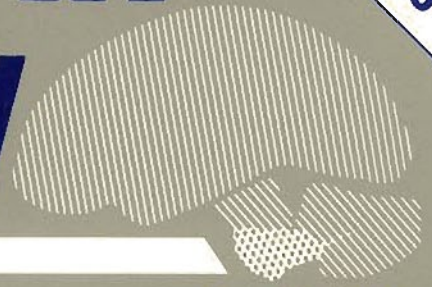# Seminars in Neurology

INCLUDING CME CREDITS

Volume 21, Number 3

September 2001

Editor in Chief
Robert M. Pascuzzi, M.D.

Mitochondrial Disorders

Seminars in Neurology

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Metabolic Testing in Mitochondrial Disease

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ABSTRACT

Mitochondrial oxidative phosphorylation (OXPHOS) disorders are a heterogeneous group of diseases with variable expression that often pose diagnostic dilemmas. Although definitive diagnosis of these disorders usually requires a muscle biopsy and mtDNA and enzymatic testing, standard metabolic studies including organic acid and amino acid analysis often provide useful findings that support an OXPHOS disease and the need for more invasive studies. In addition, the detection of possible metabolic derangements, such as elevated lactate levels, may lead to improved long-term outcomes for affected patients through the use of various treatment regimens. Similarly, long-term yearly monitoring of diagnosed OXPHOS patients with metabolic testing is also warranted.

KEYWORDS: Mitochondrial disease, metabolic testing, metabolic screening, organic acids, amino acids, carnitine, lactate, pyruvate

Mitochondrial disorders are a heterogeneous group of diseases that affect oxidative phosphorylation (OXPHOS). The OXPHOS system consists of five protein-lipid enzyme complexes located in the mitochondrial inner membrane. Electrons generated during the catabolism of proteins, carbohydrates, and fats are collected by complexes I and II and transferred sequentially to coenzyme Q10, complex III, and complex IV. Complexes I, II, and IV use the energy produced in electron transfer to pump protons across the inner mitochondrial membrane, generating a proton gradient that is used by complex V to condense adenosine diphosphate and inorganic phosphate into adenosine triphosphate (ATP).[1,2]

Both mitochondrial and nuclear genes regulate the production of the multicomplex OXPHOS system. The human mitochondrial DNA (mtDNA), inherited exclusively through the mother via the ovum cytoplasm, is a 16,569 nucleotide pair, double-stranded, circular molecule. The mtDNA encodes for two ribosomal RNAs (rRNA), 22 transfer RNAs (tRNAs), and 13 polypeptides of the OXPHOS system. Nuclear genes encode for the remainder of the compounds comprising the OXPHOS system, as well as all of the

components of complex II. Pathogenic mutations in both nuclear and mitochondrial genes are known to be associated with disease processes and have resulted in a complex array of inheritance possibilities for mitochondrial disorders.[3] Many defects in OXPHOS are inherited in an autosomal recessive fashion, as in some cases of Leigh syndrome; a few are autosomal dominant[4]; some are sporadic (Kearns-Sayre syndrome)[5]; and others are maternally inherited such as mitochondrial encephalomyopathy lactic acidosis and strokelike episodes (MELAS) and myoclonic epilepsy and ragged-red fibers (MERRF).[6,7]

The cytoplasmic location of mitochondria results in the unique maternal inheritance pattern in which transmission of mtDNA occurs exclusively from a mother to her children. The resulting clinical phenotype in maternally inherited pathogenic mtDNA mutations is complicated and dependent on a number of factors, including whether or not the mtDNA sequences are present in a heteroplasmic (presence of mtDNA in a cell or tissue with more than one sequence) or homoplasmic (presence of mtDNA with one sequence) fashion. The random segregation of mutant and wild-type mtDNA in dividing daughter cells when heteroplasmy exists and whether or not the mutant mtDNA reaches the critical threshold for energy production in a given cell line will determine the resulting clinical phenotype for the affected tissue and ultimately the patient. Disease expression in deleterious homoplasmic mtDNA mutations appears to be more complex and dependent on poorly understood genetic and environmental interactions.

The resulting decreased ATP production in disorders affecting OXPHOS impairs various body functions. The organ systems most affected by a decrease in energy production are the central nervous system, cardiac and skeletal muscle, liver, and kidneys.[1-3] However, although these body systems are more sensitive to a deficiency of ATP, other systems can develop problems as well. As such, individuals with mitochondrial disorders can show a wide range of symptoms including any combination of developmental delays, seizures, vision and hearing problems, autonomic nervous dysfunction, gastrointestinal difficulties including pseudo-obstruction, endocrinological problems such as diabetes and hypoparathyroidism, and failure to thrive (Table 1).[8] The presentation of a particular mitochondrial disorder can therefore be extremely variable, even among affected individuals within the same family, making a diagnosis difficult.

APPROACH TO THE DIAGNOSIS OF MITOCHONDRIAL DISORDERS
The clinical heterogeneity and complex inheritance patterns in OXPHOS disease often results in incorrect or delayed diagnosis of affected individuals. Such delay

Table 1 Variable Symptomatology in OXPHOS Disease

CNS abnormalities
   Developmental delay and regression, dementia, seizures,
      vision abnormalities, including ophthalmoplegia and
      cataracts, sensorineural hearing loss, autonomic nervous
      system dysfunction, peripheral neuropathy
Cardiomyopathy and arrythmias
Liver dysfunction and failure
Myopathy
Renal abnormalities
   Fanconi syndrome
GI Disturbances
   Motility problems, including pseudo-obstruction and
      diarrhea, pancreatitis
Endocrine dysfunction
   Diabetes mellitus, hypoparathyroidism, hypothalamic
      hypogonadism, adrenal abnormalities
Other
   Failure to thrive, short stature, anemia, lipomas

can lead to failure of successful treatment of presymptomatic or early-stage disease processes seen with these disorders and results in increased morbidity and mortality for patients. In addition, familial variability often results in the failure to recognize significant recurrence risks that can lead to the subsequent birth of affected children to unsuspecting couples and families. The accurate diagnosis of such patients is therefore dependent on heightened awareness of suggestive clinical symptomatology, complete and detailed family histories, and interpretation of a battery of oftentimes complicated testing results.

Although the clinical symptoms seen in patients affected with OXPHOS diseases are often variable, certain findings, patterns of abnormalities, or histories can be highly suggestive of these disorders. For example, mitochondrial disease should be suspected in any patient with seemingly unrelated multisystem problems. Others present with rare abnormalities often not seen in conjunction with other diseases (Table 2). For instance, strokes in children or young adults, especially associated with any other neurological difficulties, could indicate MELAS.[6] Ophthalmoplegia would be of concern for Kearns-Sayre syndrome,[5] and movement disorders could suggest Leigh disease. Patients and families often report a history of "brownout" periods with intercurrent illnesses or surgery where normal functioning is decreased or lost until some time after wellness returns. On occasion, the patient may demonstrate a permanent loss of functioning following such stressors. Affected individuals may also develop a worsening of problems, such as an increase in seizure activity, or demonstrate difficulties, such as metabolic acidosis, requiring med-

Table 2 Symptoms Suggestive of OXPHOS Disease

Strokelike episodes
Movement disorder
Ophthalmoplegia
Episodic encephalopathy

ical intervention during seemingly minor illnesses. In general, patients with OXPHOS disease demonstrate long-term regression of skills and functioning. A lack of such history makes the diagnosis of OXPHOS disease for a given patient much less likely.

A detailed extended family history is essential in helping the practitioner decipher sometimes subtle clues contained in a pedigree. An awareness of the clinical variability of OXPHOS diseases is crucial to prevent misinterpretation of family data, assignment of incorrect recurrence risks, and misdiagnosis. This is clearly demonstrated in a multigeneration family affected with the ATPase 6 gene 8993 T to G NARP (neuropathy, ataxia, and retinitis pigmentosa) mutation described by Tatuch et al.[9] The proband's parents sought genetic counseling prior to his conception because of a maternal history of Leigh disease. They were given negligible risks based on nonconsanguinity and a supposed autosomal recessive inheritance of the disorder. Following further investigation for his ataxia and developmental delay, the proband was found to have the NARP mutation. His younger sibling, born several months following his diagnosis, was also found to have the mutation and died from complications of his disease during early childhood. Further review of the family history noted that the maternal great-grandmother had late-onset retinitis pigmentosa and ataxia. All affected individuals in this pedigree were maternally related, indicating a maternal inheritance pattern associated with a significantly greater recurrence risk than autosomal recessive inheritance.

In some cases, careful review of clinical symptoms and family history will result in identification of a described OXPHOS phenotype with subsequent confirmation of diagnosis by genetic testing on leukocyte and platelet mtDNA. However, most patients identified at risk for these diseases will also require procedures such as metabolic testing, skeletal muscle histchemistry, immunohistochemistry, electron microscopy, OXPHOS analysis on mitochondria isolated from skeletal muscle, and genetic screening for mutations in skeletal muscle mtDNA. Metabolic testing generally involves analysis of blood lactate and pyruvate, plasma carnitine, urine organic acids, blood and urine amino acids, and in some cases cerebrospinal fluid (CSF). Diagnostic information obtained from this complex battery of testing varies and can be difficult to interpret.

INTERPRETATION OF STANDARD METABOLIC TESTING

Patients who are being evaluated for OXPHOS disease typically undergo a multitude of standard metabolic tests that can be helpful not only in supporting the diagnosis but in determining the multisystem extent of the disease process and therefore possible treatment options. This battery includes blood lactate and pyruvate, plasma carnitine, blood and urine amino acids, and urine organic acids, and should include CSF studies such as lactate and pyruvate if the central nervous system is involved (Table 3). Urine metabolic screens provide limited information and are not useful in either supporting the diagnosis of or detecting multisystem problems in mitochondrial disorders.

The significance of a number of testing results, in particular blood and CSF lactate and pyruvate values, can only be appreciated in light of an understanding of the normal metabolism of lactate and pyruvate, and lactate production in OXPHOS disease. During normal glycolysis and cellular respiration, glucose is converted into pyruvate through a multistep process. Pyruvate can also be generated during the catabolism of the amino acids alanine, serine, glycine, and cysteine. Pyruvate acts as a substrate for final ATP production in the electron transport chain following its conversion to acetyl CoA and subsequent entrance into the Kreb cycle. This process generates high energy electrons in the form of NADH and FADH2, and results in the production of 38 ATP molecules for each molecule of glucose metabolized. Under anaerobic conditions, pyruvate is shunted away from the Kreb cycle and converted to lactate through the action of lactate dehydrogenase, an NADH-dependent enzyme. This alternative pathway only generates two molecules of ATP per molecule of glucose.

In OXPHOS disorders, NADH generated during the catabolism of various substrates is unable to deposit its electrons in the defective respiratory pathway, leading to its accumulation. This accumulation then drives the conversion of pyruvate to lactate, resulting in an increase in lactate and pyruvate and in the lactate-to-pyruvate ratio. Disorders of pyruvate metabolism, namely pyruvate dehydrogenase deficiency and pyruvate carboxylase deficiency, can also cause a secondary rise in pyruvate and lactate because of interference in the conversion of pyruvate to other compounds. However,

Table 3 Standard Metabolic Testing in Evaluation of OXPHOS Disease

Blood lactate and pyruvate
Blood carnitine
Urine organic acids
Blood and urine amino acids
CSF lactate and pyruvate

these latter disorders typically do not cause an increased ratio of lactate to pyruvate.

Although absolute increases in blood lactate and pyruvate, and in some cases CSF lactate and pyruvate, and/or in their ratio is suggestive of OXPHOS disease (ratio greater than 20), other disorders and circumstances can cause similar findings. Glycogen metabolism disorders, organic acidemias including propionic and methylmalonic acidemias, liver damage, and values collected after a seizure or during status epilepticus may also show elevated lactate/pyruvate (L/P) ratios.[10] Therefore, interpretation of L/P ratios should take into account a wide variety of aspects that include oxygenation status, cellular perfusion, and hepatic function. This is clearly demonstrated by the findings of Trijbels et al[10] who report a fivefold increase in blood lactate levels when children are resistant to venipuncture. They recommend placement of an indwelling intravenous cannula for collection of lactate after 45 minutes of bed rest.[10] Although impractical for most outpatient investigations, this merely reflects the sensitivity of lactate and pyruvate testing to other factors.

This group also reviewed blood, urine, and CSF data collected on 23 patients with known defects in the pyruvate dehydrogenase complex or in the complexes of the respiratory chain. They determined that blood lactate values for any given patient could be intermittently elevated, stressing the need for repeated measurements. In addition, they noted that CSF lactate was most consistently elevated, particularly in patients with neurological symptoms, making this fluid the most reliable for the detection of abnormal patient lactate concentrations.[10]

In unpublished data, we have demonstrated that quantitative values of blood and CSF lactate and pyruvate are more accurately measured enzymatically and not by gas chromatography-mass spectrometry (GCMS) methods.

In addition to the diagnostic value of blood and CSF lactate and pyruvate levels, these specific tests are also useful in the management of patients with OXPHOS disease because a number of treatment options, including the use of coenzyme Q10 and dichloroacetate, have been shown to improve patient outcome in a number of instances.[11,12]

Plasma carnitine is another example of a metabolic test that provides both diagnostic and management information for patients with OXPHO disease. L-carnitine is essential for the transport of long-chain fatty acids into the mitochondrial for beta-oxidation.[13] In addition, carnitine acts as a "carrier" for various acyl compounds and allows for their nontoxic excretion through the urine.[14] Although synthesized by the human body from lysine through a series of reactions that include vitamin C, nicotinic acid, vitamin B6, and iron, the carnitine pool, indicated mainly by the plasma concentration, depends on dietary intake.[15] Red meat is the main source of carnitine in the normal diet; in general, fruits, vegetables, and white meats have low carnitine concentrations or none at all.[16]

The measurement of carnitine typically involves the determination of total, free, and esterified fractions of carnitine. A free-to-total carnitine ratio is also typically calculated. Various primary and secondary disease processes have been shown to be associated with changes in these levels.[17] A decreased free-to-total carnitine ratio with normal absolute values indicates the presence of an acyl compound binding with free carnitine to form an esterified compound. These compounds can include an acetyl group as seen with ketosis or any number of other abnormal metabolites that can accumulate with inborn errors of metabolism or valproate therapy. Significant decreases in both free and total carnitine values with a normal ratio can occur and are most commonly of dietary or nutritional origin or, on occasion, can be due to prematurity. Rarely, primary systemic carnitine deficiency can cause profound decreases in free and total carnitine. Increases in carnitine values may be seen with the use of supplemental carnitine or, rarely, with carnitine palmitoyl transferase (CPT) I deficiency, a disorder associated with coma, seizures, hepatomegaly, and hypoketotic hypoglycemia.

Several reports have described a deficiency in plasma carnitine in a number of patients with OXPHOS disease.[18,19] In one study, 21 of 48 patients (43.8%) with a mitochondrial myopathy were found to have elevated esterified carnitine levels with a concurrent decrease in free carnitine. Four of the 21 patients were also shown to have both free and total carnitine deficiencies in plasma. All 21 patients were treated with supplemental carnitine. Muscle weakness improved in 19 of 20 patients, failure to thrive in four of eight, encelphalopathy in one of nine, and cardiomyopathy in eight of eight patients.[18]

The secondary carnitine deficiency in OXPHOS disease is thought to be caused by an impairment of beta oxidation. The increased NADH/NAD(+) ratio generated by reduced flux through the respiratory chain inhibits beta oxidation, producing secondary carnitine deficiency while increasing reactive oxygen species that deplete alpha-tocopherol (vitamin E). A deficiency in both carnitine and alpha-tocopherol causes impairment in production of docosahexaenoic (22:6n-3)-containing phospholipids through a recently elucidated mitochondrial pathway. These phospholipid compounds are known to be crucial in brain, retina, heart and skeletal muscle function. Thus, the carnitine deficiency in OXPHOS diseases may contribute significantly to the pathophysiology of these disorders. The authors postulate supplementation with doxosahexaenoic acid and alpha-tocopherol may prove beneficial to improving the outcome of patients with OXPHOS disorders.[20]

Blood and urine amino acids are also helpful in evaluating such patients. Blood amino acids will often

show an increase in alanine in patients with OXPHOS disease. This increase in blood alanine is caused by transamination of accumulated pyruvate to alanine. If hyperalaninemia is found lactate should be measured, if not previously done. However, a patient with obviously increased lactate levels in blood may have a normal alanine concentration. Urine amino acids in some patients with OXPHOS disease will show a generalized aminoaciduria reflecting a disturbance in the ATP-dependent renal tubular function of the kidney.[21]

Urine organic acid analysis in patients with OXPHOS disease may demonstrate increases in a number of compounds including lactate, pyruvate, and the Kreb cycle intermediates, particularly alpha ketoglutarate. However, similar increases in these compounds can be seen if the patient is suffering from poor perfusion or dehydration at the time of sample collection. Several more recent reports indicate that the organic acids tiglyglycine and 2-oxoadipic acid may also be markers for OXPHOS disease.[22,23]

Tiglyglycine, an intermediate product of the catabolism of the branched-chain amino acid isoleucine, is increased in the urine of patients with beta-ketothiolase deficiency or with disorders of propionate metabolism and can be seen on routine organic acid analysis. Using a stable isotope dilution mass spectrometric assay for tiglyglycine, Bennett et al[22] detected the presence of this compound in the urine of five of six patients with enzyme-confirmed OXPHOS disease, suggesting that it may also be a potential marker for these disorders. Although this stable isotope technique is not employed in standard organic acid analysis, the detection of tiglyglycine in the urine of patients with suspected OXPHOS disease undergoing routine organic acid analysis would support this diagnosis. The presence of 2-oxoadipic aciduria and 2-aminoadipic aciduria has been reported in only one patient with confirmed Kearns-Sayre syndrome who presented at 2 years of age with ketosis and acidosis, progressing to coma.[23] Because these abnormal metabolites disappeared in her urine samples prior to the onset of her complete heart block, retinopathy, and ophthalmoplegia at age 9 years, their significance as a marker for OXPHOS disease is unclear.

Although the detection of abnormalities on any of these tests (Table 4) may be helpful in supporting an underlying disorder in OXPHOS, normal results do not exclude the possibility. The intermittent nature of some testing abnormalities, factors influencing testing results, and knowledge of possible age-related norms must be considered when reviewing testing results and determining their significance. Ultimately, definitive diagnosis is usually dependent on confirmatory mtDNA testing or histological, immunohistochemical, and enzymatic results from analysis of tissue, typically muscle. Although other body tissues can theoretically be used for enzymatic and pathological analysis, some abnormalities are not detectable in skin fibroblast cultures[24,25] and other mitochondria-rich tissues, including the heart, are not readily or feasibly accessible. Because testing artifacts can be associated with frozen specimens, enzymatic analysis of OXPHOS activity should be completed on mitochondria isolated from fresh muscle biopsy tissue.[26]

LONG-TERM FOLLOW-UP TESTING

Following definitive diagnosis of OXPHOS disease for a given patient, the implications for long-term multisystem dysfunction and disease must be considered. Because of the often ubiquitous nature of the OXPHOS defect and/or the inability to effectively determine the extent of body system involvement based on definitive testing, usually limited to one tissue, intermittent medical evaluations and multisystem screening are warranted. The testing targets the body systems most affected by defects of OXPHOS disease and also includes screening for treatable difficulties known to be associated with these disorders, such as diabetes (Table 5). The frequency of testing is partially patient-dependent but, as a general rule, should occur yearly.

The practical application of screening is demonstrated by a case report of two patients diagnosed with pre- or minimally symptomatic hypoparathyroidism, a rare but known complication of OXPHOS disease. Patient 1, with Kearns-Sayre syndrome, complained of fatigue and some mild muscle weakness only despite a serum calcium of 6.9 mg/dL (normal 8.5 to 9.5), phosphorus of 6.9 mg/dL (normal 2.7 to 4.5), and parathyroid hormone (PTH) <10 pg/mL (normal >15) with normal vitamin D and magnesium levels. Patient 2, with a complex I deficiency, had normal calcium and phosphorus levels but a low PTH level. Although we elected to monitor the calcium and phosphorus levels in patient 2, treatment of patient 1 with vitamin D and calcium supplements resulted in normalization of her calcium and phosphate levels and an improvement of her fatigue.[27] Significant complications, including tetany and cardiac arrhythmias, were prevented by this early detection and treatment.

Finally, some of the multisystem testing may not yield results useful for immediate therapeutic management but may provide insight into prognosis for a given

Table 4   Metabolic Testing Abnormalities Supportive of OXPHOS Disease

Increased blood and/or CSF lactate and pyruvate and/or ratio

Decreased plasma carnitine

Increased blood alanine

Generalized aminoaciduria

Increase lactate, pyruvate, Kreb cycle intermediates, tiglyglycine, and 2-oxoadipic acid on organic acid analysis

Table 5   Suggested Follow-up Testing in OXPHOS Disease

Central nervous system
  Developmental testing—yearly or as appropriate
  Audiology/BAER—yearly
  Ophthalmology—yearly
  +/− MRI brain scans—every 1–3 years or as appropriate
  +/− EEG—every 1–3 years or as appropriate
Heart
  ECG—every 1–2 years
  Echocardiogram—every 1–2 years
Liver
  Liver function tests (AST, ALT, alkaline phosphatase,
    bilirubin)—yearly
Kidney
  Renal functions (BUN, creatinine)—yearly or as appropriate
  Renal tubular functions (urinalysis, urine amino acids)—
    yearly or as appropriate
Endocrinologic
  Calcium, phosphorus, thyroid function tests, and fasting
    glucose—yearly
Metabolic
  Blood lactate
  Blood carnitine

patient and affect long-term care planning. In particular, brain scans are obtained for this purpose.

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