Biomarkers of Abnormal Energy Metabolism in Children with Autism Spectrum Disorder

Richard E. Frye, MD, PhD*
Division of Autism Research, Department of Pediatrics Arkansas Children’s Hospital Research Institute, Little Rock, AR

Biomarkers of mitochondrial disease were studies in 133 consecutive autism spectrum disorder patients to determine the prevalence of abnormalities in biomarkers of mitochondrial disease. Biomarkers included traditional biomarkers of mitochondrial disease (lactate, alanine), fatty-acid oxidation defects (acyl-carnitine panel) and recently described novel biomarkers of detecting mitochondrial dysfunction in individuals with autism spectrum disorder (alanine-to-lysine ratio, creatine kinase, aspartate transaminase). Biomarkers were collected in the morning fasting state. Abnormal biomarker values were verified by repeat testing. For those with abnormal acyl-carnitine panels, secondary disorders of fatty acid metabolism were ruled out. Abnormalities in lactate, alanine-to-lysine ratio and acyl-carnitine panels occurred in over 30% of children on initial testing. Among the patients with abnormal biomarkers who had repeated testing, abnormalities were confirmed about half of the time except for alanine which was only confirmed 20% of the time. Elevation in alanine-to-lysine ratio was associated with epilepsy and elevation in multiple acyl-carnitines was associated with regression. In order to confirm the significance of certain biomarkers, a wide variety of mitochondrial biomarker values were compared between specific subgroups of children with abnormal biomarkers and matched children without any abnormalities in biomarkers. Lactate, alanine-to-lysine ratio and acyl-carnitine panel groups demonstrated abnormalities in multiple mitochondrial biomarkers, confirming the validity of these biomarkers of mitochondrial dysfunction. This study demonstrates that multiple biomarkers of mitochondrial dysfunction are elevated in a significant portion of children with autism spectrum disorder and lend support to the notion that disorders of energy production may affect a significant subset of children with autism.


Key Words: autism, mitochondrial disease, biomarkers

INTRODUCTION
Mitochondrial disease (MD) may be associated with autism spectrum disorder (ASD).1,2 A recent meta-analysis by Rossignol and Frye found that 5% of children with ASD meet criteria for classic MD and that children diagnosed with ASD and MD (ASD/MD) have clinical characteristics distinct from the general ASD population, thereby supporting the existence of an ASD/MD subgroup.2 This meta-analysis also found that about 30% of children in the general ASD population exhibited biomarkers consistent with mitochondrial dysfunction, although it is not known how many of these patients in these studies qualify for a classic MD diagnosis since additional clinical and laboratory findings supporting the diagnosis of classic MD were not reported. A recent study compared electron transport chain (ETC) function in lymphocytes between ASD and typically developing controls to determine if children with ASD manifested mitochondrial dysfunction.3 This study reported that 80% of the children with ASD demonstrated lower than normal ETC function, consistent with mitochondrial dysfunction.

The majority of studies that have estimated ASD/MD prevalence in the general ASD population have used the modified Walker criterion to define MD.4 However, there are some serious limitations to using criterion, especially when examining children with ASD. This criterion relies on a significant reduction in ETC complex function and known mitochondrial DNA mutations.4 However, only 23% of children with ASD/MD have a known mitochondrial DNA mutation5 and reports have noted that some children with ASD/MD have ETC over-activity rather than deficiencies.5,6 Thus, Frye and Rossignol1 suggest that the Morava et al criterion,7 a standard criterion that considers a wide variety of clinical, metabolic, imaging and morphological findings, may be more sensitive for diagnosing MD in children with ASD.

MD was first confirmed on muscle biopsy in a series of children with ASD by Fillano et al8 in the syndrome called
HEADD (hypotonia, epilepsy, autism, and developmental delay). In 2006 Poling et al found that 38% to 47% of patients with ASD who were referred to a pediatric neurology clinic manifested abnormal elevations in non-specific serum markers for defects of oxidative phosphorylation. A recent study demonstrates that 65% of children with ASD who were referred for a neurometabolic work-up demonstrated an oxidative phosphorylation defect.

Although these previous studies demonstrated that subpopulations of children with ASD can be identified that manifest MD, each study has used a limited set of biomarkers. For example, Poling et al examined aspartate aminotransferase (AST), lactate and creatine kinase (CK). Oliveira et al examined lactate and Weissman et al examined lactate, pyruvate, AST, alanine aminotransferase, alanine, CK and urine organic acids. Despite this concentration on biomarkers of respiratory chain defects, others have reported metabolic biomarkers more consistent with fatty-acid oxidation defects. For example, Filipek et al reported that over one-third of children with ASD demonstrated total carnitine levels greater than 1 standard deviation below the mean and Clark-Taylor and Clark-Taylor reported a child with abnormal elevation in long chain acyl-carnitine levels.

Some have criticized biomarker studies because of the fact that some testing (e.g., lactic acid) may be prone to high rates of false positive. Another limitation of these biomarker studies is that many studies only look at isolated biomarkers, so it is difficult to say if the various biomarkers of MD are from the same subpopulation of children with ASD/MD or whether there are subgroups of children with ASD/MD that manifest different biomarkers. In the current study we sought to answer some of these questions by examining a wide variety of biomarkers in large series of children with ASD and determining if they were consistently elevated and whether they corresponded to abnormalities in other biomarkers of abnormal mitochondrial function.

METHODS

Biomarkers for MD included traditional biomarkers (lactate, alanine) and recently described biomarkers (alanine-tolysine ratio, CK, AST) and markers for fatty acid oxidation disorders (acyl-carnitine). Because acyl-carnitines are measured as a panel, the panel was only considered abnormal if there were 3 or more individual acyl-carnitines that were abnormally elevated. All biomarkers were collected via venopuncture in the morning during a fasting state from 133 consecutive patients who presented to a medically-based autism clinic for evaluation. Due to laboratory or collection error, some children did not have results for some biomarkers. If any biomarkers were found to be abnormal, repeat testing of the biomarker was recommended to confirm the abnormality. Not all patients complied with repeat testing; however, it is assumed that the repeat testing on the subgroup that did undergo repeat testing was representative of the complete group. All parents and/or guardians signed a consent approved by the Institutional Review Board at the University of Texas Health Science Center (Houston, TX) to allow their child’s medical information to be abstracted into an anonymous database. Child assent was obtained when appropriate.

The prevalence of biomarkers being abnormalities was calculated in several ways. First the prevalence of each biomarker being abnormal one time was calculated. Second, the percent of individuals confirmed to have abnormal biomarkers were calculated. Confirmation was obtained by repeat testing of the biomarker. The final prevalence of biomarker abnormality was calculated by multiplying the prevalence of being abnormal once by the percent of individuals with confirmed abnormalities. This minimized any bias introduced from particular individuals failing to obtain repeat testing.

For the group of children with a particular confirmed abnormal biomarker, the prevalence for various neurodevelopmental diagnoses were examined. Specifically, during clinical evaluation, the child’s neurodevelopmental diagnosis was reevaluated using the Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR). Children were separated into those with classic autistic disorder (AD) or pervasive developmental disorder not otherwise specified (PDD-NOS) as opposed to children with motor delays and AD/PDD-NOS. The diagnosis of speech or language delay verses an ASD diagnosis was also considered as were other diagnosis such as attention deficit disorder with or without hyperactivity. The percentage of each of these categories for the children with consistently abnormal biomarkers was calculated and compared to a group of ASD children without any biomarker abnormalities. The prevalence of regression and epilepsy was also examined for each subgroup.

For individuals with consistent elevation in acyl-carnitines, secondary disorder of fatty acid metabolism including biotin, biotinidase, carnitine, zinc and copper deficiency were ruled out as was a general increase in fatty acids. Some children with consistent abnormalities underwent standard clinical workup for MD including muscle biopsy or genetic testing. Nuclear DNA gene abnormalities were examined in some patients using an oligonucleotide array comparative genomic hybridization analysis that tested for deletions or duplications in approximately 180 nuclear genes involved in mitochondria function, including genes involved in fatty-acid oxidation, carnitine metabolism, mtDNA biogenesis, maintenance, transcription and translation, or ETC complex assembly (MitoMapSM, Baylor Medical Genetics Laboratory, Houston, TX). Mitochondrial DNA was examined in some patients by sequencing of the entire mitochondrial DNA genome to look for rare and common mutations, insertions or deletions that could cause MD (Baylor Medical Genetics Laboratory, Houston, TX).

For each group of children with consistent elevations in a particular biomarker, the values for all of the other biomarkers examined, as well as urine organic acids and ammonia, was compared to a control group of ASD children.
who did not demonstrate any abnormal elevations in biomarkers and were matched as closer as possible on age and gender. A 2-tailed t-test with heteroscedastic assumption and alpha set to < 0.01 was used for this latter analysis to determine statistical significance. Only laboratory values that differed significantly were reported. Finally, the overlap in the biomarker subgroups was calculated.

Table 1. Prevalence of Biomarker Abnormalities. Both the total number of patients and the percent of the total are provided except for prevalence which is a calculated value.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Total Tested</th>
<th>Abnormal at Least Once</th>
<th>Patients with Abnormalities Tested Twice</th>
<th>Abnormal Twice</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>96</td>
<td>34 (35%)</td>
<td>20 (59%)</td>
<td>9 (45%)</td>
<td>15.9%</td>
</tr>
<tr>
<td>Alanine</td>
<td>94</td>
<td>8 (9%)</td>
<td>5 (63%)</td>
<td>1 (20%)</td>
<td>1.7%</td>
</tr>
<tr>
<td>AST</td>
<td>113</td>
<td>20 (18%)</td>
<td>14 (70%)</td>
<td>8 (57%)</td>
<td>10.1%</td>
</tr>
<tr>
<td>CK</td>
<td>81</td>
<td>11 (14%)</td>
<td>4 (36%)</td>
<td>2 (50%)</td>
<td>6.8%</td>
</tr>
<tr>
<td>Alanine-to-Lysine Ratio</td>
<td>98</td>
<td>39 (40%)</td>
<td>20 (51%)</td>
<td>8 (40%)</td>
<td>15.9%</td>
</tr>
<tr>
<td>Acyl-carnitine</td>
<td>58</td>
<td>25 (40%)</td>
<td>10 (44%)</td>
<td>6 (60%)</td>
<td>23.8%</td>
</tr>
</tbody>
</table>

Table 2. Clinical neurodevelopmental diagnoses among subsets patients with abnormal biomarkers and an ASD control group without abnormal biomarkers. Both number of subjects and percent of total are provided.

<table>
<thead>
<tr>
<th></th>
<th>AD/PDD-NOS with motor delay</th>
<th>Isolated Delay</th>
<th>Speech Delay</th>
<th>Autistic Disorder</th>
<th>PDD-NOS</th>
<th>Other Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate (n=9)</td>
<td>4 (44%)</td>
<td>0 (0%)</td>
<td>1 (12%)</td>
<td>4 (44%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>AST (n=8)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (25%)</td>
<td>5 (63%)</td>
<td>1 (13%)</td>
<td></td>
</tr>
<tr>
<td>Alanine-to-Lysine Ratio (n=8)</td>
<td>2 (25%)</td>
<td>1 (13%)</td>
<td>2 (25%)</td>
<td>3 (38%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Acyl-carnitine (n=6)</td>
<td>0.0%</td>
<td>1 (17%)</td>
<td>2 (33%)</td>
<td>3 (50%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>ASD Control (n=9)</td>
<td>3 (33%)</td>
<td>0 (0%)</td>
<td>6 (67%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Clinical characteristics among subsets patients with abnormal biomarkers and an ASD control group without abnormal biomarkers. Both number of subjects and percent of total are provided.

<table>
<thead>
<tr>
<th></th>
<th>Regression</th>
<th>Epilepsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate (n=9)</td>
<td>2 (22%)</td>
<td>3 (33%)</td>
</tr>
<tr>
<td>AST (n=8)</td>
<td>3 (38%)</td>
<td>1 (13%)</td>
</tr>
<tr>
<td>Alanine-to-Lysine Ratio (n=8)</td>
<td>2 (25%)</td>
<td>6 (75%)</td>
</tr>
<tr>
<td>Acyl-carnitine (n=6)</td>
<td>4 (67%)</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>ASD Control (n=9)</td>
<td>5 (55%)</td>
<td>3 (33%)</td>
</tr>
</tbody>
</table>

RESULTS

Biomarker Prevalence

Table 1 outlines the prevalence of consistent elevations in biomarkers of energy metabolism. Abnormalities in lactate, alanine-to-lysine ratio and acyl-carnitines were very prevalent in the population studied, with abnormalities occurring over 30% of children screened. For most of the biomarkers, abnormalities were confirmed in about half of the cases except for alanine which was only confirmed in only 20% of the cases.

Clinical characteristics of subgroups with abnormal biomarker

The distribution of neurodevelopmental diagnosis for each subset of children with confirmed abnormal biomarkers as well as a control group of ASD children with no abnormal biomarkers is presented in Table 2. The subgroup of children that had elevated lactate demonstrated a much higher rate of motor delay as compared to the other groups while children with acyl-carnitine elevations did not have any children with
motor delays. Table 3 reviews the clinical characteristics for each subset of children with abnormal biomarkers as well as a control group of children with no abnormal biomarkers. Note the high rate of regression in the subgroup with elevations in acyl-carnitines and the high rate of seizures in the subgroup with elevated alanine-to-lysine ratio.

Metabolic characteristics of subgroups with abnormal biomarkers
In order to better understand the relationship between biomarkers of mitochondrial dysfunction and to validate these biomarkers as representing abnormal mitochondrial metabolism we compared all biomarkers within each subgroup to a group of children with ASD without any abnormalities in any biomarkers. To maintain valid statistical comparison, only groups that contained six or more patients were included. These included children with abnormal lactic acid, AST, alanine-to-lysine ratio and acyl-carnitine panels. The results for each of these subgroups will be discussed below.

Figure 1. Metabolic biomarkers which demonstrate significant differences between a subgroup with consistently elevated lactic acid and a control group of children without metabolic abnormalities. As compared to the control group, the subgroup of children with consistently elevated lactic acid demonstrated significantly higher values for venous lactic acid and ammonia and urine 2-methyl-3-hydroxybutyric acid.

The subgroup of children with a consistently abnormal fasting lactic acid were found to have, on average, a significantly higher venous lactic acid, venous ammonia and urine 2-methyl-3-hydroxybutyric acid, as compared to children with ASD with no metabolic abnormalities (See Figure 1). The significant elevation in venous lactic acid is expected but this also verifies that these children do indeed have a significant elevation when compared to other children with ASD. Ammonia elevations are not uncommonly seen in children with MD, making this abnormality consistent with the notion that this subgroup of children may have MD. Although 2-methyl-3-hydroxybutyric acid at high levels in
the urine is seen in the disorder 2-methyl-3-hydroxybutyric aciduria, a disease of isoleucine metabolism, other metabolic and clinical abnormalities consistent with this disorder were not found in these patients. Since 2-methyl-3-hydroxybutyric is a precursor to acetyl-CoA, it can accumulate if the citric acid cycle is inefficient or slow. Thus, an abnormality in citric acid cycle metabolism probably explains its relatively higher level in the urine in this subgroup. Two of the nine children in this group had mitochondrial DNA sequencing and examination of nuclear mitochondrial genes with the MitoMet array but all were negative, suggesting that a common genetic abnormality was not responsible for the metabolic abnormalities in this subgroup of children.

The subgroup of children with abnormally elevated AST values demonstrated a higher serum AST and lower urine 5-oxoproline than children without any metabolic abnormalities (See Figure 2). The significant elevation in AST is expected but this also verifies that these children do indeed have a significant elevation when compared to other children with ASD. 5-oxoproline, also known as pyroglutamate, is a metabolite of the gamma-glutamyl cycle which is involved in glutathione utilization and recovery. Decreased 5-oxoproline suggests decreased glutathione availability potentially from glutathione depletion. Glutathione depletion could result in a reduced ability of the liver to protect itself against oxidative stress and a reduced ability to neutralize toxins. This could result in liver dysfunction, resulting in an increased AST. Thus, these data suggest that AST elevations in this cohort of ASD patients was probably not directly related to MD but rather due to abnormal increases in oxidative stress.

**Figure 3.** Metabolic biomarkers which demonstrate significant differences between a subgroup of children with consistently elevated alanine-to-lysine ratio and a control group of children without metabolic abnormalities. As compared to the control group, the group of children with consistently abnormal alanine-to-lysine ratio demonstrated significantly higher values for venous alanine (left graph) and venous alanine-to-lysine ratio and lactate and urine pyruvate (right graph).

**Figure 4.** Metabolic biomarkers which demonstrate significant differences between a subgroup of children with consistent elevations in multiple acyl-carnitines and a control group of children without metabolic abnormalities. As compared to the control group, the group of children with consistent abnormal acyl-carnitines demonstrated significantly higher values for C5OH, C12, C14:1 C14OH and C16 acyl-carnitines and lower urine 3-OH-3-methylglutaric acid.
The subgroup of children found to have a consistent elevation in alanine-to-lysine ratio demonstrated a higher alanine, alanine-to-lysine ratio, venous lactate and urine pyruvate than children without any metabolic abnormalities (See Figure 3). The significant elevation in the alanine-to-lysine ratio is expected but verifies that these children do indeed have a significant elevation when compared to other children with ASD. All of these biomarkers are directly related to MD, suggesting that this is indeed a biomarker of MD. One of the eight children in this group had mitochondrial DNA sequencing which was normal, suggesting that a common genetic abnormality was not responsible for the metabolic abnormalities in these children.

Children with consistent elevations in acyl-carnitines were found to have higher C5OH, C12, C14:1 C14OH and C16 acyl-carnitines and lower urine 3-OH-3-methylglutaric acid than children without any abnormalities in metabolic biomarkers (See Figure 4). Thus, carnitines associated with both short and long, but not medium, chain fatty acids were elevated. This pattern is not consistent with a fatty-acid oxidation disorder as elevations in medium chain acyl-carnitines would also be expected to be elevated in a long-chain fatty acid oxidation disorder, while elevations in long-chain fatty acids would not be expected to be elevated in a short-chain fatty acid oxidation disorder. However, this pattern is consistent with a recently developed rodent model of ASD in which intraventricular propionic acid injections causes ASD type behavior, mitochondrial dysfunction and a unique pattern of short and long, but not medium, chain acyl-carnitine elevations. 17 3-hydroxy-3-methylglutaryl is a metabolite of acetyl-CoA, the starting point of the citric acid cycle. This suggests that the citric acid cycle is working inefficiently in this group of children with multiple abnormal acyl-carnitines. Three of the six children in this group had examination of nuclear mitochondrial genes with the MitoMet array and one patient had mitochondrial DNA sequencing but all genetic testing was negative, suggesting that a common genetic abnormality was not responsible for the metabolic abnormalities in these children. One patient had a muscle biopsy. The electron microscopy demonstrated an increased number of mitochondria and mild degeneration of membranous organelles, a finding that has been reported in other children with ASD and MD.1,2 Light microscopy demonstrated mild-to-moderate type II atrophy.

**Overlap of subgroups with abnormal biomarkers**

The biomarkers that were validated in the previous section were examined to determine overlap between the subgroups of children with these abnormal biomarkers. Of the nine individuals with consistent elevations in lactate, two (22%) demonstrate a consistent elevation in alanine-to-lysine ratio and one (12%) demonstrated consistent acyl-carnitine panel abnormalities. Of the eight individuals with consistent elevations in alanine-to-lysine ratio, two (25%) demonstrate a consistent elevation in lactate. Of the six individuals with acyl-carnitine panel elevations, one (17%) also demonstrated a consistent elevation in lactate. Thus, although there was some overlap between groups, most of the individuals in each subgroup were unique to that particular subgroup, suggesting that each biomarker may be independently useful.

**DISCUSSION**

In this study we examined a wide variety of biomarkers of MD in children evaluated in a medically-based autism clinic. We chose to concentrate on six biomarkers of MD: lactate, alanine, alanine-to-lysine ratio, CK, AST and the acyl-carnitine panel. Overall we found that three biomarkers, lactate, alanine-to-lysine ratio and acyl-carnitine panel, were abnormal in a large percentage of children with ASD and that elevations in these biomarkers could be verified as abnormal in about half of the cases. This not only suggests that these biomarkers should be examined in children with ASD but that it is important to verify these biomarker abnormalities with follow-up testing before proceeding to an extensive MD workup.

The groups of biomarkers with a high rate of being consistently abnormal were further investigated to verify that they truly represented a subgroup of children with MD. By examining other biomarkers of MD in the subgroups of children with these consistent abnormalities we verified that three of the four subgroups examined did indeed represent subgroups of children with ASD and mitochondrial metabolic abnormalities. Consistent abnormalities in lactate, alanine-to-lysine ratio and acyl-carnitine elevations appeared to be associated with other biomarkers of MD while elevations in AST appear to be associated with abnormalities in glutathione metabolism.

Each of the subgroups identified to represent mitochondrial metabolism abnormalities appears to have unique clinical characteristics. ASD children with consistent elevations in lactate appear to have a high incidence of motor delays. This corresponds to a recent meta-analysis that reviewed all reported cases of ASD/MD3 which demonstrated an overall high prevalence of motor delays. Since traditional MD evaluations, including two of three studies examining the prevalence of MD in ASD,11,18 use lactate as a primary biomarker for identifying children with MD, the data from this study confirm that children with ASD and elevated lactate may indeed have a high prevalence of motor delay. Consistent elevations in alanine-to-lysine ratio appear to be associated with epilepsy in our cohort of children with ASD. This is consistent with abnormalities associated with alanine-to-lysine ratio elevations such as ETC complex I deficiency, a respiratory chain abnormality associated with epilepsy.19 The subgroup of children with abnormalities in acyl-carnitine appeared to have a high incidence of regression. This is consistent with the fact that the pattern of acyl-carnitine abnormalities identified in this study are similar to the propionic acid rodent model of ASD17 and that propionic acid can be produced by Clostridia, a bacteria species that is found to be of increased incidence in the stool of children with regressive-type ASD.20 Peroxisomal disorders can result elevations in very long chain fatty-acid, but have not been considered in the current study; the role of such disorders could also be important in ASD and should be considered in the future.
The fact that the three primary biomarkers of MD verified in this study do not appear to have significant overlap suggests that there are significant subgroups of children with ASD/MD. Further research will need to further verify these biomarkers and investigate characteristics of these subgroups. Some have questioned whether ASD or MD should be considered the primary disorder in children with ASD/MD. The diagnosis of ASD is based on a constellation of symptoms regardless of the underlying cause. The DSM-IV-TR criterion for the diagnosis of ASD does not exclude a medical condition. In fact, it is not uncommon for children with ASD to be diagnosed with underlying genetic, metabolic, or neurologic disorders—for example, Tuberous Sclerosis, Prader-Willi syndrome, Fragile X, Phenylketonuria, or cerebral palsy. Thus, at this point, it is most appropriate to consider this as a dual diagnosis of both MD and ASD. Hopefully in the future, the connection between these two diagnoses will be better elucidated so their etiology can be better understood and such diseases can be better treated or even prevented.

These data lend support to the notion that disorders of energy production may affect a subset of children with ASD. This study demonstrates that several biomarkers of energy production are elevated in children with ASD and that these may represent independent groups of children. This suggests that the proportion of children with ASD and disorders of energy production may be higher than previous estimates as such estimates are based on a limited set of biomarkers, usually only lactate.

CONFLICT OF INTEREST
None.

SUPPORTS
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REFERENCES