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Evaluation and Treatment of Patients with Autism and Mitochondrial Disease*

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I. Introduction and Background

Our clinical experience at Kennedy Krieger Institute over the last 15 years has shown that a deficiency of mitochondrial complex I is a common cause of regressive autism. Although some clinical characteristics of mitochondrial disease, such as mild gross motor delay and hypotonia, are sometimes manifest, the abnormalities typically are subtle and not appreciated until there is loss of language and regression of social development, most commonly at the time of an otherwise simple childhood infection. Most children with autism secondary to mitochondrial disease (“AMD”) experience a single episode of injury, while a few suffer two or more periods of regression during a characteristic window of vulnerability between 12 and 30 months. The subsequent natural history of AMD is typical for regressive autism, with most children showing partial recovery between 3 and 10 years. The principal clinical differences between AMD and non-regressive autism are, variably, a mild myopathy, abnormal fatigue, and, occasionally, minor motor seizures in the years following the first episode of injury. Others with biochemically defined AMD experience a period of only developmental stagnation lasting several months or more between ages 12 and 30 months and show overall better recovery than those who experience a severe autistic regression during this period of neurological fragility. More noteworthy, but uncommonly identified, are sibs of AMD individuals who have all the biochemical features of AMD with no or only minimal developmental or behavioral abnormalities, such as ADHD or obsessive-compulsive disorder.

While permanent developmental losses in AMD can be substantial, especially in the few individuals who suffer more than one episode of regression, recovery can be almost complete in some children when treatment is started early after the first episode of regression, and a partial response to metabolic therapy remains possible indefinitely. Treatment of AMD includes augmentation of residual complex I activity with carnitine, thiamine, nicotinamide, and pantothenate, and protection against free radical injury with several antioxidants, including vitamin C, vitamin E, alpha-lipoic acid, and coenzyme Q10 (CoQ10).

Inheritance of classical mitochondrial disorders often follows a pattern of “maternal inheritance” of the mitochondrial genome, and rare individuals with autism who carry apparently pathologic mtDNA mutations have been reported [1,2]. In contrast, none of our multiplex AMD families shows a pattern of inheritance of autism spectrum disorders (“ASDs”) consistent with mtDNA inheritance, nor has mtDNA mutations testing, including complete mtDNA sequencing, revealed pathologic mutations. In two non-consanguineous families, however, affected first cousins were related through their fathers, while in several other pedigrees a mother or

* Note: This information is presented as personal practice parameters at Kennedy Krieger Institute solely to aid other physicians who wish to evaluate children with autism spectrum disorders for a mitochondrial disease or related metabolic disorder. While every effort has been made to make this information as accurate as possible, this summary reflects the clinical experience with a single institution’s autism population and diagnostic laboratories and, therefore, may differ substantially from experience elsewhere. Prepared: June 13, 2009

father manifested signs of Asperger syndrome or severe ADHD, raising the question of variable penetrance and expression of an autosomal dominant disorder.

Although a deficiency of mitochondrial complex I may be the most common identifiable cause of regressive autism, the relatively mild biochemical abnormalities often are missed by “routine” metabolic testing. In some cases, all test results are in the normal range for the laboratory, but abnormal ratios of metabolites offer clues to the diagnosis. Moreover, because the characteristic mild elevation of lactate occurs only under certain nutritional conditions, the diagnosis can easily be missed when screening by random blood lactate levels, or a mild elevation of lactate will be attributed to a “tourniquet effect.” However, when blood and urine samples are carefully collected under defined nutritional conditions and interpreted based on fasting-specific norms, the diagnosis of AMD can be made without ambiguity and without need for a muscle biopsy.

The identification of patients with AMD has now become routine Kennedy Krieger Institute, in part because of its specialization in both ASD and metabolic diseases and in part because of the availability of on-site biochemical testing. However, with an understanding of the characteristic biochemistry and natural history of AMD and how to standardize sample collection for metabolic testing, making a diagnosis of AMD is straightforward and can be undertaken by clinicians using any reference laboratory able to perform standard metabolic testing. To aid other clinicians in identifying this common cause of regressive autism but subtle metabolic disorder, the following provides a detailed description of the natural history, biochemical diagnosis, and treatment of AMD as practiced at our institution.

II. Diagnosis of Autism with Mitochondrial Disease

A. Measurement of Lactate and Pyruvate. Complex I deficiency associated with regressive autism is manifest biochemically by an intermittent, mild lactic acidemia and a normal lactate/pyruvate ratio. A distinct elevation of lactate often is evident only during the first hour or two after a meal. At other times, the lactate level may be only disproportionately increased for the degree of fasting but remains below the laboratory’s upper limit of normal and, therefore, is not recognized as abnormal. In addition, because the specific block in mitochondrial complex I in AMD is quite proximal in the electron transport chain, the lactate/pyruvate ratio is normal or even mildly decreased because of increased utilization of substrates that enter distal to the metabolic block. Unfortunately, because pyruvate is an unstable compound, an increased level of pyruvate can be missed unless the sample is immediately deproteinized and assayed, or deproteinized and frozen at -70 °C and assayed within a week, requirements that are rarely followed by hospital or commercial laboratories. However, because pyruvate is in equilibrium with its corresponding amino acid, alanine, the level of alanine can be used as a stable and reliable proxy for pyruvate. On a molar basis, the level of alanine (usually reported in $\mu\text{mol/L}$) is 3 times the level of pyruvate, usually reported in mmol/L . For example, an alanine level of 450 $\mu\text{mol/L}$ is equal to a pyruvate level of 0.15 mmol/L (1.4 mg/dL), which is the upper limit of normal in most laboratories.

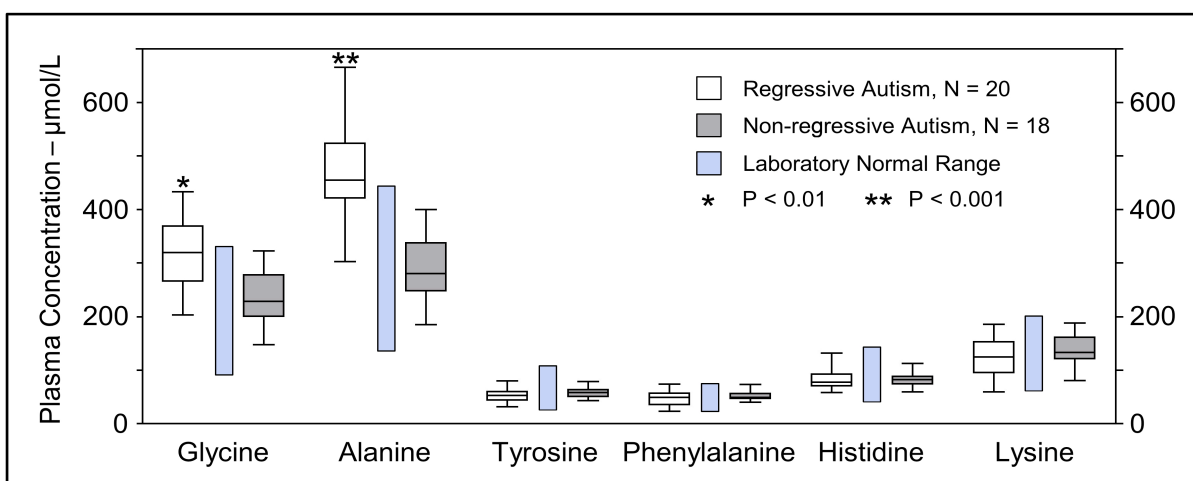
Because the levels of lactate and pyruvate rise and fall with meals, especially in complex I deficiency, the interpretation of lactate and pyruvate measurements must take into account the degree of fasting. Most commercial and hospital laboratories report a broad range of normal for a blood lactate level, typically between 0.5 and 2.0 to 2.5 mmol/L , without reference to the length of fasting. However, the assessment of lactate levels is no different from the interpretation of blood glucose levels relative to the duration of fasting. For example, a lactate level greater than 2.0 mmol/L after overnight fasting is approximately twice normal, whereas a level of 3.0 mmol/L an hour after a full meal can be physiologically normal. In children with AMD, the blood lactate level is rarely more than 3.5 mmol/L at any time, and too often a mildly increased lactate level between 2.5 and 3.5 mmol/L is dismissed as an artifact of collection or processing when a follow-up lactate level happens to be within the laboratory’s normal range.

B. Measurement of Alanine and other Amino Acids The major limitation to using alanine as a proxy for pyruvate in randomly drawn blood samples is that the level of alanine rises rapidly after meals and can be disproportionately increased relative to pyruvate for the first hour or two of the postprandial absorptive period. For many hours thereafter, the level of alanine slowly decreases as a larger proportion of alanine enters gluconeogenesis, but the level remains in equilibrium with the blood pyruvate level until the next meal. Because alanine often is the only amino acid relatively increased in a child with AMD, the ratio of alanine to

an amino acid whose level typically is not affected by a mitochondrial disease, such as lysine, phenylalanine, or tyrosine, provides a marker for an abnormally increased pyruvate level that is more reliable than the absolute level of alanine. After the postprandial amino acid surge has passed, the level of lysine usually falls below 150 $\mu\text{mol/L}$ and the level of phenylalanine and tyrosine are both below 60 $\mu\text{mol/L}$. When a blood sample is drawn 3 to 5 hours after a regular meal, the levels of pyruvate and alanine in a child with AMD remain disproportionately increased. In this time window, and during more extended fasting as well, the ratio of alanine to lysine is greater than 3.0, and the ratio of alanine to phenylalanine + tyrosine is greater than 3.5. (These same sampling windows—3 to 4 hours fasting for children under 5 years and 4 to 5 hours fasting for school-aged children and adults—should be observed for screening any patient for an amino acid abnormality). At those times, the plasma amino acid levels provide a valuable window on the function of mitochondria, which are essential participants in the regulation of most plasma amino acid levels. Unfortunately, AMD is just one of many phenotypes of biochemically mild mitochondrial disease of childhood, including “idiopathic” cerebral palsy, infantile striatal necrosis, and various movement disorders, that are missed for the same reasons.

Although the ratio of alanine to lysine is the most convenient parameter to calculate when scanning an amino acid profile for evidence of a mitochondrial disorder, the level of lysine occasionally itself is disproportionately increased in a few mitochondrial disorders (strikingly so in Reye syndrome) in which lysine transport is disturbed. Similarly, hepatocellular abnormalities in Alpers disease and mtDNA depletion disorders (e.g., deficiencies of POLG1, DGUOK, MPV17; [3]) can cause a disproportionate increase in the level of tyrosine. In the absence of these disease artifacts, phenylalanine + tyrosine equals approximately 80% of lysine, a useful check on the validity of the alanine/lysine ratio. Fortunately, the levels of lysine, phenylalanine, and tyrosine in AMD deviate only infrequently from these ratios except by incorrect timing of samples, extended fasting or, occasionally, hepatocellular dysfunction due to treatment with multiple liver-metabolized drugs. Assessing alanine/amino acid ratios rather than just the absolute level of alanine also is important because many laboratories give inappropriate upper limits for alanine, often as high as 600 $\mu\text{mol/L}$, which would never be normal except between 30 and 90 minutes after a meal. In addition, many laboratories do not determine their own normal ranges for amino acid levels but use data taken from published studies, some of which used blood samples collected without regard to length of fasting.

Another useful amino acid marker for AMD and other forms of complex I deficiency is a disproportionately or absolutely increased level of glycine, which like alanine requires oxidation by complex I [4]. Except in patients treated with valproate, the level of glycine, like that of lysine, rises and falls with the nutritional state. When blood is drawn in the 3 to 5-hour postprandial period, the level of glycine in normal individuals is close to the mean level given by most laboratories. In contrast, in many children with AMD, the glycine level is 50 to 100% higher than expected, as shown below.



Glutamine, serine, and glutamate are also sometimes increased absolutely or relatively. However, unless a plasma sample is rapidly deproteinized and analyzed, even slight deamidation of the 20-fold higher level of glutamine causes a spurious increase in its deamidation product, glutamate. The increase in serine is more variable and most likely reflects “back-up” in the serine degradative pathway leading to pyruvate, whereas the increase in glutamate and glutamine may be secondary to increased levels of 2-ketoglutarate, as discussed below. Mild hyperammonemia, usually less than twice the upper limit of normal, sometimes occurs and may increase further following a meal or after overnight fasting. This mild degree of hyperammonemia, however, does not appear to be clinically important and in our experience does not require protein restriction.

C. Urine Organic Acid Quantification Although urinary organic acid abnormalities in mitochondrial disorders usually are diagnostically non-specific for the site of the metabolic block, the urinary organic acid profile nonetheless can be diagnostically helpful as a screening test for many mitochondrial diseases, including AMD. In addition to lactic aciduria, most patients with mitochondrial diseases have increased urine levels of one or more citric acid cycle (CAC) intermediates. In patients who excrete abnormal amounts of all CAC intermediates, the apparent cause of the organic aciduria is an abnormal redox potential or a primary block in the CAC, causing a failure of transport and/or metabolism of CAC intermediates produced outside the mitochondria. In contrast, individuals with AMD usually have increased urine levels of only 2-ketoglutarate. The increased levels of 2-ketoglutarate (2KG), a five-carbon homolog of pyruvate, could arise from inhibition of 2KG dehydrogenase by increased pyruvate levels, which is the cause of the 2KG aciduria characteristic of pyruvate dehydrogenase deficiency. Like the serum pyruvate level, the urinary 2KG level is only modestly increased, usually about two to three-fold higher than normal, but disproportionately increased for the degree of fasting in a morning fasting urine sample. However, because 2KG is poorly extracted by some methods for urine organic acid analysis, some laboratories detect or report only much greater elevations in 2KG than typically found in AMD. In contrast, laboratories that extract 2KG after “oximation” or use a column extraction method usually are able to quantify and report accurately the mild increases in 2KG found in most patients with AMD. Another common problem in interpreting organic acid results is that the upper limit of normal given for the level of 2KG is inappropriately high, often because the strong age dependency of 2KG urine levels is lost in the compilation of a normal range for young children. For example, a random urine 2KG level of 200-mg/g creatinine is quite normal for a 6-month-old infant but distinctly abnormal for an 18-month-old toddler being tested for AMD. Moreover, like the measurement of lactate levels in blood, the level of 2KG in a morning fasting urine sample should be lower than the level in a daytime sample, whereas, in AMD, the opposite is often found.

Children with biochemically less common variants of AMD sometimes have a generalized increase in urinary CAC intermediates, especially fumarate and aconitate. Other have an increase in the level of 3-methylglutaconate, a compound that when increased usually reflects impaired mitochondrial transport. (Note that, apart from certain defects of branched-chain amino acid catabolism, most urinary 3-methylglutaconate is derived not from leucine, but from extra-mitochondrial isoprenoid metabolism, and requires transport into mitochondrial for catabolism). Children with ASDs who have these varied organic acid patterns are not common and do not respond as well to treatment as AMD patients with these biochemical profiles more characteristic of complex I deficiency. In addition, such atypical children with AMD may have a mild, non-progressive tremor and a higher incidence of minor motor and major motor seizures.

D. Other Laboratory Measurements Although many mitochondrial diseases manifest some degree of metabolic acidosis, at most there is only a slight anion-gap acidosis in AMD, and that occurs infrequently. However, because AMD often includes a mild myopathy, the level of creatine kinase can be increased two or three fold. In addition, the level of AST is mildly increased out of proportion to ALT, giving an AST/ALT ratio between 2 and 2.5, presumably secondary to mitochondrial dysfunction in muscle. While an increased AST/ALT ratio has less specificity in AMD than other biochemical markers, it is rarely normal and, therefore, is a diagnostically useful element of the complete biochemical profile of AMD. The clinically and biochemically mild muscle abnormalities of AMD are manifest in fresh muscle biopsy samples as a surprisingly significant deficiency of complex I, and sometimes complexes I + III [2]. However, with the

better recognition of the complex I type of metabolic profile in AMD, and with the response to carnitine treatment as an additional a diagnostic test, we now consider diagnostic muscle biopsies to be contraindicated in this patient population because of the occasional child with mitochondrial disease who suffers substantial anesthesia-related brain injury. Moreover, the rapidly increasing number of molecular tests available for diagnosing both nuclear- and mitochondrial DNA-encoded mitochondrial diseases and the knowledge that the results of a muscle biopsy rarely change mitochondrial treatment argue for this less-injurious approach to diagnosis and treatment. Complete sequencing of the mitochondrial genome is already available, and sequencing of all nuclear-encoded complex I genes should be offered in the near future.

In almost all patients with “typical” AMD, the cranial MRI is normal, but a few patients have had T2 hyperintensities in the putamen ± caudate, which correlates with a poorer long-term outcome. In contrast, the small subgroup of patients with autistic behaviors due to pyruvate dehydrogenase deficiency characteristically have MRI lesions limited to the globus pallidus, which is the predominant site of basal ganglial injury in very few pediatric metabolic diseases. These include methylmalonic aciduria, succinic semialdehyde dehydrogenase deficiency (4-hydroxybutyric aciduria,) and pantothenate kinase associated neuropathy (“PKAN,” formerly Hallervorden-Spatz syndrome), none of which is likely to present like AMD.

E. Natural History of Autism with Mitochondrial Disease The natural history of AMD and the events surrounding the period of regression are as important as the biochemical abnormalities in establishing the diagnosis. Before regression, all affected children have had normal or even advanced language and cognitive development and no neurological abnormalities apart from mildly delayed gross motor milestones and hypotonia in a few. Regression often can be dated to a specific event, most often a simple childhood illness, such as otitis media, streptococcal pharyngitis, or viral syndrome, or, rarely, an immunization, most often the MMR vaccine or the former DPT. The common feature of all identified precipitants is inflammation. Regression occurs either acutely during the illness or within 14 days of immunization with the MMR attenuated virus vaccine. Regression is otherwise typical for autism and includes acute or subacute loss of language, onset of perseverative behaviors, and loss of eye contact and other social skills. Although neurological regression in many mitochondrial diseases and other metabolic disorders often occurs because of illness-associated fasting, most children with AMD continue to eat normally during the crisis. Moreover, regression during an illness can occur whether or not there is fever. The nature of the regression and its timing suggest that mitochondrial failure is caused by immune-mediated destabilization of mitochondria as part of a TNF-alpha/caspase-mediated apoptosis cascade [5]. Because “steady state” loading of complex I in brain is close to 50% [6,7], if a child had a 50% reduction in complex I activity due to haploinsufficiency for a complex I null mutation, just a 5 or 10% further reduction in mitochondrial activity could cause neurons to cross the threshold for energy failure and cell death.

The well-defined role of nutritional factors in modulating the inflammatory response and the shift from animal fats to vegetable-derived fats in western diets are important factors to consider in the cause and treatment of AMD. The increase in the consumption of pro-inflammatory omega-6 fatty acids in infancy and early childhood over the last generation has been particularly striking. The established role of inflammation in causing mitochondrial destabilization [8,9] could explain an increasing incidence of regressive autism in individuals who have otherwise asymptomatic variants of complex I deficiency, which may have specific adaptive function in host defense and cognitive development [10]. In this respect, AMD, which in our experience is the cause of most regressive autism, could be another inflammatory disorder among several that have seen a markedly increased incidence over the last 20 to 30 years: asthma, inflammatory bowel disease, atopic dermatitis, eosinophilic gastroenteritis, and type I diabetes [11]. The recognition of inflammation as an apparently common cause of regression in AMD recommends the use of anti-inflammatory agents, including ibuprofen and leukotriene receptor inhibitors (i.e. montelukast, zafirlukast), to prevent further injury in children with AMD. For example, the recently reported increased risk for post-MMR autistic regression in children given pro-oxidant acetaminophen [12] could also be interpreted as an increased risk for developmental regression in those who were not given ibuprofen. Moreover, the effect of the gradual elimination of aspirin use in children between the 1980s and 1990s following the Reye syndrome epidemic

may have contributed to the rise in the incidence of autism, although, epidemiologically, aspirin elimination alone is not likely to be a major factor in the rising incidence of regressive autism.

Although most patients with AMD have a discrete episode of acute or subacute language loss and social regression, some will manifest only relative stagnation of development for a period of several months to a year or more. At least 90% of such events—developmental regression or stagnation—occur in a window of vulnerability between 12 and 30 months. This epoch in cerebral cortical development straddles the predicted peak of NMDA receptor density in the frontal cortex and the basal ganglia [13,14]. As embodied in the Henneberry excitotoxicity hypothesis [15], any impairment of neuronal mitochondrial function increases susceptibility to NMDA-mediated glutamate excitotoxicity. Children with more severe defects of complex I probably exceed the threshold of NMDA-mediated and other neuronal toxicity much earlier in the first year, if not at or before birth, and can suffer progressive neurological deterioration from multiple inflammatory events. In contrast, children with AMD appear to reach a threshold for injury only late in the first year and, compared to classical forms of complex I deficiency, suffer less severe injury limited to those aspects of cortical development, such as acquisition of language and social skills, most active at that time. Consistent with the overall greater severity of injury in children with AMD-tremor syndrome, basal ganglial lesions can occur in this subset of regressive autism, although progressive deterioration after age 3 years has not. The hypothesis that neuronal damage in AMD is a threshold phenomenon mediated through NMDA glutamate toxicity when cortical NMDA receptor density is high would explain the usually non-progressive nature of AMD after the period of greatest vulnerability between 12 and 30 months. The non-progressive nature of typical regressive autism in later years and the minimally abnormal biochemistry doubtless dissuade many clinicians from undertaking a more detailed search for a metabolic disorder as the cause of the child's developmental disability. Such minimal biochemical abnormalities cause the metabolic basis of other childhood neurodevelopmental disabilities to escape identification and, therefore, effective treatment.

F. Genetics of Autism with Mitochondrial Disease Genetic studies of AMD have not progressed very far. However, autosomal dominant inheritance in several families is suggested by some parents' having significant social skill deficits, Asperger syndrome, or even a period of developmental regression or stagnation between ages 12 and 30 months. Apparent autosomal dominant inheritance is evident in two families with the AMD plus tremor variant, in which there are affected first cousins related through at least one male. Although autosomal recessive inheritance remains possible in families with only one affected child or sibship, haploinsufficiency of a complex I subunit is plausible cause for an autosomal dominant mitochondrial disease, because the "threshold" for neuronal injury in the brain is only slightly more than a 50% decrease in complex I activity [6,7]. Theoretically, haploinsufficiency of a null or near-null mutation of one of the 36 nuclear-encoded subunits of complex I could cause a critical deficiency of complex I activity in the cerebral cortex. A clinically significant deficiency of complex I activity could also be caused by homozygosity or compound heterozygosity for two milder complex I subunit mutations or even by digenic heterozygosity, i.e. mutations causing partial loss of activity in two different complex I subunits.

Although a few reports link various mtDNA mutations to cases of autism [1,2,16], none of the AMD families we have identified has had evidence for mitochondrial inheritance or a family history that suggests mtDNA-like pathology in other individuals. Even with the currently available complete mtDNA sequencing, we have not found either a pathologic mtDNA mutation or a recurrent low frequency polymorphism in our AMD patients. In view of the non-progressive nature of AMD beyond the first few years, a mutation in the mitochondrial genome would not be expected, because most mtDNA disorders are intrinsically progressive and, especially beyond the first decade, typically have ragged red fibers on muscle biopsy, findings present in none of our patients. Mutation in one or more subunits of mitochondrial complex I in AMD also is suggested by the often immediate response to carnitine, which activates latent complex I by the same NDUSF7/phosphatase-kinase system that activates pyruvate dehydrogenase [17,18]. Although immediate behavioral improvement with carnitine treatment in a child with regressive autism makes complex I deficiency the most likely cause, the similar effect of carnitine to activate latent pyruvate dehydrogenase complex

recommends consideration of pyruvate dehydrogenase deficiency in the child with atypical autism and substantial postprandial lactic acidemia.

In addition to identifying an occasional child with pyruvate dehydrogenase deficiency and autism, appropriately timed evaluation of patients with autistic spectrum disorders for mitochondrial disease has revealed amino acid or organic abnormalities that define both known and novel metabolic disorders. A well-timed amino acid profile will disclose most genetic hyperammonemias, of which the most likely to cause language delay, social deficits, and illness-associated regression is X-linked ornithine transcarbamylase deficiency in heterozygous females. Defects in mitochondrial amino acid transport affecting amino acids closely linked to the citric acid cycle, such as glutamate, glutamine, aspartate, and asparagine, theoretically could cause critical deficiencies of mitochondrial energy metabolism during periods of increased metabolic demand, and we have identified two patients who appear to have novel defects in this area. Several other metabolic diseases that overlap phenotypically with autism, such as disorders of creatine synthesis or transport [19], are known but usually are non-regressive and not detected by the below outlined protocol for diagnosis of AMD. However, tests for other metabolic causes of autism, if indicated, can be added to the AMD diagnostic protocol. For example, the accuracy of combined blood and urine testing for X-linked creatine transporter deficiency is greater if collected after overnight fasting.

Another relatively common X-linked genetic disorder with a natural history and, sometimes, biochemical findings similar to that of AMD is Rett syndrome, caused by functional deficiency of MECP2, a protein involved in DNA methylation and gene expression. Although most patients with Rett syndrome can be distinguished clinically from other ASD patients, Rett syndrome in its early phase of regression before their characteristic behaviors have developed can be difficult to distinguish from AMD, both clinically and biochemically. The probable pathological over-pruning of cortical neurons that occurs in AMD has been demonstrated in Rett syndrome [20], and the age at which autistic regression occurs in Rett syndrome is similar to that of AMD. In addition, recent studies of the *Mecp2*-deficient mouse show over-expression of *Uqcrc1*, a subunit of complex III that could be increased to compensate for deficient activity of complex I [21]. Therefore, clinicians should consider MECP2 testing for female patients with regressive autism in whom biochemical signs of mitochondrial dysfunction are found. An interesting possibility that remains to be tested is whether or not early treatment of Rett syndrome as a mitochondrial disease could limit the degree of intellectual and social regression that typically occurs during the same window of vulnerability as seen in AMD.

III. Diagnostic Protocol for AMD and other Mitochondrial Disorders

A. Samples and Sample Timing. With the goal of making a biochemical diagnosis of AMD without subjecting a child to a muscle biopsy and, thereby, a potentially damaging anesthetic procedure, we have developed an outpatient diagnostic protocol that requires only blood and urine collections and that, in most cases, establishes a clear biochemical difference between AMD and other metabolic disorders or collection artifacts. The success of the protocol depends on careful timing of sample collection and interpretation of results using biochemical norms for different times relative to the last meal. The full protocol requires three samplings: 1) after overnight fasting, 2) just before lunch (3 to 5-hour fasting sample), and 3) 60 to 90 minutes after a full lunch. The patient arrives at the testing center after fasting for three hours beyond the child's usual overnight fasting period, before which the first morning void has been collected and saved for urine organic acid analysis unless it can be replaced with the second morning fasting void collected before the first blood draw. Following the first blood draw, the child has a regular breakfast and returns for the second blood draw before lunch and at least 3 hours after finishing breakfast, having had nothing but water following breakfast. After a lunch containing approximately 0.75 g/kg protein, the patient returns to have a third blood sample drawn between 60 and 90 minutes after lunch. Because the full protocol requires three blood draws from children who often are difficult to phlebotomize, we sometimes limit the blood studies to just preprandial and postprandial samples drawn by the physician or an expert phlebotomist. If the first blood draw is omitted, the AM fasting urine sample is still collected. Although a venous catheter can be placed for blood sampling, this

rarely lasts for all three draws in a small child. Using EMLA cream and a 23 or 25-gauge butterfly needle in a small dorsal hand vein can be almost painless and efficient even for 10 mL blood draws if the child is given ibuprofen before and the needle is rinsed with heparin.

Collection 1: Morning fasting samples

- Quantitative plasma amino acids
- Blood lactate level
- Creatine kinase
- Comprehensive metabolic profile
- Quantitative urinary organic acids
- Vitamin E, CoQ10, and selenium levels (baseline levels)
- Blood cholesterol-lipoprotein profile
- Blood and urine studies for creatine deficiency disorders (optional)

Collection 2: 3 to 5 hours fasting (pre-lunch)

- Quantitative plasma amino acids
- Blood lactate level
- Acylcarnitine profile (if not done previously)

Collection 3: 60 to 90 minutes after a 0.75 g/kg protein lunch

- Quantitative urinary organic acids (anytime up to 4 hours after lunch)
- Blood ammonia level
- Blood lactate level

B. Interpretation of Tests and Follow-up Testing The interpretation of the test results has been reviewed, in part, in several sections above. Because one often fails to obtain enough blood for all studies at one of the sampling times, the missed tests can be repeated on another day observing the same timing. If only a urine sample is missed, then the parents can be sent home with collection supplies and instructed to keep the urine sample in the freezer until it can be delivered.

If the clinical and laboratory studies are consistent with AMD, treatment is begun as soon as plans for follow-up developmental assessments can be made. The specific follow-up plan devised depends largely on the clinical experts and assessment tools available. Because there can be substantial improvement in language and behavior even while the laboratory parameters remain unchanged, we do not routinely repeat the full protocol for biochemical follow-up in our typical AMD patients. For limited biochemical comparison pre- vs. post-treatment, we usually obtain a single preprandial (3 to 5 h fasting) blood draw for amino acids, lactate, vitamin E and CoQ10 about 4 months after starting treatment, by which time all cofactors should be at therapeutic levels. However, depending on the baseline laboratory results, one of the other two blood draws may yield a better profile for follow-up, especially in some of the atypical AMD patients. The lack of biochemical improvement despite obvious clinical gains in some patients suggests that mitochondrial function in liver and muscle (which determine most blood metabolite levels) often is already maximally activated, and that the benefit of treatment derives largely from increasing the levels of mitochondrial cofactors in the brain. In children with abnormal fatigue, the apparently greater muscle involvement may permit meaningful serial testing of, for example, creatine kinase, AST/ALT ratio, and a 15-minute resting lactate level as markers for muscle mitochondrial function. In other children, the fatigue is largely cerebral, and markers of muscle involvement can be normal. In older children who can sit for extended testing, the mental fatigue is most evident in declining subtest scores over the testing period, and one or more of the final subtests can be repeated as the first tests on another day to show objectively the effect of mental fatigue.

IV. Treatment of Autism with Mitochondrial Disease

The goals for treatment of AMD due to complex I deficiency are:

- 1) Augment residual complex I activity
- 2) Enhance natural systems for protection of mitochondria from reactive oxygen species
- 3) Avoid conditions known to impair mitochondrial function or increase energy demands, such as prolonged fasting, inflammation, and the use of drugs that inhibit complex I.

A. Augmenting residual complex I activity The first treatment goal is addressed by providing pharmacological amounts of L-carnitine and pantothenate, the vitamin precursor of coenzyme A. Complex I is unique among the five primary mitochondrial respiratory chain complexes in being regulated by a specific phosphorylation-dephosphorylation system sensitive to the intramitochondrial free-CoA/Acyl-CoA ratio [17]. The specific kinase inactivates the NDUFS7 (PSST) subunit of complex I by phosphorylation and serves in the same capacity to inactivate nearby pyruvate dehydrogenase subunits [18]. Supplemental carnitine enhances the conversion of acyl-CoAs to free CoA + acylcarnitines, thereby raising the intramitochondrial free CoA/acyl-CoA ratio and activating the phosphatase that reverses the inhibitory phosphorylation of NDUFS7. Pharmacological amounts of pantothenic acid increase the synthesis of free CoA in mitochondria [22], which increases further the free-CoA/acyl-CoA ratio. Raising the free-CoA/acyl-CoA ratio recruits more functional complex I units to compensate for the partial deficiency of complex I. Because complex I is the rate limiting step in the mitochondrial respiratory chain for most substrates, each percentage increase in complex I activity should be followed by a substantial fraction of that percentage increase in mitochondrial ATP synthesis.

Although we sometimes include thiamine and nicotinamide (vitamin precursor of nicotinamide adenine dinucleotide, “NAD,” used by complex I) in vitamin compounds for more severe deficiencies of complex I, their value in treating AMD is not as certain as it is in other forms of complex I deficiency. Therefore, because both nicotinamide and thiamine can be stomach irritants, and because thiamine adds a bitter taste and strong odor to liquid vitamin preparations, we usually do not include thiamine and nicotinamide in our initial vitamin prescription for AMD, although this aspect of AMD treatment needs more study. While the rationale for adding NAD-forming nicotinamide to the vitamin combination for complex I deficiency is clear, how thiamine can augment the activity of complex I, which does not require thiamine, is not obvious. Nevertheless, thiamine responsive complex I deficiency has been reported both in patients and in vitro, and thiamine deficiency causes a secondary complex I deficiency [23,24]. Because the increased levels of 2-ketoacids (pyruvate and 2KG) increase the need for thiamine, thiamine-responsive complex deficiency could reflect a relative thiamine deficiency. For this reason, we recommend a regular daily multivitamin tablet in all patients with mitochondrial disease, and other metabolic disorders in which there is substantially increased flux through a vitamin-dependent pathway.

An occasional immediate sign of the effectiveness of carnitine in children with more severe, non-AMD complex I deficiencies is hyperactivity or even increased seizure activity. However, children who react in this way often show other signs, such as more rapid recovery from seizures and increased alertness, that indicate that the hyperactivity stems from increased cerebral energy availability and, therefore, that long-term benefit from carnitine supplementation can be anticipated. For AMD patients who have seizures, we begin carnitine at 20% of the usual 50 mg/kg/d TID dose and increase to the full dose, as tolerated, in several steps over one to two months. We have not seen the same hyperactivity effect when pantothenate is added later to a vitamin compound, but such an effect is theoretically possible. Because of the high fat solubilities of vitamin E and CoQ10, the antioxidant combination (discussed below) requires 2 to 3 months to reach effective levels, depending in part on the amount of a child’s fat tissue and efficiency of intestinal absorption. As a result, one can separate the effects of complex I activation by carnitine + pantothenate from the effects of the antioxidant mixture if both treatment components are started at the same time. Nevertheless, we usually start carnitine alone first, because carnitine usually is prescribed separately from the vitamin suspension, and because distinguishing a gastrointestinal irritant effect of other vitamins from carnitine-induced hyperactivity could be

difficult in a child with an ASD. Because some AMD patients on muscle biopsy are found to have a combined deficiency of complex I and complex III, a more rapid effect of CoQ10 is theoretically possible, but we have not seen that.

B. Enhancing protection from damage by reactive oxygen species The second component of mitochondrial vitamin therapy addresses the problem of reactive oxygen species. There is much theoretical and experimental support for the proposition that increased production of reactive oxygen species caused by a block in electron transport leads to additional injury of mitochondria and, consequently, cycles of increasing mitochondrial damage and worsening mitochondrial function. Although there are many targets within mitochondria for peroxidative damage, the best-characterized and most important effects are on the inner mitochondrial membrane and, especially, the oxidation-sensitive linoleic acid residues in cardiolipin and other membrane phospholipids. Most effective in preventing peroxidative damage of mitochondrial membranes are antioxidants such as CoQ10 and vitamin E, which target to the inner mitochondrial membrane and the electron transport chain complexes because of their extreme hydrophobicity and natural sites of action in the mitochondrial inner membrane. Vitamin C has the dual ability to reduce directly vitamin E chromanoxyl radicals generated in the process of capturing reactive oxygen species [25] and to donate electrons to complex IV from CoQ10.* Freely diffusing molecules of CoQ10, a key electron shuttle from complexes I and II to complex III, also have an intrinsic ability to neutralize reactive oxygen species including chromanoxyl radicals, although less efficiently than vitamin E [26]. Alpha-lipoic acid, an essential cofactor for 2-ketoacid dehydrogenases, has a role similar to that of vitamin E in the inner mitochondrial membrane but can also enhance the catabolism of 2-ketoacids, which accumulate behind a block in the complex I NADH dehydrogenase required for 2-ketoacid dehydrogenase function [27]. Depending on the location of the block in complex I (proximal vs. distal to the iron-sulfur clusters), augmenting residual complex I activity theoretically could increase the production of free radicals in some forms of complex I deficiency. However, because the molecular genetic etiology of a mitochondrial disease in young children is only infrequently identified, and because the biological cost of augmentation of complex I activity can be increased production of reactive oxygen species, providing antioxidant protection to patients with any known or suspected abnormality of the electron transport chain is prudent. Moreover, because most reactive oxygen species produced by mitochondria are believed to come from complex III, the uncertain nature of the complex III deficiency sometimes found in AMD muscle biopsies also recommends providing antioxidant protection for patients with AMD.

Combining the first and second parts of the treatment plan, the following is a typical prescription for treating AMD:

L-Carnitine	50 mg/kg/d	α -Lipoic acid	10 mg/kg/d
Coenzyme Q10	10 mg/kg/d	Pantothenate	10 mg/kg/d
Vitamin C	30 mg/kg/d	Nicotinamide	7.5 mg/kg/d (optional)
Vitamin E	25 IU/kg/d	Thiamine	15 mg/kg/d (optional)

The choice of these cofactors and the dosages have derived over the years from treating more typical mitochondrial diseases, such as Leigh disease, and they are not necessarily followed exactly as given above, especially in older adolescents and adults, for whom dosages are calculated based on a weight of 40 kg as a starting point. Most major commercial reference laboratories can now measure serum levels of both CoQ10 and vitamin E, which are important for monitoring treatment. The therapeutic goal for CoQ10 is 3 to 4 mg/L, with a typical normal range of 0.5 – 2.0 mg/L. For vitamin E, the goal is between 120 and 150% of the upper limit of normal, which typically is between 10 and 15 mg/L, depending on age. Because both CoQ10 and

* Note that the adverse effects reported in some studies with high-dose vitamin E, about which parents may express concern, likely were caused by the failure to provide a mechanism, such as co-supplementation with CoQ10 and vitamin C, to reduce Vitamin E chromanoxyl radicals, which themselves can be damaging pro-oxidants. This is important for reassuring parents who might be concerned about the use of pharmacological amounts of vitamin E in treating AMD.

vitamin E are carried on VLDL and LDL particles, the patient's lipoprotein profile should be obtained at the outset of treatment to determine if the goals for CoQ10 and vitamin E levels need to be normalized to lipoprotein levels if LDL or especially VLDL is abnormally high or low. For this reason, also, vitamin E and CoQ10 levels are best measured in fasting blood samples.

L-carnitine, usually prescribed as a 10% liquid formulation (Carnitor) for small children, also is available in 330 mg tablets and, when needed, generic L-carnitine can be compounded as the free powder with the other vitamins. The vitamins can be given as commercial tablets and capsules, specially compounded capsules containing all vitamins, or as a uniform suspension prepared by a compounding pharmacist. All vitamins or compounds are given three times a day with meals. Liquid Carnitor, a prescription medication in the US, also can be combined with the other vitamins, which sometimes qualifies the cost of the mixture for coverage by some medical insurance carriers. However, compounding with liquid Carnitor increases considerably the volume of a somewhat bitter-tasting vitamin suspension, and therefore some parents prefer to give liquid Carnitor separately. Although most insurance companies deny coverage for any compound that is available over the counter, more insurance companies today accept vitamin cocktails, which are costly, as the "standard of care" for mitochondrial diseases, since literature supporting their beneficial effects and legal cases deciding in favor of vitamin compounds as standard-of-care are increasing. Therefore, whereas payment for treatment of otherwise undefined autism with a vitamin preparation would be denied coverage, biochemical evidence of a mitochondrial disease, even in the absence of muscle biopsy data, together with a letter of justification from a physician can qualify a vitamin compound for coverage by some insurance companies.

C. Avoiding conditions known to impair mitochondrial function Certain general medical measures for treatment of mitochondrial diseases should also be considered. As overnight fasting progresses from glycogenolysis to predominantly gluconeogenesis by the early morning hours, mitochondrial oxidative metabolism must increase to process gluconeogenic amino acids through the citric acid cycle for gluconeogenesis and to convert liberated ammonia into urea. Illnesses, especially when accompanied by fever, increase the metabolic demands placed on mitochondria, while elements of a normal inflammatory response, such as increased levels of TNF-alpha, can directly impair mitochondrial function [8,9]. In addition, after 12 hours of fasting and after shorter periods of overnight fasting by a child who ate poorly the previous day, levels of cytoplasmic free fatty acids begin to rise and directly impair the function of complex I. Although, individually, these mitochondrial stressors might have clinically unimportant effects on mitochondrial function, their cumulative effect can be sufficient to cause neurological injury. Therefore, these potential causes of mitochondrial impairment require that caloric intake be closely monitored during illnesses, and that intravenous nutrition be started early in the course of a vomiting illness, especially during the age-dependent window of vulnerability characteristic of AMD and many other mitochondrial disorders. Hospitalization to provide adequate fluids and nutrition during acute illnesses often can be avoided with the use of ondansetron (Zofran) to limit nausea and vomiting.

Another important clinical observation is that many children with mitochondrial diseases are more symptomatic (irritability, weakness, abnormal lethargy) in the morning until they have had breakfast, although this phenomenon is not as common in AMD as it is in other mitochondrial diseases. In some children, early morning symptoms can be a consequence of compromised mitochondrial function, whereas, in others, a normal rise in epinephrine consequent to a falling blood glucose level in the early morning hours can elicit agitation, ataxia, tremors, or difficulty waking. In children who normally sleep more than 10 hours at night, significant mitochondrial destabilization can occur by the morning and be evident in biochemical tests, although this is less common in AMD than in other mitochondrial disorders. When early morning signs of disease are observed or suspected, giving uncooked cornstarch (1 g/kg; 1 tbsp = 10g) at bedtime effectively shortens the overnight fasting period. Uncooked cornstarch, usually given in cold water, juice (other than orange juice), yogurt, or pudding, provides a slowly digested source of carbohydrate that, in effect, shortens overnight fasting by 4 to 5 hours.

Equally important to recognize is that children and adults with mitochondrial disorders are, in general, more dependent on amino acids to maintain needed levels of citric acid cycle intermediates for energy

metabolism and gluconeogenesis when they fast beyond their available glycogen stores. In addition to using bedtime cornstarch to limit overnight fasting-induced depression of amino acid levels, maintaining normal amino acid levels during illnesses should be part of the care plan for AMD. Severe depression of amino acid levels during illness-induced anorexia has effects on ATP synthesis little different from that of hypoglycemia, and prolonged use of carbohydrate-only fluids and foods during catabolic illnesses causes insulin-induced further lowering of amino acid levels, sometimes causing a metabolic coma. When illnesses are severe enough to require hospitalization, a source of protein amino acids, including if necessary hyperalimentation, should be started no later than 24 hours after hospitalization. Much encephalopathy, both transient and permanent, can be avoided by close attention to this aspect of amino acid nutrition. While most children with AMD who show early morning symptoms appear to reclaim a normal fasting tolerance in the second half of the first decade, a few can retain a life-long sensitivity to fasting, especially if muscle mass is low.

V. Measures to Prevent Injury in Mitochondrial Disorders

A. Infections and Inflammation As noted above, an important consideration for treatment of AMD is that “normal” inflammation can impair mitochondrial function. Although most infections cannot be avoided, certain measures can limit the risk of injury during infection or other causes of inflammation. First, whereas the earlier, highly inflammatory “cellular” forms of pertussis vaccine (DPT) could readily precipitate mitochondrial injury, the currently used acellular pertussis vaccine (DTaP) appears to be safe and, in our experience, has not been associated with autistic regression. We believe it is much better to immunize with DTaP than risk infection with highly inflammatory and potentially damaging community-acquired pertussis. While we have not seen regression in AMD in recent years clearly associated in time with the standard immunizations given in the first year, the MMR vaccine has been temporally associated, if rarely, with regression in AMD and other mitochondrial diseases when given in the second year. Doubtless some of these regressions are coincidental, since the usual age for giving the MMR falls within the typical window of vulnerability for AMD regression. In some children, however, MMR-suspected regression has coincided with the peak inflammatory response on days 8 to 10 post-immunization, as measured by IL-10 levels [28]. Unfortunately, the falling rates of immunization with MMR in the United States and other countries all but guarantees that major outbreaks of measles, mumps, and rubella will occur in the near future. As a result, the goal is now changing to prevention of severe injury by the naturally acquired diseases by not withholding MMR vaccination and by providing anti-inflammatory protection for the duration of the cytokine response to the immunization. Because AMD is only one of many biochemically mild mitochondrial diseases first recognized in a patient because of illness-induced injury, it is hoped that the rapid advances in molecular diagnostic testing will speed the identification of the causative nuclear DNA mutations and make possible the early identification of in at-risk children.

As one way to address concerns about vaccine-related regression in children with mitochondrial disorders, suppression of the inflammatory response with a leukotriene receptor antagonist, such as montelukast (Singulair) or zafirlukast (Accolate), as well as ibuprofen, can attenuate the anti-mitochondrial elements of the immune response, as discussed above, without affecting the primary immunological response to attenuated virus vaccines. Although for most children with AMD the risk for injury was unknown until regression occurred, preventative measures can be undertaken when there is a family history of regressive autism or other suspicion of mitochondrial disease, such as delayed gross motor milestones. For example, for diseases with spontaneous activation of inflammation (hyper IgD syndrome, Mediterranean fever) and for certain classical metabolic disorders (maple syrup urine disease, methylmalonic aciduria), we have found that acute or daily treatment with a leukotriene receptor antagonist can greatly diminish the inflammatory-catabolic response and allow a more benign course of the illness. In the hyper IgD syndrome variant of mevalonic aciduria, for example, montelukast all but eliminates the monthly febrile crises in most affected individuals. Therefore, treatment with montelukast or zafirlukast should be considered in selected patients, such as those who suffered an early inflammation/infection-related regression and who are still in the vulnerable age range.

B. Nutritional Factors Diet is another variable to consider in the treatment of AMD. Vegetable oils that are “pro-inflammatory” due to low levels of omega-3 (n-3) fatty acids and increased amounts of linoleic acid and other omega-6 (n-6) fatty acids today predominate in infant formulas and most prepared foods, largely because

of nutritional recommendations to avoid animal fats containing saturated fatty acids and cholesterol. The serious consequences of this trend are now being felt. A study in 2000 [29] showed that two- to four-month-old breast-fed infants had more than twice the level of docosahexaenoic acid (C22:6n-3) and higher levels of most other n-3 fatty acids compared to formula-fed infants, although immunological consequences of the difference could not be demonstrated using limited immunological assays in that particular study. While the average child may suffer no obvious ill effects from diets deficient in n-3 fatty acids, the possible pro-inflammatory effect of these diets could be a contributing factor to infection-induced regressive autism in a child who has a metastable mitochondrial disorder. Moreover, in view of a recent study that associated decreased synthesis of cholesterol with rare cases of non-regressive autism [30], the early termination of breast-feeding and the major shift in infant diets toward low-cholesterol vegetable fats could be contributing factors to the apparent rise in the incidence of both regressive and non-regressive autism. Indeed, studies over the last two decades have shown that absence or early termination of breast-feeding is associated with higher rates of autism [31]. The simplest way to assure an adequate amount of C22:6n-3 and related fatty acids for children on typical vegetable-oil enriched diets is to provide an oil supplement, such as flaxseed oil, which is enriched in the precursors for C20 and C22 n-3 fatty acids, or salmon oils, which contain substantial amounts of DHA and EPA and a relatively low mercury content compared to many other fish species.

C. Medications Certain behavior medications used in the treatment of ASD are inhibitors of complex I and, therefore, warrant consideration in treating children with AMD. Although these medications appear to have little effect on overall energy metabolism in individuals with normal mitochondria, clinically significant compromise of mitochondrial function can occur when complex I is impaired and relatively high doses of the more inhibitory drugs are prescribed. The complex I-inhibiting drugs most likely to be used in the treatment of ASD include both typical and atypical neuroleptics, such as risperidone (Risperdal), haloperidol, and some SSRIs. Although these medications are used most often in older children who are beyond the vulnerable period for autistic regression, this theoretical risk should be considered when prescribing older generation neuroleptics, such as haloperidol and related drugs, with a higher risk for development of tardive dyskinesias. These older neuroleptics have been shown to inhibit complex I activity in direct proportion to their propensity to cause tardive dyskinesia [32]. However, there is no evidence that the newer “atypical” neuroleptics, such as risperidone and quetiapine, which have a low risk for extrapyramidal damage, are contraindicated in children with AMD and other mitochondrial diseases. Indeed one of the commonly used atypical neuroleptics, risperidone, has been shown to possibly protect against mitochondrial injury via modulation of damaging stress-induced calcium influxes into mitochondria [33].

VI. References

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