doi:10.1093/brain/awx168 BRAIN 2017: 140; 2530–2540 | 2530

BRAIN
A JOURNAL OF NEUROLOGY

UPDATE

Monitoring clinical progression with mitochondrial disease biomarkers

Hannah E. Steele,[1] Rita Horvath,[1] Jon J. Lyon[2] and Patrick F. Chinnery[3,4]

Mitochondrial disorders are genetically determined metabolic diseases due to a biochemical deficiency of the respiratory chain. Given that multi-system involvement and disease progression are common features of mitochondrial disorders they carry substantial morbidity and mortality. Despite this, no disease-modifying treatments exist with clear clinical benefits, and the current best management of mitochondrial disease is supportive. Several therapeutic strategies for mitochondrial disorders are now at a mature preclinical stage. Some are making the transition into early-phase patient trials, but the lack of validated biomarkers of disease progression presents a challenge when developing new therapies for patients. This update discusses current biomarkers of mitochondrial disease progression including metabolomics, circulating serum markers, exercise physiology, and both structural and functional imaging. We discuss the advantages and disadvantages of each approach, and consider emerging techniques with a potential role in trials of new therapies.

1 Wellcome Trust Centre for Mitochondrial Research, Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, NE1 3BZ, UK
2 GlaxoSmithKline, Molecular Safety and Disposition, Ware, SG12 0DP, UK
3 Department of Clinical Neurosciences, University of Cambridge, Cambridge Biomedical Campus, Cambridge CB2 0QQ, UK
4 MRC Mitochondrial Biology Unit, Cambridge Biomedical Campus, Cambridge CB2 0QQ, UK

Correspondence to: Patrick F Chinnery
Department of Clinical Neurosciences, University of Cambridge, Cambridge Biomedical Campus, Cambridge CB2 0QQ, UK
E-mail: pfc25@cam.ac.uk

Keywords: biomarkers; mitochondrial disease; disease progression; mtDNA; mitochondrial encephalomyopathy

Abbreviations: MELAS = mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MRS = magnetic resonance spectroscopy; NMR = nuclear magnetic resonance; OEF = oxygen extraction fraction

Introduction

Mitochondrial disorders are genetically determined metabolic diseases due to a biochemical deficiency of the respiratory chain that affect around 1 in 4300 of the population in the UK (Gorman *et al.*, 2015). Given that multisystem involvement and disease progression are common features, mitochondrial disorders carry substantial morbidity and are associated with excess premature death (Kaufmann *et al.*, 2011). Despite this burden, a recently published Cochrane review did not identify any disease-modifying treatments of benefit (Pfeffer *et al.*, 2012), and current best management of mitochondrial disease is therefore supportive. Consequently there is an unmet need for treatments that modify the underlying biochemical deficit and disease trajectory.

However, the development of new therapeutic strategies presents major challenges for the scientific, pharmaceutical, academic and clinical communities (Food and Drug Administration, 2004). For rare diseases, including mitochondrial disorders, these issues are magnified through the geographical dispersion of patients, the use of

Received February 1, 2017. Revised April 24, 2017. Accepted May 14, 2017. Advance Access publication August 3, 2017

heterogeneous patient groups in both interventional and natural history studies to date, and historically at least, a perceived lack of return on financial investment for the pharmaceutical industry (Pfeffer *et al.*, 2013). As biomarker identification provides a means of overcoming some of these barriers—for example, enabling prospective compounds to be assessed in a practical timescale, or facilitating patient subgroup categorization—their development has been afforded high scientific priority (Food and Drug Administration, 2004).

Biomarkers are widely defined as: 'A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic response(s) to a therapeutic intervention' (Biomarkers Definitions Working Group, 2001). With increasing scientific focus on developing biomarkers, an expansive nomenclature has emerged (Biomarkers Definitions Working Group, 2001; Altar *et al.*, 2008; Wagner, 2008). 'Drug-related' biomarkers are those identifying novel pathways, or enabling assessment of drug-target interactions; while 'disease-related' biomarkers reflect the presence or absence of disease, aid disease stratification, guide prognosis or inform disease natural history (Fig. 1). Following identification of a potential biomarker, it should be both validated and qualified: where validation refers to the process of determining assay reliability, and qualification describes the process of linking a biomarker with biological processes and clinical endpoints. Where biomarkers have a specific purpose, in a specific patient cohort, their impact on clinical trial design is outlined in Fig. 1.

Traditionally, biomarkers in mitochondrial diseases have been used to improve diagnostic accuracy or target those who should undergo invasive investigation. However, as next generation sequencing techniques progressively improve the diagnostic process (Taylor *et al.*, 2014), alternative uses of biomarkers can be increasingly explored. As several therapeutic strategies for mitochondrial disorders are now at a mature preclinical stage (Bogacka *et al.*, 2005; Lagouge *et al.*, 2006; Yatsuga and Suomalainen, 2012), and are making the transition into early-phase patient trials (Table 1), it is the lack of validated biomarkers for disease progression that currently presents the biggest challenge in developing new therapies for patients.

The unmet need for surrogate markers of disease progression becomes clear when considering three key clinical features of mitochondrial diseases. First, the disorders are notoriously heterogeneous—even within genetically homogeneous groups. Second, acute relapses are frequently experienced; and third, baseline progression tends to occur slowly over a number of years. In the context of clinical trials, and particularly early phase trials that tend to be conducted over short timescales (e.g. 6 weeks to 6 months), these factors present significant barriers in determining therapeutic efficacy. Given this, our review will focus on scientific approaches to identify biomarkers of clinical disease progression in mitochondrial disorders with a unique emphasis on emerging preclinical techniques. We are aware that mitochondrial abnormalities may be secondary to various cellular processes including calcium metabolism, neurode generation and various metabolic diseases; however, the role of mitochondria in these diseases needs further investigations (Pyle *et al.*, 2015). Therefore, we focus this update on primary mitochondrial disorders.

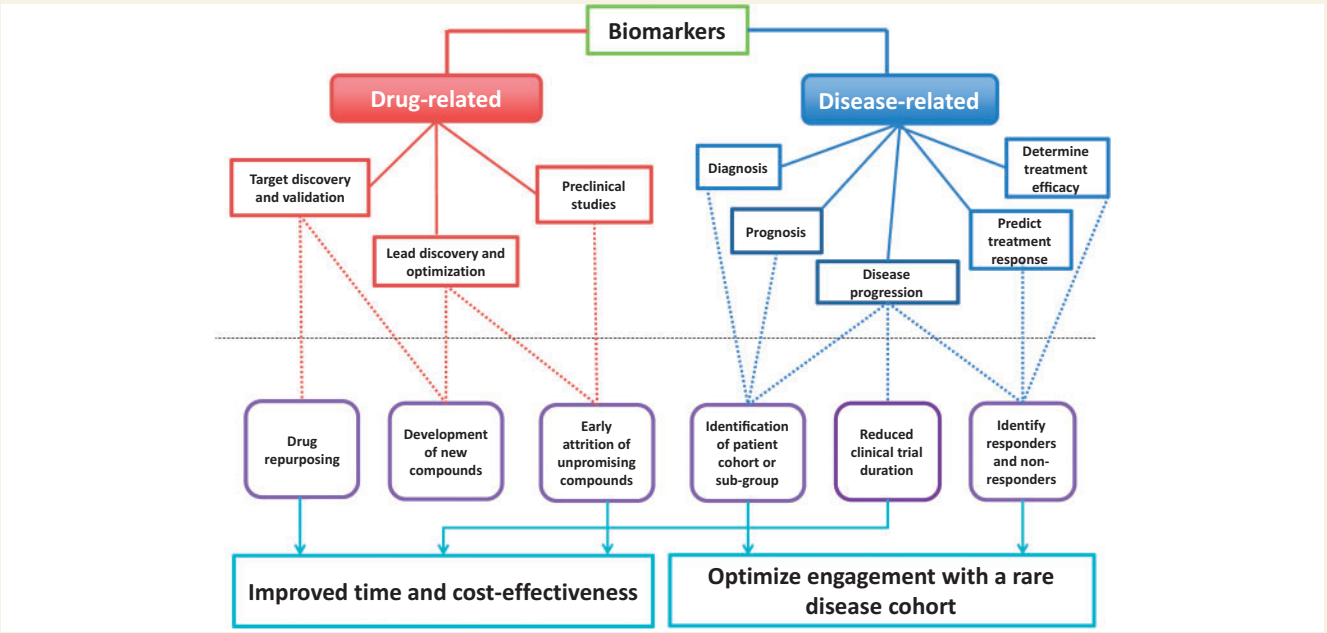

Figure 1 Potential impact of biomarkers on clinical trials.

Table 1 Active clinical trials of potential disease modifying agents for primary mitochondrial diseases, ClinicalTrials.gov April 2017

Study title	Phase	Design	IMP
The effect of arginine and citrulline supplementation on endothelial dysfunction in mitochondrial diseases	II	R, PC, DB, CO	Arginine, citrulline
Study to assess the efficacy and safety of Raxone in LHON patients (LEROS)	IV	OL	Idebenone
An exploratory, double-blind, randomized, placebo-controlled, single-center, two-way cross-over study with KH176 in patients with the mitochondrial DNA tRNALeu(UUR) m.3243A>G mutation and clinical signs of mitochondrial disease	II	R, PC, DB, CO	KH173
A study of bezafibrate in mitochondrial myopathy	II	OL	Bezafibrate
RTA 408 capsules in patients with mitochondrial myopathy - MOTOR	II	R, PC, DB	RTA408
Efficacy study of GS010 for the treatment of vision loss up to 6 months from onset in LHON due to the ND4 mutation (RESCUE)	III	R, Sham C, DB	GS010
EPI-743 for metabolism or mitochondrial disorders	II	R, PC, DB, CO	EPI-743
MNGIE allogeneic hematopoietic stem cell transplant safety study (MASS)	I	OL	Hematopoietic allogeneic stem cells
A study investigating the safety, tolerability, and efficacy of elamipretide (MTP-131) topical ophthalmic solution for the treatment of Leber's hereditary optic neuropathy	II	R, PC, DB	MTP-131
Safety study of an adeno-associated virus vector for gene therapy of Leber's hereditary optic neuropathy (LHON) caused by the G11778A mutation (LHON GTT)	I	OL	scAAV2-P1ND4v2
Long term safety and efficacy study of EPI-743 in children with Leigh syndrome	II	R, PC, DB	EPI-743

CO = crossover; DB = double blinded; IMP = investigational medicinal product; OL = open label; PC = placebo controlled; R = randomized; Sham C = sham controlled.

Metabolomics

A fundamental feature of primary mitochondrial disorders is deficient oxidative phosphorylation, with both up- and downstream metabolic perturbations arising secondarily. Abnormalities in lactate, pyruvate, creatine kinase, amino acids and carnitines are firmly established in the clinical investigation of mitochondrial diseases. However, their diagnostic sensitivity and specificity is poor (Petty *et al.*, 1986; Campos *et al.*, 1993; Jackson *et al.*, 1995; Sim *et al.*, 2002; Jeppesen *et al.*, 2006*a*; Haas *et al.*, 2008; Suomalainen *et al.*, 2011; Yamada *et al.*, 2012; Davis *et al.*, 2013) and there are limited natural history studies studying change in relation to disease progression. To date, metabolomic approaches have been highly successful in identifying potential biomarkers in a diverse range of disorders including: cancers (Puchades-Carrasco *et al.*, 2013; Rocha *et al.*, 2015; Cui *et al.*, 2016), vascular disease (Jove *et al.*, 2015; Naz *et al.*, 2015), renal transplantation (Kienana *et al.*, 2015), respiratory diseases (Adamko *et al.*, 2015), immunological disorders (Young *et al.*, 2013; Saegusa *et al.*, 2014), liver disease (Gao *et al.*, 2015), and diabetes (Balderas *et al.*, 2013; Drogan *et al.*, 2015). With secondary mitochondrial dysfunction arising in several of these conditions (Wallace, 2012; Yu *et al.*, 2012; Begriche *et al.*, 2013; Cloonan and Choi, 2016), alongside the association of diabetes with primary mitochondrial diseases, the application of metabolomics in genetically determined mitochondrial cohorts is timely and holds great potential.

For a multi-systemic disorder, a particularly attractive feature of metabolomic analysis is that it can be undertaken using CSF, blood, urine, saliva, or solid biopsy samples. It is unknown at present which of these is optimal in mitochondrial disorders specifically, but non-invasive (urine/saliva) or minimally invasive (blood) samples would facilitate repeated measurements in both longitudinal and therapeutic settings. Initial study of the urinary proteome and metabolome in patients with heterogeneous mitochondrial disorders identified key differences between carriers, healthy controls and those manifesting symptoms (Hall *et al.*, 2015).

Analysis can be undertaken using either nuclear magnetic resonance spectrometry (NMR spectrometry) or mass spectrometry (MS) techniques. NMR spectrometry can utilize samples in both solid and liquid states; is highly reproducible, and is better at analyte quantification than mass spectrometry (Emwas, 2015). However, mass spectrometry techniques have significantly higher sensitivity than NMR spectrometry, enabling detection of analytes at low concentrations (femto–attomolar) thereby facilitating recognition of metabolites not traditionally measured in routine clinical practice, with a relevant example being the alteration of sphingomyelins and phosphatidylcholines in a cohort with Leber's hereditary optic neuropathy (Chao de la Barca *et al.*, 2016).

Furthermore, data arising from metabolomics studies can be used in a variety of clinically meaningful ways. In addition to genotype or phenotype specific cohort analysis identifying specific disease manifesting and carrier

'signatures' (Hall *et al.*, 2015), longitudinal 'n-of-1' studies can be undertaken to facilitate analysis of metabolomic change with an individual's clinical disease progression (Alonso *et al.*, 2015). The ability to combine these approaches is particularly attractive and could help with the presymptomatic identification of 'disease onset' in carrier individuals—a factor of clear importance for clinical trial design.

Circulating serum markers

Circulating cytokines

In the past 5 years, serum fibroblast growth factor-21 (FGF-21) and serum growth and differentiation factor-15 (GDF-15) (Kajiyama *et al.*, 1989; Suomalainen *et al.*, 2011; Davis *et al.*, 2013; Kalko *et al.*, 2014; Fujita *et al.*, 2015; Yatsuga *et al.*, 2015) have emerged as two promising diagnostic biomarkers for mitochondrial diseases. Identified first in mouse models of mitochondrial disease they were subsequently validated in patient cohorts. Presently, both markers are more sensitive and specific than currently used clinical diagnostic markers of mitochondrial disorders but are yet to be incorporated into formal diagnostic pathways (Suomalainen *et al.*, 2011; Yatsuga *et al.*, 2015; Davis *et al.*, 2016). In part, this has been due to concerns that both have been associated with a range of non-mitochondrial disorders, encompassing obesity, cancer, renal disease, diabetes, and liver disease, with the latter two frequently co-existing in patients with mitochondrial disorders (Semba *et al.*, 2012; Chow *et al.*, 2013).

Although preliminary data suggested that FGF-21 may correlate with disease severity and disease progression (Suomalainen *et al.*, 2011), this was not subsequently substantiated in adult cohorts with the m.3243A > G mutation (Koene *et al.*, 2014, 2015). Their utility in determining disease progression and severity therefore needs further assessment in broader, well characterized mitochondrial cohorts. Additionally, it is not known whether they are influenced by therapeutic agents, but both markers should be considered for inclusion as study endpoints in relevant clinical trials, particularly as emerging work suggests that GDF-15, and FGF-21 in particular, appear to be more specific markers for mitochondrial disorders arising due to mitochondrial translation and mtDNA maintenance defects, as opposed to those resulting from impaired respiratory chain complex or assembly factors (Lehtonen *et al.*, 2016).

MicroRNAs

MicroRNAs are non-coding genomic regions, around 20 nucleotides long, that control gene expression through transcription silencing. Several studies have used serum microRNAs in the diagnosis of inherited muscle disease (Cacchiarelli *et al.*, 2011; Endo *et al.*, 2013; Hu *et al.*, 2014), and serum circulating, muscle-specific microRNAs have been linked to disease progression in myotonic dystrophy (Koutsoulidou *et al.*, 2015). Distinctive microRNA patterns have also been associated with various metabolic processes including non-alcoholic fatty liver disease (Leti *et al.*, 2015), diabetes (Raffort *et al.*, 2015), brown adipogenesis (Zhang *et al.*, 2015), as well as exercise capacity in healthy individuals (Mooren *et al.*, 2014). Their interaction with the mitochondrial genome has not been fully elucidated but a recent study in cybrid cells carrying the m.3243A > G mutation identified that microRNA-9/9* patterns associated with mitochondrial disorder phenotypes [mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and myoclonic epilepsy with ragged-red fibres (MERRF)] (Meseguer *et al.*, 2015). Given that serum samples are straightforward to collect, further study of microRNAs in relation to mitochondrial disease phenotype and disease progression would be rapidly feasible.

Exercise physiology

The functional assessment of mitochondrial aerobic capacity in the adult patient population has been extensively studied using exercise physiology for over 10 years. Key differences are seen in peak oxygen consumption (peak VO_2), peak power (W_{max}), and peak arterial-venous oxygen difference between individuals with mitochondrial disorders and healthy controls (Jeppesen *et al.*, 2003, 2006*b*; Bates *et al.*, 2013). As an indicator of oxygen uptake from the capillary network during circulation, the reduced peak arterial-venous oxygen difference seen in those with mitochondrial disorders is believed to be a key mechanism underpinning the widespread experience of exercise intolerance (Jeppesen *et al.*, 2006*b*; Bates *et al.*, 2013).

To date, exercise testing has been used both to support a diagnosis of mitochondrial disease (Jensen *et al.*, 2002) and to demonstrate efficacy of treatment (Drinkard *et al.*, 2010; Glover *et al.*, 2010), including when exercise is used as a therapy itself (Taivassalo *et al.*, 1998, 2001, 2006; Jeppesen *et al.*, 2006*a*, *b*, 2009; Murphy *et al.*, 2008; Bates *et al.*, 2013). To our knowledge, no longitudinal studies using exercise physiology parameters as markers of disease progression have been undertaken in a mitochondrial cohort to date. While exercise testing is safe in mitochondrial populations, there are several potential limitations with its use in this way. First, studies have largely focused on those with myopathic symptoms and application to other mitochondrial phenotypes is likely to require further exploration. Second, little is published on the exercise capacity of children with mitochondrial disorders, although a small study reviewing exercise as a therapeutic intervention did not identify problems with the exercise itself (Schreuder *et al.*, 2010). Third, participants with cardiac involvement and significant intellectual or physical disabilities would be unable to exercise at the level required for a valid test.

Finally, the test requires specialist equipment and trained staff to administer which could limit its widespread use and reliability in longitudinal settings.

In addition to maximal exercise testing, recent work on gait physiology has identified distinct abnormalities in the m.3243A > G and m.8344A > G mitochondrial populations (Galna *et al.*, 2014). These characteristics correlate with disease severity and furthermore, affected individuals can be distinguished from healthy controls at an early disease stage.

Imaging

Imaging findings are being increasingly identified in a broad range of neuromuscular disorders, including as markers of disease progression (Morrow *et al.*, 2013, 2016). The main focus is on the measurement of muscle volume, and the relative amount of intramuscular fat and water (reviewed in Carlier *et al.*, 2016). The majority of studies to date have been carried out in patients with inherited muscular dystrophies and inflammatory myopathies, but similar features have also been shown in mitochondrial diseases, suggesting potential applications for the diagnosis and monitoring of these disorders. However, large longitudinal studies have not been performed to date. The array of imaging available to study the impact of mitochondrial disorders encompasses structural imaging using CT or magnetic resonance, to functional imaging using magnetic resonance spectroscopy (MRS) and PET. All modalities are established in diagnostic and research settings and provide non-invasive, quantitative measurements in a variety of tissues, making it attractive to patients and researchers alike, particularly as stronger magnetic fields (7 T) and novel software algorithms enable shorter scan times.

Structural imaging

Structural brain imaging using MRI is well described in mitochondrial disorders. While common clinical features include cerebral and cerebellar atrophy, bilateral high signal in deep grey structures, leukoencephalopathy and stroke-like episodes in non-vascular territories (reviewed in Saneto *et al.*, 2008); these findings are non-specific and highly variable (Tzoulis *et al.*, 2006; Engelsen *et al.*, 2008), making them unsuitable for further development as biomarkers. In contrast, extra-ocular muscle T_2 signal in individuals with chronic progressive external ophthalmoplegia (CPEO) correlates with eye movement restriction thereby providing a quantitative method of assessing disease severity (Yu-Wai-Man *et al.*, 2013; Pitceathly *et al.*, 2016). Similarly, in a cohort with the m.3243A > G mutation, structural cardiac abnormalities have been identified in the absence of both high symptom load and cardiac abnormality on routine clinical tests (Hollingsworth *et al.*, 2012). Furthermore, the specific pattern of cardiac involvement appears dependent on patient genotype and is distinct from more common causes of cardiac impairment (Florian *et al.*, 2015). Longitudinal studies using these modalities would be relatively straightforward, and both would be potentially attractive as trial endpoints in appropriate cohorts.

Functional imaging with existing techniques

Magnetic resonance spectroscopy

MRS enables the quantitative assessment of tissue metabolites in steady state, with ^{31}P and ^{1}H spectra most commonly used in mitochondrial cohorts. ^{1}H enables capture of tissue-specific metabolites such as lactate, choline, and *N*-acetyl aspartate, while ^{31}P captures the relative proportions of phosphorus metabolites. Although skeletal muscle can be examined by ^{31}P-MRS in resting, exertional or post-exertional states, the evaluation of oxidative capacity depends on measuring the phosphocreatine recovery time—a direct reflection of mitochondrial ATP production—following phosphocreatine depletion through exercise.

^{31}P-MRS on skeletal muscle has identified key differences in tissue bioenergetics in a range of neuromuscular disorders, including mitochondrial disease (Lodi *et al.*, 1999, 2004*b*; Cea *et al.*, 2002; Jeppesen *et al.*, 2007). To date these metabolic alterations have been used to support a diagnosis of mitochondrial disease (Walker *et al.*, 1996; Bernier *et al.*, 2002) and as an endpoint in several therapeutic studies (Penn *et al.*, 1992; Barbiroli *et al.*, 1995, 1997; Kornblum *et al.*, 2005). However, some involved single patients only (Penn *et al.*, 1992; Barbiroli *et al.*, 1995) while in others, trial design was not optimal (Pfeffer *et al.*, 2012). Similar abnormalities of tissue bioenergetics have also been identified using ^{31}P-MRS of cardiac muscle in those carrying the m.3243A > G mutation (Lodi *et al.*, 2004*a*), and have been used to assess the effectiveness of an exercise intervention programme (Bates *et al.*, 2013). Longitudinal studies correlating clinical disease progression in mitochondrial cohorts with findings from ^{31}P-MRS studies are notably lacking.

While impaired ATP production and elevated lactate levels are established findings of brain MRS studies (Barbiroli *et al.*, 1993; Kaufmann *et al.*, 2004; Lindroos *et al.*, 2009), little has been published on the use of brain MRS to monitor disease progression or to study the effects of pharmaceutical agents (Barbiroli *et al.*, 1999; Lee *et al.*, 2010). Recent work using ^{1}H-MRS brain imaging has identified fundamental differences between healthy controls, those manifesting disease due to the m.3243A > G mutation, presymptomatic individuals converting to affected individuals and presymptomatic individuals not converting (Weiduschat *et al.*, 2014); once more supporting the notion of specific disease state 'signatures' (Hall *et al.*, 2015). Natural history studies to further understand this in relation to both symptom progression and in broader mitochondrial patient populations would be timely and

highly relevant given current interest in biomarker development.

Positron emission tomography

In contrast to MRS, which provides steady state measurement of metabolites, PET measures metabolic flux, thereby permitting the study of tissue metabolic and haemodynamic properties. Several radioisotopically labelled metabolites are relevant to the study of mitochondrial disorders and include ^{15}O, 2-deoxy-2 ^{18}F-fluoro-D-glucose (FDG) and ^{11}C pyruvate enabling the study of tissue-specific bioenergetics. To date, PET has identified a variety of metabolic abnormalities in the mitochondrial disease population. MELAS patients with the m.3243A>G point mutation are the best studied population, with reports of PET imaging of heart (Arakawa *et al.*, 2010), brain (Nariai *et al.*, 2001; Shelly *et al.*, 2007; Lindroos *et al.*, 2009) and muscle tissue (Arakawa *et al.*, 2010; Rodan *et al.*, 2015), both in background states (Lindroos *et al.*, 2009), acute post stroke-like episodes (Shelly *et al.*, 2007; Ikawa *et al.*, 2009), and in response to potential therapies (Arakawa *et al.*, 2010). Key findings from cerebral studies include: global impairment of cerebral oxygen metabolic rate ($CMRO_2$) (Nariai *et al.*, 2001; Lindroos *et al.*, 2009) and regional glucose hypometabolism of the occipito-parietal regions (Nariai *et al.*, 2001; Shelly *et al.*, 2007). These perturbations are supported by findings from heterogeneous mitochondrial populations, which include reduced molar ratio (of glucose and oxygen) (Frackowiak *et al.*, 1988); impairment of $CMRO_2$ (Frackowiak *et al.*, 1988; Shishido *et al.*, 1996); and reduced cerebral metabolic ratio glucose (CMRglu) (Frackowiak *et al.*, 1988; Haginoya *et al.*, 2016), including in a family with mitochondrial neurogastrointestinal encephalopathy (MNGIE) with no clinically overt CNS features (Lehnhardt *et al.*, 2008). Additionally, perturbations in cerebral oxygen extraction fraction (OEF) have been identified, with the OEF representing the percentage of oxygen removed from the blood by tissue during the passage through the capillary network (Frackowiak *et al.*, 1988; Lindroos *et al.*, 2009). It is therefore analogous to the 'arterial-venous oxygen difference' measured during sub-maximal exercise testing, although no studies exist that examine the relationship between the two. Further study could therefore be warranted although PET scanning has several limitations impeding its widespread use in both clinical and research settings. In particular, due to the use of radioactive isotopes (Duncan *et al.*, 1995), there are no natural history studies of mitochondrial populations, and restricted studies in children with mitochondrial disorders.

Functional MRI

These limitations have provided impetus to develop magnetic resonance protocols that facilitate study of tissue haemodynamics. MRI using gradient echo sampling of spin echo (GESSE) sequences has emerged as a technique enabling quantitative assessments of cerebral haemodynamics, in particular the OEF (He and Yablonskiy, 2007). Key advantages of OEF measurement over cerebral blood flow, are its relative uniformity despite regional variations in cerebral blood flow or oxygen metabolic rate (Gusnard *et al.*, 2001) and its interindividual stability (He and Yablonskiy, 2007). Initial application in a small MELAS (m.3243A>G) cohort demonstrated reduced cerebral OEF irrespective of relationship to stroke-like episode, with further reduction in OEF in the acute and subacute phases of the stroke-like episode (Yu *et al.*, 2013).

Similar techniques to enable measurement of skeletal muscle OEF have already been applied in small cohorts of healthy individuals (Zheng *et al.*, 2014; Wang *et al.*, 2016) and future assessment in patients with mitochondrial disorders would be relevant given the reduction in tissue oxygen extraction (peak arterial-venous oxygen difference) widely identified during sub-maximal exercise tests in these cohorts (Taivassalo *et al.*, 2006). Should the muscle OEF technique be further assessed and validated, it could have advantage over submaximal exercise testing in a mitochondrial cohort because of its application to those unable to exercise, for example, those with significant weakness, cardiovascular disease, children or intellectual disabilities.

Functional imaging with novel techniques

With conventional FDG-PET and NMR spectroscopy both lacking the necessary sensitivity to identify substrates in low tissue concentrations, researchers have developed a relatively new MRI technique—dynamic nuclear polarization (DNP). DNP facilitates real time functional imaging, using ^{13}C-MRS, of substrates and their metabolites in existence at low tissue concentrations—such as reactive oxygen species—*in vivo* (Ardenkjaer-Larsen *et al.*, 2003). Although hyperpolarized [1-^{13}C] pyruvate has been the most widely studied substrate, enabling both functional imaging of key tumour metabolites (Brindle *et al.*, 2011; Nelson *et al.*, 2013) and cellular response to chemotherapy (Day *et al.*, 2007; Ward *et al.*, 2010), use of hyperpolarized [1-^{13}C] glucose permits more direct study of the glycolytic pathway.

To date, a key limitation in the technique has been the short half-life of the hyperpolarization period (10–40 s for pyruvate; <1 s for glucose), necessitating the ensuing metabolic processes to occur rapidly, and resulting image generation to occur in <2 min (Brindle *et al.*, 2011). Deuteration is one means of overcoming this challenge and recently, hyperpolarized, deuterated [U-^{2}H, U-^{13}C] glucose was used to image glycolysis in real time (Rodrigues *et al.*, 2014). Initial results suggest the technique allows the sensitive study of lactate accumulation in murine cancer models pre- and post-chemotherapy.

Such techniques, although relatively early in development, have clear application in mitochondrial disease, potentially providing a minimally invasive, yet quantitative, means of diagnosis—as well as a way of monitoring the

systemic, or indeed tissue-specific, impact of a given treatment. DNP is under active development in cancer medicine and requires further validation in man before preliminary assessments are made in mitochondrial disease specifically.

Preclinical work is also underway using novel PET ligands, for example 1^8F-BCPP-EF, to enable quantitative analysis of complex I activity. To date the technique has been used following induced ischaemia in monkey brain (Tsukada, 2014; Tsukada *et al.*, 2014) and its application to primary dysfunction of mitochondria has not been established. However, the use of ligands with the ability to interrogate specific mitochondrial complexes may in future enable the non-invasive assessment of respiratory chain function.

Emerging techniques

Small molecule reporters

Small molecule reporters enable the quantifiable measurement of mitochondrial function, mitochondrial-specific metabolites and reactive oxygen species generation *in vivo*. Tailor-made probes, administered intravenously to an intact organism, accumulate within mitochondria and react with a substrate of interest. In doing so, the probes are modified to produce an 'exomarker' (exogenous marker), which can then be extracted to enable its quantitative analysis, and inferences to be made about the reacting substrate. Currently, the technique is in preclinical development, with analysis of the exomarker using mass spectrometry only being feasible following destruction of the organism (Logan *et al.*, 2014). However, it is anticipated that further study of exomarkers in urine may facilitate analysis of probes *in vivo* and facilitate future work in animal models and eventually, humans.

Although current focus of this technique is in the assessment of acquired mitochondrial dysfunction arising due to ischaemic and reperfusion insults (Chouchani *et al.*, 2014), the potential application to primary respiratory chain disorders is evident. Such techniques would provide an attractive way of ensuring drug delivery to mitochondria and quantifying drug activity (Porteous *et al.*, 2010; Hoogewijs *et al.*, 2016); as well as hypothetically determining optimal drug dosing for individual patients, or facilitating the direct assessment of mitochondrial function in relation to disease progression.

Cutaneous respirometry

The ability to objectively measure respiratory chain function *in vivo*, non-invasively, and without need for imaging, has the potential to revolutionize the follow-up and treatment of those with mitochondrial disorders. Cutaneous respirometry has been developed by a Dutch research team investigating mitochondrial dysfunction arising due to sepsis (Harms *et al.*, 2015). The device, which sits over the sternum, can measure both mitochondrial oxygen tension ($mitoPO_2$) and oxygen consumption ($mitoVO_2$). It does this by using the oxygen dependent optical properties of protoporphyrin-IX, a haem precursor synthesized within mitochondria, known as the PpIX-triplet state lifetime technique. Testing in healthy volunteers is at an early stage, but has confirmed that $mitoPO_2$ and $mitoVO_2$ measurements are viable (Harms *et al.*, 2016). Furthermore, aside from minor local skin reactions, the device was well tolerated. The application to mitochondrial patients is evident and would be particularly suitable, following further assessment of reliability and validity, in the setting of a natural history study, clinical trial or even routine clinic appointment.

Discussion

While mitochondrial disorders unite on a final common metabolic pathway, their heterogeneous, multi-systemic and fluctuating nature provides particular challenges in the identification of biomarkers correlating with overall disease progression. Functional imaging studies and exercise physiology are well established for the diagnosis of mitochondrial disorders, and both converge on impaired tissue oxygen extraction. However, their role in measuring disease progression is less clear, and much could be gained through well designed longitudinal studies of genotyped cohorts using these modalities. The combination of bio-energetic and structural imaging is particularly promising, particularly in evaluating mitochondrial cardiomyopathy (Ng *et al.*, 2016), but, the need for specialist equipment and personnel conceivably limits their use. In contrast, circulating serum markers, such as FGF-21 and GDF-15; microRNA analysis and metabolomic assessment are appealing because the samples are relatively easy to collect in both paediatric and adult mitochondrial populations. However, it is currently not clear whether any serum or blood-based biomarkers will be useful as endpoints in clinical efficacy trials, particularly in groups that are genetically and clinically heterogeneous, or where a tissue-specific phenotype of interest falls within a multi-system mitochondrial disease syndrome. Longitudinal studies in patient cohorts are required to clarify whether these approaches will have a role in the future, or whether the integration of different datasets using multivariate statistical methods, for example, may help identify collections of markers that together better reflect the complex nature of disease progression. Furthermore, as 'signatures' reflecting carrier status have already been identified (Weiduschat *et al.*, 2014; Hall *et al.*, 2015), the field needs to consider whether inclusion of asymptomatic carrier individuals in observational and/or interventional studies should be facilitated.

Patients and their families want the least burdensome and clear means to monitor progression of their disease so that clinicians can provide timely and appropriate support. Clinicians and industry partners urgently need new

biomarkers to facilitate clinical development of promising novel treatments. How best to reach these goals and capitalize on the advances in biomarker techniques discussed is a critical issue that needs to be collectively addressed by clinicians, scientists, industry partners and patients, to ensure a strategic, integrated and acceptable approach is taken.

Funding

P.F.C. 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 Wellcome Trust Centre for Mitochondrial Research (096919Z/11/Z), 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. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. R.H. is a Wellcome Trust Investigator (109915/Z/15/Z), who receives support from the 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). GlaxoSmithKline provides support for H.E.S. as part of their Early Talent Review Board (ETRB) Clinical Fellow program.

References

ClinicalTrials.gov. Available from: https://clinicaltrials.gov/ (20 April 2017, date last accessed).

Adamko DJ, Nair P, Mayers I, Tsuyuki RT, Regush S, Rowe BH. Metabolomic profiling of asthma and chronic obstructive pulmonary disease: a pilot study differentiating diseases. J Allergy Clin Immunol 2015; 136: 571–80.e3.

Alonso A, Marsal S, Julia A. Analytical methods in untargeted metabolomics: state of the art in 2015. Front Bioeng Biotechnol 2015; 3: 23.

Altar CA, Amakye D, Bounos D, Bloom J, Clack G, Dean R, et al. A prototypical process for creating evidentiary standards for biomarkers and diagnostics. Clin Pharmacol Ther 2008; 83: 368–71.

Arakawa K, Kudo T, Ikawa M, Morikawa N, Kawai Y, Sahashi K, et al. Abnormal myocardial energy-production state in mitochondrial cardiomyopathy and acute response to L-arginine infusion. C-11 acetate kinetics revealed by positron emission tomography. Circ J 2010; 74: 2702–11.

Ardenkjaer-Larsen JH, Fridlund B, Gram A, Hansson G, Hansson L, Lerche MH, et al. Increase in signal-to-noise ratio of > 10,000 times in liquid-state NMR. Proc Natl Acad Sci USA 2003; 100: 10158–63.

Balderas C, Ruperez FJ, Ibanez E, Senorans J, Guerrero-Fernandez J, Casado IG, et al. Plasma and urine metabolic fingerprinting of type 1 diabetic children. Electrophoresis 2013; 34: 2882–90.

Barbiroli B, Frassineti C, Martinelli P, Iotti S, Lodi R, Cortelli P, et al. Coenzyme Q10 improves mitochondrial respiration in patients with mitochondrial cytopathies. An *in vivo* study on brain and skeletal muscle by phosphorous magnetic resonance spectroscopy. Cell Mol Biol 1997; 43: 741–9.

Barbiroli B, Iotti S, Lodi R. Improved brain and muscle mitochondrial respiration with CoQ. An *in vivo* study by 31P-MR spectroscopy in patients with mitochondrial cytopathies. Biofactors 1999; 9: 253–60.

Barbiroli B, Medori R, Tritschler HJ, Klopstock T, Seibel P, Reichmann H, et al. Lipoic (thioctic) acid increases brain energy availability and skeletal muscle performance as shown by *in vivo* 31P-MRS in a patient with mitochondrial cytopathy. J Neurol 1995; 242: 472–7.

Barbiroli B, Montagna P, Martinelli P, Lodi R, Iotti S, Cortelli P, et al. Defective brain energy metabolism shown by *in vivo* 31P MR spectroscopy in 28 patients with mitochondrial cytopathies. J Cereb Blood Flow Metab 1993; 13: 469–74.

Bates MG, Newman JH, Jakovljevic DG, Hollingsworth KG, Alston CL, Zalewski P, et al. Defining cardiac adaptations and safety of endurance training in patients with m.3243A>G-related mitochondrial disease. Int J Cardiol 2013; 168: 3599–608.

Begriche K, Massart J, Robin MA, Bonnet F, Fromenty B. Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease. Hepatology 2013; 58: 1497–507.

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.

Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 2001; 69: 89–95.

Bogacka I, Xie H, Bray GA, Smith SR. Pioglitazone induces mitochondrial biogenesis in human subcutaneous adipose tissue *in vivo*. Diabetes 2005; 54: 1392–9.

Brindle KM, Bohndiek SE, Gallagher FA, Kettunen MI. Tumor imaging using hyperpolarized 13C magnetic resonance spectroscopy. Magn Reson Med 2011; 66: 505–19.

Cacchiarelli D, Legnini I, Martone J, Cazzella V, D'Amico A, Bertini E, et al. miRNAs as serum biomarkers for Duchenne muscular dystrophy. EMBO Mol Med 2011; 3: 258–65.

Campos Y, Huertas R, Bautista J, Gutierrez E, Aparicio M, Lorenzo G, et al. Muscle carnitine deficiency and lipid storage myopathy in patients with mitochondrial myopathy. Muscle Nerve 1993; 16: 778–81.

Carlier PG, Marty B, Scheidegger O, Loureiro de Sousa P, Baudin PY, Snezhko E, et al. Skeletal muscle quantitative nuclear magnetic resonance imaging and spectroscopy as an outcome measure for clinical trials. J Neuromuscul Dis 2016; 3: 1–28.

Cea G, Bendahan D, Manners D, Hilton-Jones D, Lodi R, Styles P, et al. Reduced oxidative phosphorylation and proton efflux suggest reduced capillary blood supply in skeletal muscle of patients with dermatomyositis and polymyositis: a quantitative 31P-magnetic resonance spectroscopy and MRI study. Brain 2002; 125 (Pt 7): 1635–45.

Chao de la Barca JM, Simard G, Amati-Bonneau P, Safiedeen Z, Prunier-Mirebeau D, Chupin S, et al. The metabolomic signature of Leber's hereditary optic neuropathy reveals endoplasmic reticulum stress. Brain 2016; 139: 2864–76.

Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature 2014; 515: 431–5.

Chow WS, Xu A, Woo YC, Tso AW, Cheung SC, Fong CH, et al. Serum fibroblast growth factor-21 levels are associated with carotid atherosclerosis independent of established cardiovascular risk factors. Arterioscler Thromb Vasc Biol 2013; 33: 2454–9.

Cloonan SM, Choi AM. Mitochondria in lung disease. J Clin Invest 2016; 126: 809–20.

Cui M, Wang Q, Chen G. Serum metabolomics analysis reveals changes in signalling lipids in breast cancer patients. Biomed Chromatogr 2016; 30: 42–7.

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 2013; 81: 1819–26.

Davis RL, Liang C, Sue CM. A comparison of current serum biomarkers as diagnostic indicators of mitochondrial diseases. Neurology 2016; 86: 2010–15.

Day SE, Kettunen MI, Gallagher FA, Hu DE, Lerche M, Wolber J, et al. Detecting tumor response to treatment using hyperpolarized 13C magnetic resonance imaging and spectroscopy. Nat Med 2007; 13: 1382–7.

Drinkard BE, Keyser RE, Paul SM, Arena R, Plehn JF, Yanovski JA, et al. Exercise capacity and idebenone intervention in children and adolescents with Friedreich ataxia. Arch Phys Med Rehabil 2010; 91: 1044–50.

Drogan D, Dunn WB, Lin W, Buijsse B, Schulze MB, Langenberg C, et al. Untargeted metabolic profiling identifies altered serum metabolites of type 2 diabetes mellitus in a prospective, nested case control study. Clin Chem 2015; 61: 487–97.

Duncan DB, Herholz K, Kugel H, Roth B, Ruitenbeek W, Heindel W, et al. Positron emission tomography and magnetic resonance spectroscopy of cerebral glycolysis in children with congenital lactic acidosis. Ann Neurol 1995; 37: 351–8.

Emwas AH. The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. Methods Mol Biol 2015; 1277: 161–93.

Endo K, Weng H, Naito Y, Sasaoka T, Takahashi A, Fukushima Y, et al. Classification of various muscular tissues using miRNA profiling. Biomed Res 2013; 34: 289–99.

Engelsen BA, Tzoulis C, Karlsen B, Lillebo A, Laegreid LM, Aasly J, et al. POLG1 mutations cause a syndromic epilepsy with occipital lobe predilection. Brain 2008; 131 (Pt 3): 818–28.

Florian A, Ludwig A, Stubbe-Drager B, Boentert M, Young P, Waltenberger J, et al. Characteristic cardiac phenotypes are detected by cardiovascular magnetic resonance in patients with different clinical phenotypes and genotypes of mitochondrial myopathy. J Cardiovasc Magn Reson 2015; 17: 40.

Food and Drug Administration. Innovation or stagnation: challenge and opportunity on the critical path to new medical products. 2004.

Frackowiak RS, Herold S, Petty RK, Morgan-Hughes JA. The cerebral metabolism of glucose and oxygen measured with positron tomography in patients with mitochondrial diseases. Brain 1988; 111 (Pt 5): 1009–24.

Fujita Y, Ito M, Kojima T, Yatsuga S, Koga Y, Tanaka M. GDF15 is a novel biomarker to evaluate efficacy of pyruvate therapy for mitochondrial diseases. Mitochondrion 2015; 20: 34–42.

Galna B, Newman J, Jakovljevic DG, Bates MG, Schaefer AM, McFarland R, et al. Discrete gait characteristics are associated with m.3243A>G and m.8344A>G variants of mitochondrial disease and its pathological consequences. J Neurol 2014; 261: 73–82.

Gao R, Cheng J, Fan C, Shi X, Cao Y, Sun B, et al. Serum metabolomics to identify the liver disease-specific biomarkers for the progression of hepatitis to hepatocellular carcinoma. Sci Rep 2015; 5: 18175.

Glover EI, Martin J, Maher A, Thornhill RE, Moran GR, Tarnopolsky MA. A randomized trial of coenzyme Q10 in mitochondrial disorders. Muscle Nerve 2010; 42: 739–48.

Gorman GS, Schaefer AM, Ng Y, Gomez N, Blakely EL, Alston CL, et al. Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. Ann Neurol 2015; 77: 753–9.

Gusnard DA, Raichle ME, Raichle ME. Searching for a baseline: functional imaging and the resting human brain. Nat Rev Neurosci 2001; 2: 685–94.

Haas RH, Parikh S, Falk MJ, Saneto RP, Wolf NI, Darin N, et al. The in-depth evaluation of suspected mitochondrial disease. Mol Genet Metab 2008; 94: 16–37.

Haginoya K, Kaneta T, Togashi N, Hino-Fukuyo N, Kobayashi T, Uematsu M, et al. FDG-PET study of patients with Leigh syndrome. J Neurol Sci 2016; 362: 309–13.

Hall AM, Vilasi A, Garcia-Perez I, Lapsley M, Alston CL, Pitceathly RD, et al. The urinary proteome and metabonome differ from normal in adults with mitochondrial disease. Kidney Int 2015; 87: 610–22.

Harms FA, Bodmer SI, Raat NJ, Mik EG. Cutaneous mitochondrial respirometry: non-invasive monitoring of mitochondrial function. J Clin Monit Comput 2015; 29: 509–19.

Harms FA, Stolker RJ, Mik EG. Cutaneous respirometry as novel technique to monitor mitochondrial function: a feasibility study in healthy volunteers. PLoS One 2016; 11: e0159544.

He X, Yablonskiy DA. Quantitative BOLD: mapping of human cerebral deoxygenated blood volume and oxygen extraction fraction: default state. Magn Reson Med 2007; 57: 115–26.

Hollingsworth KG, Gorman GS, Trenell MI, McFarland R, Taylor RW, Turnbull DM, et al. Cardiomyopathy is common in patients with the mitochondrial DNA m.3243A>G mutation and correlates with mutation load. Neuromuscul Disord 2012; 22: 592–6.

Hoogewijs K, James AM, Smith RA, Gait MJ, Murphy MP, Lightowlers RN. Assessing the delivery of molecules to the mitochondrial matrix using click chemistry. Chembiochem 2016; 17: 1312–16.

Hu J, Kong M, Ye Y, Hong S, Cheng L, Jiang L. Serum miR-206 and other muscle-specific microRNAs as non-invasive biomarkers for Duchenne muscular dystrophy. J Neurochem 2014; 129: 877–83.

Ikawa M, Okazawa H, Arakawa K, Kudo T, Kimura H, Fujibayashi Y, et al. PET imaging of redox and energy states in stroke-like episodes of MELAS. Mitochondrion 2009; 9: 144–8.

Jackson MJ, Schaefer JA, Johnson MA, Morris AA, Turnbull DM, Bindoff LA. Presentation and clinical investigation of mitochondrial respiratory chain disease. A study of 51 patients. Brain 1995; 118 (Pt 2): 339–57.

Jensen TD, Kazemi-Esfarjani P, Skomorowska E, Vissing J. A forearm exercise screening test for mitochondrial myopathy. Neurology 2002; 58: 1533–8.

Jeppesen TD, Duno M, Schwartz M, Krag T, Rafiq J, Wibrand F, et al. Short- and long-term effects of endurance training in patients with mitochondrial myopathy. Eur J Neurol 2009; 16: 1336–9.

Jeppesen TD, Quistorff B, Wibrand F, Vissing J. 31P-MRS of skeletal muscle is not a sensitive diagnostic test for mitochondrial myopathy. J Neurol 2007; 254: 29–37.

Jeppesen TD, Schwartz M, Frederiksen AL, Wibrand F, Olsen DB, Vissing J. Muscle phenotype and mutation load in 51 persons with the 3243A>G mitochondrial DNA mutation. Arch Neurol 2006a; 63: 1701–6.

Jeppesen TD, Schwartz M, Olsen DB, Vissing J. Oxidative capacity correlates with muscle mutation load in mitochondrial myopathy. Ann Neurol 2003; 54: 86–92.

Jeppesen TD, Schwartz M, Olsen DB, Wibrand F, Krag T, Duno M, et al. Aerobic training is safe and improves exercise capacity in patients with mitochondrial myopathy. Brain 2006b; 129 (Pt 12): 3402–12.

Jove M, Mauri-Capdevila G, Suarez I, Cambray S, Sanahuja J, Quilez A, et al. Metabolomics predicts stroke recurrence after transient ischemic attack. Neurology 2015; 84: 36–45.

Kajiyama M, Kawamura I, Fujita A, Hamamoto K, Nishi Y, Kitano A, et al. A case of mitochondrial encephalomyopathy with cardiomyopathy due to decreased complex I and IV activities [in Japanese]. No to Hattatsu [Brain Dev] 1989; 21: 369–73.

Kalko SG, Paco S, Jou C, Rodriguez MA, Meznaric M, Rogac M, et al. Transcriptomic profiling of TK2 deficient human skeletal muscle suggests a role for the p53 signalling pathway and identifies

growth and differentiation factor-15 as a potential novel biomarker for mitochondrial myopathies. BMC Genomics 2014; 15: 91.

Kaufmann P, Engelstad K, Wei Y, Kulikova R, Oskoui M, Sproule DM, et al. Natural history of MELAS associated with mitochondrial DNA m.3243A > G genotype. Neurology 2011; 77: 1965–71.

Kaufmann P, Shungu DC, Sano MC, Jhung S, Engelstad K, Mitsis E, et al. Cerebral lactic acidosis correlates with neurological impairment in MELAS. Neurology 2004; 62: 1297–302.

Kienana M, Lydie ND, Jean-Michel H, Binta D, Matthias B, Patrick E, et al. Elucidating time-dependent changes in the urinary metabolome of renal transplant patients by a combined H NMR and GC-MS approach. Mol Biosyst 2015; 11: 2493–510.

Koene S, de Laat P, van Tienoven DH, Vriens D, Brandt AM, Sweep FC, et al. Serum FGF21 levels in adult m.3243A > G carriers: clinical implications. Neurology 2014; 83: 125–33.

Koene S, de Laat P, van Tienoven DH, Weijers G, Vriens D, Sweep FC, et al. Serum GDF15 levels correlate to mitochondrial disease severity and myocardial strain, but not to disease progression in adult m.3243A > G carriers. JIMD Rep 2015; 24: 69–81.

Kornblum C, Schroder R, Muller K, Vorgerd M, Eggers J, Bogdanow M, et al. Creatine has no beneficial effect on skeletal muscle energy metabolism in patients with single mitochondrial DNA deletions: a placebo-controlled, double-blind 31P-MRS crossover study. Eur J Neurol 2005; 12: 300–9.

Koutsoulidou A, Kyriakides TC, Papadimas GK, Christou Y, Kararizou E, Papanicolaou EZ, et al. Elevated muscle-specific miRNAs in serum of myotonic dystrophy patients relate to muscle disease progress. PLoS One 2015; 10: e0125341.

Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, et al. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell 2006; 127: 1109–22.

Lee SK, Kim J, Kim HD, Lee JS, Lee YM. Initial experiences with proton MR spectroscopy in treatment monitoring of mitochondrial encephalopathy. Yonsei Med J 2010; 51: 672–5.

Lehnhardt FG, Horvath R, Ullrich R, Kracht L, Sobesky J, Moller-Hartmann W, et al. Altered cerebral glucose metabolism in a family with clinical features resembling mitochondrial neurogastrointestinal encephalomyopathy syndrome in association with multiple mitochondrial DNA deletions. Arch Neurol 2008; 65: 407–11.

Lehtonen JM, Forsstrom S, Bottani E, Viscomi C, Baris OR, Isoniemi H, et al. FGF21 is a biomarker for mitochondrial translation and mtDNA maintenance disorders. Neurology 2016; 87: 2290–9.

Leti F, Malenica I, Doshi M, Courtright A, Van Keuren-Jensen K, Legendre C, et al. High-throughput sequencing reveals altered expression of hepatic microRNAs in nonalcoholic fatty liver disease-related fibrosis. Transl Res 2015; 166: 304–14.

Lindroos MM, Borra RJ, Parkkola R, Virtanen SM, Lepomaki V, Bucci M, et al. Cerebral oxygen and glucose metabolism in patients with mitochondrial m.3243A > G mutation. Brain 2009; 132 (Pt 12): 3274–84.

Lodi R, Cooper JM, Bradley JL, Manners D, Styles P, Taylor DJ, et al. Deficit of *in vivo* mitochondrial ATP production in patients with Friedreich ataxia. Proc Natl Acad Sci USA 1999; 96: 11492–5.

Lodi R, Rajagopalan B, Blamire AM, Crilley JG, Styles P, Chinnery PF. Abnormal cardiac energetics in patients carrying the A3243G mtDNA mutation measured *in vivo* using phosphorus MR spectroscopy. Biochim Biophys Acta 2004a; 1657: 146–50.

Lodi R, Tonon C, Valentino ML, Iotti S, Clementi V, Malucelli E, et al. Deficit of *in vivo* mitochondrial ATP production in OPA1-related dominant optic atrophy. Ann Neurol 2004b; 56: 719–23.

Logan A, Cocheme HM, Li Pun PB, Apostolova N, Smith RA, Larsen L, et al. Using exomarkers to assess mitochondrial reactive species *in vivo*. Biochim Biophys Acta 2014; 1840: 923–30.

Meseguer S, Martinez-Zamora A, Garcia-Arumi E, Andreu AL, Armengod ME. The ROS-sensitive microRNA-9/9* controls the expression of mitochondrial tRNA-modifying enzymes and is involved in the molecular mechanism of MELAS syndrome. Hum Mol Genet 2015; 24: 167–84.

Mooren FC, Viereck J, Kruger K, Thum T. Circulating microRNAs as potential biomarkers of aerobic exercise capacity. Am J Physiol Heart Circ Physiol 2014; 306: H557–63.

Morrow JM, Matthews E, Raja Rayan DL, Fischmann A, Sinclair CD, Reilly MM, et al. Muscle MRI reveals distinct abnormalities in genetically proven non-dystrophic myotonias. Neuromuscul Disord 2013; 23: 637–46.

Morrow JM, Sinclair CD, Fischmann A, Machado PM, Reilly MM, Yousry TA, et al. MRI biomarker assessment of neuromuscular disease progression: a prospective observational cohort study. Lancet Neurol 2016; 15: 65–77.

Murphy JL, Blakely EL, Schaefer AM, He L, Wyrick P, Haller RG, et al. Resistance training in patients with single, large-scale deletions of mitochondrial DNA. Brain 2008; 131 (Pt 11): 2832–40.

Nariai T, Ohno K, Ohta Y, Hirakawa K, Ishii K, Senda M. Discordance between cerebral oxygen and glucose metabolism, and hemodynamics in a mitochondrial encephalomyopathy, lactic acidosis, and strokelike episode patient. J Neuroimaging 2001; 11: 325–9.

Naz S, Calderon AA, Garcia A, Gallafrio J, Mestre RT, Gonzalez EG, et al. Unveiling differences between patients with acute coronary syndrome with and without ST elevation through fingerprinting with CE-MS and HILIC-MS targeted analysis. Electrophoresis 2015, doi: 10.1002/elps.201500169, in press.

Nelson SJ, Kurhanewicz J, Vigneron DB, Larson PE, Harzstark AL, Ferrone M, et al. Metabolic imaging of patients with prostate cancer using hyperpolarized [1-(1)(3)C]pyruvate. Sci Transl Med 2013; 5: 198ra08.

Ng YS, Grady JP, Lax NZ, Bourke JP, Alston CL, Hardy SA, et al. Sudden adult death syndrome in m.3243A > G-related mitochondrial disease: an unrecognized clinical entity in young, asymptomatic adults. Eur Heart J 2016; 37: 2552–9.

Penn AM, Lee JW, Thuillier P, Wagner M, Maclure KM, Menard MR, et al. MELAS syndrome with mitochondrial tRNA(Leu)(UUR) mutation: correlation of clinical state, nerve conduction, and muscle 31P magnetic resonance spectroscopy during treatment with nicotinamide and riboflavin. Neurology 1992; 42: 2147–52.

Petty RK, Harding AE, Morgan-Hughes JA. The clinical features of mitochondrial myopathy. Brain 1986; 109 (Pt 5): 915–38.

Pfeffer G, Horvath R, Klopstock T, Mootha VK, Suomalainen A, Koene S, et al. New treatments for mitochondrial disease-no time to drop our standards. Nat Rev Neurol 2013; 9: 474–81.

Pfeffer G, Majamaa K, Turnbull DM, Thorburn D, Chinnery PF. Treatment for mitochondrial disorders. Cochrane Database Syst Rev 2012, doi: 10.1002/14651858.CD004426.pub3, in press.

Pitceathly RD, Morrow JM, Sinclair CD, Woodward C, Sweeney MG, Rahman S, et al. Extra-ocular muscle MRI in genetically-defined mitochondrial disease. Eur Radiol 2016; 26: 130–7.

Porteous CM, Logan A, Evans C, Ledgerwood EC, Menon DK, Aigbirhio F, et al. Rapid uptake of lipophilic triphenylphosphonium cations by mitochondria *in vivo* following intravenous injection: implications for mitochondria-specific therapies and probes. Biochim Biophys Acta 2010; 1800: 1009–17.

Puchades-Carrasco L, Lecumberri R, Martinez-Lopez J, Lahuerta JJ, Mateos MV, Prosper F, et al. Multiple myeloma patients have a specific serum metabolomic profile that changes after achieving complete remission. Clin Cancer Res 2013; 19: 4770–9.

Pyle A, Nightingale HJ, Griffin H, Abicht A, Kirschner J, Baric I, et al. Respiratory chain deficiency in nonmitochondrial disease. Neurol Genet 2015; 1: e6.

Raffort J, Hinault C, Dumortier O, Van Obberghen E. Circulating microRNAs and diabetes: potential applications in medical practice. Diabetologia 2015; 58: 1978–92.

Rocha CM, Barros AS, Goodfellow BJ, Carreira IM, Gomes A, Sousa V, et al. NMR metabolomics of human lung tumours reveals distinct

metabolic signatures for adenocarcinoma and squamous cell carcinoma. Carcinogenesis 2015; 36: 68–75.
Rodan LH, Wells GD, Banks L, Thompson S, Schneiderman JE, Tein I. L-arginine affects aerobic capacity and muscle metabolism in MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-Like Episodes) syndrome. PLoS One 2015; 10: e0127066.
Rodrigues TB, Serrao EM, Kennedy BW, Hu DE, Kettunen MI, Brindle KM. Magnetic resonance imaging of tumor glycolysis using hyperpolarized 13C-labeled glucose. Nat Med 2014; 20: 93–7.
Saegusa J, Irino Y, Yoshida M, Tanaka S, Kogata Y, Kageyama G, et al. GC/MS-based metabolomics detects metabolic alterations in serum from SLE patients. Clin Exp Rheumatol 2014; 32: 148.
Saneto RP, Friedman SD, Shaw DW. Neuroimaging of mitochondrial disease. Mitochondrion 2008; 8: 396–413.
Schreuder L, Peters G, Nijhuis-van der Sanden R, Morava E. Aerobic exercise in children with oxidative phosphorylation defects. Neurol Int 2010; 2: e4.
Semba RD, Sun K, Egan JM, Crasto C, Carlson OD, Ferrucci L. Relationship of serum fibroblast growth factor 21 with abnormal glucose metabolism and insulin resistance: the Baltimore Longitudinal Study of Aging. J Clin Endocrinol Metab 2012; 97: 1375–82.
Shelly MJ, Kelly P, O'Connell MJ. FDG-PET imaging in the investigation of homonymous hemianopia in a patient with MELAS syndrome. Clin Nucl Med 2007; 32: 479–80.
Shishido F, Uemura K, Inugami A, Tomura N, Higano S, Fujita H, et al. Cerebral oxygen and glucose metabolism and blood flow in mitochondrial encephalomyopathy: a PET study. Neuroradiology 1996; 38: 102–7.
Sim KG, Carpenter K, Hammond J, Christodoulou J, Wilcken B. Acylcarnitine profiles in fibroblasts from patients with respiratory chain defects can resemble those from patients with mitochondrial fatty acid beta-oxidation disorders. Metabolism 2002; 51: 366–71.
Suomalainen A, Elo JM, Pietilainen KH, Hakonen AH, Sevastianova K, Korpela M, et al. FGF-21 as a biomarker for muscle-manifesting mitochondrial respiratory chain deficiencies: a Diagnostic Study. Lancet Neurol 2011; 10: 806–18.
Taivassalo T, De Stefano N, Argov Z, Matthews PM, Chen J, Genge A, et al. Effects of aerobic training in patients with mitochondrial myopathies. Neurology 1998; 50: 1055–60.
Taivassalo T, Gardner JL, Taylor RW, Schaefer AM, Newman J, Barron MJ, et al. Endurance training and detraining in mitochondrial myopathies due to single large-scale mtDNA deletions. Brain 2006; 129 (Pt 12): 3391–401.
Taivassalo T, Shoubridge EA, Chen J, Kennaway NG, DiMauro S, Arnold DL, et al. Aerobic conditioning in patients with mitochondrial myopathies: physiological, biochemical, and genetic effects. Ann Neurol 2001; 50: 133–41.
Taylor RW, Pyle A, Griffin H, Blakely EL, Duff J, He L, et al. Use of whole-exome sequencing to determine the genetic basis of multiple mitochondrial respiratory chain complex deficiencies. JAMA 2014; 312: 68–77.
Tsukada H. The use of (1)(8)F-BCPP-EF as a PET probe for complex I activity in the brain. Methods Enzymol 2014; 547: 417–31.
Tsukada H, Ohba H, Nishiyama S, Kanazawa M, Kakiuchi T, Harada N. PET imaging of ischemia-induced impairment of mitochondrial complex I function in monkey brain. J Cereb Blood Flow Metab 2014; 34: 708–14.
Tzoulis C, Engelsen BA, Telstad W, Aasly J, Zeviani M, Winterthun S, et al. The spectrum of clinical disease caused by the A467T and W748S POLG mutations: a study of 26 cases. Brain 2006; 129 (Pt 7): 1685–92.
Wagner JA. Strategic approach to fit-for-purpose biomarkers in drug development. Ann Rev Pharmacol Toxicol 2008; 48: 631–51.
Walker UA, Collins S, Byrne E. Respiratory chain encephalomyopathies: a diagnostic classification. Eur Neurol 1996; 36: 260–7.
Wallace DC. Mitochondria and cancer. Nat Rev Cancer 2012; 12: 685–98.
Wang C, Zhang R, Zhang X, Wang H, Zhao K, Jin L, et al. Noninvasive measurement of lower extremity muscle oxygen extraction fraction under cuff compression paradigm. J Magn Reson Imaging 2016; 43: 1148–58.
Ward CS, Venkatesh HS, Chaumeil MM, Brandes AH, Vancriekinge M, Dafni H, et al. Noninvasive detection of target modulation following phosphatidylinositol 3-kinase inhibition using hyperpolarized 13C magnetic resonance spectroscopy. Cancer Res 2010; 70: 1296–305.
Weiduschat N, Kaufmann P, Mao X, Engelstad KM, Hinton V, DiMauro S, et al. Cerebral metabolic abnormalities in A3243G mitochondrial DNA mutation carriers. Neurology 2014; 82: 798–805.
Yamada K, Toribe Y, Yanagihara K, Mano T, Akagi M, Suzuki Y. Diagnostic accuracy of blood and CSF lactate in identifying children with mitochondrial diseases affecting the central nervous system. Brain Dev 2012; 34: 92–7.
Yatsuga S, Fujita Y, Ishii A, Fukumoto Y, Arahata H, Kakuma T, et al. Growth differentiation factor 15 as a useful biomarker for mitochondrial disorders. Ann Neurol 2015; 78: 814–23.
Yatsuga S, Suomalainen A. Effect of bezafibrate treatment on late onset mitochondrial myopathy in mice. Hum Mol Genet 2012; 21: 526–35.
Young SP, Kapoor SR, Viant MR, Byrne JJ, Filer A, Buckley CD, et al. The impact of inflammation on metabolomic profiles in patients with arthritis. Arthritis Rheum 2013; 65: 2015–23.
Yu-Wai-Man C, Smith FE, Firbank MJ, Guthrie G, Guthrie S, Gorman GS, et al. Extraocular muscle atrophy and central nervous system involvement in chronic progressive external ophthalmoplegia. PLoS One 2013; 8: e75048.
Yu E, Mercer J, Bennett M. Mitochondria in vascular disease. Cardiovasc Res 2012; 95: 173–82.
Yu L, Xie S, Xiao J, Wang Z, Zhang X. Quantitative measurement of cerebral oxygen extraction fraction using MRI in patients with MELAS. PLoS One 2013; 8: e79859.
Zhang H, Guan M, Townsend KL, Huang TL, An D, Yan X, et al. MicroRNA-455 regulates brown adipogenesis via a novel HIF1an-AMPK-PGC1alpha signaling network. EMBO Rep 2015; 16: 1378–93.
Zheng J, An H, Coggan AR, Zhang X, Bashir A, Muccigrosso D, et al. Noncontrast skeletal muscle oximetry. Magn Reson Med 2014; 71: 318–25.