:814–23.

34 Davis RL, Liang C, Edema-Hildebrand F, Riley C, Needham M, Sue CM. Fibroblast growth factor 21 is a sensitive biomarker of mitochondrial disease. *Neurology* Published Online First: 18 October 2013. doi:10.1212/01.wnl.0000436068.43384.ef

35 Davis RL, Liang C, Sue CM. A comparison of current serum biomarkers as diagnostic indicators of mitochondrial diseases. *Neurology* 2016;**86**:2010–5.

36 Abadi A, Glover EI, Isfort RJ, Raha S, Safdar A, Yasuda N, Kaczor JJ, Melov S, Hubbard A, Qu X, Phillips SM, Tarnopolsky M. Limb immobilization induces a coordinate down-regulation of mitochondrial and other metabolic pathways in men and women. *PLoS One* 2009;**4**:e6518.

37 McKiernan P, Ball S, Santra S, Foster K, Fratter C, Poulton J, Craig K, McFarland R, Rahman S, Hargreaves I, Gupte G, Sharif K, Taylor RW. Incidence of Primary Mitochondrial Disease

in Children Younger Than 2 Years Presenting With Acute Liver Failure. *J Pediatr Gastroenterol Nutr* 2016;**63**:592–7.

38 Lane M, Boczonadi V, Bachtari S, Gomez-Duran A, Langer T, Griffiths A, Kleinle S, Dineiger C, Abicht A, Holinski-Feder E, Schara U, Gerner P, Horvath R. Mitochondrial dysfunction in liver failure requiring transplantation. *J Inherit Metab Dis* 2016;**39**:427–36.

39 Berger A, Mayr JA, Meierhofer D, Fötschl U, Bittner R, Budka H, Grethen C, Huemer M, Kofler B, Sperl W. Severe depletion of mitochondrial DNA in spinal muscular atrophy. *Acta Neuropathol* 2003;**105**:245–51.

40 Fourcade S, Ferrer I, Pujol A. Oxidative stress, mitochondrial and proteostasis malfunction in adrenoleukodystrophy: A paradigm for axonal degeneration. Free Radic. Biol. Med. 2015;**88**:18–29.

41 Frye RE, Cox D, Slattery J, Tippett M, Kahler S, Granpeesheh D, Damle S, Legido A, Goldenthal MJ. Mitochondrial Dysfunction may explain symptom variation in Phelan-McDermid Syndrome. *Sci Rep* 2016;**6**. doi:10.1038/srep19544

42 Hui J, Kirby DM, Thorburn DR, Boneh A. Decreased activities of mitochondrial respiratory chain complexes in non-mitochondrial respiratory chain diseases. *Dev Med Child Neurol* 2006;**48**:132–6.

43 Niyazov DM, Kahler SG, Frye RE. Primary Mitochondrial Disease and Secondary Mitochondrial Dysfunction: Importance of Distinction for Diagnosis and Treatment. Mol. Syndromol. 2016;**7**:122–37.

44 Garcia-Cazorla A, Serrano M, Perez-Duenas B, Gonzalez V, Ormazabal A, Pineda M, Fernandez-Alvarez E, Campistol JM, Artuch RM. Secondary abnormalities of neurotransmitters in infants with neurological disorders. *Dev Med Child Neurol* 2007;**49**:740–4.

45 Prince J, Jia S, Båve U, Annerén G, Oreland L. Mitochondrial enzyme deficiencies in Down's syndrome. *J Neural Transm - Park Dis Dement Sect* 1994;**8**:171–81.

46 Kleefstra T, Wortmann SB, Rodenburg RJT, Bongers EMHF, Hadzsiev K, Noordam C, van den Heuvel LP, Nillesen WM, Hollody K, Gillessen-Kaesbach G, Lammens M, Smeitink JAM, van der Burgt I, Morava E. Mitochondrial dysfunction and organic aciduria in five

patients carrying mutations in the Ras-MAPK pathway. *Eur J Hum Genet* 2011;**19**:138–44.

47 Panneman DM, Smeitink JA, Rodenburg RJ. Mining for mitochondrial mechanisms: linking known syndromes to mitochondrial function. *Clin Genet* Published Online First: 2017. doi:10.1111/cge.13094

48 Frye RE. 15q11.2-13 Duplication, Mitochondrial Dysfunction, and Developmental Disorders. *J Child Neurol* 2009;**24**:1316–20.

49 Maynard TM, Meechan DW, Dudevoir ML, Gopalakrishna D, Peters AZ, Heindel CC, Sugimoto TJ, Wu Y, Lieberman JA, LaMantia AS. Mitochondrial localization and function of a subset of 22q11 deletion syndrome candidate genes. *Mol Cell Neurosci* 2008;**39**:439–51.

50 Witters P, Saada A, Honzik T, Tesarova M, Kleinle S, Horvath R, Goldstein A, Morava E. Revisiting mitochondrial diagnostic criteria in the new era of genomics. *Genet Med* 2017;**00**:1–8.

51 Wolf NI, Smeitink JA. Mitochondrial disorders: a proposal for consensus diagnostic criteria in infants and children. *Neurology* 2002;**59**:1402–5.

52 Calvo SE, Clauser KR, Mootha VK. MitoCarta2.0: An updated inventory of mammalian mitochondrial proteins. *Nucleic Acids Res* 2016;**44**:D1251–7.

53 Smith AC, Robinson AJ. MitoMiner v3.1, an update on the mitochondrial proteomics database. *Nucleic Acids Res* 2016;**44**:D1258–61.

54 Frazier AE, Thorburn DR, Compton AG. Mitochondrial energy generation disorders: genes, mechanisms and clues to pathology. *J Biol Chem* 2017;:jbc.R117.809194.

55 Ormondroyd E, Mackley MP, Blair E, Craft J, Knight JC, Taylor JC, Taylor J, Watkins H. "Not pathogenic until proven otherwise": perspectives of UK clinical genomics professionals toward secondary findings in context of a Genomic Medicine Multidisciplinary Team and the 100,000 Genomes Project. *Genet Med* 2018;**20**:320–8.

56 McCormick EM, Kenyon L, Falk MJ. Desmin common mutation is associated with multi-systemic disease manifestations and depletion of mitochondria and mitochondrial DNA. *Front Genet* 2015;**6**:1–5.

57 Camara Y, Carreno-Gago L, Martin MA, Melia MJ, Blazquez A, Delmiro A, Garrabou G,

Moren C, Diaz-Manera J, Gallardo E, Bornstein B, Lopez-Gallardo E, Hernandez-Lain A, San Millan B, Cancho E, Rodriguez-Vico JS, Marti R, Garcia-Arumi E. Severe TK2 enzyme activity deficiency in patients with mild forms of myopathy. *Neurology* 2015;**84**:2286–8.

58 Elliott HR, Samuels DC, Eden JA, Relton CL, Chinnery PF. Pathogenic mitochondrial DNA mutations are common in the general population. *Am J Hum Genet* 2008;**83**:254–60.

59 Parikh S, Goldstein A, Karaa A, Koenig MK, Anselm I, Brunel-Guitton C, Christodoulou J, Cohen BH, Dimmock D, Enns GM, Falk MJ, Feigenbaum A, Frye RE, Ganesh J, Griesemer D, Haas R, Horvath R, Korson M, Kruer MC, Mancuso M, McCormack S, Raboisson MJ, Reimschisel T, Salvarinova R, Saneto RP, Scaglia F, Shoffner J, Stacpoole PW, Sue CM, Tarnopolsky M, Van Karnebeek C, Wolfe LA, Cunningham ZZ, Rahman S, Chinnery PF. Patient care standards for primary mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet Med* Published Online First: 27 July 2017. doi:10.1038/gim.2017.107

60 Parikh S, Goldstein A, Koenig MK, Scaglia F, Enns GM, Saneto R, Irina Anselm, Abigail Collins, Bruce HC, Suzanne DD, David Dimmock, Marni JF, Jaya Ganesh, Carol Greene, Andrea LG, Richard Haas, Stephen GK, John Kamholz, Fran Kendall, Mark SK, Andre Mattman, Margherita Milone, Dmitriy Niyazov, Phillip LP, Tyler Reimschisel, Ramona Salvarinova-Zivkovic, Katherine Sims, MarkTarnopolsky, Chang-Yong Tsao, Johan van Hove, Laurence Walsh, Lynne AW. Practice patterns of mitochondrial disease physicians in North America. Part 2: Treatment, care and management. *Mitochondrion* 2013;**13**:681–7.

Tables

Table 1 Differential diagnosis of selected phenotypes commonly associated with mitochondrial disease

Phenotype	Mitochondrial cause	Limited differential diagnosis
Dystonia	Leigh syndrome, deafness-dystonia syndrome, other mitochondrial encephalomyopathies	Biotinidase deficiency, thiamine transporter deficiency 2, *ADAR* mutations (Aicardi- Goutières syndrome 6), organic acidaemias (especially glutaric aciduria type I), NBIA, acute (viral) necrotising encephalopathy, mutations in *NUP62*, *RANBP2* and *PDE8B*, primary genetic dystonias
Epileptic encephalopathy	Alpers-Huttenlocher syndrome, many other mitochondrial disorders	Many genetic epileptic encephalopathies, including Dravet syndrome and *KCNQ2* mutations, Pyridoxine dependent epilepsies (Antiquitin deficiency, PNPO deficiency), viral encephalitis
Progressive myoclonic epilepsy	MERRF	Ramsay Hunt syndrome, Unverricht-Lundborg disease, Lafora body disease, sialidosis, *PRICKLE1* mutations
Leukoencephalpathy	Complex I deficiency, Complex II deficiency, SURF1 deficiency (rarely), disorders of mitochondrial translation and Fe-S cluster assembly	Vanishing white matter disease, lysosomal storage disorders, Canavan disease, Alexander disease, Pelizaeus-Merzbacher(-like), hypo/dysmyelination
Ataxia	*ADCK3* mutations, ataxia-neuropathy syndromes e.g. SCAE, MIRAS, MERRF, NARP, disorders of coenzyme Q_{10} biosynthesis	Spinocerebellar ataxias, CAPOS syndrome
Demyelination	MNGIE	ADEM, multiple sclerosis
Peripheral neuropathy	Mutations in *POLG, MPV17, KARS* and *SURF1*; part of multisystem disease in many mitochondrial disorders, e.g. MNGIE	Other non-mitochondrial genetic causes of Charcot-Marie-Tooth syndromes, riboflavin transporter deficiency, toxic neuropathies, critical illness

Ptosis and ophthalmoplegia	PEO, KSS, MNGIE, MELAS	Some congenital myopathies, pseudo upgaze impairment in *OPMD*, horizontal gaze palsy and scoliosis (*ROBO3* mutation)
Optic neuropathy	LHON, ADOA, Leigh syndrome	Toxic optic neuropathy (e.g. methanol, cyanide, tobacco)
Hypertrophic cardiomyopathy with lactic acidosis	Complex I deficiency, *TMEM70* mutations, Sengers syndrome (AGK deficiency), disorders of mitochondrial translation	Viral infection
Dilated cardiomyopathy with lactic acidosis	Barth syndrome, disorders of mitochondrial phospholipid remodelling, other mitochondrial cardiomyopathies	Viral infection
Exocrine pancreatic insufficiency	Pearson syndrome	Cystic fibrosis
Diabetes and deafness	MIDD, other mtDNA mutations	Type II diabetes mellitus with incidental nonsyndromic deafness
Sideroblastic anaemia	Pearson syndrome, MLASA, TRNT1 deficiency, *PUS1* or *YARS2* mutations	Blackfan-Diamond syndrome, Schwachman-Diamond syndrome, X-linked sideroblastic anaemia
B cell immune deficiency	TRNT1 deficiency	Primary immunodeficiency disorder
Liver failure	Mitochondrial DNA (mtDNA) depletion syndromes,	NBAS, LARS and IARS deficiencies, viral infection, lysosomal storage disorders, other syndromic genetic conditions
Renal tubulopathy/failure	Pearson and Kearns-Sayre syndromes, *RMND1*-related disease	Gitelman syndrome, Fanconi Bickel (*SLC2A2* mutations) syndrome, other syndromic genetic conditions
Myopathy	Part of multisystem disease in many mitochondrial disorders especially mtDNA depletion syndromes	Congenital muscular dystrophies, myositis, many other disorders

Rhabdomyolysis	Mitochondrial myopathies (e.g. *MTCO1*, *MTCO2*, *MTCO3* and *MTCYB* mutations)	*LPIN1* mutations, fatty acid oxidation defects (VLCAD, LCHAD), TANGO deficiency, glycolytic defects, toxic, post-exercise
Low copper	Cytochrome oxidase deficiency	Menkes, *SLC33A1* mutations
Complex multisystem disorders	Many mitochondrial disorders, particularly in childhood	Congenital disorders of glycosylation, peroxisomal disorders, lysosomal storage disorders, other syndromic genetic conditions

Key:
ADEM acute disseminated encephalomyelitis
ADOA autosomal dominant optic atrophy
CAPOS cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss
Fe-S iron-sulphur
KSS Kearns-Sayre syndrome
LHON Leber hereditary optic neuropathy
MERRF myoclonic epilepsy with ragged red fibers
MIDD maternally inherited diabetes and deafness
MIRAS mitochondrial recessive ataxia syndrome
MLASA myopathy, lactic acidosis, sideroblastic anemia
MNGIE mitochondrial neurogastrointestinal encephalomyopathy
NBIA neurodegeneration with brain iron accumulation
PEO progressive external ophthalmoplegia
SCAE spinocerebellar ataxia with epilepsy

Table 2 Mitochondrial Dysfunction Identified in Other Genetic Disorders

Disorder	Mitochondrial defect	Reference by PubMed ID number
AOA1 (*APTX* mutations)	Coenzyme Q_{10}	15699391
Desminopathy	CS, mtDNA depletion (35%)	26097489
Dravet syndrome (*SCN1A* mutations)	Variable OXPHOS deficiencies	20392657; 21906962
EXOSC3 and *EXOSC8* related diseases	Low Complex I and pyruvate dehydrogenase activities, low mtDNA copy number, increased expression of mitochondrial genes	28687512; 24989451
GLUT1 deficiency	Complex I	22156785
GM3 synthase deficiency	Respiratory chain dysfunction in fibroblasts and liver	22990144
LCHADD	Complex III, COX	16417669
Limb immobilization	COX and CS	19654872
Lysosomal diseases: GM1-gangliosidosis, mucopolysaccharidosis IIIC, multiple sulfatase deficiency, Krabbe disease, Gaucher disease, Niemann Pick disease type C	Multiple OXPHOS deficiencies attributed to excessive production of mitochondrial reactive oxygen species and dysregulated calcium homeostasis with mitochondria-induced apoptosis and neurodegeneration	28132808
MADD (*ETFDH, ETFA* or *ETFB* mutations)	Complex I and II deficiencies; Riboflavin and Coenzyme Q_{10} responsive	17412732
Molybdenum co-factor deficiency	COX	16417669
MTHFR mutations	Complex I deficiency	21131308
Multiple carboxylase deficiency	Complex III	16417669
NBIA (PKAN)	Complex III	16417669
Neonatal haemochromatosis	Complex III (liver)	16417669
Neuroferritinopathy (*FTL1)*	Complex I or multiple Complex deficiency	17142829
NPHS3 (PLCE1 deficiency)	COX	21365190
Neuronal Ceroid Lipofuscinosis (*CLN2* and *CLN3*-related)	Partial deficiency in fatty acid oxidation enzymes and the storage of subunit c of mitochondrial ATP synthase in fibroblasts	8971698
ORAI1 related disease	Impaired lipid metabolism and fatty acid oxidation in skeletal muscle, heart and liver due to abnormal store-operated Ca^{2+} entry	28132808
Organic acidemias	Coenzyme Q_{10}, multiple OXPHOS deficiencies and free radical induced oxidative damage	21329767; 28753922; 28753922
Ras/MAPK pathway mutations	Variable OXPHOS deficiencies	26097489
Rett syndrome (*MECP2* mutations)	Variable OXPHOS deficiencies	26741492
SCAR10	Coenzyme Q_{10}	25182700

Spinal muscular atrophy	Complexes I-IV, mtDNA depletion	12557011; 25844556
STXBP1 mutation (*de novo*)	Complex I	25418441
Zellweger syndrome	Complexes II+III, COX	25287621; 28753922

Key: AOA1 oculomotor apraxia type 1, COX cytochrome *c* oxidase, CS citrate synthase, GLUT1 glucose transporter 1, LCHADD long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency, MADD multiple acyl-CoA dehydrogenase deficiency, MAPK mitogen activated protein kinases, mtDNA mitochondrial DNA, NBIA neuronal brain iron accumulation, NPHS3 nephrotic syndrome type 3, OXPHOS oxidative phosphorylation, PKAN pantothenate kinase, SCAR10 autosomal recessive spinocerebellar ataxia-10,

Table 3 Limitations of Testing

Current Limitations of Biochemical Testing
Imperfect sensitivity and specificity
Secondary mitochondrial dysfunction leading to abnormal results
Inter-lab variability of methods and reference ranges
Challenges with tissue processing

Current Limitations of Genetic Testing
Incomplete understanding of the role of the entire genome in mitochondrial function
Novel genes still being identified
Interpretation of nuclear and mtDNA variants of uncertain significance
Lack of understanding of tissue-specificity of mtDNA mutations
Unclear relevance of low heteroplasmy levels of pathogenic mtDNA mutations
mtDNA deletions and depletion may be observed in non-mitochondrial disease

Table 4 Potential Harms Arising from a Diagnosis of "Possible" Mitochondrial Disease

Ending diagnostic odyssey prematurely
Missing potentially treatable disorders
Psychological burden of mitochondrial disease diagnosis: parent/patient fear of progressive or degenerative disorder
Inaccurate recurrence risk counseling
Inappropriate preventative care
Unnecessary medical interventions at times of catabolic stress
Avoidance of needed medications owing to fear of mitochondrial toxicity
Inappropriate reproductive decisions taken

Table 5 Terminology to avoid when a mitochondrial diagnosis is uncertain
"Possible," "Probable" or "Suspected" Mitochondrial Disease
Mitochondrial Myopathy
Mitochondrial Cytopathy
Mitochondrial Metabolism Disorder
Defect of Mitochondrial Metabolism

Supplementary Material

Biochemical Testing Concerns

Once a mitochondrial disease is suspected, most testing protocols begin with assessing lactic acid, plasma or CSF amino acids and urine organic acids. Elevations of plasma lactic acid and certain amino acids (such as alanine, glycine, proline or threonine) or urinary organic acids (such as malate, fumarate or 3-methylglutaconic acid) may indicate mitochondrial dysfunction. Concerns regarding their sensitivity and specificity have been discussed previously, and they remain imperfect biomarkers in assessing mitochondrial dysfunction. However they continue to be routinely obtained since they are relatively inexpensive and may be used to guide the need for further testing.[1] Some have reported that when collected properly and considered in the correct clinical context, elevations in lactate may selectively have specificity as high as 83-100%[2–4] although this is not always the case.[5] Nonetheless, permanent urinary excretion of 3-methylglutaconic acid, if associated with a suggestive clinical picture, is usually a good indicator of PMD.[6] FGF21 and GDF15 are cytokines more recently associated with mitochondrial dysfunction in cases of myopathy but also have less than optimal sensitivity and specificity, often being elevated in non-mitochondrial diseases including diabetes, hypothyroidism, overfeeding, sepsis and other genetic diseases.[2, 7–13]

While evaluation of suspected mitochondrial disease now often quickly moves to molecular genetic testing, functional studies of the mitochondrial electron transport chain (ETC) are still important in many situations. However, in many centers they are no longer the first line test when mitochondrial disease is suspected. Functional assays include ETC enzymology via spectrophotometry, measurement of the presence of selected protein components within complexes and super-complexes via western blots and blue-native gel electrophoresis, and oxygen consumption rates using various substrates and inhibitors.

False positive results may be seen due to challenges in tissue processing and test application including improper tissue collection or handling, the impact of local anesthetic on results,[14] and utilizing reference ranges intended for quadriceps muscle for muscle tissue collected from other sites. ETC assays may have little to no margin between patient and control ranges and can also differ between different laboratories and make inter-laboratory comparison of test results extremely challenging. Multiple global health factors impact results, including exercise or the lack thereof, effects of chronic illness, and age of the patient. Secondary and potentially reversible ETC functional defects have also been observed following limb immobilisation,[15] and in liver failure from non-mitochondrial causes.[16, 17] ETC deficiency has been seen in ischaemia/reperfusion injury, sepsis and trauma.[18–20]

Even once these factors are seemingly accounted for, functional studies may lead to misdiagnoses and mistaken labels of a "possible" mitochondrial disease, because other genetic conditions may sometimes be associated with a secondary mitochondrial dysfunction.[21] For instance, multiple ETC deficiencies and mtDNA depletion have been reported in Spinal Muscular Atrophy (SMA).[22] Downregulated muscle mitochondrial biogenesis has been shown to underlie this mitochondrial dysfunction in SMA.[23] Abnormal accumulation of very long-chain fatty acids in X-linked adrenoleukodystrophy (ALD) leads to a defect in mitochondrial respiration and biogenesis with chronic redox imbalance, which in turn is partly responsible for neurodegeneration.[24] Other examples exist for Phelan-McDermid syndrome, Down syndrome, Zellweger syndrome, the rasopathies and a variety of other conditions.[25–30] Mechanistic links for this secondary dysfunction have been described for very few of these disorders .[31] Thus, evidence of biochemical dysfunction on functional testing cannot provide a conclusive diagnosis of mitochondrial disease.

Functional mitochondrial studies do serve several key roles. As genetic testing remains imperfect, biochemical testing may help to better elucidate the degree of mitochondrial dysfunction and potentially define the pathogenicity of genetic variants of uncertain significance (VUS). Functional tests can also provide further evidence when genomic studies

identify a pathogenic mutation in a single allele for an autosomal recessive condition with a compatible clinical phenotype and there is a concern that a second pathogenic disease allele may not have been identified by exome sequencing.[32]

Genetic Testing Concerns

The advent of rapid, relatively low cost NGS technologies has allowed for a genetic diagnosis to be made in many more patients with PMD. Accurate genetic diagnosis allows physicians and affected families to better understand the condition and for the provision of appropriate genetic counseling. For some disorders where the natural history is better known, clinicians and families can more accurately predict the disease course and provide appropriate clinical management and preventative care.[33]

However, genetic testing in mitochondrial disease remains limited by aspects of tissue specificity of mutations in mtDNA, heteroplasmy, lack of understanding of the entire genome, VUS findings and an ever-growing list of nuclear disease genes. In the absence of functional testing, pathogenicity can only be assigned to genetic variants that have previously been observed to cause the same or highly similar phenotype in an unrelated family.[34]

<u>Testing mtDNA and tissue specificity</u>

MtDNA can now be accurately sequenced in its entirety for a relatively low cost. However, mtDNA heteroplasmy, with a varied distribution of point mutations or deletions in various post-mitotic tissues (e.g. muscle or brain), may lead to difficulties with diagnosis when assessing rapidly dividing tissues such as blood, where levels of mutated mtDNA may be extremely low. NGS has improved sensitivity so that it is now possible to detect levels of heteroplasmy of less than 5% in blood DNA. However, testing in blood cannot accurately determine mtDNA copy number or mtDNA deletions in a majority of cases.

In patients with suspected mitochondrial disease, the finding of low level heteroplasmy of a pathogenic mtDNA mutation does little to assist the clinician in determining if the mutation is

clinically relevant and if so, how the prognosis is affected. Low level heteroplasmy in blood does not exclude a pathogenic level of heteroplasmy in another tissue, especially if the patient's symptoms are primarily muscle or brain related. In disorders that have been well characterized with clear heteroplasmy:phenotype relationships (e.g. MERRF m.8344G>A, MELAS m.3243A>G, NARP m.8993T>C/G) the finding of low level heteroplasmy (< 5 %) in blood is not always likely to be associated with neurological disease; however, assessment in other tissues is still recommended if the phenotype is compatible.

Incidental findings of mtDNA mutations at low level heteroplasmy are not uncommon, especially since ~ 1/200 asymptomatic people may carry a low level heteroplasmic pathogenic mtDNA variant in blood.[35–37] Such variants are now even detected in patients tested by WES in whom mitochondrial disease may not have been strongly on the differential diagnosis list. It is quite easy to mistakenly attribute clinical relevance to low-level heteroplasmic mtDNA mutations due to a clinician's anchoring and confirmation bias even though it may not be the actual cause of a given patient's constellation of medical problems.

Assessing mtDNA in other tissues such as muscle, liver or urinary epithelial cells may help when pathogenic mutations are not detected in blood or only low levels of a pathogenic mutation are found. MtDNA point mutation heteroplasmy analysis in urine provides another non-invasive, reliable and relatively inexpensive methodology that has been validated against skeletal muscle heteroplasmy, although testing is not available on a clinical basis in all regions.[38] Some mtDNA point mutations and large-scale or multiple deletions may only be detected in muscle or liver in some patients. Long-range PCR is the preferred method for detecting deletions as Southern blot analysis lacks sufficient sensitivity to detect low levels of heteroplasmic deletions. Southern blot analysis remains useful for clarifying the type of rearrangement in patients with duplicated or deleted mtDNA.[39] Interpretation is complicated, as normal aging may lead to a low level of multiple mtDNA deletions in tissues including muscle, and accumulation of mtDNA deletions may be accelerated in other muscle disorders, particularly sporadic inclusion body myositis.

MtDNA copy number analysis for mtDNA depletion is also not yet routinely measurable by NGS or accurately quantifiable or always represented in blood. Such testing may become viable and cost effective via whole genome sequencing (WGS) or other NGS approaches in the near future. Currently, the most widely used approach if mtDNA depletion is suspected is to perform a quantitative real-time PCR assay, preferably in an affected tissue, although unaffected tissue (e.g. skeletal muscle in *POLG*-related Alpers-Huttenlocher syndrome) may still demonstrate a significant, albeit clinically silent, mtDNA depletion. Interpretation of mtDNA copy number data is critically dependent on appropriate age and tissue matched normal control ranges,[40] which can be difficult to obtain, thereby limiting applicability. Results may be equivocal even when normal control ranges are available. To complicate matters, mtDNA depletion has been identified in non-mitochondrial diseases including desminopathies, Parkinson disease, age-related changes in paraspinal muscles and as a consequence of antiretroviral therapy.[41–45]

Heteroplasmic variants

As mtDNA has a higher mutation rate than the nuclear genome, many individuals have rare or private mtDNA polymorphisms. Determining the pathogenicity of these polymorphisms remains challenging. Interpreting the genetic results in the light of clinical and laboratory findings and the family history may be helpful, but still may not provide a conclusive answer. MtDNA haplotyping may assist in establishing pathogenicity,[46] and the same mutation arising independently several times and co-segregating with clinical features may support a causal role.[47]

Even when pathogenicity is suspected, phenotypic variability remains pronounced. This is the case for many of the less common or novel pathogenic mutations in the mt-tRNA genes. Distinguishing novel pathogenic mutations from benign polymorphisms remains difficult. Research based testing to interrogate the pathogenicity of heteroplasmic mutations – via single fibre and/or transmitochondrial cybrid studies – remains costly and labour intensive,[48] and is not possible for most patients.

Testing nuclear genes

Since the mitochondrial genome only encodes 13 mitochondrial proteins (and 24 tRNA and rRNA genes required for the intramitochondrial synthesis of these 13 proteins), nuclear genes encode almost the entire mitochondrial proteome. New genes impacting mitochondrial function and leading to human disease continue to be discovered. Many of the known disease-causing genes may be tested via gene panels and whole exome sequencing (WES) although the ability to detect a clearly pathogenic mutation in suspected PMD is imperfect, with a reported diagnostic yield ranging from 25-75%, dependent upon study design.[49–53]

WGS is beginning to be utilized beyond research-based testing but our current understanding remains limited. Many ongoing WGS analyses are restricted to assessing variants in the approximately 2% of the genome that represents the exome, owing to a very limited understanding of the consequences of sequence variants in other regions (promoters, deep intronic regions and other non-coding regions). Much of what the genome holds in regard to causing primary mitochondrial disease remains to be seen. This lack of a complete understanding of and the inability to meaningfully analyze variants in all the genomic elements involved in mitochondrial function greatly contributes to our inability to make genetic diagnoses in 100% of cases with suspected PMD.

Using current approaches, many suspected cases, both pediatric and adult, may still remain without a genetic diagnosis following WES or WGS.[54, 55] This figure will decrease as sequencing quality, bioinformatic analysis, and our ability to interpret variants throughout the genome improves. In addition, supplementary approaches such as RNA sequencing of the transcriptome and proteomics and metabolomics approaches are proving helpful in increasing the genetic diagnosis rate.[5, 55]

Nuclear gene variants

It is now well known that when analysing large numbers of genes by WES or WGS, tens of thousands of coding sequence variants will be identified in any given individual. Even after bioinformatic filtering of common benign polymorphisms, hundreds of putative significant variants remain. Interpreting these variants constitutes a huge challenge. In practice, one or more VUS is routinely identified. A multi-disciplinary team approach is necessary for optimal interpretation, bringing together clinical, biochemical, histological and genetic expertise. In the case of known mitochondrial disease genes, concordance with clinical and biochemical features can be complicated by weak genotype-phenotype correlation and the continuing widening of phenotype associated with a particular gene as more cases are identified. Further studies, including RNA sequencing or protein functional studies, may be necessary although these follow-up studies are often only available in a research setting.

For nuclear VUS, variant prioritisation following bioinformatic analysis[56–58] and database interrogation (e.g. 1000 Genomes,[55] ClinVar,[59] ExAC/gnomAD,[60] Medical Genome Reference Bank, MITOMAP,[61] MSeqDR,[62]), as well as classification using the American College of Medical Genetics (ACMG) criteria,[34] a search of known attributes of the affected gene and its transcriptional/translational products can inform an approach toward confirmation of pathogenicity.

The simplest approach for ascribing conditional pathogenicity is investigating segregation of a variant with disease in family members. This may be difficult when considering multisystemic mitochondrial diseases that can display high levels of phenotypic heterogeneity for the same gene or variant.[63] If familial segregation information is inconclusive or cannot be obtained, investigating larger cohorts of phenotypically similar patients for the same genotype may provide support for pathogenicity. As mentioned above, this may be difficult in the absence of a consistent genotype-phenotype correlation and additionally in the setting of a rare disease. In any case, segregation data must be accompanied by empirical evidence of a functional defect, requiring a synthesis of clinical tests and laboratory experiments to establish a pathological basis for the VUS. This may require a research laboratory with specialist interests in the gene or

disease in question and can be aided by the availability of patient-derived tissue samples or cell lines for investigations.

Validation of VUS pathogenicity is determined by the nature of the variant in question (missense, nonsense, splicing, indel, copy number variation, structural variant, etc.), the affected gene and its associated function and the biological/pathological relevance. Experimental evidence supporting pathogenicity (in addition to clinical presentation, phenotype/genotype correlation and theoretical evidence) could include imaging studies, biomarker measurements, or functional approaches in patient-derived tissue and cell lines or model organisms.

Functional biochemical testing in muscle or other tissues may help better ascertain the relevance of selected VUS and remains a necessary tool in some patients. Interpreting VUS remains an ongoing challenge although there are guidelines on how to better categorize these.[34] These are important considerations for accurate diagnosis and counselling of patients with PMD disorders, particularly at a time when reproductive choice has been expanded to include preimplantation genetic diagnosis and more recently mitochondrial donation.

<u>mtDNA VUS</u>

Novel mtDNA variants pose unique challenges for clinical correlation and interpretation given the challenges of performing functional validation studies, broad heterogeneity of PMD clinical phenotypes, and unknown threshold effects necessary for a given variant to manifest disease in particular organs. However, additional information at both bioinformatic and clinical levels can be readily attained to further understand the potential functional impact of a given mtDNA variant.

Several readily available bioinformatics tools now exist that can be used to efficiently gather information about novel mtDNA variants. For example, mvTool,[64] hosted on the

Mitochondrial Disease Sequence Data Resource (MSeqDR[62]), provides population level frequency data for mtDNA variants, a compilation of *in silico* variant pathogenicity predictor tools, and a collection of expert curated pathogenicity data from multiple sources including MITOMAP[61] and HmtDB.[65]
Guidelines and recommendations for mtDNA variants curation have also been developed by an expert panel to aid clinicians and molecular geneticists in proper interpretation. (Standards and guidelines for mitochondrial DNA variant interpretation. [Elizabeth M. McCormick et al.. Human Mutation 2018 (accepted; under revision)]

Valuable information may also be gained from pursuing diagnostic testing of the mtDNA variant in question in multiple tissues from both the proband and family members, although this is not always possible due to family preferences and insurance restrictions. A higher level of heteroplasmy in more clinically symptomatic tissues provides strong supporting evidence for the likely pathogenicity of a given mtDNA variant.

Traditionally, the gold-standard definitive study to assess pathogenicity of a heteroplasmic mtDNA mutation was to generate a transmitochondrial cell line, by fusion of an enucleated patient cell with a rho zero cell lacking mtDNA, to determine whether a biochemical defect is transferred with the patient mtDNA.[66]

REFERENCES

1 Parikh S, Goldstein A, Koenig MK, Scaglia F, Enns GM, Saneto R, Anselm I, Cohen BH, Falk MJ, Greene C, Gropman AL, Haas R, Hirano M, Morgan P, Sims K, Tarnopolsky M, Van Hove JLK, Wolfe L, DiMauro S. Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet Med* 2015;**17**:689–701.

2 Suomalainen A, Elo JM, Pietiläinen KH, Hakonen AH, Sevastianova K, Korpela M, Isohanni P, Marjavaara SK, Tyni T, Kiuru-Enari S, Pihko H, Darin N, Õunap K, Kluijtmans LAJ, Paetau A, Buzkova J, Bindoff LA, Annunen-Rasila J, Uusimaa J, Rissanen A, Yki-Järvinen H, Hirano M, Tulinius M, Smeitink J, Tyynismaa H. FGF-21 as a biomarker for muscle-manifesting mitochondrial respiratory chain deficiencies: A diagnostic study. *Lancet Neurol* 2011;**10**:806–18.

3 Davis RL, Liang C, Edema-Hildebrand F, Riley C, Needham M, Sue CM. Fibroblast growth factor 21 is a sensitive biomarker of mitochondrial disease. *Neurology* Published Online First: 18 October 2013. doi:10.1212/01.wnl.0000436068.43384.ef

4 Tarnopolsky M, Stevens L, MacDonald JR, Rodriguez C, Mahoney D, Rush J, Maguire J. Diagnostic utility of a modified forearm ischemic exercise test and technical issues relevant to exercise testing. *Muscle Nerve* 2003;**27**:359–66.

5 Rahman J, Rahman S. Mitochondrial medicine in the omics era. *Lancet (London, England)* 2018;**391**:2560–74.

6 Wortmann SB, Kluijtmans LA, Rodenburg RJ, Sass JO, Nouws J, van Kaauwen EP, Kleefstra T, Tranebjaerg L, de Vries MC, Isohanni P, Walter K, Alkuraya FS, Smuts I, Reinecke CJ, van der Westhuizen FH, Thorburn D, Smeitink JA, Morava E, Wevers RA. 3-Methylglutaconic aciduria--lessons from 50 genes and 977 patients. *J Inherit Metab Dis* 2013;**36**:913–21.

7 Kim KH, Kim SH, Min YK, Yang HM, Lee JB, Lee MS. Acute exercise induces FGF21 expression in mice and in healthy humans. *PLoS One* 2013;**8**:e63517.

8 Gariani K, Drifte G, Dunn-Siegrist I, Pugin J, Jornayvaz FR. Increased FGF21 plasma levels in humans with sepsis and SIRS. *Endocr Connect* 2013;**2**:146–53.

9 Salehi MH, Kamalidehghan B, Houshmand M, Aryani O, Sadeghizadeh M, Mossalaeie MM. Association of fibroblast growth factor (FGF-21) as a biomarker with primary mitochondrial disorders, but not with secondary mitochondrial disorders (Friedreich Ataxia). *Mol Biol Rep* 2013;**40**:6495–9.

10 Lee Y, Park YJ, Ahn HY, Lim JA, Park KU, Choi SH, Park do J, Oh BC, Jang HC, Yi KH. Plasma FGF21 levels are increased in patients with hypothyroidism independently of lipid profile. *Endocr J* 2013;**60**:977–83.

11 Suomalainen A, Elo JM, Pietilainen KH, Hakonen AH, Sevastianova K, Korpela M, Isohanni P, Marjavaara SK, Tyni T, Kiuru-Enari S, Pihko H, Darin N, Ounap K, Kluijtmans LA, Paetau A, Buzkova J, Bindoff LA, Annunen-Rasila J, Uusimaa J, Rissanen A, Yki-Jarvinen H, Hirano M, Tulinius M, Smeitink J, Tyynismaa H. FGF-21 as a biomarker for muscle-manifesting mitochondrial respiratory chain deficiencies: a diagnostic study. *Lancet Neurol* 2011;**10**:806–18.

12 Davis RL, Liang C, Sue CM. A comparison of current serum biomarkers as diagnostic indicators of mitochondrial diseases. *Neurology* 2016;**86**:2010–5.

13 Yatsuga S, Fujita Y, Ishii A, Fukumoto Y, Arahata H, Kakuma T, Kojima T, Ito M, Tanaka M, Saiki R, Koga Y. Growth differentiation factor 15 as a useful biomarker for mitochondrial disorders. *Ann Neurol* 2015;**78**:814–23.

14 Janssen AJ, Smeitink JA, van den Heuvel LP. Some practical aspects of providing a diagnostic service for respiratory chain defects. *Ann Clin Biochem* 2003;**40**:3–8.

15 Abadi A, Glover EI, Isfort RJ, Raha S, Safdar A, Yasuda N, Kaczor JJ, Melov S, Hubbard A, Qu X, Phillips SM, Tarnopolsky M. Limb immobilization induces a coordinate down-regulation of mitochondrial and other metabolic pathways in men and women. *PLoS One* 2009;**4**:e6518.

16 McKiernan P, Ball S, Santra S, Foster K, Fratter C, Poulton J, Craig K, McFarland R, Rahman S, Hargreaves I, Gupte G, Sharif K, Taylor RW. Incidence of Primary Mitochondrial Disease in Children Younger Than 2 Years Presenting With Acute Liver Failure. *J Pediatr Gastroenterol Nutr* 2016;**63**:592–7.

17 Lane M, Boczonadi V, Bachtari S, Gomez-Duran A, Langer T, Griffiths A, Kleinle S, Dineiger

C, Abicht A, Holinski-Feder E, Schara U, Gerner P, Horvath R. Mitochondrial dysfunction in liver failure requiring transplantation. *J Inherit Metab Dis* 2016;**39**:427–36.

18 Murphy MP. Understanding and preventing mitochondrial oxidative damage. *Biochem Soc Trans* 2016;**44**:1219–26.

19 Stepien KM, Heaton R, Rankin S, Murphy A, Bentley J, Sexton D, Hargreaves IP. Evidence of Oxidative Stress and Secondary Mitochondrial Dysfunction in Metabolic and Non-Metabolic Disorders. *J Clin Med* 2017;**6**:71.

20 Kilbaugh TJ, Karlsson M, Byro M, Bebee A, Ralston J, Sullivan S, Duhaime AC, Hansson MJ, Elmér E, Margulies SS. Mitochondrial bioenergetic alterations after focal traumatic brain injury in the immature brain. *Exp Neurol* 2015;**271**:136–44.

21 Pyle A, Nightingale HJ, Griffin H, Abicht A, Kirschner J, Baric I, Cuk M, Douroudis K, Feder L, Kratz M, Czermin B, Kleinle S, Santibanez-Koref M, Karcagi V, Holinski-Feder E, Chinnery PF, Horvath R. Respiratory chain deficiency in nonmitochondrial disease. *Neurol Genet* 2015;**1**. doi:10.1212/NXG.0000000000000006

22 Berger A, Mayr JA, Meierhofer D, Fötschl U, Bittner R, Budka H, Grethen C, Huemer M, Kofler B, Sperl W. Severe depletion of mitochondrial DNA in spinal muscular atrophy. *Acta Neuropathol* 2003;**105**:245–51.

23 Ripolone M, Ronchi D, Violano R, Vallejo D, Fagiolari G, Barca E, Lucchini V, Colombo I, Villa L, Berardinelli A, Balottin U, Morandi L, Mora M, Bordoni A, Fortunato F, Corti S, Parisi D, Toscano A, Sciacco M, DiMauro S, Comi GP, Moggio M, Di Mauro S, Comi GP, Moggio M. Impaired muscle mitochondrial biogenesis and myogenesis in spinal muscular atrophy. *JAMA Neurol* 2015;**72**:666–75.

24 Fourcade S, Ferrer I, Pujol A. Oxidative stress, mitochondrial and proteostasis malfunction in adrenoleukodystrophy: A paradigm for axonal degeneration. Free Radic. Biol. Med. 2015;**88**:18–29.

25 Frye RE, Cox D, Slattery J, Tippett M, Kahler S, Granpeesheh D, Damle S, Legido A, Goldenthal MJ. Mitochondrial Dysfunction may explain symptom variation in Phelan-McDermid Syndrome. *Sci Rep* 2016;**6**. doi:10.1038/srep19544

26 Hui J, Kirby DM, Thorburn DR, Boneh A. Decreased activities of mitochondrial respiratory

chain complexes in non-mitochondrial respiratory chain diseases. *Dev Med Child Neurol* 2006;**48**:132–6.

27 Niyazov DM, Kahler SG, Frye RE. Primary Mitochondrial Disease and Secondary Mitochondrial Dysfunction: Importance of Distinction for Diagnosis and Treatment. Mol. Syndromol. 2016;**7**:122–37.

28 Garcia-Cazorla A, Serrano M, Perez-Duenas B, Gonzalez V, Ormazabal A, Pineda M, Fernandez-Alvarez E, Campistol JM, Artuch RM. Secondary abnormalities of neurotransmitters in infants with neurological disorders. *Dev Med Child Neurol* 2007;**49**:740–4.

29 Prince J, Jia S, Båve U, Annerén G, Oreland L. Mitochondrial enzyme deficiencies in Down's syndrome. *J Neural Transm - Park Dis Dement Sect* 1994;**8**:171–81.

30 Kleefstra T, Wortmann SB, Rodenburg RJT, Bongers EMHF, Hadzsiev K, Noordam C, van den Heuvel LP, Nillesen WM, Hollody K, Gillessen-Kaesbach G, Lammens M, Smeitink JAM, van der Burgt I, Morava E. Mitochondrial dysfunction and organic aciduria in five patients carrying mutations in the Ras-MAPK pathway. *Eur J Hum Genet* 2011;**19**:138–44.

31 Panneman DM, Smeitink JA, Rodenburg RJ. Mining for mitochondrial mechanisms: linking known syndromes to mitochondrial function. *Clin Genet* Published Online First: 2017. doi:10.1111/cge.13094

32 Simon MT, Ng BG, Friederich MW, Wang RY, Boyer M, Kircher M, Collard R, Buckingham KJ, Chang R, Shendure J, Nickerson DA, Bamshad MJ, Van Hove JLK, Freeze HH, Abdenur JE. Activation of a cryptic splice site in the mitochondrial elongation factor GFM1 causes combined OXPHOS deficiency. *Mitochondrion* 2017;**34**:84–90.

33 Parikh S, Goldstein A, Karaa A, Koenig MK, Anselm I, Brunel-Guitton C, Christodoulou J, Cohen BH, Dimmock D, Enns GM, Falk MJ, Feigenbaum A, Frye RE, Ganesh J, Griesemer D, Haas R, Horvath R, Korson M, Kruer MC, Mancuso M, McCormack S, Raboisson MJ, Reimschisel T, Salvarinova R, Saneto RP, Scaglia F, Shoffner J, Stacpoole PW, Sue CM, Tarnopolsky M, Van Karnebeek C, Wolfe LA, Cunningham ZZ, Rahman S, Chinnery PF. Patient care standards for primary mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet Med* Published Online First: 27 July 2017.

doi:10.1038/gim.2017.107

34 Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015;**17**:405–24.

35 Elliott HR, Samuels DC, Eden JA, Relton CL, Chinnery PF. Pathogenic mitochondrial DNA mutations are common in the general population. *Am J Hum Genet* 2008;**83**:254–60.

36 Bitner-Glindzicz M, Pembrey M, Duncan A, Heron J, Ring SM, Hall A, Rahman S. Prevalence of Mitochondrial 1555A→G Mutation in European Children. *N Engl J Med* Published Online First: 2009. doi:10.1056/NEJMc0806396

37 Manwaring N, Jones MM, Wang JJ, Rochtchina E, Howard C, Mitchell P, Sue CM. Population prevalence of the MELAS A3243G mutation. *Mitochondrion* 2007;**7**:230–3.

38 Poulton J, Morten KJ, Weber K, Brown GK, Bindoff L. Are duplications of mitochondrial DNA characteristic of Kearns-Sayre syndrome? *Hum Mol Genet* 1994;**3**:947–51.

39 Poulton J, Holt IJ. Mitochondrial DNA: Does more lead to less? *Nat Genet* 1994;**8**:313–5.

40 Calvo SE, Clauser KR, Mootha VK. MitoCarta2.0: An updated inventory of mammalian mitochondrial proteins. *Nucleic Acids Res* 2016;**44**:D1251–7.

41 McCormick EM, Kenyon L, Falk MJ. Desmin common mutation is associated with multi-systemic disease manifestations and depletion of mitochondria and mitochondrial DNA. *Front Genet* 2015;**6**:1–5.

42 Scruggs ER, Dirks Naylor AJ. Mechanisms of zidovudine-induced mitochondrial toxicity and myopathy . *Pharmacology* 2008;**82**:83–8.

43 Pyle A, Anugrha H, Kurzawa-Akanbi M, Yarnall A, Burn D, Hudson G. Reduced mitochondrial DNA copy number is a biomarker of Parkinson's disease. *Neurobiol Aging* 2015;**38**:216.e7-216.e10.

44 Campbell GR, Reeve A, Ziabreva I, Polvikoski TM, Taylor RW, Reynolds R, Turnbull DM, Mahad DJ. Mitochondrial DNA deletions and depletion within paraspinal muscles. *Neuropathol Appl Neurobiol* 2013;**39**:377–89.

45 Poulton J, Sewry C, Potter CG, Bougeron T, Chretien D, Wijburg FA, Morten KJ, Brown G. Variation in mitochondrial DNA levels in muscle from normal controls. Is depletion of mtDNA in patients with mitochondrial myopathy a distinct clinical syndrome? *J Inherit Metab Dis* Published Online First: 1995. doi:10.1007/BF00711367

46 Yarham JW, Al-Dosary M, Blakely EL, Alston CL, Taylor RW, Elson JL, McFarland R. A comparative analysis approach to determining the pathogenicity of mitochondrial tRNA mutations. *Hum Mutat* 2011;**32**:1319–25.

47 Dombi E, Diot A, Morten K, Carver J, Lodge T, Fratter C, Ng YS, Liao C, Muir R, Blakely EL, Hargreaves I, Al-Dosary M, Sarkar G, Hickman SJ, Downes SM, Jayawant S, Yu-Wai-Man P, Taylor RW, Poulton J. The m.13051G>A mitochondrial DNA mutation results in variable neurology and activated mitophagy. Neurology. 2016;**86**:1921–3.

48 Caporali L, Iommarini L, La Morgia C, Olivieri A, Achilli A, Maresca A, Valentino ML, Capristo M, Tagliavini F, Del Dotto V, Zanna C, Liguori R, Barboni P, Carbonelli M, Cocetta V, Montopoli M, Martinuzzi A, Cenacchi G, De Michele G, Testa F, Nesti A, Simonelli F, Porcelli AM, Torroni A, Carelli V. Peculiar combinations of individually non-pathogenic missense mitochondrial DNA variants cause low penetrance Leber's hereditary optic neuropathy. *PLoS Genet* 2018;**14**. doi:10.1371/journal.pgen.1007210

49 Calvo SE, Compton AG, Hershman SG, Lim SC, Lieber DS, Tucker EJ, Laskowski A, Garone C, Liu S, Jaffe DB, Christodoulou J, Fletcher JM, Bruno DL, Goldblatt J, Dimauro S, Thorburn DR, Mootha VK. Molecular diagnosis of infantile mitochondrial disease with targeted next-generation sequencing. *Sci Transl Med* 2012;**4**:118ra10.

50 Wortmann SB, Koolen DA, Smeitink JA, van den Heuvel L, Rodenburg RJ. Whole exome sequencing of suspected mitochondrial patients in clinical practice. *J Inherit Metab Dis* 2015;**38**:437–43.

51 Taylor RW, Pyle A, Griffin H, Blakely EL, Duff J, He L, Smertenko T, Alston CL, Neeve VC, Best A, Yarham JW, Kirschner J, Schara U, Talim B, Topaloglu H, Baric I, Holinski-Feder E, Abicht A, Czermin B, Kleinle S, Morris AAM, Vassallo G, Gorman GS, Ramesh V, Turnbull DM, Santibanez-Koref M, McFarland R, Horvath R, Chinnery PF. Use of whole-exome sequencing to determine the genetic basis of multiple mitochondrial respiratory chain

complex deficiencies. *Jama* 2014;**312**:68–77.

52 Lieber DS, Calvo SE, Shanahan K, Slate NG, Liu S, Hershman SG, Gold NB, Chapman BA, Thorburn DR, Berry GT, Schmahmann JD, Borowsky ML, Mueller DM, Sims KB, Mootha VK. Targeted exome sequencing of suspected mitochondrial disorders. *Neurology* 2013;**80**:1762–70.

53 Kohda M, Tokuzawa Y, Kishita Y, Nyuzuki H, Moriyama Y, Mizuno Y, Hirata T, Yatsuka Y, Yamashita-Sugahara Y, Nakachi Y, Kato H, Okuda A, Tamaru S, Borna NN, Banshoya K, Aigaki T, Sato-Miyata Y, Ohnuma K, Suzuki T, Nagao A, Maehata H, Matsuda F, Higasa K, Nagasaki M, Yasuda J, Yamamoto M, Fushimi T, Shimura M, Kaiho-Ichimoto K, Harashima H, Yamazaki T, Mori M, Murayama K, Ohtake A, Okazaki Y. A Comprehensive Genomic Analysis Reveals the Genetic Landscape of Mitochondrial Respiratory Chain Complex Deficiencies. *PLoS Genet* 2016;**12**. doi:10.1371/journal.pgen.1005679

54 Kremer LS, Bader DM, Mertes C, Kopajtich R, Pichler G, Iuso A, Haack TB, Graf E, Schwarzmayr T, Terrile C, Koňaříkova E, Repp B, Kastenmüller G, Adamski J, Lichtner P, Leonhardt C, Funalot B, Donati A, Tiranti V, Lombes A, Jardel C, Gläser D, Taylor RW, Ghezzi D, Mayr JA, Rötig A, Freisinger P, Distelmaier F, Strom TM, Meitinger T, Gagneur J, Prokisch H. Genetic diagnosis of Mendelian disorders via RNA sequencing. *Nat Commun* 2017;**8**. doi:10.1038/ncomms15824

55 1000 Genomes Project Consortium, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR. A global reference for human genetic variation. *Nature* 2015;**526**:68–74.

56 Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, del Angel G, Levy-Moonshine A, Jordan T, Shakir K, Roazen D, Thibault J, Banks E, Garimella K V., Altshuler D, Gabriel S, DePristo MA. From fastQ data to high-confidence variant calls: The genome analysis toolkit best practices pipeline. *Curr Protoc Bioinforma* Published Online First: 2013. doi:10.1002/0471250953.bi1110s43

57 Paila U, Chapman BA, Kirchner R, Quinlan AR. GEMINI: Integrative Exploration of Genetic Variation and Genome Annotations. *PLoS Comput Biol* 2013;**9**. doi:10.1371/journal.pcbi.1003153

58 McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GRS, Thormann A, Flicek P, Cunningham F. The Ensembl Variant Effect Predictor. *Genome Biol* 2016;**17**. doi:10.1186/s13059-016-0974-4

59 Landrum MJ, Lee JM, Benson M, Brown G, Chao C, Chitipiralla S, Gu B, Hart J, Hoffman D, Hoover J, Jang W, Katz K, Ovetsky M, Riley G, Sethi A, Tully R, Villamarin-Salomon R, Rubinstein W, Maglott DR. ClinVar: Public archive of interpretations of clinically relevant variants. *Nucleic Acids Res* 2016;**44**:D862–8.

60 Lek M, Karczewski KJ, Minikel E V., Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper DN, Deflaux N, DePristo M, Do R, Flannick J, Fromer M, Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won HH, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elosua R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG. Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 2016;**536**:285–91.

61 Lott MT, Leipzig JN, Derbeneva O, Michael Xie H, Chalkia D, Sarmady M, Procaccio V, Wallace DC. MtDNA variation and analysis using Mitomap and Mitomaster. *Curr Protoc Bioinforma* Published Online First: 2013. doi:10.1002/0471250953.bi0123s44

62 Shen L, Diroma MA, Gonzalez M, Navarro-Gomez D, Leipzig J, Lott MT, van Oven M, Wallace DC, Muraresku CC, Zolkipli-Cunningham Z, Chinnery PF, Attimonelli M, Zuchner S, Falk MJ, Gai X. MSeqDR: A Centralized Knowledge Repository and Bioinformatics Web Resource to Facilitate Genomic Investigations in Mitochondrial Disease. *Hum Mutat* 2016;**37**:540–8.

63 Crimmins D, Morris JG, Walker GL, Sue CM, Byrne E, Stevens S, Jean-Francis B, Yiannikas C, Pamphlett R. Mitochondrial encephalomyopathy: variable clinical expression within a single kindred. *J Neurol Neurosurg Psychiatry* 1993;**56**:900–5.

64 Shen L, Attimonelli M, Bai R, Lott MT, Wallace DC, Falk MJ, Gai X. MSeqDR mvTool: A mitochondrial DNA Web and API resource for comprehensive variant annotation, universal nomenclature collation, and reference genome conversion. Hum. Mutat. 2018. doi:10.1002/humu.23422

65 Clima R, Preste R, Calabrese C, Diroma MA, Santorsola M, Scioscia G, Simone D, Shen L, Gasparre G, Attimonelli M. HmtDB 2016: Data update, a better performing query system and human mitochondrial DNA haplogroup predictor. *Nucleic Acids Res* 2017;**45**:D698–706.

66 King MP, Attardi G. Human cells lacking mtDNA: Repopulation with exogenous mitochondria by complementation. *Science (80-)* Published Online First: 1989. doi:10.1126/science.2814477

Case 1 Basal ganglia disease

Two sisters from a consanguineous family had a severe neurodegenerative disorder, characterized by infantile onset of profound intellectual disability, failure to thrive associated with severe feeding difficulties, seizures and dystonia. Pertinent investigations in the older proband included normal urine amino and organic acids, plasma acylcarnitines and blood lactate. CSF lactate was also normal. Nerve conduction studies were suggestive of an axonal neuropathy. Brain magnetic resonance imaging (MRI) at 13 months was notable for bilateral basal ganglia hyperintensities and cerebral atrophy. Muscle histology was nonspecifically abnormal. Muscle complex IV activity was 48% of the control mean relative to citrate synthase, and in liver was 32% of normal relative to citrate synthase. She died at 26 months of age. Her younger sister exhibited a very similar onset and course, but has survived, currently aged 13 years, albeit with profound intellectual disability, upper airway abnormalities and poor growth requiring gastrostomy feeding. A brain MRI at seven months of age also showed bilateral symmetrical basal ganglia hyperintensities. She did not have any biopsies but her serum FGF21 was elevated at 1400 pg/ml (normal < 300). Together, these features were highly suggestive of an underlying mitochondrial electron transport chain (ETC) disorder primarily affecting complex IV. However whole genome sequencing revealed a homozygous variant in the *SLC39A8* gene (Riley et al 2017), which was recently identified to cause a manganese transporter disorder also impacting glycosylation. (Boycott et al 2015). Manganese is a cofactor for manganese superoxide dismutase, without which there is an increase in oxidative stress and decrease in ROS scavenging. (Riley et al 2017 PMID: 27995398) Subsequent analysis of serum transferrin isoforms revealed a profile consistent with the expected type II congenital disorder of glycosylation pattern.

Case 2 Ophthalmoplegia and myopathy

Two brothers (68 and 80 years old) from a non-consanguineous family were evaluated for progressive ptosis, ophthalmoplegia, dysphagia and mild limb-girdle muscle weakness since their 50s. Pedigree analysis was suggestive of maternally inherited autosomal dominant disease, with no clinical anticipation. Blood tests, including creatine kinase (CK) and lactate,

were normal in both brothers. Electromyography (EMG) studies revealed myopathic changes, and a muscle biopsy performed in the older brother (at the age of 70 years) showed rare ragged red and cytochrome *c* oxidase (COX) negative fibers (5% for both). Long-range PCR analysis revealed multiple mtDNA deletions in muscle. However, next generation sequencing (NGS) analysis of genes involved in mtDNA maintenance was negative. Further analysis of the *PABPN1* gene revealed expansion of 13 GCN trinucleotide repeat in the first exon, thus shifting the diagnosis to oculopharyngeal muscular dystrophy (OPMD). OPMD and myotonic dystrophy are two genetic illnesses in addition to many mitochondrial disorders where ptosis, incomplete ophthalmoplegia, dysphagia and myopathy are common features. As NGS technology improves, the nucleotide repeat disorders will be more easily identified.

Case 3 Lactic acidosis and atrophy of the putamen

A one-year-old boy was evaluated for hypotonia and motor regression. He was normal at birth but late in the first year of life he was found to have lost motor tone. He had undergone extensive biochemical evaluation prior to his initial assessment at the specialist center. Physical examination was normal other than extreme hypotonia. He had no speech but was attentive and smiled socially. On direct enquiry, mother had not observed any crystals in his urine. MRI brain showed mild parenchymal atrophy and volume loss bilaterally in the putamen and thalamus. Blood lactic acid was elevated at 6 mmol/L. A muscle biopsy was performed, showing normal histology but the enzymatic activity of complex I (I+III assay) was 25% of the mean control. He was treated with coenzyme Q_{10}, levocarnitine and a B vitamin complex for presumed complex I disease. His examination over the next few years showed him to be an alert and visually interactive child who did not develop language function and had continued severe hypotonia with progressive but mild dystonia. At the age of 5 years, he arrived for a routine examination wearing gloves, to protect his fingers from a recent onset of biting behavior (self-mutilation). The uric acid level was elevated and hypoxanthine phosphoribosyl-transferase enzyme activity was less than 10% of control values. Genetic testing was not performed but the patient's clinical course followed that typical of Lesch-Nyhan disease. The history of progressive hypotonia with evolving dystonia, language delay, lactic acidosis, deep

gray matter loss and generalized brain parenchymal volume loss, together with the low complex I ETC activity seemed to fit together with a primary mitochondrial disorder. With our current knowledge regarding altered concentration of nucleotide pools affecting mtDNA replication, it seems likely that the abnormal purine concentrations could result in mtDNA depletion, although this was not confirmed in this patient.

Case 4 Cognitive decline with ataxia and dystonia

The patient, a 20-year-old male, was born at term (weight 3.01kg) to unrelated parents following an uncomplicated pregnancy and normal delivery. He displayed mild early gross motor development delay (crawled at 14 months, walked at 18 months) but language development was normal.

He first presented to the paediatric service at the age of 6 years with an episode suggestive of a nocturnal tonic-clonic seizure. A history of 'blank spells' from age 6 months was noted by his mother. Neurological examination was normal but an electroencephalogram (EEG) demonstrated bilateral independent foci in both temporal regions and he was commenced on carbamazepine. His school performance was above the average expected for his age group. He remained under follow up and these events were eventually deemed non-epileptic, and carbamazepine was weaned.

Between the ages of 10 and 12 years, there was emerging concern that the quality of his school-work had declined. However, neuropsychological assessment at age 12 years demonstrated that he was still working within the normal expected range, albeit at the lower end (10^{th} centile for verbal comprehension; 5^{th} centile perceptual reasoning; 55^{th} centile for working memory). However, by the age of 14 years there was a clear and objective deterioration. He had also started tripping frequently; was struggling to button, zip or tie laces; his speech had declined; and there was prominent daytime somnolence.

On clinical examination there were no dysmorphic or neuro-cutaneous signs. Cranial nerve examination, including eye movements, was normal. Tone was normal, but reflexes were brisk throughout, plantar responses were down-going. No sensory abnormalities were evident. He had a clumsy gait, with an inability to tandem walk, mild dystonic posturing and subtle choreiform movements of the upper limbs. Cerebellar dysarthria, and finger-nose, heel-shin and truncal ataxia were noted. Cognitive assessment revealed emotional lability and poverty of mentation.

MRI brain demonstrated bilateral symmetrical increased signal in the white matter adjacent to the posterior horns of the lateral ventricles. Routine laboratory tests were normal including ferritin, transferrin isoforms, thyroid function, cholesterol, triglycerides, lipids, copper, ceruloplasmin, lysosomal enzymes, very long chain fatty acids, vitamin E, biotinidase, intermediate metabolites, lactate, pyruvate, ammonia, urate, amino acids, urinary organic acids and mucopolysaccharide screen. Genetic tests for DRPLA, Friedreich ataxia, spinocerebellar ataxias (SCA1, 2, 3, 6, 7 and 17) and Huntington disease were negative. No concerning features were identified on slit lamp examination. Visual evoked potentials (VEP) and electroretinogram (ERG) were normal.

At age 15 years, the patient was referred to a regional neuro-genetic clinic where a mitochondrial aetiology was explored, given the clinical features and imaging findings. Thiamine was commenced but without clinical improvement. Muscle biopsy was undertaken for ETC enzyme analysis which identified a complex I deficiency (0.051 U/UCS; normal 0.104+0.036) with low complex I:II ratio (0.05;, normal 0.52-0.95). Muscle histochemistry was normal. Array CGH was normal and further genetic testing for mutations in *POLG, MTATP6, MTATP8* and *MTTK* (associated with MERRF phenotypes) were all negative.

At clinical review at 18 years of age, symptoms had progressed and the patient was having increasing problems with falls, speech, and cognitive decline. There was a history suggestive of

mild cataplexy with heightened emotion and giggling triggering brief head drop. Examination revealed a mixed dystonic-ataxic gait, myoclonic jerks and a supranuclear gaze palsy.
Given the clinical features, a sample for plasma oxysterols was sent. Cholestane-3b, 5a, 6b-triol was found to be elevated at 55.4 ng/ml (normal range 8.1-37.7 ng/ml) and pathogenic compound heterozygous mutations were identified in *NPC1* (c.3182T>C;p.lle1061Thr and c.3566A>G;p.Glu1189Gly) which confirmed the diagnosis of Niemann-Pick disease type C. The patient was referred for commencement of miglustat therapy.

Photograph and video of the patient at 18 years of age.



Case 5: mtDNA pathogenic mutation with low level heteroplasmy in blood
A 43-year-old Romanian woman presented with normal early development except for lifelong clumsiness and progressive multi-systemic disease since her 20s. Her disease was progressive and severe, including complex epilepsy since age 20 with generalized seizures and then myoclonic jerks with diffuse slowing and multi-focal periodic lateralizing epileptiform discharges on electroencephalogram, mild cortical atrophy on computed tomography (CT) at age 40 and more diffuse cortical atrophy on brain MRI at the time of her first stroke at age 42. She had recurrent acute "metabolic" strokes in the posterior parietal and medial occipital-

temporal cortex at age 42 with residual hemiplegia, hypertonia, walker or wheelchair dependence, dysarthria, and permanent left peripheral vision loss. She developed cognitive decline from her mid-30s including psychosis, delusions and functioning estimated at a second-grade level. She also had migraines. Additional systemic manifestations include vertical and horizontal ophthalmoplegia, bilateral progressive sensorineural hearing loss onset at 30, left ventricular hypertrophy (LVH), a patent foramen ovale, nausea and vomiting with stress, irritable bowel syndrome, gastroesophageal reflux disease, bilateral renal cysts, and early morning carbohydrate craving and cloudy cognition that improved with eating. Testing showed elevated lactate intermittently in blood (4 mM; normal < 2.2) and CSF (4.2 mM; normal < 2.2), and mildly elevated glutaryl dicarboxylic acid on plasma acylcarnitine analysis.

Her family history was significant in that her mother had type II diabetes diagnosed at age 56 and an episode of paralysis at age 56, poor balance with apparent peripheral neuropathy, cortical atrophy, sudden complete hair loss in her 30s with thinner hair regrowth over time, and sensorineural hearing loss onset in her 70s. Her maternal grandmother had extreme fatigue.

Muscle biopsy at age 42 showed mild myofiber size variation, hypertrophic and atrophic fibers, one necrotic fiber, rare degenerating but no regenerating fibers, an unquantified number of ragged red fibers, targetoid and small dark fibers on NADH-TR staining, significant type II > I fiber atrophy, increased SDH positive fibers, and 5-10% COX negative fibers. No biochemical or genetic testing was performed on muscle.

A low level (8%) of the mitochondrial DNA (mtDNA) mutation m.3243A>G that causes Mitochondrial Encephalopathy Lactic Acidosis and Stroke-like episodes (MELAS) syndrome was detected in her blood. This same variant was subsequently found by highly sensitive ARMS qPCR analysis to be at similarly low levels (4% in blood, 7% in urine) in her mother. Urine heteroplasmy testing in the proband revealed 60% m.3243A>G mutant heteroplasmy load, which was clinically significant and confirmed the diagnosis of MELAS spectrum disorder.

Typical heteroplasmy levels in symptomatic patients are in the 25-90% range. However, a wide range of multi-systemic diseases that poorly fit into a pre-defined clinical syndrome can often be seen.[1, 2] It is well-known that the m.3243A>G mutation load is selected against and decreases over time in blood at the same time it may persist or increase in other tissues such as urine, which reflects shed renal cells.[3–6] A software tool to model the expected tissue heteroplasmy levels in individuals with the m.3243A>G mutation in blood is available at the Newcastle Wellcome Trust website (www.newcastle-mitochondria.com/).

Case 6: mtDNA VUS

A 19-year-old Caucasian man presented with progressive multi-system involvement. Several features were attributed to a congenital open neural tube defect (L5/S1 myelomeningocele) including complete paraplegia of lower extremities, lack of bladder and bowel control, and Chiari malformation requiring shunt placement. However, additional multisystem involvement unexplained by the myelomeningocele was ultimately found to be related to a novel pathogenic mtDNA variant (Schrier et al., 2012). As previously reported, these manifestations included developmental delay and intellectual disability, hypotonia, bilateral severe mixed hearing loss, moderate to severe LVH, Wolff-Parkinson-White syndrome, severe retinal dystrophy, bilateral dense nuclear cataracts requiring extraction, hypopituitarism, growth deficiency, type II diabetes mellitus, hypogonadism, truncal obesity, and osteopenia. Family history was non-contributory. The proband's mother was healthy until her death in a motor vehicle accident in her early 30s.

Metabolic screening studies showed persistently elevated blood alanine (492 nmol/mL and 708.9 nmol/mL; normal 88-440) and intermittently elevated blood lactate (1.54 mM and 2.8 mM; normal <2). Skeletal muscle biopsy findings in biceps (proband recently received botox injections to both quadriceps) were consistent with a mitochondrial myopathy (unquantified number of ragged red fibers and absence of COX staining in scattered fibers; electron microscopy revealing irregular branched mitochondria, focal mitochondrial collections, and

mitochondrial lipid deposits). ETC enzyme activities were within normal limits and citrate synthase was increased (159% of mean).

Clinical molecular testing available at the time (2007-2008) was unrevealing, including karyotype, microarray, *BBS1* and *BBS2* gene sequencing, mtDNA common point mutation and deletion analysis in blood, and mtDNA deletion and duplication analysis in muscle. A homoplasmic novel variant, m.12264C>T in *MTTS2* encoding mitochondrial tRNA-serine 2, was identified in muscle by mtDNA whole genome sequencing. Research-based mtDNA heteroplasmy quantitation was performed in blood, buccal, muscle, and extracted cataract samples from the proband and blood and buccal samples from the proband's father and maternal grandmother. The variant was found to be homoplasmic in the proband's muscle, extracted cataract, and buccal sample, and heteroplasmic in blood (34%). His father did not have the mtDNA variant. His maternal grandmother was found to have the m.12264C>T variant at 1% heteroplasmy level in blood and 18% heteroplasmy level in her buccal sample. His mother was deceased and a sample was not available for testing.

Case 7: OPA1 VUS

A 67-year-old Caucasian male presented with fatigue, disturbed sleep, muscle pain, weakness and numbness. Clinical examination revealed lower limb distal muscle weakness and wasting, peripheral neuropathy, mild sensorineural hearing loss, mild bilateral ptosis, dyspnea, dysphagia and dysarthria with bulbar and respiratory muscle weakness, as well as dyslipidaemia and elevated transaminase levels. A muscle biopsy showed histological changes consistent with mitochondrial myopathy, including increased subsarcolemmal staining with Gomori trichrome and COX-negative fibres (quantity not known). The family history was unremarkable and did not indicate a likely mode of inheritance.

Serum levels of FGF21 were elevated at 798, 691 and 812 pg/ml (control range < 350 pg/ml on longitudinal sampling, potentially supporting a muscle-manifesting mitochondrial disease. Long-range PCR of the mitochondrial genome showed a 16.5kb amplicon with no evidence of large-

scale deletions. Deep sequencing of the entire mtDNA amplified by long-range PCR revealed no significant variants. Bioinformatic analysis of known mitochondrial and neuromuscular disease genes from whole genome sequencing (WGS) of blood genomic DNA revealed a heterozygous, in-frame, 18 nucleotide deletion in *OPA1* (NM_015560.2(*OPA1*):c.113_130del18 (p.Arg38_Ser43del)) that was confirmed by Sanger sequencing on a separate blood sample. The deletion locates to exon 2 of *OPA1* and potentially interferes with the mitochondrial targeting sequence of the protein. There are reports of at least 9 other individuals with the same deletion in ClinVar, however it is classified as a VUS owing to conflicting descriptions of associated pathogenicity and phenotype (https://www.ncbi.nlm.nih.gov/clinvar/variation/214916/#supporting-observations).

OPA1 encodes a mitochondrial inner-membrane protein with dynamin-related GTPase activity that is required for maintenance of cristae structure (in doing so regulating apoptosis), the mitochondrial reticulum (by mediating mitochondrial fusion) and the mitochondrial genome. Autosomal dominant pathogenic variants in *OPA1* (OMIM 605290) are associated with Optic Atrophy 1 (Phenotype MIM 165500), an optic neuropathy affecting retinal ganglion cells and leading to blindness, and also dominant optic atrophy plus (Phenotype MIM 125250), which has systemic manifestations (e.g. hearing loss, myopathy and neuropathy) in addition to optic atrophy. As the patient described here had no evidence of optic atrophy he was not initially considered for *OPA1* genetic testing. However, his symptoms do fit with the extraocular manifestations of dominant optic atrophy plus.

Case 8: Spasticity and mitochondrial dysfunction

The now 43-year old patient presented in the early 1990s, during late adolescence, with new onset academic difficulties in high school. Over the next few years she had difficulty with ambulation. Her examination when first evaluated about 8 years after her symptoms began revealed mild cognitive dysfunction, dysarthria, spasticity and neuropathy. Her initial brain MRI was normal but her biochemical evaluation revealed consistently elevated pipecolic acid levels (range of 2.7-4.7 uM (control 0.7-2.5). Detailed evaluation for known peroxisomal disorders

was performed with the advice and assistance of experts in this group of disorders, and was not revealing. A liver biopsy was performed in 1999 to confirm what was believed to be a peroxisomal disorder. The EM of the liver demonstrated normal peroxisomal appearances but massive and abnormally shaped mitochondria with paracrystalline inclusions. A subsequent muscle biopsy showed ragged blue fibers (amount not specified) and a biochemical defect in ETC Complex I, I+III and III (amounts not specified). The patient did not have consistent abnormalities in lactic acid, blood amino acids or urine organic acids, but because of the histopathology and biochemical findings, the working diagnosis was believed to be a mitochondrial disease. Over the next 13 years the patient underwent genetic testing in a sequential fashion that included screening for common mtDNA mutations, Southern blot for mtDNA deletions, whole genome mtDNA sequencing, and sequencing of numerous individual nuclear genes. This testing did not reveal any mutations that would explain her phenotype. Her clinical symptoms worsened and the patient currently has difficulty speaking in sentences because of both cognitive limitations and dysarthria. She spends most of her day in a wheelchair, with a limited ability to ambulate. In addition to the spasticity and neuropathy her clinical findings now include retinal degeneration, scoliosis, pes cavus, myopathy, ataxia and parkinsonism (stooped posture, masked facies and bradykinesia). The brain MRI shows moderate generalized volume loss. In 2012 sequence analysis of a large panel of nuclear-encoded mitochondrial genes (> 250 genes) was normal. In late 2013 WES was reported to show a number of possible candidate mutations. These included compound heterozygous mutations in *TIMM44*. Both mutations were predicted *in silico* to be pathogenic, but this mitochondrial gene had not been reported to be associated with any clinical disease. In addition, a nonsense mutation was found in *SPG11*, which was also present in the patient's father, but not mother. Because only one mutation was identified in *SPG11* (a gene whose defects typically cause autosomal recessive disease) and a phenotype that partially matched the phenotype (no cerebellar atrophy or thinning of the corpus callosum was identified), both *SPG11* and *TIMM44* remained candidate genes. Further genetic evaluation was delayed because of costs and the need for insurance approval to pay for the testing but in 2015 del/dup analysis of *SPG11* was performed and revealed the previously identified c.1744G>T

(p.Glu5882*) mutation, also identified in the father, as well as partial deletion of the gene including exons 17 and 18, which was found in her mother a year later. The gene product of *SPG11* controls the assembly of the ribosomes in the mitochondria and is associated with the clinical illness autosomal recessive spastic paraplegia type 11 (OMIM 604360), which is a good fit for this patient's illness. The liver pathology and biochemistry reported in this case suggest this gene, and disease, could be considered as a primary mitochondrial disorder, although it is not known if the mutations in *TIMM44* play a role in the patient's phenotype. Future technological advances may help to address this question as the patient and her family wish to pursue this question. Of note, her GDF15 is elevated at 955 pg/mL (control range < 750). This case illustrates a two-decade journey through technological advancements that eventually led to the diagnosis, possibly led astray by unexpected laboratory findings. Had the initial evaluation not identified the consistent elevation in pipecolic acid, the liver biopsy would not have been performed and the subsequent evaluation would likely not have led down a mitochondrial pathway. An explanation for the elevation in pipecolic acid remains unclear. If the initial clinical suspicion led towards a spastic paraparesis evaluation, the results of genetic testing would have likely stumbled across the same technological issues, specifically early massively parallel sequencing failed to identify many deletions, duplications or insertions.

REFERENCES

1 Chin J, Marotta R, Chiotis M, Allan EH, Collins SJ. Detection rates and phenotypic spectrum of m.3243A>G in the MT-TL1 gene: A molecular diagnostic laboratory perspective. *Mitochondrion* 2014;**17**:34–41.

2 Kaufmann P, Engelstad K, Wei Y, Kulikova R, Oskoui M, Battista V, Koenigsberger DY, Pascual JM, Sano M, Hirano M, DiMauro S, Shungu DC, Mao X, De Vivo DC. Protean phenotypic features of the A3243G mitochondrial DNA mutation. *Arch Neurol* 2009;**66**:85–91.

3 McDonnell MT, Schaefer AM, Blakely EL, McFarland R, Chinnery PF, Turnbull DM, Taylor RW. Nonivasive diagnosis of the 3243A > G mitochondrial DNA mutation using urinary epithelial cells. *Eur J Hum Genet* 2004;**12**:778–81.

4 Shanske S, Pancrudo J, Kaufmann P, Engelstad K, Jhung S, Lu J, Naini A, DiMauro S, De Vivo DC. Varying loads of the mitochondrial DNA A3243G mutation in different tissues: Implications for diagnosis. *Am J Med Genet* 2004;**130 A**:134–7.

5 Whittaker RG, Blackwood JK, Alston CL, Blakely EL, Elson JL, McFarland R, Chinnery PF, Turnbull DM, Taylor RW. Urine heteroplasmy is the best predictor of clinical outcome in the m.3243A>G mtDNA mutation. *Neurology* 2009;**72**:568–9.

6 Rahman S, Poulton J, Marchington D, Suomalainen A. Decrease of 3243 A-->G mtDNA mutation from blood in MELAS syndrome: a longitudinal study. *Am J Hum Genet* 2001;**68**:238–40.