



THE MICROBIOME IN AUTISM SPECTRUM DISORDER

Gastrointestinal dysfunction in autism spectrum disorder: the role of the mitochondria and the enteric microbiome

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Autism spectrum disorder (ASD) affects a significant number of individuals worldwide with the prevalence continuing to grow. It is becoming clear that a large subgroup of individuals with ASD demonstrate abnormalities in mitochondrial function as well as gastrointestinal (GI) symptoms. Interestingly, GI disturbances are common in individuals with mitochondrial disorders and have been reported to be highly prevalent in individuals with co-occurring ASD and mitochondrial disease. The majority of individuals with ASD and mitochondrial disorders do not manifest a primary genetic mutation, raising the possibility that their mitochondrial disorder is acquired or, at least, results from a combination of genetic susceptibility interacting with a wide range of environmental triggers. Mitochondria are very sensitive to both endogenous and exogenous environmental stressors such as toxicants, iatrogenic medications, immune activation, and metabolic disturbances. Many of these same environmental stressors have been associated with ASD, suggesting that the mitochondria could be the biological link between environmental stressors and neurometabolic abnormalities associated with ASD. This paper reviews the possible links between GI abnormalities, mitochondria, and ASD. First, we review the link between GI symptoms and abnormalities in mitochondrial function. Second, we review the evidence supporting the notion that environmental stressors linked to ASD can also adversely affect both mitochondria and GI function. Third, we review the evidence that enteric bacteria that are overrepresented in children with ASD, particularly *Clostridia* spp., produce short-chain fatty acid metabolites that are potentially toxic to the mitochondria. We provide an example of this gut–brain connection by highlighting the propionic acid rodent model of ASD and the clinical evidence that supports this animal model. Lastly, we discuss the potential therapeutic approaches that could be helpful for GI symptoms in ASD and mitochondrial disorders. To this end, this review aims to help better understand the underlying pathophysiology associated with ASD that may be related to concurrent mitochondrial and GI dysfunction.

Keywords: *autism spectrum disorders*; *Clostridia* spp.; *electron transport chain*; *enteric bacteria*; *fatty acid metabolism*; *gastrointestinal*; *mitochondrial dysfunction*; *oxidative stress*; *propionic acid*; *short-chain fatty acids*

Responsible Editor: Tore Midtvedt, Karolinska Institutet, Sweden.

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Received: 1 February 2015; Revised: 9 April 2015; Accepted: 10 April 2015; Published: 7 May 2015

The autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by impairments in communication and social interactions along with restrictive and repetitive behaviors (1). In the United States, ASD is now estimated to affect 1 in 68 individuals (2). Even though ASD is formally defined by a specific set of behaviors, the disorder has a wide heterogeneous spectrum of behavioral manifestations. Despite

the behavioral definition, many children with ASD have co-morbid medical conditions including gastrointestinal (GI) symptoms (3), seizures and epilepsy (4), attention deficits (5), anxiety (6), allergies (7) and mitochondrial disease (8, 9).

The etiology of ASD is not known at this time. Even though only a minority of individuals with ASD has inherited single gene or chromosomal defects, a great deal

of ASD research has traditionally concentrated on genetic causes of ASD (10). Recently, other areas of research have led to the increasing recognition that several physiological abnormalities are related to ASD. For example, an increasing number of research studies support evidence that metabolic disturbances, immune dysregulation, oxidative stress, and toxicant exposures could be linked to ASD (11, 12). Identification of these abnormalities is important as they may lead to screening, treatment and possibly even prevention strategies. For example, understanding which toxicant exposures could be linked to ASD could help identify important genetic vulnerabilities and could possibly lead to the development of prevention strategies (13). Furthermore, understanding metabolic disturbances could lead to identification of biomarkers and targeted treatments for specific metabolic abnormalities, which is important since treatments for metabolic disturbances have been shown to foster improvements in ASD symptoms (14–17).

Abnormalities in mitochondrial function are one of the most prevalent metabolic disturbances associated with ASD. A meta-analysis estimated that a significant subset of children with ASD manifest biomarkers of mitochondrial dysfunction (8) with some studies estimating that as many as 50%+ of children with ASD may manifest biomarkers of mitochondrial dysfunction when unique biomarkers, such as specific patterns of acylcarnitine abnormalities, are included (18, 19). Other studies that carefully examined electron transport chain (ETC) function in immune cells derived from children with ASD suggest that up to 80% of children with ASD exhibit some degree of abnormal ETC function (20, 21).

Mitochondrial dysfunction appears pervasive in ASD pathophysiology. Studies have demonstrated physiologic and genetic markers of mitochondrial dysfunction in the postmortem ASD brain (22–27). Mitochondrial dysfunction may be associated with several genetic syndromes that are highly associated with ASD, including Rett syndrome (28–30), Phelan–McDermid syndrome (31), 15q11-q13 duplication syndrome (32, 33), Septo-optic dysplasia (34) Down's syndrome (35, 36), and organic acidemias (37, 38). Many animal models of ASD also demonstrate the pervasive nature of mitochondrial dysfunction and its putative role in the pathophysiology of the disorder. Interestingly, mitochondrial dysfunction has been demonstrated in animal models induced by exogenous toxicants such as the propionic acid adult rodent model (39, 40) and the prenatal valproic acid exposure rodent model (41), as well as in genetic animal models of ASD, including the Rett syndrome (28), phosphatase and tensin homolog gene haploinsufficiency (42), and Angelman syndrome (43) rodent models of ASD.

The literature contains several reports of novel mitochondrial disorders in individuals with ASD. Several reports have documented that ETC activity is markedly

greater than normal for complex I in muscle (44) and complex IV in muscle (45, 46), skin (19), and brain (47). Related to this, studies from our laboratory have demonstrated that a subset of approximately one-third of the lymphoblastoid cell lines derived from ASD patients has increased mitochondrial respiratory activity (48, 49) as compared to control and other ASD lymphoblastoid cell lines. This subset of ASD cell lines exhibit increased vulnerability to oxidative challenges as compared to other ASD cell lines and controls, suggesting that these cell lines may be more vulnerable to endogenous and exogenous stressors that increase reactive species. Another novel mitochondrial disorder associated with ASD that is possibly linked to the enteric microbiome (19) will be described in this review.

Mitochondria are best known for their role in producing cellular energy. Thus, in individuals with disorders of mitochondrial function, their most affected body organs and systems are those that have the highest energy demand, including the central and peripheral nervous system, GI tract, muscles, and immune system. Interestingly, these are some of the same organs and systems commonly affected in children with ASD (9). Individuals with ASD who also have mitochondrial dysfunction are reported to have more severe behavioral and cognitive disabilities and are prone to neurodevelopmental regression compared to those with ASD without mitochondrial dysfunction (8, 50–52). Mitochondrial dysfunction may also explain the wide variety of medical abnormalities associated with ASD (9). In a recent meta-analysis, a review of all published cases of individuals with ASD and mitochondrial disease demonstrated that certain particular medical abnormalities are seen with a strikingly high prevalence in children with ASD and mitochondrial disease (8). In fact, while the prevalence of GI disorders in the general ASD populations was found to be 20%, the prevalence of GI disorders in individuals reported to have ASD with mitochondrial disease was 74%. This was also higher than children with mitochondrial disease without ASD, of which 39% were reported to have GI disorders. Thus, there may be a unique link between mitochondrial disease, GI disorders, and ASD.

This review will highlight the connections between GI disorders and mitochondrial abnormalities with reference to ASD. There appears to be at least three possible connections between the GI tract and mitochondrial abnormalities specific to ASD, which need not be mutually exclusive. First, mitochondrial dysfunction itself could result in GI dysfunction. Second, there are common exposures to environmental stressors that are associated with ASD that can affect both the mitochondria and the GI tract. Third, cell wall agents (i.e. lipopolysaccharide) (53) or metabolites from enteric bacteria (19, 40) could disrupt mitochondrial function. Each of these connections will be discussed in detail. We will also discuss the

potential treatments that may be effective for improving GI symptoms, given the mitochondrial pathophysiology we have highlighted.

Mitochondrial disease is associated with GI disorders

There is a strong association between GI symptoms and mitochondrial disease. In this section, we will review the evidence for the association between mitochondrial disease and GI disorders without specific reference to ASD. This will provide a framework to further discuss the link between GI symptomatology and mitochondrial dysfunction in ASD discussed later in this article. Several well-defined mitochondrial diseases with genetic underpinnings are strongly associated with GI abnormalities. We review the link between specific mitochondrial diseases and GI disorders, the importance of mitochondrial function for enterocyte function, and the evidence for GI abnormalities in mitochondrial disease in general.

Mitochondrial neurogastrointestinal encephalopathy syndrome is characterized by progressive GI dysmotility leading to pseudo-obstruction and severe GI symptomatology. This disorder is caused by a mutation in the *TYMP* gene, a gene for thymidine phosphorylase, which results in nucleotide pool imbalances and mitochondrial deoxyribonucleic acid (mtDNA) depletion. Interestingly, pseudo-obstruction is also caused by mutations in the nuclear *POLG* and *TMEM70* genes. The *POLG* gene codes for the mtDNA polymerase gamma that is responsible for replication of human mtDNA, and mutations in *POLG* also cause mtDNA depletion. The *TMEM70* gene codes for ETC complex V; complex V is responsible for making the energy carrier of the cell known as adenosine triphosphate (ATP). Researchers examining the prevalence of mitochondrial dysfunction in adults with chronic intestinal pseudo-obstruction have found that 15 of the 80 adult patients studied (19%) demonstrated biochemical and/or histological findings consistent with a mitochondrial disease (54). Five demonstrated *TYMP* mutations, five demonstrated *POLG* mutations, and two demonstrated mtDNA mutations consistent with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS). Genetic defects were not found in the remaining three patients with biochemical and/or histological evidence of mitochondrial disease. Almost all of the patients identified with a mitochondrial disorder also demonstrated neurological abnormalities. Another study examining eight children with chronic intestinal pseudo-obstruction could not find a genetic mutation that could account for a mitochondrial disease (55) so it was presumed unlikely in this cohort. However, the small sample size was a significant limitation to this latter study. In addition, the absence of a genetic mutation does not rule out mitochondrial disease. Indeed, functional testing of mitochondrial enzymes, which was not

performed, would be needed to evaluate the true function of the mitochondria.

Another GI disorder that has been linked to mitochondrial dysfunction is cyclic vomiting syndrome. Cyclic vomiting syndrome has been associated with MELAS (56, 57), Kearns–Sayre syndrome (58), large mtDNA deletions (59), *3010A* and *16519T* mtDNA polymorphisms (60, 61), the *A3243G* mtDNA mutation (62), and greater homoplasmic sequence variants in the mtDNA termination associated sequence (63). It also has a pattern of maternal inheritance consistent with mitochondrial disease (64). Several studies have suggested that the genetic basis of this disorder is different in children as compared to adults (64, 65). One study that looked at the prevalence of mitochondrial disorders in patients with cyclic vomiting syndrome found that 38% of patients had abnormalities in blood and/or urine suggesting evidence of mitochondrial dysfunction (66). Finally, effective treatments for cyclic vomiting syndrome overlap treatments used for mitochondrial disease including co-enzyme Q10, riboflavin, niacin, L-carnitine, and lipoic acid (67–69). Thus, cyclic vomiting syndrome appears to be strongly linked to mitochondrial dysfunction in a significant number of cases.

Several mitochondrial diseases are associated with progressive liver dysfunction (70). In fact, about 10–20% of childhood mitochondrial diseases involve liver dysfunction (71, 72). Perhaps, the most well known is Alpers–Huttenlocher syndrome, a syndrome caused by *POLG* mutations that presents with refractory seizures, developmental regression, and liver dysfunction (73). This disorder is well known because valproic acid can trigger acute liver failure leading to death (74). Like *POLG* mutations, other genetic abnormalities that impair mtDNA replication, including *C10orf2*, *DGUOK*, *MPV17*, *PEO1*, and *SUCLG1*, are part of the hepatocerebral mitochondrial DNA depletion syndromes, which are syndromes that include prominent liver failure (70, 75, 76). Genetic defects resulting in defects of mitochondrial protein synthesis also cause liver failure (70, 77). One of these mitochondrial protein synthesis abnormalities caused by *TRMU* gene mutations is unique in that the infantile-onset acute liver failure can spontaneously remit in many cases (78).

Pancreatic dysfunction is also a significant feature of some mitochondrial disorders (70). The best known of these is, perhaps, Pearson syndrome, which is a mitochondrial disorder caused by large-scale mtDNA rearrangements, which are characterized by exocrine pancreatic insufficiency, macrocytic anemia, and lactic acidosis (79). However, other mitochondrial disorders caused by mtDNA mutations have also been reported to involve pancreatic dysfunction (80). Endocrine pancreatic dysfunction in the form of diabetes mellitus has also been linked to several mitochondrial diseases (81, 82) and research has suggested that insulin resistance, the basic

abnormality in type 2 diabetes, involves mitochondrial dysfunction (83).

Interestingly, the organic acidurias, which are disorders involving metabolic enzymatic defects resulting in accumulation of organic acids, appear to directly or indirectly involve mitochondrial dysfunction (38) and commonly involve GI symptoms. For example, propionic acidemia and other branched-chain organic acidurias, such as methylmalonic aciduria, isovaleric aciduria, and maple syrup urine disease, commonly manifest vomiting during acute crises and can manifest pancreatitis in severe cases (84–86), whereas ethylmalonic encephalopathy is characterized by chronic diarrhea (87). Interestingly, propionic acid, which is significantly elevated in propionic acidemia, can have direct effects on GI physiology. In animal models, propionic acid reduces gastric motility (88), directly stimulates longitudinal colonic smooth muscle contractions (89), induces rapid large amplitude phasic contractions followed by tonic contractions in the distal colon via serotonin and prostaglandin release (90), and dilates colonic arteries resulting in a trophic effect on intestinal mucosa (91). This suggests that non-specific GI symptoms associated with propionic acidemia such as feeding refusal, vomiting, weight loss, and abdominal distension could be a direct effect of propionic acid on GI motility through a disruption in mitochondrial function.

Mitochondrial function may also be important at the level of the enterocytes. Perturbed enterocyte mitochondrial function is believed to initiate inflammation and relapses in inflammatory bowel disease, potentially through disrupting the balance between the enterocyte and the endogenous enteric microbiome (92) or by disrupting the active transport of luminal substrates or ion flux across the cell membrane. Disruption of mitochondrial function may be responsible for a wide range of disorders involved in enterocyte dysfunction, including (a) enterocyte dysfunction following traumatic brain injury (93, 94); (b) small bowel injury related to non-steroidal anti-inflammatory drug exposure (95–97), surgical manipulation (98, 99), intestinal allergic reaction (100), and carnitine deficiency (101); (c) cyclooxygenase inhibitor-induced enteropathy (102); (d) small bowel dysfunction in chronic alcoholics with liver disease (103), and rodent models of cirrhosis (104) and obstructive jaundice (105); (e) *Clostridium perfringens* enterotoxin-mediated enterocyte cell death (106, 107); (f) *Clostridium difficile* toxin A (108); (g) methotrexate-induced enteritis (109); and finally, (h) cases of villous atrophy (110, 111).

Several studies have taken a broader look at GI function in mitochondrial disorders in children. A case series described six children who initially presented with GI dysmotility within 2 weeks of life who experienced later onset of neurological symptoms. All were found to have significant ETC abnormalities without identifiable mtDNA mutations or neuropathic GI abnormalities (112). In

another case series of 36 children with diagnosed mitochondrial disorders, 20 (56%) were found to have GI abnormalities (113). Lastly, a rather elegant study found that, of 26 children with mitochondrial disorders, gastric emptying was delayed in 69% and intestinal transit time was prolonged in 46% (114). Thus, even in general mitochondrial disorders, GI symptoms seem to be common.

Interestingly, variations in mitochondrial function that do not cause frank disease may also be related to GI abnormalities. For example, one study found that specific mtDNA polymorphisms were associated with variations in satiation, rate of gastric emptying, GI pain symptoms, and specific types of irritable bowel syndrome (115). Thus, the connection between mitochondrial dysfunction and GI abnormalities is rather strong. Given that it is likely that a large proportion of children with ASD have abnormal mitochondrial function, it is possible that many of the comorbid GI abnormalities associated with ASD could be related, at least in part, to mitochondrial dysfunction.

Environmental and iatrogenic exposures can disrupt both GI and mitochondrial function

Both the mitochondria and GI tract are sensitive to environmental and iatrogenic exposures, and some of the exposures that can affect both the mitochondria and GI tract have been linked to ASD. In this section, we will review the known iatrogenic and environmental exposures that are associated with ASD and affect both GI and mitochondrial function.

There are many examples of iatrogenic exposures that have been linked to GI abnormalities, mitochondrial dysfunction, and ASD. Valproic acid is also known to cause hepatotoxicity (116) and pancreatitis (117) as well as being linked to mitochondrial disease and dysfunction (118), and exposure *in utero* increases the risk of developing ASD (119). In fact, the prenatal valproic acid exposure rodent model of ASD has been shown to manifest mitochondrial dysfunction (41). However, valproic acid is one of the few medications with good evidence for treating seizures and behavioral symptoms in children with ASD with a good safety profile (4), so its mechanism and effect on the mitochondria are likely to be complex and dependent on age or stage of development. Both prenatal and perinatal acetaminophen exposure (120) as well as acetaminophen exposure during childhood (121) have been associated with the development of ASD. Acetaminophen has also been associated with GI problems, including acute upper GI emergencies (122) and liver toxicity (123), and is believed to cause hepatotoxicity through its effect on the mitochondria (124). Exposures to antibiotics, either early in life (125) or during pregnancy (126), have been linked to the later developmental of ASD, and some have suggested that early antibiotic exposure could cause ASD (127, 128). Many believe that

mitochondria are evolutionarily derived from bacteria, leading to the potential for some antibacterial agents such as antibiotics to negatively influence host mitochondria (129–131). Indeed, antibiotics, particularly quinolones, aminoglycosides, and beta-lactams, some of which are not uncommonly used to treat childhood and maternal infections, can cause mitochondrial dysfunction (132) and are well known to cause adverse GI effects as well as altering the developing infant gut microbiome (132–134). Lastly, proton-pump inhibitors, which are used to treat gastroesophageal reflux, a disorder not uncommonly associated with ASD, impair the transportation of carnitine, potentially interrupting carnitine metabolism (135). In addition, proton-pump inhibitors can alter the pH of the GI tract. Since various bacteria grow optimally at a narrow pH range, proton-pump inhibitors can alter the enteric microbiome.

Several environmental exposures that have been linked to ASD can cause mitochondrial dysfunction and GI abnormalities. Pesticides and heavy metals that have been linked to ASD (13) also cause mitochondrial dysfunction and GI abnormalities. For example, in rodents, chlorpyrifos induces mitochondrial ultrastructure changes (136) and reduces mitochondrial enzyme function (137, 138), while chronic chlorpyrifos exposure increases gut permeability and bacterial translocation in rodents (139). Mercury induces mitochondrial-dependent apoptosis in human (140) and murine (141) cells, induces mitochondrial dysfunction in rodent liver and brain tissue (142), impairs the carnitine transporter (143), and reduces mitochondrial enzyme function in cells from murine (141, 144, 145) and zebrafish (146). Mercury causes dysfunction of human intestinal epithelium cells (147) and has been linked to chronic atrophic gastritis (148) while it also appears to significantly change the bacterial community structures in the gut microbiome in animal models (149).

Thus, from this brief review of some environmental exposures that have been linked to ASD, it is clear that certain such exposures have also been associated with both mitochondrial dysfunction and GI abnormalities. Although most studies have not looked at a causative link between GI abnormalities and mitochondrial function, given the fact that mitochondrial dysfunction can cause GI abnormalities, including gastric dysmotility, hepatic and pancreatic dysfunction, and abnormalities in enterocyte function, it is possible that mitochondrial dysfunction is the primary abnormality leading to the GI dysfunction associated with these exposures.

Alterations in the enteric microbiome may induce mitochondrial dysfunction

The human digestive tract is host to a complex array of intestinal bacterial flora that outnumber host cells by a factor of at least 10 to 1, and over 100 to 1 regarding the amount of genetic material. This ecosystem, termed the

enteric microbiome, behaves as a functional organ and produces a diverse array of bioactive metabolic products capable of entering the systemic circulation. It is important to note that the enteric microbiome and its metabolic products are not static and can be altered throughout the life cycle of the individual, with the first 18 months of life being an especially important time for the development of a stable enteric microbial ecosystem (150). The metabolic products from the enteric microbiome can have profound and dynamic effects on host metabolism, immune function, and gene expression in many organ systems, including the gut and brain (151–153).

Many authors have proposed a connection between the gut microbiome and ASD (39, 133, 134, 151, 154–156). There are several studies that point to overrepresentation of enteric bacteria, particularly *Clostridia* spp., in children with ASD (157–161), especially those with a regressive ASD phenotype (162, 163) and/or those children who present with GI symptoms at or before the onset of ASD symptoms (164). In addition, a small clinical trial demonstrated that treatment with vancomycin, an antibiotic aimed at decreasing *Clostridia* spp., is transiently effective in treating ASD symptoms (165).

Clostridia spp. are producers of the short-chain fatty-acid propionic acid (39, 40) following the fermentation of dietary carbohydrates and some proteins. Propionic acid, as well as other short-chain fatty-acid bacterial fermentation products (e.g. butyric and acetic acid), are compounds that are increasingly recognized as being important in the maintenance of health and have been implicated as possible contributing factors for certain disease processes (40, 166, 167). In particular, propionic acid can modulate cell signaling (e.g. specific free fatty acid G-protein-coupled receptors) (168, 169), cell–cell interactions (e.g. gap junctions) (170), gene expression (e.g. histone deacetylase inhibition) (171, 172), immune function (173), and neurotransmitter synthesis and release (174) as well as influence mitochondrial (175) and lipid (176, 177) metabolism. Interestingly, neurodevelopmental abnormalities that include ASD features are seen in individuals with impaired propionic acid metabolism (175, 178, 179). Furthermore, elevated propionic acid levels are present in the stool from individuals with ASD (180).

Interestingly, propionic acid is also endogenously present or added as a food preservative to a wide variety of foods including refined wheat and dairy products (181–187). Of note, propionic acid and its chemical derivatives have increasing use in agriculture and the food industry (166) and occur naturally in many foods (e.g. Swiss cheese). It is a major animal silage and food preservative in wheat and dairy products, either as a sodium or calcium salt (188, 189). Propionic acid is also produced by adding high fructose corn syrup substrate to propionibacteria cultures, which are then inoculated into foods. Inulin propionate has recently been suggested as a weight

loss agent (190, 191), and aspartame is known to increase propionic acid levels in rodent gut flora. Nitropropionic acid, a derivative of propionic acid produced by many plants and fungi, is a potential contaminant of processed rice and also produced in ruminant gut. It is a potent mitochondrial toxin, capable of causing neurotoxicity and administration in rodents is an acceptable model for Huntington's chorea (192). In addition, propionate is an important naturally occurring intermediate of odd-chain length fatty-acid oxidation.

Recently, we have developed an animal model of ASD (39, 40). In the initial model, brief intracerebroventricular pulsed infusions of propionic acid into adult animals produced reversible (~30 min) bouts of ASD-type behaviors including altered social interactions (193), stereotyped behavior (194), tics (194), and hyperactivity (194, 195) as well as other cognitive and sensorimotor deficits (196) [see Fig. 1 of (40) for behavioral videos of propionic acid rodent model of ASD, link: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3747729/figure/F0001/>]. The propionic acid rodent model of ASD also demonstrates biological abnormalities associated with ASD such as reactive astrogliosis and activated microglia (193, 194, 196) as well as abnormalities in redox, lipid, phosphatidylethanolamine, mitochondrial, acyl-carnitine, and carnitine metabolism

(177, 194, 195, 197). Electrographic abnormalities are also seen, specifically epileptiform-like spikes in the hippocampus, neocortex, and basal ganglia, with discharges in the basal ganglia associated with measurable extrapyramidal behavioral abnormalities (194).

Further development of this model shows that brief intracerebroventricular infusions of propionic acid into adolescent rats results in similar abnormalities in behavior, including abnormal restricted and repetitive behavior, altered social behavior, object vs. social preference, and cognitive abnormalities as well as ASD-like immunohistochemical evidence of innate neuroinflammation (198). To further validate this developmental model of ASD, animals were briefly exposed systemically to propionic acid prenatally and postnatally. Such exposure was found to alter conditioned taste and place avoidance (199), acoustic startle response and pre-pulse inhibition (200) and induced an increase in anxiety, repetitive, and impaired social behavior (201, 202), in rats as adolescents in a sexually dimorphic manner. In addition, propionic acid, and to a lesser extent butyric acid, modulates the expression of genes associated with ASD, including cell adhesion molecules (neurexin 1 and neuroligin), neurotransmitter systems, mitochondrial, redox, immune, and FMR1 genes in PC12 cells (172).

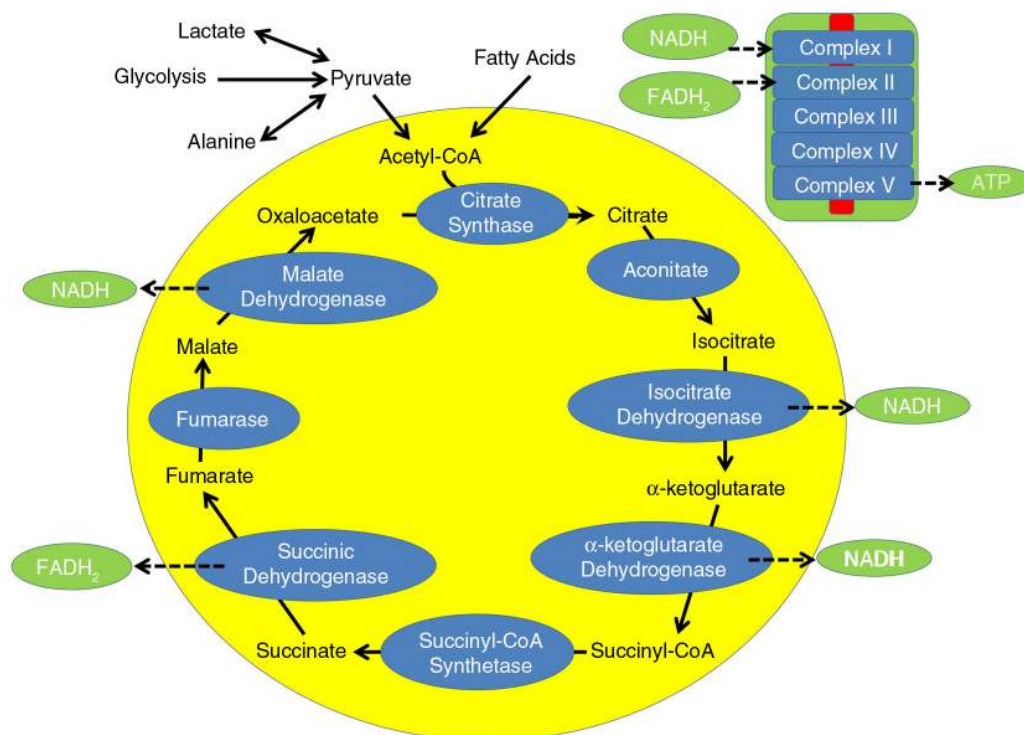


Fig. 1. The tricarboxylic acid cycle during typical metabolism. Carbohydrates and fatty acids enter the cycle as acetyl-CoA and through a series of enzymatic steps produce energy utilizing two electron carriers, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂). NADH and FADH₂ are metabolized by complex I and complex II, respectively, of the electron transport chain (ETC). Complex V of the ETC produces adenosine triphosphate (ATP), the energy carrier of the cell.

One of the unique biochemical markers of this rodent model was also independently reported in children with ASD (18), suggesting that this model has predictive validity. A pattern of abnormalities in fatty-acid metabolism characterized by elevations in short-chain and long-chain, but not medium-chain, acyl-carnitines were found in brain tissue of adult rats intracerebroventricularly infused with propionic acid (177). A similar pattern of elevation in acyl-carnitines was reported in children with ASD in a study that reviewed a wide range of metabolic markers from 133 consecutive patients evaluated in a medically based autism clinic (18). A standardized metabolic screening algorithm was used (203, 204) and abnormalities were verified with repeat testing (203). The workup included screening for fatty-acid oxidation defects by measuring a fasting acyl-carnitine panel. Consistent acyl-carnitine abnormalities were present in 24% of the patient sample. When the individuals with acyl-carnitine abnormalities were pooled, a specific pattern of abnormalities in acyl-carnitines was found that paralleled the acyl-carnitine elevation in the adult rodent propionic acid model of ASD: short-chain and long-chain acyl-carnitines but not medium-chain acyl-carnitines were elevated. Interestingly, 67% of this subset of children with ASD demonstrated neurodevelopmental regression, which is a particularly high rate compared to the general ASD population [about 25% (8)].

To further verify this pattern of abnormalities, we reviewed 213 patients with ASD seen in a medically based clinic who underwent a metabolic evaluation similar to the one noted above (19). Of the 213 patients screened, 17% were found to have consistent elevations in acyl-carnitines. When the data were combined across patients, C4OH, C14, and C16:1 were found to be significantly elevated at 186%, 226%, and 131% of the upper limit of normal. This subset of ASD patients with consistent elevations in short and long acyl-carnitines (CESLAC) was evaluated further.

Further testing of individuals with ASD and CESLAC ruled-out secondary causes of fatty-acid oxidation deficiencies including multiple carboxylase deficiencies, zinc deficiency, elevated copper, generalized hyperlipidemia or hypercholesterolemia, and hypoglycemia. Other biochemical markers for mitochondrial disorders were found in some CESLAC patients. Citrate and/or isocitrate was elevated in most CESLAC patients, and lactate was elevated in about half of the CESLAC patients. Genetic testing demonstrated a 22q13.1-q13.33 duplication in one (31) and suspicious novel maternally inherited homoplasmic variants of unknown significance in the cytochrome *b* gene in two (205). Thus, overall, no clear genetic factors seem to be consistent within these CESLAC patients that would explain their pattern of metabolic abnormalities.

Five CESLAC patients who underwent muscle biopsy had abnormal muscle histology, and electron microscopy of the muscle demonstrated abnormal mitochondria. ETC

studies conducted on the muscle (206) demonstrated a partial defect in ETC complex I and complex I + III. The reason for the pattern of ETC abnormalities, particularly a decrease in ETC complex I function, is not exactly clear, but one of the hypothesized mechanisms in the context of elevated propionic acid has been outlined in our recent publication (19). The significant inhibition of ETC complex I may be closely related to a decrease in the production of the reduced form of nicotinamide adenine dinucleotide (NADH) since NADH is the driving force for ETC complex I (Fig. 1). We believe that this is due to decreased activity of two enzymes in the citric acid cycle that produce NADH, specifically, isocitrate dehydrogenase and α -ketoglutarate dehydrogenase (Fig. 1). The reason for this is outlined below.

Using acetyl-CoA, propionic acid can produce propionyl-CoA, a compound that can be further metabolized to methylmalonyl-CoA (Fig. 2). Methylmalonyl-CoA is important because it can enter the citric acid cycle after being metabolized into succinyl-CoA (Fig. 2). Thus, high levels of propionic acid can be used to bypass the first four enzymes of the citric acid cycle, thereby functionally 'short circuiting' the citric acid cycle (Fig. 2). This results in a bypass of important steps of the citric acid cycle (including two enzymes that make NADH) and elevates succinyl-CoA concentrations that can further negatively modulate these bypassed enzymes through several mechanisms. First, high levels of succinyl-CoA will result in high levels of citric acid cycle intermediates that precede it in the cycle, such as α -ketoglutarate, isocitrate, and citrate (the latter two were found to be elevated in this ASD patient cohort) because their breakdown is stoichiometrically inhibited by high levels of their breakdown products. Second, succinyl-CoA directly inhibits citrate synthase, the first step in the citric acid cycle (Fig. 2) as well as α -ketoglutarate dehydrogenase, the citric acid cycle enzyme that produces it and an enzyme that produces NADH (Fig. 2). In addition, elevated levels of α -ketoglutarate will inhibit isocitrate dehydrogenase, the citric acid cycle enzyme that produces it and another enzyme that produces NADH (Fig. 2).

Thus, an increased succinyl-CoA concentration as a result of high levels of propionic acid can shut down the first half of the citric acid cycle and prevent the production of two of the three NADH molecules typically produced by the citric acid cycle. As ETC complex I transfers electrons from NADH, whereas ETC complex II transfers electrons from flavin adenine dinucleotide in the hydroquinone form (FADH₂), it is not surprising that ETC complex I is found to be underactive (especially relative to ETC complex II) in the muscle of CESLAC patients.

The elevations in acyl-carnitines in CESLAC patients suggest that at least some specific parts of fatty-acid oxidation are inhibited. This is important as each turn of

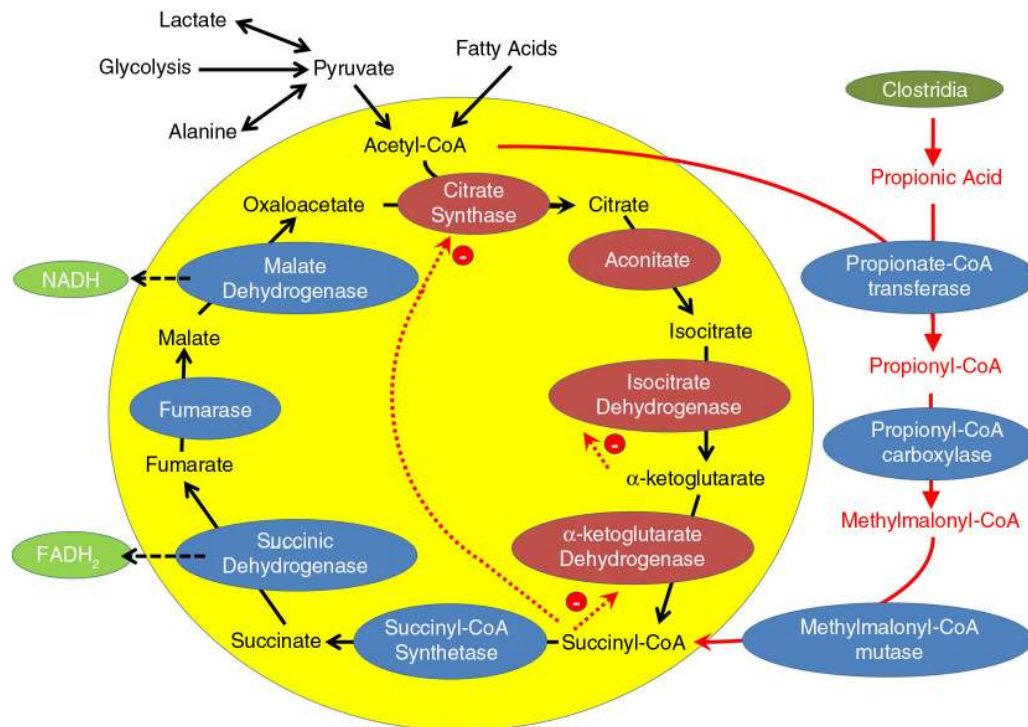


Fig. 2. The tricarboxylic acid cycle during high levels of propionic acid. Propionic acid, presumably derived from *Clostridia* spp., is metabolized to propionyl-CoA using acetyl-CoA. Propionyl-CoA is further metabolized into methylmalonyl-CoA, which enters the tricarboxylic acid cycle as succinyl-CoA. Succinyl-CoA inhibits the first and fourth enzyme in the tricarboxylic acid cycle. In this manner, propionic acid may ‘short circuit’ the tricarboxylic acid cycle, thereby reducing the production of nicotinamide adenine dinucleotide (NADH). This decrease in NADH is hypothesized to cause the decrease in complex I activity measured in the patients with consistent elevations in short and long acyl-carnitines (CESLAC).

β -oxidation (the key reaction of fatty acid oxidation) produces three NADH for each two FADH₂, so inhibition of β -oxidation will further decrease NADH relative to FADH₂. The products of fatty-acid oxidation and how they enter the citric acid cycle are dependent on the length of the fatty acid, specifically whether the fatty acid is odd or even in length. Each turn of β -oxidation shortens the fatty acid by a length of two carbons and produces one acetyl-CoA. This is the same for both odd and even length fatty acids until the last step when the fatty acid is reduced to either a two- or three-carbon fatty acid. If the fatty acid is even in length, then the last step in the breakdown of the fatty acid produces an acetyl-CoA, while the last step in the breakdown of an odd length fatty acid results in propionyl-CoA. The significant elevations in the acyl-carnitines in this cohort were composed of even length fatty acids, suggesting that the breakdown of acetyl-CoA is inhibited relative to propionyl-CoA. This is consistent with the notion that the alternative pathway that ‘short circuits’ the citric acid cycle is upregulated. The fact that acetyl-CoA breakdown may be dependent on the availability of propionic acid when this alternative pathway is upregulated means that

β -oxidation may be stoichiometrically inhibited if propionic acid is not available. If the source of propionic acid is the metabolic fermentation products of certain enteric bacteria, then the level of propionic acid will depend on enteric factors that may vary from day-to-day and hour-to-hour, such as diet, transcolonal uptake, or GI transport time. The reason why medium-chain fatty acids are not particularly elevated in this cohort is not clear at this time. Examination of the β -oxidation pathway in fibroblasts did not demonstrate any consistent abnormalities across patients examined (207), again pointing to an acquired functional inhibition of the fatty-acid oxidation pathway rather than a fixed deficit in this pathway. Overall, the elevation in acyl-carnitines in CESLAC patients appear to not be due to enzymatic defects but rather due to a functional abnormality related to altered metabolism.

CESLAC patients also demonstrated significant changes in glutathione metabolism suggesting increased oxidative stress and increased cellular vulnerability to reactive oxygen and nitrogen species. This finding could be related to the findings of mitochondrial dysfunction. First, aconitase, the second enzyme in the citric acid cycle, is redox

sensitive and its activity has been shown to correlate with the glutathione redox ratio in the brain of individuals with ASD (22). Second, ETC complexes I and III are inhibited by reactive oxygen species. Thus, the abnormal redox state of the cell could also be inhibiting the specific mitochondrial enzymes that appear to be dysfunctional in CESLAC patients and in a similar manner could affect the mitochondria in the brain tissue in the propionic acid animal model of ASD (19, 40, 195).

Interestingly, ETC function in fibroblasts from the CESLAC patients was almost opposite from the results from the muscle, with complex I activity being elevated rather than depressed. However, fibroblasts are grown in culture for approximately 6 weeks, thereby representing mitochondrial function without the influences of any systemic metabolites or modulators, whereas muscle ETC function is derived from freshly frozen muscle that essentially reflects the influences of the body *in situ*. Thus, the fibroblast results suggest that the enzymes themselves are not dysfunctional, but rather that they are being actively inhibited by the *in situ* environment of the human body.

Thus, the detailed examination of CESLAC patients reveals unique changes in ETC and citric acid cycle function consistent with the excess metabolic flux of propionic acid (19). Theoretically, propionic acid can be overproduced by the overrepresented species of *Clostridia* found in the GI tract of children with ASD (39, 157, 180) and could result in mitochondrial dysfunction. Systematic effects of propionic acidemia, an organic acidemia in which propionic acid builds up excessively, are being recognized to be due, in part, to mitochondrial dysfunction (38).

Several lines of evidence link propionic acid with ASD. The propionic acid rodent model of ASD demonstrates ASD-like behavioral abnormalities as well as other biochemical and immune abnormalities associated with ASD (40). Our recent study demonstrated that short-chain fatty acids, such as propionic acid, can modulate the expression of many ASD implicated genes, including those involved in mitochondrial function (172). Propionic acid can have direct effects on GI motility (88–90) and perfusion (91) reminiscent of GI abnormalities associated with ASD (40, 194). Interestingly, behavioral and GI symptoms in ASD have been reported to improve when simple carbohydrates, which can be fermented by enteric bacteria to produce propionic acid, are eliminated from the diet (40, 208) or when propionic acid producing bacteria are transiently eradicated by antibiotics (165). This is clearly a novel area of ASD research that requires further study in patients with ASD and with additional animal studies.

Leading toward potential treatment

Given these insights into the relationship between mitochondrial dysfunction and GI abnormalities in ASD, there may be therapeutic avenues that can be explored for

at least some children with ASD and these underlying conditions.

Given that propionic acid is a short-chain fatty acid that can complex with L-carnitine, increased propionic acid production could also potentially explain the common relative carnitine deficiency documented in children with ASD (19, 39, 40). Interestingly, clinical trials, including two double-blind placebo-controlled trials, have demonstrated that ASD symptoms can be improved with L-carnitine treatment (33, 209–215), with one trial demonstrating that the improvement in symptoms was related to the improvement in the L-carnitine levels in the blood. However, L-carnitine also increases the transport of fatty acids across the enterocytes into the body and has been suggested to be potentially detrimental if unhealthy fatty acids are ingested (216). Thus, it is important to consider that supplementation with L-carnitine could increase the flux of propionic acid from the gut into the body in the presence of bacteria that produce this metabolite. This could theoretically explain some of the adverse effects seen with L-carnitine clinical trials in some children with ASD. Acetyl L-carnitine, which transports the ‘beneficial’ acetate moiety for driving TCA function, may theoretically be superior, but this remains to be explored. Still, it is possible that propionic acid could potentially be used as a fuel for the mitochondria, if mitochondrial metabolism was able to adapt accordingly, and this might be dependent on the form in which it is delivered. For example, propionyl-L-carnitine, but not L-carnitine or propionate, is therapeutic to intestinal pathology in ulcerative colitis (217). Lastly, the GI pathology in a rodent model of colitis induced by early exposure to propionic acid or trinitrobenzene sulfonic acid was reduced by pre-administration of L-carnitine (218, 219).

Interestingly, the ketogenic diet has been shown to improve ASD-like symptoms in mouse models of ASD, including the BTBR (220), EL (221), and prenatal valproic acid exposure (41) mouse models. The ketogenic diet has been rated as very effective for controlling seizures and improving behavior and cognition in children with ASD (222–224) and has been recommended for children with ASD, especially those with epilepsy, in several systematic and expert literature reviews (4, 225). The ketogenic diet has been found to regulate key signaling pathways that both enhance mitochondrial function and promote neuroprotection (226) and is recommended for a wide variety of mitochondrial disorders (227, 228). With special reference to the novel mitochondrial disorder we have described in the CESLAC patients, several lines of research suggest that the ketogenic diet may be particularly useful for complex I defects (229, 230). Interestingly, the finding that the main high affinity blood–brain barrier transporter of propionic acid can be competitively inhibited by hydroxybutyrate (231) gives a potential mechanism where ketogenic diets may work through

competitive inhibition of propionic acid transport-mediated entry into the brain.

Effective treatments for cyclic vomiting syndrome, a disorder that most likely has heavy mitochondrial underpinnings, especially in children, overlap with treatments used for mitochondrial disease including co-enzyme Q10, riboflavin, niacin, L-carnitine, and lipoic acid (67–69). Some of these same treatments have reportedly been found to improve ASD symptoms, including L-carnitine (33, 209–215), a multivitamin containing riboflavin, niacin and coenzyme Q10 (232, 233), and ubiquinol (234).

Several heavy metals have been associated with ASD (13). There is growing interest in using typical enteric microbiome flora such as *Lactobacillus*, which have a natural ability to bind to toxic heavy metals as a means for protecting against exposures (235). In an open-label trial, *Lactobacillus rhamnosus GR* was protective against increasing levels of blood mercury and arsenic in pregnant women, but not children (236). Given the wide use of probiotics in children with ASD and the growing excitement as a result of recent animal models of ASD (237), further research in this area might be promising.

Melatonin is widely used for assisting with sleep initiation in children with ASD and has been shown to be effective for improving sleep in several controlled clinical trials (238, 239). Besides the hormonal effects of melatonin, it also appears to have antioxidant and anti-inflammatory properties as well as modulating mitochondrial function (240). Interestingly, melatonin is produced in and has receptors throughout the GI tract (240) and has been shown to modulate serotonin-induced contractions and gastric glandular mucosal blood flow (241). Melatonin is being investigated for its role in protection and healing of the GI tract (242). Thus, melatonin could be especially helpful for children with ASD who have comorbid GI and mitochondrial abnormalities.

Small bowel injury related to surgical manipulation appears to involve mitochondrial dysfunction within enterocytes (98, 99). Several animal studies have suggested that supplementation with metabolic intermediates can protect the bowel from injury in animal models through a mitochondrial mechanism. Supplementation with α -ketoglutarate improves intestinal morphology, antioxidant capacity, and parameters of mitochondrial energy production in the lipopolysaccharide-induced intestinal damage animal model (243). Interestingly, α -ketoglutarate can be converted into glutamate, another metabolic intermediate and a neurotransmitter, by mitochondrial glutamate dehydrogenase in the GI tract. Glutamine is an important nutrient and fuel for the GI tract during illness, stress, and injury and can be produced by glutamate and ammonia. Pretreatment with glutamine prior to surgical gut manipulation in rodents prevents gut and lung injury due to oxidative and inflammatory processes and protects enterocyte mitochondria (244). Interestingly,

the protective effect of enterocyte mitochondria during surgical stress may also be related to nitric oxide production as pretreatment with L-arginine protects enterocyte mitochondria from the effects of intestinal manipulation, but not when nitric oxide synthase is inhibited (245).

Conclusion

This manuscript outlines several of the connections between mitochondrial dysfunction, enteric microbiome abnormalities, and GI abnormalities in relation to ASD. There are many potential interactions between basic biological mechanisms and disease etiologies, which can result in symptoms of ASD and GI tract abnormalities as well as mitochondrial dysfunction. Considering the effect of the microbiome and its metabolic products adds another layer of complexity onto an already complex story. Understanding the interaction between ASD and GI symptoms in the context of mitochondrial dysfunction can provide a greater understanding of the underlying pathophysiology and how biological abnormalities are related to the complex behavioral manifestations characteristic of ASD. Considering the potential etiologies that can cause mitochondrial dysfunction, ASD and/or GI tract dysfunction can provide insight into the possible etiological factors contributing to ASD. Lastly, considering treatments that can address these underlying abnormalities may lead to the development of new treatments that reduce symptoms and improve cellular metabolism.

Acknowledgments

The authors express their heartfelt thanks to countless parents and caregivers of persons with autism and to David Patchell-Evans, for his tireless devotion to persons with autism, and his daughter, Kilee Patchell-Evans.

Conflict of interest and funding

The authors have no conflicts of interest or financial disclosures to declare. This research was supported by the Arkansas Biomedical Institute (REF), Autism Canada (DFM), Autism Research Institute (DFM, REF), and GoodLife Children's Charities (DFM).

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