Biomarkers Subgroup Guidance Document

Mitochondrial Disease Biomarkers		
Clinical Assessments		
Height; Weight; Head Circumference	Refer to the Physical Exam CRF for guidance	(Parikh et al. 2017)
Hearing	Refer to Audiology Outcomes Subgroup Hearing Loss in Mitochondrial Disease CRF for guidance	(Parikh et al. 2017; Parikh et al. 2013)
Vision	Refer to Ophthalmology Outcomes Subgroup Ophthalmology Test Guidance Document	(Parikh et al. 2017; Parikh et al. 2013)
Cardiac Evaluation	Refer to Exercise Physiology Subgroup Echocardiogram, EKG, Holter Exam and Cardiac MRI CRFs for guidance	(Parikh et al. 2017; Parikh et al. 2013)
Cycle Ergometry	The characterization of exercise intolerance in mitochondrial disease is performed using cycle ergometry with measurements of VO_2, VCO_2, respiratory exchange ratio (RER = VCO_2/VO_2), heart rate, minute ventilation, rating of perceived exertion, and cardiac output. VO_2 max correlates with the mtDNA mutation load in exercising muscle, suggesting that the mutation load, rather than the genotype, determines the oxidative capacity of skeletal muscle in mitochondrial myopathies. Therefore, measurement of VO_2 max via cycle ergometry is a non-invasive and effective method to assess oxidative capacity in the skeletal muscle of patients with mitochondrial myopathy. Refer to Exercise Physiology Subgroup Staged Exercise Tolerance Test CRF for guidance	(Bergs et al. 2022; Bhatia, Cohen, and N 2021; Jeppesen et al. 2021; Kurihara et al. 2022)
Indirect Calorimetry	Indirect calorimetry (oxygen consumption, VO_2) in patients with mitochondrial disease shows elevated resting energy expenditure (REE) or hypermetabolism, predicting a more accelerated biological aging.	(Sturm et al. 2023)
Serum / Plasma		
Acylcarnitines	Carnitine plays an essential role in the transfer of long-chain fatty acids into the mitochondria for beta-oxidation. The elevated NAD+ /NADH ratio that can occur in mitochondrial diseases can cause secondary inhibition of NADH-generating reactions. In particular, long-chain hydroxyl-acyl-CoA dehydrogenase enzymatic activity can be inhibited by high NADH concentration. Furthermore, the mitochondrial trifunctional protein is bound to complex I, and this interaction can be disrupted by genetic defects affecting complex I. These events may lead to the accumulation of long-chain hydroxyacylcarnitines. Quantitative measurement of plasma acylcarnitine levels is a clinical assay that may include the analysis of free carnitine. The use of free to total carnitine ratio as a marker of mitochondrial disease has been recommended as an adjuvant screening tool as acylcarnitines may accumulate in mitochondrial disease due to	(Suomalainen 2011; Haas et al. 2007; Mancuso et al. 2009; Longo, Amat di San Filippo, and Pasquali 2006)

	impaired oxidation. Secondary carnitine deficiencies can occur in mitochondrial diseases in the setting of renal Fanconi syndrome.	
Amino Acids	Elevated alanine, and proline can be observed in mitochondrial diseases as they suggest a persistent lactate increase. Hyperprolinemia is the results of the inhibition of proline dehydrogenase by elevated lactate. Elevated plasma alanine levels, when present, may be a useful indicator of long-standing lactate and pyruvate accumulation because alanine is in equilibrium with pyruvate through alanine aminotransferase. Alanine can be affected by the prandial state. Alanine/lysine, alanine/(phenylalanine+tyrosine), alanine/leucine and proline/leucine ratios provide improved specificity and exclude spurious elevations. Decreased citrulline is a feature of some mitochondrial diseases, including MT-ATP6 mitochondrial disease.	(Bedoyan et al. 2020; Haas et al. 2008; Kowaloff et al. 1977; Tise et al. 2023)
Ammonia	Hyperammonemia can occur in the context of mtDNA depletion syndromes presenting with a hepatocerebral phenotype. Some of these disorders may have hepatic involvement that can be severe, and in some patients, hepatic failure occurs triggered by an infection or the use of sodium valproate therapy (e.g., Alpers syndrome). Furthermore, hyperammonemia may occur in the setting of TMEM70 deficiency and mitochondrial carbonic anhydrase VA deficiency.	(Parikh et al. 2009)
CBC	A complete blood count with differential should be considered annually in patients with mitochondrial disease. Sideroblastic anemia is a known feature of several mitochondrial diseases, including Pearson syndrome and MLASA. Neutropenia has been reported in Barth syndrome. Leukopenia, thrombocytopenia, and pancytopenia have been reported although not as frequently. Patients at higher risk of anemia or bone marrow suppression (such as Pearson syndrome) should have a complete blood count checked more frequently, based on the patient's clinical course.	(Parikh et al. 2017)
Cell-Free mtDNA (cf-mtDNA)	Cell-free mtDNA (cf-mtDNA) has been mostly evaluated in plasma samples as a potential biomarker that may be increased in necrosis, apoptosis, tumors, or inflammation. Increased plasma levels of cf-mtDNA were found in acute events or progression of neurodegeneration in longitudinal assessments of patients with MELAS syndrome. Cf-mtDNA were found to be higher in a cohort of patients with single mtDNA deletion and mtDNA depletion syndromes than in controls.	(Maresca et al. 2020; Trumpff et al. 2021; Trifunov et al. 2021)
CPK	The determination of CK activity is a commonly used assay in the investigation of skeletal muscle disease. Patients with mitochondrial disease can have increases in CPK or even episodes of rhabdomyolysis. Mitochondrial myopathies do not lead to marked increases in creatine kinase at baseline except for TK2 related mitochondrial DNA depletion syndrome. An initial evaluation of muscle function will require measuring	(Davis et al. 2013; Haas et al. 2007; Suomalainen 2011; Parikh et al. 2017)

	CK. A patient with an established myopathy may need annual CK levels.	
Creatine	Creatine plays a fundamental role in the maintenance of phosphocreatine and the replenishment of ATP in tissues with high energetic demand. Elevations in plasma creatine are specific although not sensitive to mitochondrial diseases. Patients with mitochondrial disease may have elevated creatine in plasma and low ratios of phosphocreatine/creatine in tissues. Elevated plasma creatine may be a specific but not sensitive biomarker for mitochondrial disease.	(Shaham et al. 2010; Maresca et al. 2020; Pajares et al. 2013)
Cystatin C	Based on a pediatric study of patients with mitochondrial disease, serum creatinine may not fully reflect renal function due to the relatively small body mass of patients. Cystatin C has a higher diagnostic accuracy to assess glomerular filtration rate (GFR) in mitochondrial disease. Therefore, cystatin C should be taken as the first step to evaluate glomerular filtration rate in mitochondrial diseases and should be included in the routine follow-up.	(Lee et al. 2009; Parasyri et al. 2022)
Basic Chemistries	Evaluation of a mitochondrial patient in the acute setting should include the screening of routine chemistries. Standard electrolytes (Na, K, Cl, CO_2, BUN, Creatinine) can provide insight into developing renal dysfunction new onset diabetes, and acid-base disturbances. The corrected anion gap has been shown to be significantly elevated in some patients with mitochondrial disease.	(Parikh et al. 2017)
Endocrine Testing	Annual HbA1C, fasting glucose and insulin, thyroid hormones (TSH and T4), morning cortisol, and screening for hypoparathyroidism (serum calcium, magnesium, phosphate, parathyroid hormone, vitamin D (25-OHD and 1,25-OHD); urine: creatinine, calcium, and phosphate) can be considered in individuals with mitochondrial diseases.	(Ng et al. 2022; Parikh et al. 2017)
Fibroblast Growth Factor 21 (FGF-21)	Mitochondrial diseases produce a transcriptional response mimicking starvation which includes increased expression of the metabolic regulator and hormone-like cytokine FGF-21. It leads to mobilization of lipid stores and production of ketone bodies. Several studies have shown FGF-21 levels to be elevated in patients with mitochondrial disease where myopathy is a feature. In a study serum FGF-21 proved to be a sensitive and specific pediatric mitochondrial disease biomarker and outperformed GDF-15 and lactate.	(Riley et al. 2022; Peñas et al. 2021; Chau et al. 2010; Davis et al. 2013; Gavrilova and Horvath 2013; Liang, Ahmad, and Sue 2014; Su et al. 2012; Suomalainen 2013; Suomalainen et al. 2011; Tyynismaa et al. 2010; Turnbull 2011)
Gelsolin	Gelsolin, a cytoskeletal protein that regulates actin filament assembly and disassembly, has been proposed as a potential biomarker for mitochondrial dysfunction because mitochondria are known to bind and move along microtubules and actin filaments. A study showed decreased plasma gelsolin levels in a group of patients with mitochondrial disease and suggested that the combination of this biomarker with FGF-21 and GDF-15 levels improved the diagnostic utility compared to using each one alone.	(Peñas et al. 2021; Marín-Buera et al. 2015)

Growth Differentiation Factor 15 (GDF-15)	Growth Differentiation Factor 15 (GDF-15), a member of the transforming growth factor beta superfamily, and has a role in regulating cellular response to stress and inflammation. It has been proposed as a useful biomarker for mitochondrial diseases. Although it is primarily elevated in those mitochondrial diseases affecting the muscle, it may be more sensitive in detecting mitochondrial dysfunction in other organs when compared to FGF-21	(Bermejo-Guerrero et al. 2023; Fujita et al. 2015; Yatsuga et al. 2015; Davis, Liang, and Sue 2016)
Neurofilament light chain (NF-L)	NF-L is a neuron-specific protein. It is a marker of disease activity and progression that has been evaluated in a number of different neurological conditions. A study showed that NF-L was highest in patients with multi-systemic involvement that included the central nervous system such as those with the m.3242A>G pathogenic variant in *MT-TL1*. NF-L is a marker for central nervous system involvement. Levels of NF-L may correlate with the degree of ongoing damage.	(Sofou et al. 2019; Varhaug et al. 2021)
Hepatic Panel (Albumin, Alk Phos, ALT, AST, GGT, INR, PT, PTT)	Isolated liver disease is most frequently caused by defects of mtDNA maintenance such as mtDNA depletion. Some mitochondrial diseases have hepatic involvement that can be mild to severe. In some patients, hepatic failure occurs (e.g., Alpers disease). Patients with pathogenic variants in *POLG* are at a higher risk of developing valproate-induced liver failure.	(Haas et al. 2007; Parikh et al. 2017)
Lactate	Lactate, the product of anaerobic glucose metabolism, accumulates when aerobic metabolism is impaired, which causes a shift in the oxidized-to-reduced NAD^+: NADH ratio within mitochondria (i.e., decrease in the oxidized nicotinamide-adenine dinucleotide/reduced nicotinamide-adenine dinucleotide “redox” ratio). Normal lactate does not exclude a mitochondrial disorder and increases in lactate are not specific to these diseases. Careful collection is important since a variety of difficulties with collection including prolonged tourniquet use and struggling during blood draw can elevate levels.	(Debray et al. 2007; Yamada et al. 2012; Feldman et al. 2017)
Lactate / Pyruvate Ratio	The blood lactate-to-pyruvate (L:P) ratio reflects the equilibrium between product and substrate of the reaction catalyzed by lactate dehydrogenase. The L:P ratio is correlated with the cytoplasmic NAD^+:NADH ratio and is used as a marker of the redox state. With impairment of cellular respiration, pyruvate oxidation is altered by lactate dehydrogenase resulting in an increase in the L:P ratio. In pyruvate dehydrogenase complex deficiency (PDHC deficiency), the metabolic block is upstream of the respiratory chain. The L:P ratio is within normal range. An increased L:P ratio (>25) suggests primary or secondary respiratory chain dysfunction. A ratio <25 may indicate a PDH defect in the appropriate clinical setting.	(Debray et al. 2007; Pavlu-Pereira et al. 2020; Yamada et al. 2012)
LDH		(Sharma et al. 2021)
Lipid Panel	A serum lipid panel (total cholesterol, LDL, HDL, non-HDL cholesterol,	(Clarke et al. 2013; Jacobson et al.

	and triglycerides) will enable the assessment of general lipid metabolism, which has been suggested to be influenced by mitochondrial dysfunction. Indeed, triglycerides elevation and dyslipidemia have been reported to be observed in mitochondrial disease. Lipid panel metabolites are measured by enzymatic colorimetric methods with calculations for LDL and non-HDL.	2014; Naviaux 2004)
Metabolomics	Metabolomics, or metabolic profiling, combines analytical chemistry methods and statistical analyses to quantitatively characterize the set of small molecule (typically <1500 Da) compounds in a biospecimen. Metabolic profiling can be performed by NMR or LC-MS/MS. NMR methods are non-destructive but less sensitive. LC-MS methods can be targeted to defined sets of metabolites (typically up to dozens) or untargeted which comprehensively report all measurable analytes including those of unknown chemical identity. The chromatographic approach used in an LC-MS method determines the chemical natures of the compounds that can be characterized; no single method exhaustively covers the entire metabolome. Combinations of LC-MS methods applied to a single biospecimen can together assess a wide biochemical spectrum including polar and nonpolar compounds with the total number of identified metabolites in untargeted methods reaching hundreds (and thousands if unidentified features are included). Because mitochondrial dysfunction can have wide-ranging biochemical consequences, metabolic profiling can reveal distinctive “metabolic fingerprints.” Challenges facing metabolomics studies include biological differences among participants (genetic, physiological, dietary, etc.) as well as technical variability of methodologies among labs. Importantly, measurements are in relative units and concentration determination requires developing focused calibration curve(s). Thus, careful study design, sample collection and data processing are critical. Comparison of patients with healthy or disease controls have recapitulated classic markers (lactate, pyruvate, alanine) and have also identified promising new markers. Metabolic profiling combined with therapeutic trials can also spotlight potential therapeutic markers.	(Sharma et al. 2021; Buzkova et al. 2018; Delaney et al. 2017; Pirinen et al. 2020; Ruiz et al. 2019; Thompson Legault et al. 2015)
Vitamin Levels	Essential soluble vitamins (B12, Folate, Niacin, Pyridoxal 5-phosphate, Riboflavin, Thiamine, Pantothenic acid, and Biotin) are required for proper metabolic function. Deficiencies secondary to malabsorption syndromes or malnourished states can be found in mitochondrial disease patients. Patients presenting with vitamin deficiencies may present with symptoms that exacerbate, or overlap, with mitochondrial disease. Methods to identify soluble vitamin deficiencies include liquid-chromatography-tandem mass spectrometry (LC-MS/MS) with stable isotope dilution, competitor-binding receptor assays, and competitive-	(Morava et al. 2006; Zweers et al. 2018)

	binding immunoenzymatic assays from plasma, serum or blood collected in a light-protected container.	
Pyruvate	Increases in pyruvate signals dysfunction of the cellular oxidative process. Normal pyruvate does not exclude a mitochondrial disease and increase in pyruvate are not specific to these diseases. The measurement of a lactate:pyruvate (L:P) ratio is considered a helpful tool in the evaluation of mitochondrial disease. Careful collection is important since a variety of difficulties with collection including prolonged tourniquet use and struggling during blood draw can elevate levels. In addition, whole blood pyruvic acid collection requires a special tube containing 2.5 mL of 6% perchloric acid to maintain stability. Alternative sample types, such as cerebrospinal fluid (CSF) or plasma, eliminates the need for this stabilizer. Pyruvic acid concentrations can be measured with enzyme-based spectrophotometric and GCMS methods.	(Debray et al. 2007; Yamada et al. 2012; Feldman et al. 2017; Fleischer et al. 1970)
Purines and Pyrimidines	The accumulation of specific nucleotides, such as thymidine and deoxyuridine, in plasma is an indication of imbalanced cytosolic dNTP and mitochondrial dNTP pools as there is an interchange of these nucleotides between cellular compartments. An imbalance of mitochondrial dNTPs can impair mtDNA synthesis, leading to mitochondrial disease. Biallelic variants in the gene encoding thymidine phosphorylase, which presents as mitochondrial neurogastrointestinal encephalopathy disease, is an example of a mitochondrial disease presenting with remarkably elevated plasma thymidine and deoxyuridine. These metabolites, as well as others, can be measured as a purine and pyrimidine panel by LC-MS/MS.	(Balasubramaniam, Duley, and Christodoulou 2014)
Urine		
Acylglycines	Glycine conjugation is an important detoxification system of the liver for preventing the accumulation of acyl-CoA esters in several inherited metabolic disorders as well as exogenous metabolites. Acylglycines in urine are often the direct expression of accumulation of the correspondent acyl-CoA esters in the mitochondrion from intermediate metabolism, specifically mitochondrial defects in fatty acid oxidation and branch-chain amino acid catabolism, amongst other organic acidemias. Urine acylglycines can be quantified by GCMS or LC-MS/MS.	(Bonafé et al. 2000; Gregersen 1985)
Amino Acids	Urine amino acid analysis may detect generalized aminoaciduria indicating tubular manifestations. Kidneys have a high energetic demand and contain a high density of mitochondria, making them susceptible to mitochondrial dysfunction.	(Haas et al. 2007; Suomalainen 2011; Govers et al. 2021)
Metabolomics	As noted above (see serum/plasma metabolomics), metabolomics measurements provide biochemical fingerprints consisting of dozens to thousands of analyte measurements which can be analyzed to identify	(Venter et al. 2015; Esterhuizen et al. 2019) (Kelley 1905)

	distinctive features for biomarker development. While urine is easily obtainable, a notable challenge for metabolic profiling is the concentration variability. Several studies have utilized urine metabolic profiling to identify distinguishing features in fatty acid oxidation, one-carbon metabolism, central carbon metabolism, and amino acid metabolism.	
mtDNA Heteroplasmy	For mitochondrial diseases associated with specific mtDNA variants (i.e., m.3234A>G), urinary epithelial mtDNA heteroplasmy levels are correlated with disease burden and progression after adjusting for patient sex, age, and total mtDNA content. As a less invasive matrix than blood or muscle, mtDNA heteroplasmy in urine sediment may be a useful tool for diagnosing mitochondrial disease.	(Grady et al. 2018; Whittaker et al. 2009)
Organic Acids	Urine organic acid testing is useful in the diagnosis and monitoring of patients with inborn errors of organic acid metabolism, inborn errors of amino acid metabolism, urea cycle defects, and defects of the mitochondrial respiratory chain. Organic acid analysis may fail to detect certain disorders that are characterized by minimal or intermittent metabolite excretion. Metabolic changes observed in mitochondrial diseases include increased levels of TCA intermediates, lactate, pyruvate, 3-methylglutaconic acid).	(Barshop 2004; Haas et al. 2007; Suomalainen 2011; Gill et al. 2023)
Purines and Pyrimidines	The accumulation of specific nucleotides, such as thymidine and deoxyuridine, in plasma is an indication of imbalanced cytosolic dNTP and mitochondrial dNTP pools as there is an interchange of these nucleotides between cellular compartments. An imbalance of mitochondrial dNTPs can impair mtDNA synthesis, leading to mitochondrial disease. Biallelic variants in the gene encoding thymidine phosphorylase, which presents as mitochondrial neurogastrointestinal encephalopathy disease, is an example of a mitochondrial disease presenting with remarkably elevated plasma thymidine and deoxyuridine. These metabolites, as well as others, can be measured as a purine and pyrimidine panel by LC-MS/MS.	(Balasubramaniam, Duley et al. 2014)
Urinalysis (UA)	The kidney plays a key role in the excretion of by-products of cellular metabolism, acid-base, and electrolyte balance. The high density of mitochondria in the kidney as well as its high energetic demand results in abnormalities (glomerulonephritis, glucosuria in diabetes, bilirubinuria in liver disease, etc.) from mitochondrial dysfunction often detected by urinalysis for specific gravity, proteinuria, glucose, pH, ketones, hemoglobin, nitrite, leukocyte esterase, bilirubin, and urobilinogen.	(Govers et al. 2021)
3-Methylglutaconic Acid	3-Methylglutaconic acid (3-MGA) is an intermediate of mitochondrial leucine catabolism. However, in mitochondrial diseases, and other inborn errors of metabolism, 3-MGA is an excreted biochemical marker potentially arising from a novel acetyl CoA diversion pathway that	(Jones, Klacking, and Ryan 2021; Wortmann et al. 2006; Wortmann et al. 2009)

	appears to be secondary to electron transport chain, mitochondrial lipid membrane, and metabolic dysfunction. Quantification of this metabolite in urine is often provided in urine organic acid analyses or by direct measurement methods.	
CSF		
5-Methyltetrahydrofolate	Folate is a vitamin that plays a critical role in trafficking one-carbon units in metabolic processes. In its active form, tetrahydrofolate (THF) carries methyl units in several different oxidation states and the 5-methyltetrahydrofolate (5-MTHF) form is the one required for numerous methylation reactions and is also the primary form found in CSF. Deficiency of folate in the brain may occur with either low or normal levels in the periphery. While cerebral folate deficiency can result from inherited defects in folate transporters, it has also been reported secondarily in multiple mitochondrial diseases (especially Kearns-Sayre syndrome) where levels have been reported to be very low to normal. 5-MTHF is typically quantified by HPLC with fluorescence or electrochemical detection on CSF samples that are frozen soon after collection.	(Batllori et al. 2018; Pope et al. 2019)
Amino Acids	Elevated alanine, proline, or tyrosine can be observed in mitochondrial diseases. Elevated plasma alanine levels, when present, may be a useful indicator of long-standing pyruvate accumulation.	(Guerrero-Molina et al. 2022)
Glucose (with simultaneous blood glucose)	CSF glucose levels may be decreased due to consumption by microorganisms, impaired glucose transport, or increased glycolysis. CSF glucose is normal in most mitochondrial diseases. GLUT1 deficiency syndrome is a treatable neurometabolic disorder, characterized by a low concentration of glucose in CSF and a decreased CSF to blood glucose ratio. This decrease in CSF glucose limits ATP generation by cellular energetics.	(Haas et al. 2007; Leen et al. 2013)
Lactate	Lactate concentration in CSF results from a balance between efflux and influx through the blood–brain barrier and through the plasma membrane of central nervous system cells. Lactate production is increased with defects in oxidative phosphorylation. CSF lactate concentrations were more sensitive for mitochondrial diseases than blood lactate concentrations. Both pyruvate and lactate concentrations are increased in PDH deficiency, but the L/P ratio remains normal or only slightly decreased. Measurement of CSF lactate is performed on samples that are frozen soon after collection using an enzymatic assay or with a UV method.	(Haas et al. 2007; Suomalainen 2011) (Benoist et al. 2003; Guerrero-Molina et al. 2022; Yamada et al. 2012)
Metabolomics	As noted above (see serum/plasma metabolomics), metabolomics measurements provide biochemical fingerprints consisting of dozens to thousands of analyte measurements which can be analyzed to identify distinctive features for biomarker development. There are limited studies applying metabolomics to CSF samples though one study identified	(Salvador et al. 2023)

	several potential markers for one form of mitochondrial disease.	
Neurotransmitters	Levels of CSF biogenic amines have been found to be altered in mitochondrial diseases which is thought to be due to secondary mechanisms. One study of 29 patients found high CSF levels of homovanillic acid (and low 5-methyltetrahydrofolate), indicative of dopamine dysregulation. In another study, low levels of CSF neurotransmitters have been reported in pediatric patients with severe presentations of mitochondrial diseases. These compounds can be quantified using HPLC or electrochemical methods.	(Batllori et al. 2018; Rodan, Gibson, and Pearl 2015; Garcia-Cazorla et al. 2008)
Protein	CSF is secreted by the choroid plexuses, around the cerebral vessels, and along the walls of the ventricles of the brain. Increases are observed in some disorders such as Leigh disease, Alpers syndrome, and Kearns-Sayre syndrome. CSF total protein can be measured with spectrophotometric methods.	(Haas et al. 2007)
Pyruvate	Pyruvic acid, an intermediate metabolite, plays an important role in linking carbohydrate and amino acid metabolism to the tricarboxylic acid cycle, the fatty acid beta-oxidation pathway, and the mitochondrial respiratory chain complex. Even when plasma levels of pyruvate, or lactate, are normal, CSF levels may be elevated in patients with mitochondrial disease who have CNS manifestations.	(Haas et al. 2007; Benoist et al. 2003; Suomalainen 2011; Yamada et al. 2012)
Fibroblasts		
ATP Synthesis	Measures the amount of ATP produced by ATP synthesis which is typically decreased in almost all mitochondrial diseases. Bioluminescence assay kits are available to measure ATP production in cell suspensions.	(Shepherd et al. 2006)
Blue Native Gel Electrophoresis (OXPHOS)	Clear native electrophoresis and blue native electrophoresis are microscale techniques for the isolation of membrane protein complexes. Proteins are visualized in blue native gels with Coomassie Blue G-250 dye. Blue native PAGE retains enzyme complexes in their intact and enzymatically active form. Both the amount of the fully assembled complex, and the presence of any smaller stalled assembly intermediates, can then be determined.	(Calvaruso, Smeitink, and Nijtmans 2008; Carrozzo et al. 2006)
Coenzyme Q10	Coenzyme Q10 levels can be determined by radiolabeled substrate assays looking at production. Separate subunit quinones or whole Coenzyme Q10 levels can be detected and quantified using High-performance liquid chromatography (HPLC) -Mass spectrometry and HPLC-electrochemical techniques (with standards), and ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI-MS/MS). Assays are all used is to determine Coenzyme Q10 deficiency. Moreover, assays which look at subunits that build Coenzyme Q10 can often determine enzyme/level of abnormality. One disadvantage for total Coenzyme Q10 level	(DiMauro, Quinzii, and Hirano 2007; López et al. 2006; Mollet et al. 2007; Quinzii et al. 2006; Herebian et al. 2017)

	determination is that cannot differentiate between secondary and primary deficiencies.	
High Resolution Respirometry	Live cellular respiration (Complexes I-V) allows measurement of parameters such as mitochondrial membrane potential, reserve capacity for ATP generation, and Complex I-IV substrate utilization. This testing assesses functional characteristics of intact mitochondria within living tissues.	(Cameron, Levandovskiy, MacKay, and Robinson 2004; van den Heuvel, Smeitink, and Rodenburg 2004)
Lactate / Pyruvate Ratio	The fibroblast lactate-to-pyruvate (L:P) ratio reflects the equilibrium between product and substrate of the reaction catalyzed by lactate dehydrogenase. The L:P ratio is correlated with the cytoplasmic NADH:NAD+ ratio and is used as a marker of the redox state. With impairment of cellular respiration, pyruvate oxidation is reduced, and lactate is increased, resulting in an increase in the L:P ratio. In pyruvate dehydrogenase deficiency (PDH deficiency), the metabolic block is upstream of the respiratory chain. Both pyruvate and lactate concentrations are increased in PDH deficiency, but the L/P ratio remains normal or only slightly decreased.	(Cameron, Levandovskiy, MacKay, and Robinson 2004)
OXPHOS Enzymology	OXPHOS enzymology assesses mitochondrial function by determining maximal enzymatic activity of the individual electron transport system (ETS) complexes in disrupted mitochondria by spectrophotometry. However, many aspects of mitochondrial function that occur in live cells cannot be assessed by OXPHOS enzymology.	(van den Heuvel, Smeitink, and Rodenburg 2004)
Pyruvate Dehydrogenase Enzymology	The mitochondrial pyruvate dehydrogenase complex (PDC) catalyzes the rate-limiting step in aerobic glucose oxidation and is thus integral to cellular energetics. Pyruvate dehydrogenase (PDH) deficiency is an inherited disorder of carbohydrate metabolism. PDH deficiency is due to loss-of-function mutation in one of the five component enzymes, most commonly E1α-subunit. The common clinical presentation ranges from fatal infantile lactic acidosis in newborns to chronic neurological dysfunction. Historically, pyruvate dehydrogenase specific activity is typically determined by measuring the decarboxylation of 1-14C-pyruvate to $14CO_2$ and was expressed as a unit of $14CO_2$ production per tissue mass per unit of time. A number of colorimetric kits are available for assay such that one micromole of NADH production is equal to one unit of PDH activity.	(Cameron, Levandovskiy, Mackay, Tein, et al. 2004; Schwab et al. 2005)
Seahorse Live Cell Metabolic Analysis	Seahorse respirometry is a cellular assay that provides a functional assessment of ETC function by measuring the rates of oxygen consumption and extracellular acidification. Tissue samples can include fibroblasts, muscle cells, and peripheral white blood cells and measurements can be performed on intact cells, permeabilized cells or isolated mitochondria. While measurements provide quantitative measurement of ETC parameters, methodological challenges include	(Acin-Perez et al. 2021; Ogawa et al. 2017)

	sample amount and quality as well as technical expertise. Thus, such measurements are best performed at specialized labs and with fresh, rapidly prepared sample. Fibroblasts can be obtained from punch skin biopsies which are minimally invasive. One study demonstrated that respirometry showed greater sensitivity than measurements of individual respiratory chain components.	
Leukocytes		
Coenzyme Q10 Level	Coenzyme Q10 deficiency can be detected by decreased levels. Common assay approaches as described in the fibroblast section.	(Duncan et al. 2005)
Intracellular Free Glutathione (fGSH), Oxidized Disulfide (GSSG), fGSH/GSSG Ratio	Glutathione (GSH) is the main non-protein thiol in cells. GSH functions are dependent on the redox-active thiol of its cysteine moiety that serves as a cofactor for a number of antioxidant and detoxifying enzymes. While synthesized exclusively in the cytosol from its constituent amino acids, GSH is distributed in different compartments, including mitochondria where its concentration in the matrix equals that of the cytosol. Free GSH/GSSG ratio is an indicator of redox metabolism (oxidative stress marker). Glutathione decreases in mitochondrial disease.	(Atkuri et al. 2009)
mtDNA Copy Number	Defects in mitochondrial copy number are frequently indications of abnormal mitochondrial DNA maintenance. The mutations causing this depletion are frequently encoded by nuclear genes which encode genes essential to replication of mitochondrial DNA, mitochondrial nucleotide pool, mitochondrial nucleotide import, and mitochondrial dynamics	(El-Hattab, Craigen, and Scaglia 2017)
mtDNA Deletion/Duplication	Mitochondrial DNA deletion and duplication abnormalities are typically evaluated using sequencing techniques. These can range from multi-systemic disorders to disorders of only impacting a single organ (e.g., eyes). Typically, these are inherent within the maternally inherited mitochondrial DNA and thus, not inherited from the nucleus. The impacted severity and organs/tissues of these deletions/duplication are dependent of heteroplasmy of that particular tissue. Mitochondrial DNA deletions and duplications can also be acquired if there are abnormalities in the mitochondrial DNA maintenance machinery (inherited through the nucleus).	(Broomfield et al. 2015; Poulton, Deadman, and Gardiner 1989)
Pyruvate Dehydrogenase Enzymology	The mitochondrial pyruvate dehydrogenase complex (PDC) catalyzes the rate-limiting step in aerobic glucose oxidation and is thus integral to cellular energetics. Pyruvate dehydrogenase (PDH) deficiency is an inherited disorder of carbohydrate metabolism. PDH deficiency is due to loss-of-function mutation in one of the five component enzymes, most commonly E1α-subunit. The common clinical presentation ranges from fatal infantile lactic acidosis in newborns to chronic neurological dysfunction. Historically, pyruvate dehydrogenase specific activity is typically determined by measuring the decarboxylation of 1-14C-pyruvate to $14CO_2$ and was expressed as a unit of $14CO_2$ production per tissue	(Cameron, Levandovskiy, Mackay, Tein, et al. 2004; Schwab et al. 2005)

	mass per unit of time. A number of colorimetric kits are available for assay such that one micromole of NADH production is equal to one unit of PDH activity.	
Thymidine Phosphorylase Enzymology	Enzyme assay to confirm or establish diagnosis of mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) which presents with gastrointestinal dysmotility, peripheral neuropathy, myopathy and leukoencephalopathy. Thymidine phosphorylase is important for pyrimidine pathway metabolism of thymidine. Assays available include colorimetric and HPLC.	(Lara et al. 2007; Valentino et al. 2007)
Lymphoblasts (EBV Transformed)		
ATP Synthesis		(Van Bergen et al. 2014)
High Resolution Respirometry	Live cellular respiration (Complexes I-V) allows measurement of parameters such as mitochondrial membrane potential, reserve capacity for ATP generation, and Complex I-IV substrate utilization. This testing assesses functional characteristics of intact mitochondria within living tissues.	(Van Bergen et al. 2014)
Seahorse Live Cell Metabolic Analysis	As noted above (see Seahorse Live Cell Metabolic Analysis in Fibroblast section), Seahorse respirometry can provide a functional assessment of ETC function on intact cells, permeabilized cells or isolated mitochondria. Circulating cells including platelets and leukocytes can be obtained with a blood draw and are amenable to short-term cryopreservation. Studies in animal models and humans have shown inconsistent correlation between respiratory parameters in peripheral cells with skeletal muscle. Application to patient samples has demonstrated clinical utility though noted that there was significant variability among patients.	(Acin-Perez et al. 2021; Pecina et al. 2014)
Muscle Biochemistry		
ATP Synthesis	Measures the amount of ATP produced by ATP synthesis which are typically decreased in almost all mitochondrial diseases. Bioluminescence assay kits are available to measure ATP production in cell suspensions or whole muscle preparations. This can also be assayed using MRS.	(Fiedler et al. 2016)
Blue Native Gel Electrophoresis (OXPHOS)	Clear native electrophoresis and blue native electrophoresis are microscale techniques for the isolation of membrane protein complexes. Proteins are visualized in blue native gels with Coomassie Blue G-250 dye. Blue native PAGE retains enzyme complexes in their intact and enzymatically active form. Both the amount of the fully assembled complex, and the presence of any smaller stalled assembly intermediates, can then be determined.	(Calvaruso, Smeitink, and Nijtmans 2008; Carrozzo et al. 2006; Andringa, King, and Bailey 2009; Assouline et al. 2012; Gerards et al. 2010; Pitceathly et al. 2011; Tuppen et al. 2012)
Coenzyme Q10	Coenzyme Q10 deficiency can be detected by decreased levels. Common assay approaches as described in the Fibroblast section	(DiMauro, Quinzii, and Hirano 2007; López et al. 2006)
Glutathione Content	Glutathione (GSH) is the main non-protein thiol in cells. GSH functions	(Hargreaves et al. 2005)

	are dependent on the redox-active thiol of its cysteine moiety that serves as a cofactor for a number of antioxidant and detoxifying enzymes. While synthesized exclusively in the cytosol from its constituent amino acids, GSH is distributed in different compartments, including mitochondria where its concentration in the matrix equals that of the cytosol. Glutathione decreases in mitochondrial disease.	
High Resolution Respirometry	Live cellular respiration (Complexes I-V) allows measurement of parameters such as mitochondrial membrane potential, reserve capacity for ATP generation, and Complex I-IV substrate utilization. This testing assesses functional characteristics of intact mitochondria within living tissues.	
mtDNA Copy Number	Defects in mitochondrial copy number are frequently indications of abnormal mitochondrial DNA maintenance. The mutations causing this depletion are frequently encoded by nuclear genes which encode genes essential to replication of mitochondrial DNA, mitochondrial nucleotide pool, mitochondrial nucleotide import, and mitochondrial dynamics.	
mtDNA Deletion/Duplication	Mitochondrial DNA deletion and duplication abnormalities are typically evaluated using sequencing techniques. These can range from multi-systemic disorders to disorders of only impacting a single organ (e.g., eyes). Typically, these are inherent within the maternally inherited mitochondrial DNA and thus, not inherited from the nucleus. The impacted severity and organs/tissues of these deletions/duplication are dependent of heteroplasmy of that particular tissue. Sometimes the deletions can be acquired over time impacting heteroplasmy as well. Mitochondrial DNA deletions and duplications can also be acquired if there are abnormalities in the mitochondrial DNA maintenance machinery (inherited through the nucleus).	
OXPHOS Enzymology	OXPHOS enzymology assesses mitochondrial function by determining maximal enzymatic activity of the individual electron transport system (ETS) complexes in disrupted mitochondria by spectrophotometry. However, many aspects of mitochondrial function that occur in live cells cannot be assessed by OXPHOS enzymology.	(van den Heuvel, Smeitink, and Rodenburg 2004)
Pyruvate Dehydrogenase Enzymology	The mitochondrial pyruvate dehydrogenase complex (PDC) catalyzes the rate-limiting step in aerobic glucose oxidation and is thus integral to cellular energetics. Pyruvate dehydrogenase (PDH) deficiency is an inherited disorder of carbohydrate metabolism. PDH deficiency is due to loss-of-function mutation in one of the five component enzymes, most commonly E1α-subunit. The common clinical presentation ranges from fatal infantile lactic acidosis in newborns to chronic neurological dysfunction. Pyruvate dehydrogenase specific activity is typically determined by measuring the decarboxylation of 1-14C-pyruvate to $14CO_2$ and was expressed as a unit of 14 CO_2 production per tissue	(Schwab et al. 2005; Adeva et al. 2013)

	mass per unit of time.	
Seahorse Live Cell Metabolic Analysis	Muscle biopsies have been the gold standard for respirometry measurements (see above: Fibroblast section). The high mitochondrial content and energy demand of muscle makes this tissue a clinically valuable sample to assess ETC function and for some time has been the gold standard. However, obtaining muscle samples is invasive and requires specialized apparatus and expertise.	(Gnaiger 2009; Pesta and Gnaiger 2012; Acin-Perez et al. 2021)
Muscle Histology		
Combined SDH + COX	COX deficiency, increased SDH (MELAS)	(Ross 2011; Hedberg-Oldfors et al. 2022; Murgia et al. 2019)
Cytochrome C Oxidase (COX) (Complex IV)	Complex IV (COX deficiency)	(Filosto et al. 2007; Murphy et al. 2012; Hedberg-Oldfors et al. 2022)
Gomori Trichrome	Ragged red fibers	(Filosto et al. 2007; Shelly et al. 2021; Schnitzler et al. 2017; Pant et al. 2015)
Nicotinamide Adenine Dinucleotide Tetrazolium Reductase (NADH-TR)		(Pant et al. 2015; Ravara et al. 2015)
Succinate Dehydrogenase (SDH)	Complex II	(Filosto et al. 2007; Murgia et al. 2019; Pant et al. 2015)
Genetics		
Exome Sequencing (NGS) (nDNA)	Nuclear DNA exome sequencing. While most testing is only nDNA, some NGS approaches may include mtDNA.	(Ashraf et al. 2013; Boczonadi and Horvath 2014; DaRe et al. 2013; Davit-Spraul et al. 2014; Falk et al. 2012; Farhan et al. 2014; Girotto et al. 2013; Haack et al. 2014; Hong et al. 2013; Lieber et al. 2014; Logan et al. 2014; McMillan et al. 2014; Monies et al. 2014; Morino et al. 2014; Nakajima et al. 2014; Ohtake et al. 2014; Platt, Cox, and Enns 2014; Poduri et al. 2013; Prasad et al. 2014; Rosenthal et al. 2013; Soreze et al. 2013; Spiegel et al. 2014; Tucci et al. 2014; Saisawat et al. 2014; Carroll, Brilhante, and Suomalainen 2014; Bonnen et al. 2013; Craigen et al. 2013; DiMauro et al. 2013; Gai et al. 2013; Haddad et al. 2013; Hildick-Smith et al. 2013; Imagawa et al. 2014; Neveling et al.

		2013; Persico and Napolioni 2013; Pitceathly, Rahman, et al. 2013; Pitceathly, Taanman, et al. 2013; Proverbio et al. 2013; Sarig et al. 2013; Tran-Viet et al. 2013; Auranen et al. 2013; Dinwiddie et al. 2013; Edvardson et al. 2013; Gerards et al. 2013; Gonzalez et al. 2013; Jonckheere et al. 2013; Kennerson et al. 2013; Kevelam et al. 2013; Lee et al. 2012; Lieber et al. 2013; Marina et al. 2013; Miyake et al. 2013; Nota et al. 2013; Prasad et al. 2013; Sambuughin et al. 2013; Berger et al. 2011; Calvo et al. 2012; Casey et al. 2012; Dündar et al. 2012; Elo et al. 2012; Eschenbacher et al. 2012; Garone et al. 2012; Glazov et al. 2011; Götz et al. 2011; Haack et al. 2012; Haack et al. 2013; Horvath et al. 2012; Janer et al. 2012; Keogh and Chinnery 2013; Lamperti et al. 2012; Li, Zou, and Brown 2012; Lieber et al. 2012; Lindberg et al. 2013; Marti-Masso et al. 2012; McCormick, Place, and Falk 2013; Pierson et al. 2011; Rinaldi et al. 2012; Sailer and Houlden 2012; Shamseldin et al. 2012; Siriwardena et al. 2013; Spiegel et al. 2012; Steenweg et al. 2012; Sundaram et al. 2011; Takata et al. 2011; Tyynismaa et al. 2012; Zhao et al. 2012; Barretta et al. 2023; Deen et al. 2023)
Whole Genome Sequencing	Whole genome NGS to include mtDNA in most test paradigms.	(Davis et al. 2022; Schon et al. 2021)
Gene Sequencing Panel	Mitochondrial nuclear gene panel sequencing; does not include mtDNA	(Bariş, Kırık, and Balasar 2023)
RNA Analysis	RNA sequence analysis of mitochondrial expressed genes to identify variants and their differential expression	(Yépez et al. 2022; Kuznetsova et al. 2017)
Mitochondrial Gene Expression Profiling	Measuring changes in mitochondrial gene expression in tissue or cells	(Crimi et al. 2005; He et al. 2013; Herrmann and Herrmann 2012; Yatsuga et al. 2015; Zhang et al.

		2013; Zhang and Falk 2014)
Mitochondrial Haplotype/Haplogroup	Evolutionarily related haplotype groups and phenotypic characteristics	(Hagen et al. 2013; Ridge et al. 2013; Shen-Gunther et al. 2023)
mtDNA Copy Number (Leukocytes, Liver, Muscle)	mtDNA depletion and mtDNA increases	(de Mendoza et al. 2004; Liu et al. 2006; El-Hattab, Craigen, and Scaglia 2017)
mtDNA Deletion/Duplication (Leukocytes, Liver, Muscle)	mtDNA deletion disorders; somatic mutations	(Bai and Wong 2005; El-Hattab, Craigen, and Scaglia 2017; Arbeithuber et al. 2020)
mtDNA Sequencing	Sequence analysis of mtDNA to identify variants and define heteroplasmy and homoplasmy	(Macken et al. 2023; Wang et al. 2022; Dames, Eilbeck, and Mao 2015)

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