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Review Article

Biomarkers for mitochondrial energy metabolism diseases

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Biomarkers are an indicator of biologic or pathogenic processes, whose function is indicating the presence/absence of disease or monitoring disease course and its response to treatment. Since mitochondrial disorders (MDs) can represent a diagnostic challenge for clinicians, due to their clinical and genetic heterogeneity, the identification of easily measurable biomarkers becomes a high priority. Given the complexity of MD, in particular the primary mitochondrial respiratory chain (MRC) diseases due to oxidative phosphorylation (OXPHOS) dysfunction, a reliable single biomarker, relevant for the whole disease group, could be extremely difficult to find, most of times leading the physicians to better consider a 'biosignature' for the diagnosis, rather than a single biochemical marker. Serum biomarkers like lactate and pyruvate are largely determined in the diagnostic algorithm of MD, but they are not specific to this group of disorders. The concomitant determination of creatine (Cr), plasma amino acids, and urine organic acids might be helpful to reinforce the biosignature in some cases. In recent studies, serum fibroblast growth factor 21 (sFGF21) and serum growth differentiation factor 15 (sGDF15) appear to be promising molecules in identifying MD. Moreover, new different approaches have been developed to discover new MD biomarkers. This work discusses the most important biomarkers currently used in the diagnosis of MRC diseases, and some approaches under evaluation, discussing both their utility and weaknesses.

Introduction

Biomarkers are 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, [1]). We distinguish: (i) 'disease-related' biomarkers, which reflect the presence or absence of disease and assess its severity, giving information about the disease natural history/prognosis; and (ii) 'drug-related' biomarkers, which identify novel disease pathways or drug–target interactions [2].

Mitochondria are the organelles responsible for the ATP production through the oxidative phosphorylation system (OXPHOS), which is carried out by the mitochondrial respiratory chain (MRC) in the inner membrane. The mitochondrial matrix hosts numerous metabolic pathways like the fatty acid oxidation, the Krebs cycle, and lipid and cholesterol synthesis. The term mitochondrial disorder (MD) is often used to define disorders of the energy metabolism, but it also includes diseases caused by other defective metabolic pathways localized in the mitochondria. This review is principally focussed on MRC disorders due to defective MRC, even though we will mention the other group of mitochondrial diseases related to some specific biomarkers.

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MDs are inherited, multisystem metabolic diseases due to a defective functioning of the MRC. Since their multi-organ involvement, MDs carry on a notable disease burden, require multidisciplinary management, and can often be life-threatening conditions. Effective treatments for these diseases are still missing, and the current best management for MDs is mainly supportive [2].

MDs due to OXPHOS dysfunction are clinically and genetically heterogeneous conditions and often can present a diagnostic challenge even for experienced clinicians. For this reason, patients frequently undergo invasive investigations, such as muscle biopsy. The histological hallmarks of MDs – ragged-red and/or cytochrome *c* oxidase (COX) negative muscle fibers – are found only in some patients, and can be not specific to a primary MD: for example, secondary inflammatory myopathies, such as inclusion body myositis, also show COX-negative fibers [3]. Moreover, normal histological findings in a muscle sample do not exclude MRC defects. Moreover, normal histological findings in a muscle sample do not exclude MRC defects. Some studies show that light microscopy can be normal in ∼50% of patients with confirmed mitochondrial disease. This is true especially in children [4]. Muscle biopsy is also used for functional assessment of the OXPHOS process through the measurement of the individual MRC enzyme activities. It is important to notice that normal RC activities in the skeletal muscle do not exclude the hypothesis of MD [3].

Considering the complexity of the diagnostic process and the lack of effective treatment for these disorders, the identification of potential and non-invasive biomarkers, which would facilitate the development of clinical trials, has become a high priority [2]. The ideal biomarker is detectable in easily accessible samples, i.e. blood, urine, saliva, or exhaled air, whether a subideal biomarker such as cerebrospinal fluid (CSF), muscle, liver, or heart requires more invasive procedures. Traditionally, biomarkers in MDs have been used to improve diagnostic accuracy or identify those patients who should undergo invasive investigation. Given the clinical, biochemical, and genetic complexity of MDs, a specific and reliable single biomarker, relevant for the whole disease group, may be extremely difficult to find [2,3]. Therefore, it would be better to consider a 'biosignature' for MD diagnosis, rather than a single biochemical marker.

Serum biomarkers

Individual tissues have local responses to MRC deficiency which result in the production of metabolites or signaling molecules into bloodstream, urine, or CSF: these molecules result in a disease-associated metabolic hallmark [5] (Figure 1).

In the clinical practice, the determination of plasma lactate, pyruvate, acylcarnitines, and creatine (Cr) kinase (CK), are too non-specific for MDs and do not give a significant help in many cases. This may be due to the fact that the muscle, myocardium, and brain are not affected in every mitochondrial patient or may be unaffected at the time of the investigation, and that some of these parameters depend on whether these fluids are collected at rest or during exercise, or through a proper sampling method. Depending on the presentation, amino acids, acylcarnitines, and urinary organic acids (UOA) concentrations may help to define the global picture and raise the suspicion of MD, but their values remains limited [7,8].

Here we present a description of the most important serum biomarkers of MDs.

Lactate and pyruvate

Lactate is a hydroxycarboxylic acid, which is commonly seen as a marker of ischemia and a waste product of anaerobic glycolysis. In humans, the main sources of intracellular lactate are glucose and alanine through their conversion into pyruvate. Glycolysis is the metabolic process through which a glucose molecule is consumed to obtain two pyruvate molecules consuming two NAD+; the free energy released in this process is used to produce two ATPs from two ADPs. The enzyme alanine aminotransferase (ALT), also termed as glutamate-pyruvate transaminase (GPT), catalyzes the reversible transamination of L-alanine and α-ketoglutarate to obtain pyruvate and glutamate in the cytoplasm, the endoplasmic reticulum, and the mitochondrial network [9]. Pyruvate can either be converted into lactate regenerating two NAD+ for glycolysis by a reversible oxidoreduction reaction catalyzed by the enzyme L-lactate dehydrogenase (LDH) principally located in the cytosol of human cells or can either enter oxidative metabolism in mitochondria generating more ATP. Two proteins, MPC1 and MPC2, have been recently identified as crucial for pyruvate transport inside the mitochondria in humans. The pyruvate dehydrogenase (PDH) complex catalyzes in the mitochondrial matrix the irreversible oxidative decarboxylation of pyruvate into acetyl-coenzyme A (CoA); then acetate enters the tricarboxylic acid (TCA) cycle and is metabolized in carbon dioxide producing NADH and $FADH_2$, which are reoxidized in the MRC to finally produce ATP in an oxygen consuming reaction [9] (Figure 1).

Since the metabolic clearance of lactate occurs through its oxidation to pyruvate, congenital or acquired defects of these two metabolic pathways may result in lactate accumulation; congenital disorders affecting the MRC may

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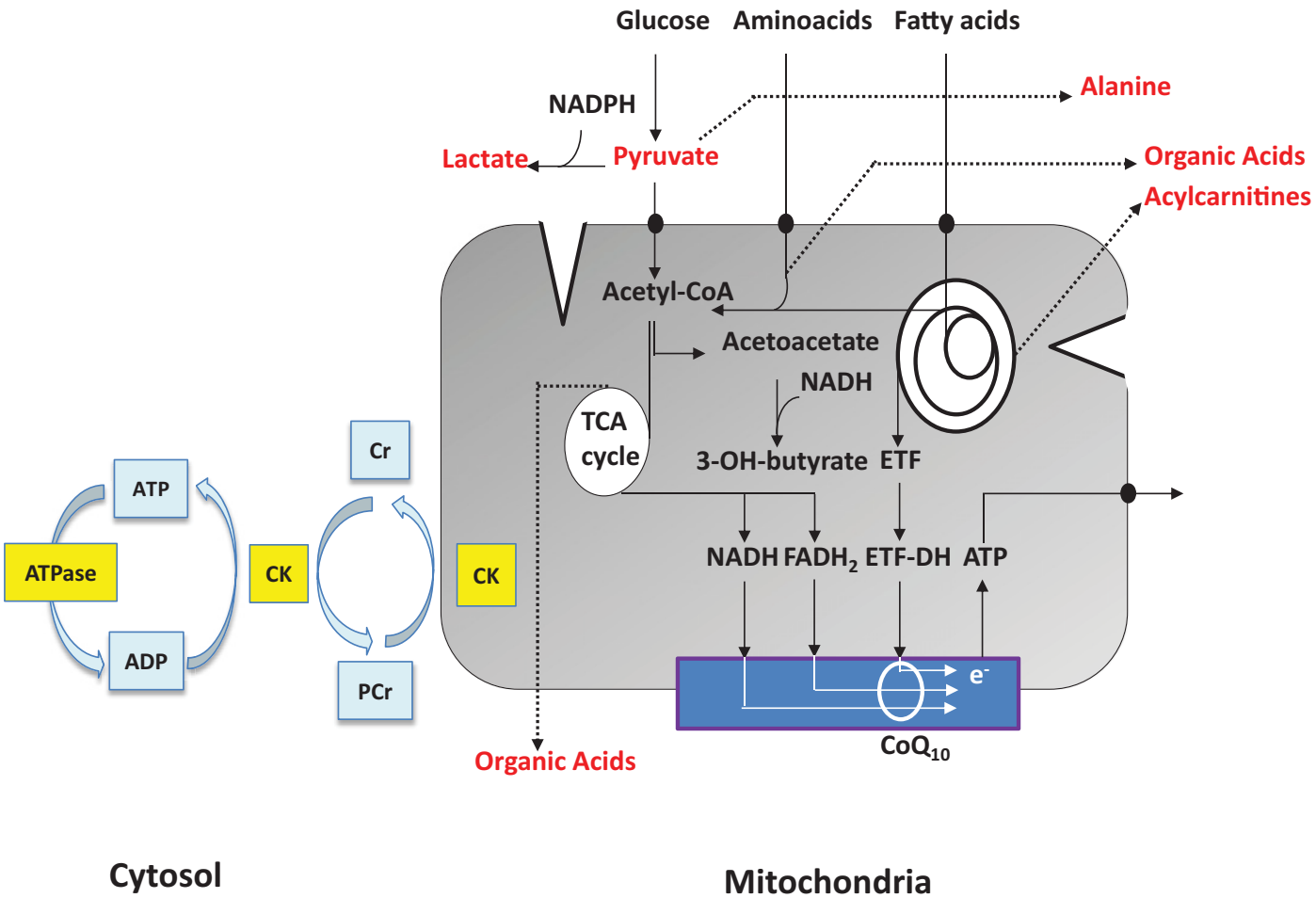

Figure 1. Metabolic pathways in mitochondria and related molecules used as biomarkers

Modified from Emma et al [6]. Abbreviations: Cr, creatine; CK, creatine kinase; CoQ10, coenzyme Q10; ETF, electron transfer flavoprotein; ETF-DH, ETF dehydrogenase.

produce lactic acidosis due to enhanced glycolysis to maintain ATP synthesis in the cytosol [9,10]. If a severe block in the pyruvate oxidation pathway occurs, and lactate cannot be adequately removed by peripheral tissues, it accumulates in the different body fluids depending upon the affected tissues.

The majority of confirmed mitochondrial oxidative defects present with a raised blood or CSF lactate and this is often associated with a raised lactate/pyruvate ratio, which means a change in the cellular redox state [11] (Table 1). Therefore, increased lactate in the serum or in the CSF can be considered a serum biomarker for MD. A fasting-related increase in plasma lactate >3 mmol/l is suggestive of MD, but artefactual increases can depend on epileptic seizure or troubled sampling (stasis, struggling child) [3] as well as improper sample treatment procedure: delays in deproteinization of samples before the analysis, induce elevation of lactate-pyruvate ratio which also showed positive correlation with pH in the pyruvate assay [12]. It is also well known that CSF lactate can be increased in central nervous system (CNS) infection, stroke, malignancy, inflammation, and seizures, which limits the power of this biomarker in the absence of other objective confirmatory findings. Lactate is the physiologic product of anaerobic energy metabolism. As exercise intensity increases, the mitochondria are unable to oxidize all the available pyruvate and the raising concentrations of pyruvate then trigger the conversion of pyruvate into lactate via the enzyme LDH [13]. Measurement of blood lactate should always take into account the working or resting state of the patient. Since muscle metabolites (e.g. lactate and ammonia) concentrations increase in the venous blood supply during and following exercise, they can be measured by progressive cycle ergometer test (PCET), which consists of a leg cycling exercise at increasing workloads until exhaustion. This test provides an objective measurement of peak functional capacity and is used in the diagnosis of metabolic myopathies [14].

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Table 1 Main biomarkers in mitochondrial disease and their pathologic and reference values

Compound	Serum/blood (μmol/l)		Urine (mmol/mol creat)		CSF (μmol/l)	
	Pathologic values	Reference values	Pathologic values	Reference values	Pathologic values	Reference values
Lactate	>2000 (B)	450–1800 (B)	>350 (<2 mo) >300 (2 mo–2 y) >130 (>2 y)	>270 (<2 mo) >200 (2 mo–2 y) >85 (>2 y)	>2000	1100–1700
Pyruvate	>130 (B)	60–100 (B)			>200	80–140
Lactate/pyruvate ratio	>17 (B)	<15 (B)			>17	<15
Cr*	>120	26.6–95.3 (<12 ys) 21.1–88.6 (12–20 ys) 19.3–49.0 (>20 ys)				
Alanine	>450 (P, S)	150–450 (P, S)	>300 (<6 mo) >150 (6 mo–7 ys)	70–250 (<6 mo) 35–165 (6 mo–1 ys) 25–130 (1–7 ys)		
CK	>200 (M) >170 (F) (S, U/l)	<200 (M) <170 (F) (S, U/l)				
Methylmalonic acid			10–500	1–11 (<4 mo) 2–13 (4 mo–2 ys) 1–4 (2–10 ys) 0–4 (>10 ys)		
Ethylmalonic acid			>25	0–15 (<2 ys) 0–9 (2–10 ys) 2–10 (>10 ys)		
3-Methylglutaconic acid			>25	<20		
3-MGAuria			40 to >1000			

Abbreviations: B, blood; F, female; M, male; mo, months; P, plasma; S, serum; U/l, units/l; ys, years; 3-MGAuria, 3-methylglutaconic aciduria. The lower and upper reference values represent the 5th and 95th percentiles.
Metabolites' reference values: see 'Physician's Guide to the Diagnosis, Treatment, and Follow-Up of Inherited Metabolic Diseases' [15].
*Plasma Cr reference values: see Boenzi et al. (2011) [16].

Increased lactate could also represent secondary mitochondrial dysfunction occurring as a result of severe liver disease. The healthy liver acts as the main consumer of lactate and contributes to 30–70% of lactate metabolism. After hepatic uptake, lactate is first converted into pyruvate and then retransformed into glucose in a process called gluconeogenesis. The process through which lactate is released from the working muscle and retransformed to glucose in the liver is called the Cori Cycle, and it releases equimolar amounts of HCO_3^-. Therefore it is important to notice that hepatic stable or acute damage can determine the increase in lactate levels and perturbation of the acid–base equilibrium [17].

Leigh syndrome (MIM #256000), a very severe MD, can often present with infantile lactic acidosis, whereas other nuclear or mtDNA defects do not present this feature [9]. A review of all published cases of nuclear-encoded complex I (CI) deficiency shows how plasma and CSF lactate were frequently elevated in the reported cases and do not seem to discriminate between different molecular genetic defects, nor any relevant difference was present between patients with nuclear subunit mutations and those with assembly factor defects; furthermore, lactate levels did not correlate with residual CI activity [18].

In PDH deficiency, laboratory investigations show increased lactate concentration in blood, urine, and CSF. Pyruvate level in blood and urine is elevated as well. Lactic acidosis in these patients is usually profound and resistant to correction with bicarbonate [19,20]. Most PDH defects display a normal redox state and thus a normal or even low lactate:pyruvate ratio (Table 1); a normal ratio can occur also in the pyruvate carboxylase deficiency (MIM #266150). Some authors report that a lactate:pyruvate ratio >25 is considered suggestive of respiratory chain dysfunction, whereas a ratio <25 is thought to be consistent with PDH deficiency [21]. A review of 371 cases of PDH deficiency showed that hyperlactatemia is a frequent, but not always present, finding in the early disease process, and is associated with a blood lactate:pyruvate ratio ≤20 [20]. On the other hand, many defects affecting CI and complex IV (CIV) do have an increase in the lactate/pyruvate ratio both in skin fibroblasts and in the patient's blood [11].

Summarizing, lactate and pyruvate are usually elevated in PDH defects and lactate:pyruvate ratio is usually normal or low. This ratio can be increased in OXPHOS defects.

Cr/CK

The Cr/CK/phosphocreatine system plays a role in the cellular homeostasis of energy. CK transfers, as an intracellular energy shuttle, high-energy phosphates from sites of *de novo* ATP production in the mitochondria to cellular sites of energy utilization [22]. Cr not only acts as a substrate but also helps in preserving the mitochondrial CK [23,24] (Figure 1).

CK can be normal or mildly increased in mitochondrial diseases (Table 1). The association of lactic acidemia and increased level of CK is highly suggestive of thymidine kinase 2 (TK2) deficiency (MIM #617069, #609560) [25].

In 2010 Shaham at al. [26] published a metabolomics study, combining biochemical analysis of spent media from cell culture with analysis of patients' plasma to identify disease biomarkers. Cell culture-defined metabolites (32 compounds) were measured in human plasma to discover that Cr was reproducibly elevated in two independent cohorts of MRC defect patients, exceeding lactate and alanine in magnitude of elevation and statistical significance [27]. A different study demonstrates that plasma Cr is not a sensitive biomarker of MD although it does present an acceptable specificity (83%) [27]. Elevated plasma Cr, together with other biomarkers, might help to reinforce the diagnosis of MD, as a biosignature. Cr reference values depends on age and they vary based on the method of analysis. Evaluation of plasma Cr must be taken into account that the normal values of healthy subjects decrease with age, therefore a Cr concentration above 100 μmol/l is not considered suggestive for MD in a newborn differently from an adult subject (Table 1).

Plasma amino acids

Amino acid quantitation can be performed on blood (plasma or serum), urine, and CSF, although testing of urine is typically only helpful in diagnosing MDs associated with renal tubulopathy. Plasma amino acids do not display a significant correlation with MDs, since most of them show a normal profile while a small proportion presents non-specific increased concentrations. Amino acids abnormalities, in particular alanine and proline, are strictly related to pyruvate increase. Excess of alanine (Table 1) together with lactate, is produced after reduction or transamination in accumulated pyruvate (Figure 1). The branched chain amino acids valine, leucine, and isoleucine can be increased in the defect of E3 subunit of PDH (MIM #246900) [28]. Citrulline might be found both decreased or increased in different MDs: citrulline low concentrations were found in complex V (CV) defects due to mutations in ATPase synthase 6 and 8 genes, associated with neuropathy, ataxia, retinitis pigmentosa (NARP, MIM #551500) [29], and/or maternally inherited Leigh Syndrome (MILS, MIM +516060), respectively [30], and in mitochondrial encephalomyopathy with stroke-like episodes (MELAS, MIM #540000) or MELAS-like syndromes [31,32]. On the contrary, citrulline high levels were found in pyruvate carboxylase deficiency 'French type' (MIM #266150), together with elevated serum ammonia [33]. Hepatopathy in association with MD could result in abnormal levels of tyrosine and methionine [34]. Moreover, defects in enzymes involved in mitochondrial Lipoate synthesis (mutations in *LIAS* gene, lipoic acid synthase) were associated with hyperglycinemia. LIAS is an iron–sulphur cluster (ISC) protein whose cofactors also participate in electron transfer reactions and are required for respiratory chain complexes I, II, and III [35]. In conclusion, taken together, the amino acid analysis is not informative to predict MDs but it is useful for the differential diagnosis between several conditions.

Acylcarnitines

Analysis of blood spot or plasma acylcarnitines is considered a useful tool for diagnosis of a large series of inherited metabolic diseases, but patients with MD showed slight alteration or normal acylcarnitines profile. The most common abnormalities were low C0 (free carnitine) and elevated C4OH (3-hyroxybutyryl-carnitine). Sometimes, elevation of medium- or long-chain acylcarnitines was detected in CV defects or in combined MRC deficiency. Long-chain acylcarnitines were found increased in ACAD9 deficiency (MIM #611126), an MD associated with CI defect [34], characterized by neurologic dysfunction, liver failure, and cardiomyopathy.

Elevated C5OH (hydroxyisovalerylcarnitine) can be found in 3-methylcrotonyl-CoA carboxylase deficiency (MCC1D, MIM #210200), an autosomal recessive inherited metabolic disease of leucine catabolism. Elevation of C3 (propionylcarnitine) together with C5OH indicates multiple carboxylase deficiency, usually due to biotinidase (MIM #253260) or holocarboxylase synthetase deficiency (MIM #253270). When molecular, genetic, or enzymatic testing for these conditions were negative, the profile could be suggestive of MT-ATP6 mutations. The MT-ATP6 protein is a part of ATP synthase enzyme, which is also known as CV, responsible for the final step of OXPHOS. The early diagnosis is relevant for patients carrying MT-ATP6 mutations because they could develop neurodegenerative disease, especially Leigh disease and NARP, as well as cardiomyopathy over time [36]. Elevation of plasma C4/C5 acylcarnitine esters is found in ethylmalonic encephalopathy (EE, MIM #602473) [37,38]. Slightly elevated concentrations of

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various short-, medium-, and long-chain acylcarnitines in dried blood spots were found in patients with defects in *FLAD1* encoding for FAD synthase, as well as in the mitochondrial FAD transporter (mutations in *SLC25A32* gene) [39].

The presence of urine 3-hydroxyisobutyryl-carnitine, is useful for differential diagnosis of two mitochondrial enzyme defects, 3-hydroxyisobutyryl-CoA hydrolase deficiency (HIBCHD, MIM #250620), a rare defect of the valine catabolic pathway associated with Leigh-like disease, and short-chain enoyl-CoA hydratase deficiency (ECHS1D, MIM #616277). It is present only in samples from ECHS1 deficiency, but not in HIBCHD [40].

Fibroblast growth factor 21

Serum fibroblast growth factor 21 (sFGF21) is a hormone-like cytokine that is involved in intermediary metabolism of carbohydrates and lipids. In the mouse model, hepatic FGF-21 is induced by peroxisomal proliferator-activated receptor-α (PPARα) and in response to fasting, but in humans the function of FGF-21 is still not completely understood. Circulating FGF-21 in humans derives mainly from the liver, but the protein is also expressed in adipocytes, myocytes, and the pancreas [7]. Some authors hold that myocytic FGF-21 is induced in a protective mechanism against metabolic stress, demonstrating its up-regulation in the heart failure and its role in inducing the expression of proteins involved in antioxidative pathways [41] or its increase in critical illness conditions [42]. This point is still controversial, since some authors have reported data against the hypothesis that fibroblast growth factor 21 (FGF21) acts as metabolic mediator of the mitochondrial stress adaptation [43].

sFGF21 was first reported as a potential biomarker in MDs since its serum concentration was found sensitive and specific for mitochondrial myopathies, correlating with disease severity and respiratory chain-deficient muscle fibers [44]. On the other hand, sFGF21 has been associated with a range of non-MDs, encompassing cancer, obesity, renal disease, diabetes, and liver disease, with the latter two frequently associated with MD [45,46]. Moreover, sFGF21 is variably increased in non-mitochondrial myopthies also [47,48] and its correlation with disease severity was not found in adult cohorts with the m.3243A4G mutation [2,49]. Recently, Lehtonen et al. (2016) [47] reported that the highest induction of FGF21 response occurs in disorders that primarily or secondarily affect mitochondrial translation such as direct mutations of translation machinery or mtDNA deletions, but not in MDs caused by mutations in structural MRC complexes or their assembly factors. This is true in patients and mouse models accumulating multiple mtDNA deletions in skeletal muscle or mice with a single large heteroplasmic mtDNA deletion. The mutant mtDNA amount correlates with sFGF21 and muscle FGF21-RNA in mito-mice, and sFGF21 in patients with single mtDNA deletions [47]. Anyway, the utility of FGF21 as a MD biomarker disease progression still needs to be determined in broader, well-characterized mitochondrial cohorts [50].

Growth differentiation factor 15

Growth differentiation factor or GDF-15 is a cytokine of the transforming growth factor β (TGF-β) superfamily, which is expressed mainly in placenta, kidney, liver, lung, pancreas, and prostate. It has an essential role in regulating the cellular response to stress signals and inflammation, being involved in suppression of inflammation in early pregnancy, cancer, and cardiovascular diseases. Moreover, GDF-15 is expressed in the choroid plexus acting as a potent neurotrophic factor for motor and sensory neurones [51]. Its exact biological functions remain poorly understood but it seems that GDF-15 is elevated by activating transcription factor 4 (*ATF4*), which is a stress-responsive gene [52].

Serum GDF15 (sGDF15) was identified in recent years as a potential diagnostic biomarker for MDs by a gene expression study in TK2-deficient human skeletal muscles [53]. The role of sGDF15 as a biomarker for MDs has been tested in large patient cohorts with different mitochondrial defects, showing its higher sensitivity and specificity than FGF21 [52]. Its measurement, combined with sFGF21 quantitation, improves the disease detection ability of either factor separately [51]. Some authors report that sGDF15 correlates with MD severity and could be used as a reliable index of disease progression [52], but Koene et al. (2015) [54] showed that sGDF15 does not correlate with disease severity in a large cohort of adult A3243G mutation carriers. On the other hand, GDF-15 seems to be more indicative of MD regardless of clinical phenotype, whereas FGF-21 sensitivity for MD is higher when muscle manifestations are present [53]. It seems that sGDF15 levels were higher in MD patients with TK2 defect or multisystem involvement, like MELAS or Pearson/KSS patients [48]. Likewise sFGF21, sGDF-15 seems to be a more specific marker for MDs due to mitochondrial translation and mtDNA maintenance defects, as opposed to those resulting from impaired respiratory chain complex or assembly factors [55]. Similar to sFGF21, the reliability and efficacy of sGDF15 as a biomarker of MDs remains to be tested in other patients' cohort.

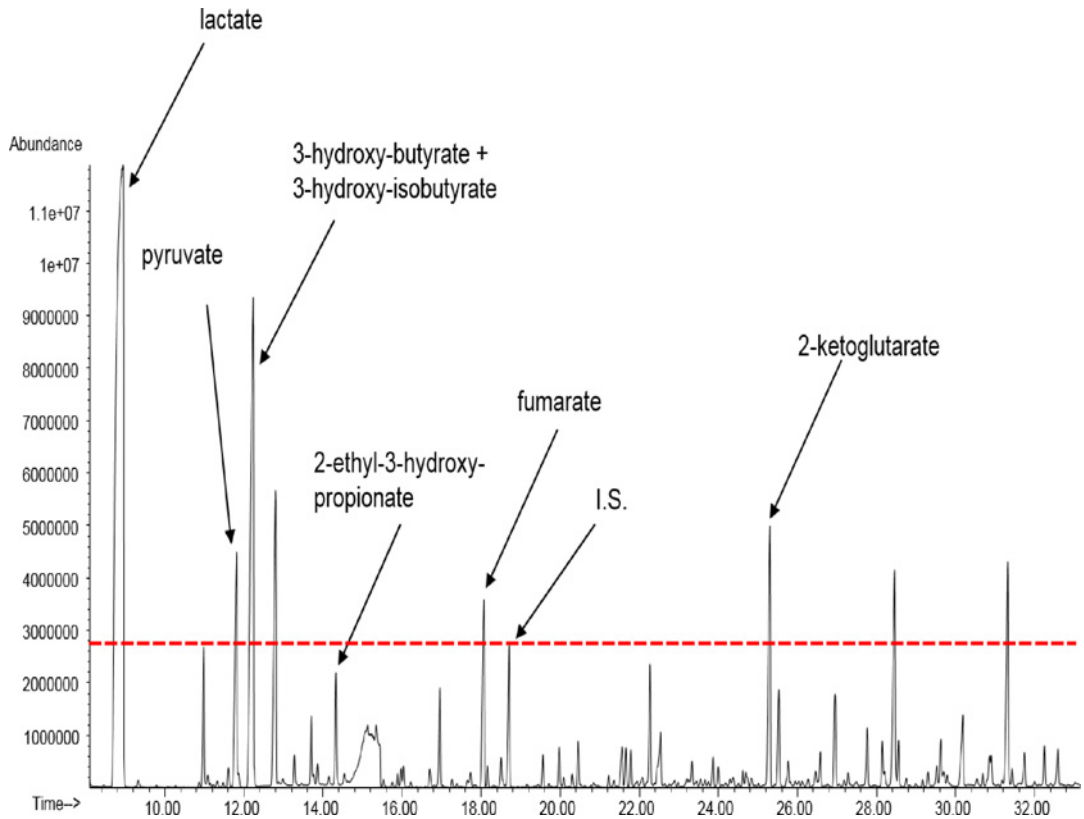

Figure 2. Example of semiquantitative UOA profile from a mitochondrial patient, by GC MS.
Modified from Emma et al. [6]. The red line stands for normal values threshold. Abbreviation: IS, internal standard: 2-phenylbutyrate.

UOA

The UOA profile in MD could be normal or abnormal to a variable extent. The analysis is quite informative, and the results could be helpful in guiding the investigation toward a specific group of mitochondrial defects.

When abnormal, UOA analysis reveals a profile characterized by large excretion of lactate, pyruvate, and/or TCA intermediates (e.g. fumarate, malate, a-ketoglutarate, citrate, isocitrate), ketones, 3-methyl-glutaconate (3-MGA), 3-methylglutarate, dicarboxylic acids, 2-ethyl-3-hydroxy-propionate, 2-methyl-2,3-dihydroxy-butyrate, tyrosine metabolites, all present in various amounts (Figure 2). Lactate and ketones (mainly 3-hydroxybutyrate and acetoacetate) are frequently found, followed by TCA and tyrosine metabolites (mainly 4-hydroxyphenylacetate). When hepatic failure occurs, whereas 3-MGA and dicarboxylic acids are less common. Occasionally, methylmalonic acid (MMA), branched chain ketoacids, glutaric acid, and acylglycines are found in traces, but their presence is not necessarily associated with a specific mitochondrial defect [30]. On the other hand, high levels of MMA, variably associated with other metabolites like TCA intermediates, can be found in mtDNA depletion syndromes MTDPS5 (MIM #612073) and MTDPS9 (MIM #245400), caused, respectively, by mutation in *SUCLA2* and *SUCLG*1 genes, although at levels (10–500 mmol/mol creatinine) that are far below those typically seen in individuals with classic MMA [56,57] (Table 1).

Ethylmalonate in urine is the main biochemical feature of EE, together with methylsuccinate, thiosulphate, and abnormal excretion of C4 and C5 (n -butyryl, isobutyryl-, isovaleryl- and 2-methylbutyryl-) acylglycines and acylcarnitines, as well as severe lactic acidosis [37,38]. However, ethylmalonic acid is not specific for *ETHE1* deficiency, also results increased in short chain acyl-CoA dehydrogenase deficiency [58] and in glutaric aciduria type II [59].

3-MGA can be mildly increased (20–40 mmol/mol creatinine) in urine of patients with MDs (i.e. due to *POLG1* mutations). In some MDs, such as 3-methylglutaconic aciduria with deafness, encephalopathy, and Leigh-like syndrome (MEGDEL, MIM #614739), Barth (MIM #302060) or mitochondrial CV deficiency, nuclear type 2 (MC5DN2, MIM #614052) syndromes, we observe a significant and consistent increase in 3-MGA excretion (40 to >1000 mmol/mol creatinine); here, the 3-methylglutaconic aciduria (3-MGAuria) can be considered as a hallmark of the phenotype and often the key to the diagnosis [60] (Table 1).

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UOA profile is useful for diagnosis of two mitochondrial enzyme defects, HIBCHD (MIM #250620), a rare defect of the valine catabolic pathway associated with Leigh-like disease, and ECHS1D (MIM #616277), in which UOA profile shows increased levels of 2-methyl-2,3-dihydroxybutyrate. The presence of urine 3-hydroxyisobutyryl-carnitine, revealed by MS analysis, is useful for differential diagnosis. It is present only in samples from ECHS1D, but not in HIBCHD [40].

Notes

Deoxyuridine and thymidine in urine and plasma could be useful for diagnosis of mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (OMIM #603041), an autosomal recessive disorder due to mutations in *TYMP* gene encoding for thymidine phosphorylase [61].

Biomarkers in functional imaging

Magnetic resonance spectroscopy (MRS) is a non-invasive functional imaging technique, which allows the quantitative assessment of tissue metabolites in steady state, commonly using ^{31}P and ^{1}H in mitochondrial cohorts. The concentration of Cr in brain tissue is relatively stable and little affected by age or by the presence of pathology, therefore in the common MRS clinical practice it is used as an internal reference for calculating metabolite ratios (lactate to Cr, L/Cr) [62].

Elevated lactate levels are established findings of brain MRS studies in MDs, but little has been published regarding the use of brain MRS to monitor disease progression or to study drug effects [2,8]. Recent work using ^{1}H-MRS brain imaging has identified fundamental differences between healthy controls, MELAS patients and pre-symptomatic individuals converting into affected individuals. The authors concluded that CSF lactate and choline might be used as biomarkers to predict the risk of carriers to develop a MELAS phenotype [63].

The applying of ^{31}P-MRS on skeletal muscle could identify important differences of tissue metabolites pattern in several neuromuscular disorders, including MDs [2,8].

Positron emission tomography (PET) measures metabolic flux in several tissues using specific radioisotopically labeled metabolites in the study of MDs (15O, 2-deoxy-2 18F-fluoro-D-glucose or FDG, and 11C pyruvate). This technique has been used mainly in MELAS patient cohorts, showing a global impairment in the cerebral oxygen consumption rate and a decreased glucose uptake in the cerebral posterior regions [64]. Moreover, some authors report that in Leigh patients, the glucose uptake was decreased in the cerebellum and basal ganglia by using the 18FDG-PET [65].

Dynamic nuclear polarization (DNP) is a promising future technique, which uses ^{13}C-MRS to provide real-time functional imaging that permits the determination of substrates in low concentrations [2]; interestingly, novel PET ligands seem to be promising in pre-clinical work to quantitate activity [2].

New approaches

In recent years, several new approaches have been tried to identify novel biomarkers for MD (see the article by Stenton and Prokisch [66] in this issue of *Essays in Biochemistry)*.

'Omics' approaches that study proteomic, metabolomic, lipidomic, and transcriptomic profiles induced by a specific gene defect, allow a global view on affected metabolic pathways and biomolecules directly or indirectly involved in pathogenic mechanisms [3]. Metabolomic and lipidomic analyses of MD models have been published, but have not yet revealed biomarkers [4]. Omics approaches have been used in a mitochondrial myopathy mouse model revealing global metabolic changes, detectable in the serum, showing how these alterations are related with pathological changes in skeletal muscles and how they change with treatment [3].

MiRNAs represent highly conserved non-coding RNAs, which control gene expression by silencing the transcription. In cybrid cells carrying the m.3243A>G mutations, Meseguer et al. [67] showed that the miRNA 9/9* pattern is associated with the MELAS or myoclonic epilepsy with ragged-red fibers (MERRF) phenotype.

Another approach is the use of 'exomarkers', which are obtained through the administration of exogenous probe compounds to the intact organims. These tailor-made probes are modified by reactive molecules of interest *in vivo* to produce a quantifiable signal; in the case of MD, exomarkers are used to measure reactive oxygen species [68]. The exomarkers approach has been used in the assessment of acquired mitochondrial dysfunction consequent to ischemic and reperfusion insults, but the potential application to primary MRC disorders is evident [2,69].

Conclusion

MDs are extremely heterogeneous, inherited conditions, which are due to defective functioning of the OXPHOS system. Even though the metabolic pathway is common, their clinical and genetic variability makes the identification of reliable and specific biomarkers quite challenging. In the clinical practice, the diagnostic algorithm of these conditions relies on the combination of phenotype, family history, imaging investigations, and laboratory measures. Serum or CSF raised lactate is a common finding in MD, but its increase is not specific for these diseases. Lactate elevation combined with specific alterations in pyruvate, acylcarnitines, or UOA profile often represents a reliable and valid 'biosignature' of MD. Lactate:pyruvate ratio can be useful in the differential diagnosis of PDH and OXPHOS defects. Consistent increase in ethylmalonic or methylmalonic acid in UOA profile is a reliable indicator of specific MDs caused by defects in *ETHE1* or *SUCLA2/SUCLG1* genes, respectively. Low plasma citrulline can be found in some MD due to mtDNA point mutations like NARP or MELAS. Newly identified serum biomakers like FGF21 and GDF15 are appealing molecules, whose sensitivity and specificity for MD has been variably assessed and still needs to be confirmed in other and large patients cohort. Both these biomarkers seem to be more indicative of MD due to mitochondrial translation and mtDNA maintenance defects. Anyway, their use as biomarkers to assess end points for clinical trials is still controversial. New approaches such as the use of miRNA or small molecules reports through omics analyses may represent a promising resource but are still under evaluation. Therefore, the identification and validation of specific and reliable biomarkers for MD become an urgent need in the scientific community and requires increasing efforts.

Summary

- Mitochondrial disorders (MDs) caused by OXPHOS dysfunction often present a diagnostic challenge even for experienced clinicians. For this reason, the identification of potential and non-invasive biomarkers has become a high priority.
- The majority of mitochondrial oxidative defects present with a raised blood or cerebrospinal fluid lactate and this is often associated with a raised lactate/pyruvate ratio. Increased lactate could be the result of secondary mitochondrial dysfunction (for example, due to liver disease) or incorrect sample processing. Lactate and pyruvate are usually elevated in pyruvate dehydrogenase defects and in these conditions, the lactate:pyruvate ratio is usually normal or low. This ratio can be increased in OXPHOS defects.
- The concomitant determination of creatine, plasma amino acids and urine organic acids helps to define a "biosignature" for mitochondrial oxidative defects.
- sFGF21 and sGDF-15 were recently identified as new potential biomarkers for MDs. They seem to be more specific for MDs due to mitochondrial translation and mtDNA maintenance defects, even though their utility as biomarkers of disease progression still needs to be determined in well characterized mitochondrial cohorts.
- Biomarkers have been revealed by functional imaging techniques, such as magnetic resonance spectroscopy (MRS), positron emission tomography (PET) dynamic nuclear polarisation (DNP).
- "Omics" approaches have been used to identify new MD biomarkers by studying proteomic, metabolomic, lipidomic and transcriptomic profiles induced by a specific gene defect

Competing interests

The authors that there are no competing interests associated with the manuscript.

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Abbreviations

CK, creatine kinase; CoA, acetyl-coenzyme A; COX, cytochrome *c* oxidase; Cr, creatine; CSF, cerebrospinal fluid; CI, complex I; CIV, complex IV; CV, complex V; ECHS1D, short-chain enoyl-CoA hydratase deficiency; EE, ethylmalonic encephalopathy; FGF21, fibroblast growth factor 21; HIBCHD, 3-hydroxyisobutyryl-CoA hydrolase deficiency; LDH, L-lactate dehydrogenase; LIAS, lipoic acid synthase; MD, mitochondrial disorder; MELAS, mitochondrial encephalomyopathy and stroke-like episode; MMA, methylmalonic acid; MRC, mitochondrial respiratory chain; MRS, magnetic resonance spectroscopy; NARP, neuropathy, ataxia, retinitis pigmentosa; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase; PET, positron emission tomography; sGDF15, serum growth differentiation factor 15; sFGF21, serum FGF-21; TCA, tricarboxylic acid cycle; TK2, thymidine kinase 2; UOA, urinary organic acid; 3-MGA, 3-methyl-glutaconate.

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