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REVIEW ARTICLE

Clinical and Molecular Characteristics of Mitochondrial Dysfunction in Autism Spectrum Disorder

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Abstract

Autism spectrum disorder (ASD) affects ~ 2% of children in the United States. The etiology of ASD likely involves environmental factors triggering physiological abnormalities in genetically sensitive individuals. One of these major physiological abnormalities is mitochondrial dysfunction, which may affect a significant subset of children with ASD. Here we systematically review the literature on human studies of mitochondrial dysfunction related to ASD. Clinical aspects of mitochondrial dysfunction in ASD include unusual neurodevelopmental regression, especially if triggered by an inflammatory event, gastrointestinal symptoms, seizures, motor delays, fatigue and lethargy. Traditional biomarkers of mitochondrial disease are widely reported to be abnormal in ASD, but appear non-specific. Newer biomarkers include buccal cell enzymology, biomarkers of fatty acid metabolism, non-mitochondrial enzyme function, apoptosis markers and mitochondrial antibodies. Many genetic abnormalities are associated with mitochondrial dysfunction in ASD, including chromosomal abnormalities, mitochondrial DNA mutations and large-scale deletions, and mutations in both mitochondrial and non-mitochondrial nuclear genes. Mitochondrial dysfunction has been described in immune and buccal cells, fibroblasts, muscle and gastrointestinal tissue and the brains of individuals with ASD. Several environmental factors, including toxicants, microbiome metabolites and an oxidized microenvironment are shown to modulate mitochondrial function in ASD tissues. Investigations of treatments for mitochondrial dysfunction in ASD are promising but preliminary. The etiology of mitochondrial dysfunction and how to define it in ASD is currently unclear. However, preliminary evidence suggests that the mitochondria may be a fruitful target for treatment and prevention of ASD. Further research is needed to better understand the role of mitochondrial dysfunction in the pathophysiology of ASD.

Key points

Clinical aspects of mitochondrial dysfunction in autism spectrum disorder (ASD) include unusual neurodevelopmental regression, especially if triggered by an inflammatory event, gastrointestinal symptoms, seizures, motor delays, fatigue and lethargy.

Many genetic abnormalities have been associated with mitochondrial dysfunction in ASD, including chromosomal abnormalities, mitochondrial DNA mutation and large-scale deletions, and mutations in both mitochondrial and non-mitochondrial nuclear genes.

Several environmental factors, including toxicants, microbiome metabolites and an oxidized microenvironment have been shown to modulate mitochondrial function in ASD tissues.

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1 Introduction

Autism spectrum disorder (ASD) affects ~ 2% of children in the United States. The cause(s) of ASD are still unknown, but evidence for a simple genetic defect is lacking [1]. The etiology of ASD likely involves environmental factors that affect cell signaling, metabolic, immune and epigenetic processes in genetically sensitive individuals [1, 2]. Of note, ASD is associated with physiological disturbances including abnormal redox and mitochondrial metabolism. In fact, between 5% and 80% of children with ASD demonstrate evidence of mitochondrial dysfunction, with many demonstrating novel types of mitochondrial dysfunction rather than classic mitochondrial disease (MD) [3–5]. This is in comparison to the general population where MD is believed to affect less than 0.1% of the population [6]. A systematic review of the literature on mitochondrial dysfunction in individuals with ASD was published in 2012 [3] based on the recognition of early reports of mitochondrial dysfunction in ASD [7–14], but an update to this systematic review has not been published in over 5 years, during which time both genetic and metabolic laboratory testing have advanced significantly and new techniques for measuring mitochondrial function have become accessible.

2 Methods

2.1 Search Strategy

A prospective protocol for this systematic review was developed a priori, and the search terms and selection criteria were chosen to capture all pertinent publications. A computer-aided search of PUBMED, Google Scholar, CINAHL, EmBase, Scopus and ERIC databases from inception through February 2018 was conducted to identify pertinent publications using the search terms 'autism', 'autistic', 'Asperger', 'ASD', 'pervasive', and 'pervasive developmental disorder' in all combinations with the terms 'mitochondria' OR 'mitochondrial' OR 'lactic' OR 'lactate' OR 'pyruvate' OR 'pyruvic' OR 'ammonia' OR 'creatine kinase' OR 'oxidative phosphorylation' OR 'phosphorylation' OR 'carnitine' OR 'acyl-carnitine' OR 'fatty acid oxidation' OR 'alanine' OR 'respiratory chain' OR 'electron transport chain' OR 'ATP' OR 'adenosine.' The references cited in identified publications were also searched to locate additional studies.

2.2 Study Selection

One reviewer (DAR) screened the titles and abstracts of all potentially relevant publications. Studies were initially included if they (1) involved individuals with ASD and (2) reported at least one finding that could indicate mitochondrial dysfunction. Abstracts or posters from conference proceedings were included if published in a peer-reviewed journal. After screening all records, 293 publications met inclusion criteria; two reviewers (DAR, REF) then independently reviewed these articles (Fig. 1). Articles were excluded if they:

1. Did not involve humans or human cells (e.g., animal models).
2. Did not present new or unique data (such as review articles or letters to the editor).
3. Presented duplicate data.
4. Reported biochemical markers related to a non-mitochondrial disorder or cellular mechanism.
5. Reported markers related to a known side effect of a medication (for example, elevated ammonia from valproic acid or rhabdomyolysis from olanzapine).

Overall, 220 studies were selected for this review. Other studies that support the discussion of MD in ASD are also referenced.

3 Summary of the Literature

3.1 Clinical Aspects

Only a limited number of studies have examined the clinical characteristics of children with ASD and MD. Weissman et al. [15] was one of the first compilations of clinical symptoms of children with ASD diagnosed with MD (ASD/MD). In their case series of 25 patients, they noted a high rate of non-neurological medical problems, including gastrointestinal (GI) dysfunction and prenatal or perinatal complications, constitutional symptoms such as excessive fatigability and exercise intolerance, early gross motor delay and unusual patterns of regression, including multiple regression and regression after 3 years of age.

Rossignol and Frye [3] reviewed the clinical characteristics of all the reported cases of children with ASD/MD and compared these to the clinical characteristics of the general ASD population (ASD/NoMD) as well as children with MD but not ASD (MD/NoASD). As compared to ASD/NoMD children, ASD/MD children had a higher rate of neurodevelopmental regression, seizures, gross

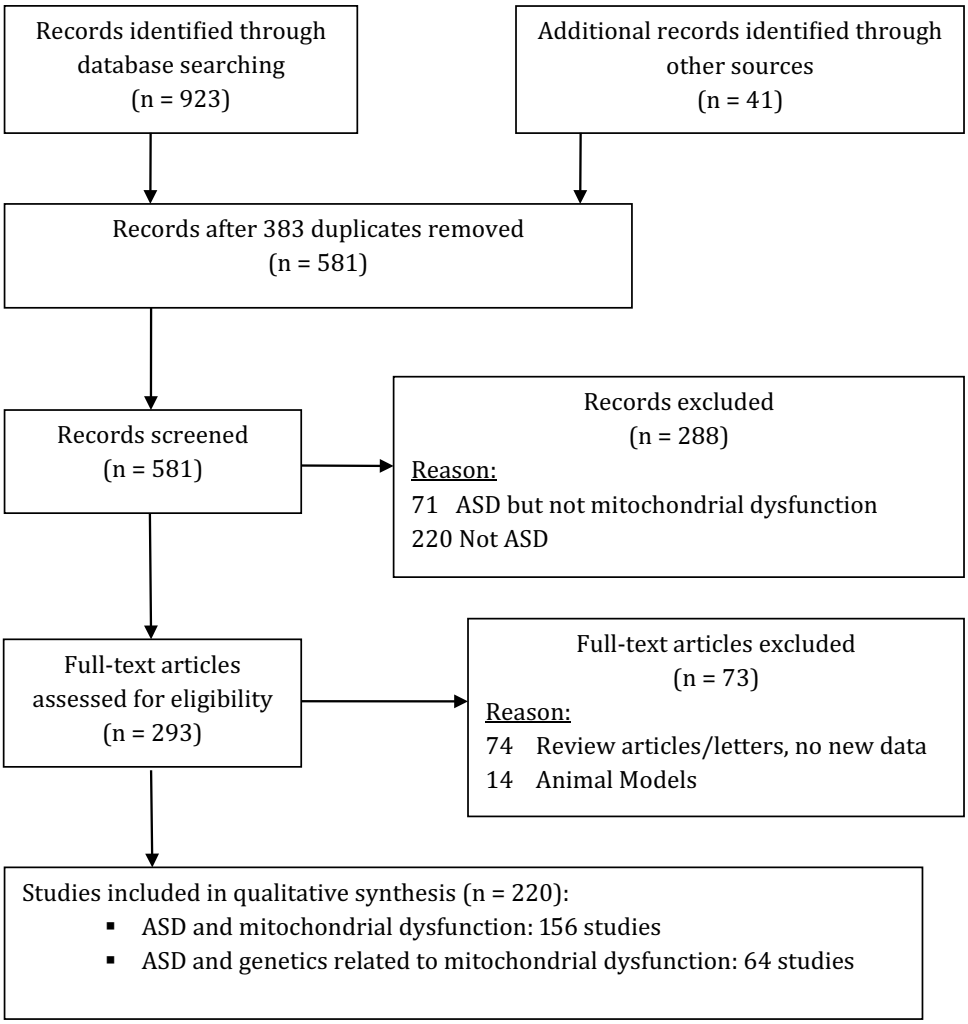

Fig. 1 Flowchart of systematic review and study selection. *ASD* autism spectrum disorder

motor delay and GI abnormalities. As compared to MD/NoASD, ASD/MD children demonstrated higher rates of fatigue and lethargy, ataxia, GI abnormalities and normal brain imaging and were less likely to have abnormal light microscopy. ASD/MD children were more likely to have elevated lactate than both MD/NoASD and ASD/NoMD groups.

Frye and Rossignol [16] pointed out that many of the clinical symptoms outlined in the Morava criteria [17], which set forth clinical diagnostic criteria for MD, overlap with characteristics of children with ASD, including developmental delays, seizures, neurodevelopmental regression, GI and endocrine abnormalities, familial recurrence and neuropathies.

In the evaluation of the etiology of a person with ASD, the presence of regression especially with recurrent episodes and multiple organ dysfunctions should prompt an extended evaluation for MD [18, 19]. Indeed, Shoffner et al. [20] demonstrated that most patients diagnosed by his center with ASD/MD experienced neurodevelopmental regression resulting in the development of ASD following an inflammatory event associated with a fever within the preceding 2 weeks.

Several case series have noted the association between mitochondrial dysfunction in ASD and epilepsy [15, 20]. Indeed, one of the first descriptions of the overlap of mitochondrial dysfunction and ASD was the HEADD syndrome, characterized by hypotonia, epilepsy, autism, and developmental delay [21]. These authors noted electron transport chain (ETC) complex deficiencies in subunits encoded by mitochondrial DNA (mtDNA) and that several patients demonstrated large-scale mtDNA deletions. Interestingly, these authors noted a high rate of ETC Complex III (C3) deficiency, which has been echoed in other case reports of children with ASD/MD and epilepsy [22, 23]. Another interesting case report linked mitochondrial dysfunction with ASD in a patient with Dravet syndrome, suggesting that even clearly genetically based epilepsy with ASD could have a mitochondrial component [24].

Lastly, one report suggests that children with ASD and classic MD are more likely to also have intellectual disabilities, suggesting early identification and treatment may be particularly useful for these children [25].

3.2 Biomarkers of Mitochondrial Disorders

3.2.1 Traditional Biomarkers of Mitochondrial Dysfunction

There are several biomarkers which are commonly used to identify individuals with MD, including lactate, pyruvate, alanine and creatine kinase. However, none of these biomarkers are very specific. In the 2012 meta-analysis of biomarkers in MD by Rossignol and Frye, several traditional biomarkers were elevated in many children with ASD. Using normal reference ranges, studies have demonstrated that ASD was associated with elevations in lactate, pyruvate, lactate-to-pyruvate ratio, alanine, creatine kinase, ammonia and aspartate aminotransferase (AST) and depression in carnitine [3]. Separate studies verified mean elevations in lactate and pyruvate as well as depressions in carnitine and ubiquinone in individuals with ASD as a group compared to contemporaneous controls [3]. Lactate, pyruvate, carnitine, creatine kinase, AST and alanine aminotransferase (ALT) all demonstrated significantly more variation in the ASD group as compared to contemporaneous controls.

Interestingly, elevation in lactate was the first indicator that children with ASD may have a mitochondrial disorder. First reported in 1985 before MD was well described in medicine, Dr Mary Coleman suggested that children with ASD might have a disorder of carbohydrate metabolism [26]. Since the meta-analysis in 2012, lactate continues to be reported as a potential biomarker for abnormal mitochondrial metabolism in children with ASD [27–30]. Despite these consistent elevations in lactate, it is important to consider that many factors can falsely elevate lactate, such as excessive muscle movement, which can occur if the child is struggling or because the tourniquet is left on too long. Thus, the search for alternative and more reliable biomarkers of mitochondrial dysfunction are being pursued for diagnosing MD in general as well as identifying children with ASD that might have mitochondrial dysfunction.

Other traditional biomarkers that have continued to be reported as abnormal in ASD include pyruvate [28], lactate-to-pyruvate ratio [31], creatine kinase [28, 30, 32], AST [27, 30], ALT [27] and ubiquinone [28, 33]. Other studies have reported abnormal urine citric acid cycle metabolites which are also biomarkers of mitochondrial dysfunction [34, 35].

Aside from elevations in alanine, some clinicians use the ratio of alanine to lysine as pioneered by Richard Kelley [36], and others have found abnormalities in other amino acids possibly related to mitochondrial metabolism [37]. In a study of 25 high-functioning males with ASD, plasma levels of arginine were elevated while 5-oxoproline was reduced [38]. A study of 60 families found elevations in plasma glutamate and aspartate [39]. Another study reported reduced plasma cysteine, tyrosine, serine, α-aminoadipic acid, carnosine and β-alanine and increased levels of glutamic acid, hydroxyproline, phosphoserine and β-amino-isobutyric acid [40]. Another study has reported elevation in γ-aminobutyric acid and glutamic acid in individuals with ASD [41]. Although plasma taurine was reported to be elevated in one study [38], another study suggested that this elevation was also found in matched related controls [39] and an another study found no differences in taurine as compared to sibling and parent controls [42]. The variability across studies in amino acid metabolites could be due to several factors. Indeed, the metabolism and transport of certain amino acids has been suggested to be abnormal in children with ASD. For example, increased transport of alanine and decreased transport of tyrosine across the cell membrane was found in fibroblast culture from 11 children with ASD [43], a genetic disorder of branched chain amino acid metabolism has been described in children with ASD [44], and abnormalities in transport of amino acids across the blood–brain barrier has also been implicated in ASD [45]. However, dietary intake and whether or not the sample was post-prandial or fasting have probably the most influence on plasma levels of amino acids.

3.2.2 Buccal Swab

Biochemical measurements of mitochondrial function can be difficult to obtain. Direct measurement of mitochondrial function by enzymology typically requires invasive biopsy, requiring anesthesia, which has risks, especially in individuals with MD. Biopsy also limits the ability to repeat the test over time to follow disease status. Goldenthal et al. [46] developed and validated the non-invasive buccal swab technique, showing that buccal tissue enzyme measurements correspond to measurements from muscle biopsy in individuals with MD. The buccal swab technique has been used to measure mitochondrial function in individuals with MD [46–48], genetic syndromes [48–50] and ASD [50–54].

Five studies have used the buccal swab technique in individuals with ASD. Four of these studies have examined citrate synthase (CS) as well as ETC Complex I (C1) and ETC Complex IV (C4) activity normalized to CS [51, 53, 54], while one study also measured ETC Complex II (C2) and C2 + C3 activity [49]. Goldenthal et al. [51] noted several unique characteristics of mitochondrial function in children with ASD who were not taking mitochondrial supplements for at least 2 weeks. First, C1 demonstrated a significantly higher variation in ASD participants as compared to a control population, with 12% and 7% of children with ASD demonstrating C1 activity lower and higher, respectively,

than the control range. There were also a significant number of individuals with ASD who demonstrated abnormal C4 activity, with 28% and 3% demonstrating C4 activity lower and higher, respectively, than the control range. The authors note that individuals with ASD and seizures more often had a deficit in C4 activity. Lastly, Goldenthal et al. [51] reported that 65% of the individuals with ASD demonstrated a C1-to-C4 ratio outside of the control reference range and that individuals with more severe ASD were more likely to have a higher C1-to-C4 ratio.

In another large study, children with ASD who were not taking any supplements or medications that could interfere with mitochondrial function at the time of testing were examined using the buccal swab technique [53]. Variation in CS, C1 and C4 activity was greater in the ASD group as compared to the control range. Significant elevations in CS, C1 and C4 outside of the reference range were found in 22%, 11% and 8% of children with ASD, respectively, which were significantly more than what would be expected by chance. Significant depression in CS, C1 and C4 activity below the reference range was found in 3%, 28% and 3% of children with ASD, respectively, which was significant only for C1. The C1-to-C4 ratio was above and below the control reference ranges in 7% and 17% of children with ASD, respectively. Overall 62% of children with ASD were found to have some abnormality in mitochondrial enzyme activity. Most interestingly, this study examined the relationship between mitochondrial enzyme activity and measures of cognitive development and behavior. Specifically, childhood development, as measured by the Vineland Adaptive Behavior Scale (VABS), was related to both C1 and C4 activity. C4 activity demonstrates an inverted U-shaped relationship to VABS subscales such that both high and low C4 activity was related to poorer scores on the VABS. In contrast, C1 activity was linearly related to the VABS such that lower C1 activity was related to lower VABS scores and higher C1 activity was related to higher VABS scores. CS activity was found to be related to Social Responsiveness Scale scores.

The largest study measuring mitochondrial enzymatic activity using the buccal swab technique in children with ASD concentrated on whether there was a significant effect of various treatments for mitochondrial disorders on enzymatic function and introduced new biomarkers of mitochondrial function [54]. Overall, the study demonstrated that supplementation with folate, cobalamin, fatty acids and antioxidants influenced mitochondrial enzymatic activity as measurable with the buccal swab technique. Secondly, the study looked at the change in the linear relationship between the three enzymes measured with the buccal swab technique. Folate supplementation resulted in a more positive relationship between CS and C1 activity and C1 and C4 activity. Cobalamin supplementation resulted in a more positive correspondence between CS and C1 activity. The authors suggested that the more positive correlations between enzyme activities indicated increased ETC coupling.

A small pilot study of 11 children with ASD has shown a correspondence between change in ETC activity, as measured by the buccal swab technique, as a consequence of treatment with a mitochondrial cocktail, and improvement in behavior [55].

The last study using the buccal swab examined a genetic syndrome known as Phelan-McDermid syndrome (del22q13) that has a high comorbidity of ASD [49]. One interesting aspect of this study was the subset of children with C1 abnormalities. Those with higher than normal C1 activity demonstrated a developmental course more consistent with ASD, while those with below normal C1 activity demonstrated a developmental course more consistent with children with MD, suggesting that the subset of children with ASD were unique in demonstrating overactive C1 activity. Additional studies utilizing the buccal swab analysis in individuals with ASD would be helpful in expanding the work in this area.

3.2.3 Biomarkers of Abnormal Fatty Acid Metabolism

Several authors have reported biomarkers representing abnormalities in fatty acid metabolism in individuals with ASD [4, 56]. Clark-Taylor and Clark-Taylor [56] were the first to report fatty acid metabolism abnormalities associated with ASD. They reported a case of a child with elevations in unsaturated fatty acid metabolites C14:1 and C14:2 along with abnormalities in citric acid cycle, ammonia and cholesterol metabolism. The authors suggested that these abnormalities were the result of a defect in long-chain acyl-CoA dehydrogenase despite the fact that there is no convincing evidence for a deficiency in this enzyme in humans. ASD patients from Saudi Arabia were found to have elevations in saturated fatty acids and depressions in polyunsaturated fatty acids as compared to age-matched controls [57]. In another study, children with ASD from Egypt were found to have lower plasma levels of polyunsaturated fatty acids, except linoleic acid, as compared to healthy controls [58]. In a small study from Canada, all children with ASD were found to have elevations in polyunsaturated long-chain fatty acids and/or saturated very long-chain fatty acids as compared to age-matched controls [59]. The frontal cortex of patients with 15q11.2-q13 duplication and ASD was found to have lipofuscin deposits as compared to age-matched controls, potentially as a product of increased lipid peroxidation [60].

Frye [4] reported the results of metabolic work-ups of 133 consecutive patients evaluated in a medically based ASD clinic using a standardized metabolic screening algorithm which included screening for fatty acid oxidation defects [61, 62]. Abnormalities found in fatty acid oxidation were unique: short-chain and long-chain acyl-carnitines were

elevated, but medium-chain acyl-carnitines were normal. Further review of 213 patients with ASD who underwent a metabolic evaluation in a medically based ASD clinic [5] found that 17% showed consistent elevations in short and long acyl-carnitines (CESLAC), with C4OH, C14 and C16:1 being statistically significantly elevated above the upper limit of normal. Overall there was no clear, consistent genetic abnormality, and the individuals with these abnormalities had a particularly high rate of neurodevelopmental regression. Muscle biopsies showed a partial defect in C1 and C1 + C3. Glutathione (GSH) metabolism abnormalities were also found. Interestingly, this same pattern of acyl-carnitine and GSH abnormalities was independently reported in the rodent propionic acid (PPA) model of ASD [63–65]. Theoretically, PPA may be overproduced by the overrepresented species of *Clostridia* found in the GI tract of some children with ASD [66–68] and could result in mitochondrial dysfunction. This finding could also explain the association between ASD and children with propionic acidemia, an inborn error of metabolism [69]. However, other studies have suggested that children with ASD and fatty acid abnormalities demonstrate a decrease in PPA in their blood [57].

Abnormalities in fatty acid metabolism may help account for the relative deficiency in carnitine associated with ASD [58, 70], and the fact that mutations in the trimethyllysine hydroxylase epsilon (TMLHE) gene, the first enzyme in carnitine biosynthesis, is a risk factor for ASD [71]. In addition, children with ASD in general [72–74] and those with the TMLHE mutations specifically [75] benefit from supplementation with L-carnitine. Clearly, further research will be needed to better understand the significance of these fatty acid abnormalities in ASD and whether they are truly linked to disruptions in the enteric microbiome.

3.2.4 Other Novel Biomarkers

Researchers from King Saud University in Saudi Arabia have been particularly active in the identification and discovery of novel biomarkers of mitochondrial dysfunction and have used receiver operating characteristic (ROC) analysis to identify combinations of biomarkers which may be diagnostic of children with ASD and mitochondrial dysfunction [28, 76, 77]. Novel biomarkers found to be potentially useful include lactate dehydrogenase [28], lactate oxidase, pyruvate kinase and hexokinase [78], Na^+/K^+ ATPase [76, 79], caspase 3 [77] and caspase 7 [28]. Although promising, many of these studies have limited samples sizes and use different combinations of biomarkers from study to study, making these studies interesting but preliminary. Selecting the most promising biomarkers and investigating them on a large sample of ASD subjects and appropriate typically developing and developmentally delayed controls will be necessary to validate this approach.

Anti-mitochondrial antibodies are a recently described biomarker. In one study of 54 children with ASD from Saudi Arabia, antibodies to the mitochondrial M2 subtype antigen were found in 52% of children with ASD (percentage positive in controls not reported), and titers of this antibody were higher in children with ASD, particularly those with more severe ASD, as compared to age- and gender-matched control participants [80]. In an independent study, antibodies against type 2 mitochondrial antigen were found to be increased in children with ASD from Greece as compared to typically developing controls [81]. In these samples, mtDNA was found to be increased in the serum from the children with ASD as compared to controls. The authors suggested that increased levels of the peptide neurotensin, which was found to be increased in children with ASD in other studies, induces the release of mtDNA, which then acts as an immune trigger.

Other potential biomarkers include growth differentiation factor 15 (GDF15) and fibroblast growth factor 21 (FGF21), which are newer mitochondrial biomarkers that possess a high specificity for MD [82]. They have yet to be studied in ASD patients.

3.3 Genetic Aspects of Mitochondrial Disorders in Autism Spectrum Disorder (ASD)

Although it has been estimated that the genetic etiology of ASD may account for up to 40% of cases [83, 84] and whole exome sequencing (WES) and chromosomal microarray analysis (CMA) studies have reported yields up to 30% [85] and 26% [86], respectively, separate clinical studies have failed to confirm this high rate of genetic disorders in children with ASD. For example, a study from Canada found that 9.3% and 8.4% of children with ASD received a molecular diagnosis using WES and CMA, respectively, resulting in only 15.8% of children with ASD receiving a molecular diagnosis [84].

The moderate rate of a molecular diagnosis is at odds with the high heritability rate associated with ASD; for example, there is a 70–90% concordance rate for monozygotic twins and up to 10% for dizygotic twins and a 25-fold increased prevalence of ASD in siblings of ASD children [87]. Thus, it is likely that the etiology of ASD is multifactorial and influenced by a complex interplay between the inherited genome and environmental effects, some of which may be related to the maternal environment [88, 89]. Epigenetic interactions which modulate the expression of nuclear [90] and mitochondrial [91] genes can be influenced by environmental factors and may also play a crucial role in ASD. These interactions must surely modulate mitochondrial function, influencing neurodevelopment. Indeed, alterations in multiple bioenergetic and metabolic genes required for mitochondrial function may lead to abnormalities in cerebral

activity, resulting in cognitive and behavioral abnormalities characteristic of ASD [92].

Mitochondrial genes can be affected by copy number variations (CNVs) or regions of homozygosity (ROH). Chromosomal regions affected by CNVs can contain genes associated with mitochondrial function and neurodevelopmental disorders. For instance, a 7q31.1 deletion/duplication disrupts the IMMP2L gene encoding an inner mitochondrial membrane protease-like protein required for processing of cytochromes inside mitochondria and is implicated in ASD [93]. The IMMP2L–DOCK4 gene region on chromosome 7 plays a role in ASD susceptibility [94], and IMMP2L deletions have been demonstrated to have an association with ASD [95]. However, deleterious point mutations in IMMP2L were not identified in a significant number of ASD patients in other studies [96]. Mitochondrial dysfunction was also demonstrated in ASD patients with other CNVs, including 15q11-q13 duplication [22, 60], 5q14.3 deletion [48], 22q13 duplication or Phelan–McDermid syndrome [49, 97] as well as chromosomal disorders such as Down syndrome [98] and X- and Y-chromosome loci rearrangements [99]. An interesting example is Phelan-McDermid syndrome, which is typically caused by a microdeletion in the 22q13 region. Although much research has concentrated on SHANK3, which is important for synaptic function, six mitochondrial genes are also present in this region and may account for symptoms associated with mitochondrial dysfunction [49].

ROH are regions that are identical in homologous regions of paired chromosomes. Small ROH reflect our common human inheritance; larger and more numerous ROH can occur because of chromosomal segments shared between consanguineous family members. ROH can include genes associated with mitochondrial function as well as cerebral synapses and neurotransmitters that are associated with ASD [100].

Primary mitochondrial disease (PMD) involves a genetic defect that results in an impairment of mitochondrial oxidative phosphorylation [101]. PMD is estimated to affect 5% of children with ASD [3], based upon three large studies [102–104]. Mutations in mtDNA are the most common genetic mutations associated with ASD/MD [3]. Numerous mtDNA point mutations [105, 106] and deletions [107] show association with ASD; large-scale mtDNA deletions have been associated with epilepsy [21]. Examples of mtDNA genes reported include MT-ATP6 [85, 108, 109], MT-ND5 [108], MT-CYB [110], MT-TK [111] and MT-TL1 [112], and MT-CO1 and MT-CO2 [113]. Mutations in mtDNA such as m.3260A > G that causes mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) have been associated with ASD [104, 112], as has primary Leber hereditary optic neuropathy mtDNA mutations [104]. ASD can be an early presentation of another MELAS mutation (m.3243A > G) [114], sometimes with a prominent manifestation of mtDNA depletion syndrome [115]. Kent et al. [116] found m.3243A > G to be a rare cause of isolated ASD. mtDNA lineages or haplogroups significantly contribute to overall ASD risk in some studies [117, 118], while other studies have not confirmed a major role for mtDNA variation in ASD susceptibility [119, 120]. Increased mtDNA copy number in leukocytes from children with ASD has been reported in six studies [121–126], while increased mtDNA damage and deletions in leukocytes from children with ASD have been reported in four studies [121, 122, 126, 127]. Interestingly, Wong et al. also found an increase in microdeletions of p53, which is a regulator of mtDNA integrity [126], leading others to suggest that changes in mtDNA may be an epiphenomenon of genetic abnormalities in nuclear DNA (nDNA) mutations [128].

nDNA gene mutations can also cause PMD. Mutations in NDUFA5 [129, 130], NDUFS4 [108], POLG [104] and SCO2 [104] have also been found to be associated with ASD. SNPs in the SLC25A12 gene have been reported by multiple authors to be strongly associated with ASD [131–143]. Mutations in SLC6A8, resulting in X-linked creatine transporter disorder including features of ASD, have been reported [144]. Heterozygous mutations in adjoining components in a multimeric complex or metabolic pathway may combine to lead to impairment through synergistic heterozygosity. This is well-described, but not yet for the mitochondrial–ASD relationship. Since these types of relationships are complex, bioinformatics platforms used to interpret WES data may not be able to detect these complex genetic interactions. In addition, novel variants of unknown significance can be difficult to interpret, and in some situations, a mutation may cause gain of function, and act in a dominant manner. A recently developed method called transmission and de novo association (TADA) analysis considers both transmitted and de novo mutations together in the same statistical model by weighting more detrimental mutations such as de novo loss-of-function mutation more heavily than an inherited loss-of-function mutation [145].

Genetic abnormalities have not been reported in most ASD/MD cases [3]. In addition, the number of children with ASD who have abnormal nutritional and mitochondrial biomarkers greatly outweighs the number who can be diagnosed with a PMD. This raises the possibility that many children with ASD may have secondary mitochondrial dysfunction (SMD). There are examples of SMD related to hereditary defects in non-mitochondrial diseases. For example, a pair of siblings with ASD-associated gene mutations in WDR45 and DEPDC5 were found to have evidence of mitochondrial dysfunction [50]. In another interesting case series of children with Dravet syndrome and SCN1A mutations, two cases were found to manifest mitochondrial dysfunction, and one of the cases had ASD [24]. In another case, lymphoblastoid cell lines (LCLs) derived from a child with ASD and

a mutation in RPL10 were found to have changes in redox-sensitive components of energy metabolism [146]. Interestingly, candidate genes implicated in ASD have been found to be enriched in modules related to mitochondrial function [147]. These cases demonstrate the importance of advanced genetic testing combined with metabolic/mitochondrial evaluation in the workup of children with ASD [71].

Mutations in non-mitochondrial genes or environmental factors may be acting via epigenetic mechanisms in ASD [101, 148]. Down-regulation of genes of mitochondrial oxidative phosphorylation and varying gene expression related to myelination, inflammation and purinergic signaling have been previously identified in ASD patients [149]. Others have found upregulation of ribosomal, spliceosomal, and mitochondrial pathways and the down-regulation of neuroreceptor-ligand, immune response and calcium signaling pathways in gene expression profiles of ASD patients [150]. One meta-analysis of over 1000 microarray samples across 12 independent studies demonstrated that genes highly ranked with consistent changes in expression in the brain suggested modulation of mitochondrial function [151]. However, one study examining expression of the C1 75-kDa subunit in blood did not find consistent changes in children with ASD [152].

Other studies have examined the expression of genes in brain tissue in ASD. Depressed expression in ETC genes in the occipital and cerebellar areas and ETC and non-ETC genes in the cingulate, thalamus and frontal areas [153] has been reported. In addition, changes in genes that control mitochondrial dynamics have been noted in the temporal lobe in ASD [154]. Still others have demonstrated brain region-specific expression alterations in mitochondrial nDNA genes such as CMYA3, MTX2, SLC25A27, DNAJC19, DNM1L, LRPPRC, SLC25A12, SLC25A14, SLC25A24 and TOMM20 in ASD patients [131, 132].

3.4 Cellular Models of Mitochondrial Dysfunction in ASD

3.4.1 Evaluating Cellular Bioenergetics Using the Seahorse Bioscience XF96 Analyzer

One of the major advances in measuring mitochondrial function is the recently introduced Seahorse 96 XF Analyzer (Agilent Technologies, Santa Clara, CA, USA) that measures oxygen consumption rate (OCR) in real time in a 96-well plate in a broad range of intact living cell types [155, 156]. The assays performed on the Seahorse aid in the elucidation of mitochondrial enzyme defects, including oxidative phosphorylation [157], glycolytic [158] and fatty acid oxidation [159] pathway abnormalities. In our experiments, we use the 96 wells to run samples in quadruplicate, include matched groups on the same plate such as tissue from children with ASD, typically developing siblings and unrelated controls, as well as test several specific manipulations of the tissue samples.

Each well contains four reagent ports which allow for the sequential injection of compounds. A schematic showing the measurement sequence of the mitochondrial assay is depicted in Fig. 2. ATP-linked respiration (ALR) represents ATP production and is determined by injecting the complex V inhibitor oligomycin. The remaining OCR represents proton-leak respiration (PLR) and non-mitochondrial respiration. Maximal respiratory capacity (MRC) is determined by injecting carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazon (FCCP). A decrease in MRC is consistent with a deficit in mitochondrial biogenesis, mtDNA damage and/or ETC inhibition. Reserve capacity (RC) determines the threshold at which bioenergetic dysfunction occurs. Specifically, when RC is zero or negative the cell cannot satisfy a bioenergetic demand [160].

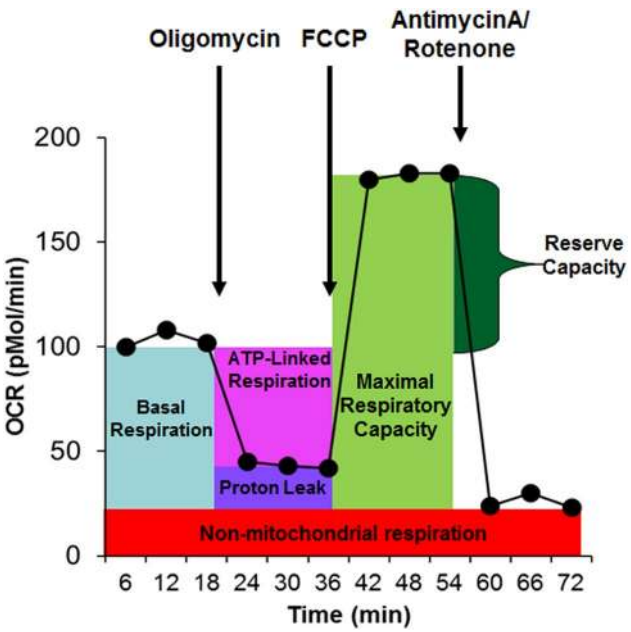

Fig. 2 The Seahorse assay measures mitochondrial function by monitoring the change in the oxygen consumption rate (OCR) as various reagents are injected into the sample of living tissue. Three measures of OCRs are obtained over an 18-min period to determine mitochondrial activity. Reagents are added to determine several parameters of mitochondrial activity. Basal respiration is initially determined as the difference between baseline OCR and non-mitochondrial OCR. Oligomycin, which is a complex V inhibitor, is added to determine the portion of basal respiration that is ATP-linked respiration and proton-leak respiration. Carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazon (FCCP), a protonophore, is added to collapse the inner membrane gradient, driving the mitochondria to respire at its maximal rate. This determines maximal respiratory capacity. Antimycin A and rotenone, which are inhibitors of complex III and I, are added to stop mitochondrial respiration to determine the non-mitochondrial respiration. Reserve capacity is calculated as the difference between basal respiration and maximal respiratory capacity

In addition, the extracellular acidification rate (ECAR), a reflection of lactate production, is also measured. From ECAR, glycolytic rate and glycolytic reserve are calculated. In addition, to obtain a measure of the relative utilization of oxidative versus glycolytic pathways, two measures are calculated. The oxidative to glycolytic ratio is calculated as basal OCR divided by basal ECAR, and the maximal oxidative capacity to glycolytic ratio is calculated as the MRC divided by the maximal glycolytic rate.

3.4.2 Primary Immune Cells

To date there are only a few published reports of aberrant mitochondrial function in peripheral blood cells from individuals with ASD [50, 121, 122, 161]. Giulivi et al. found decreased activity of C1, C2 and C4 in 80%, 60% and 30%, respectively, of lymphocytes derived from ten ASD subjects, as compared to ten control children [121]. The same group subsequently reported a significantly lower OCR as well as decreased C1 and C2 activity in granulocytes from the same cohort of children with ASD as compared to controls [122]. Both lymphocytes and granulocytes from the children with ASD exhibited significantly higher mitochondrial production of H_2O_2 as compared to controls [121, 122].

Our group has used primary cells to look at mitochondrial function in individual patients. In a recent case report, Drs Rose and Frye examined mitochondrial function in peripheral blood mononuclear cells (PBMCs) using the Seahorse analyzer in two siblings with ASD, each with a novel mutation in a different gene [50]. Our group compared these findings to mitochondrial function measured in typically developing children. PBMCs from the sibling who carried a mutation in the WDR45 gene exhibited increased mitochondrial respiration linked to ATP production, while PBMCs from the sibling who carried a DEPDC5 gene mutation exhibited increased PLR and a reduction in respiration linked to ATP production. Importantly, increased mitochondrial respiration in PBMCs from the affected sibling with the WDR45 mutation reflected significant elevations in several ETC complexes measured in contemporaneous muscle and skin biopsies, while decreased mitochondrial respiration in PBMCs from the sibling with the DEPDC5 mutation reflected decreased C4 activity as measured by the buccal swab technique.

In addition to these functional studies, several groups have reported surrogate markers of mitochondrial dysfunction in peripheral blood cells from children with ASD. Pecorelli et al. demonstrated ultrastructural changes, including densely packed and irregularly arranged cristae, in PBMC mitochondria from children with ASD [161]. Several groups have reported increased mtDNA abnormalities in peripheral blood cells in ASD [121–127].

3.4.3 Lymphoblastoid Cell Lines

LCLs are the major cellular model of mitochondrial dysfunction in ASD. LCLs are B cells transformed by the Epstein-Barr virus. They are created in a cell culture laboratory from fresh blood samples or obtained from numerous biorepositories. LCLs exhibit expression of genes in a wide range of metabolic pathways that is specific to the individual from whom the cells were derived [162].

In 2009, James et al. first reported abnormal mitochondrial function in LCLs derived from children with ASD [163]. Specifically, exposure to a nitric oxide donor induced a greater mitochondrial membrane potential reduction in ASD LCLs as compared to control LCLs, demonstrating a hypersensitivity to nitrosative stress in the ASD LCL mitochondria. ASD LCLs also demonstrated increased free radical production and more oxidized cellular and mitochondrial GSH pools, consistent with redox abnormalities reported in children with ASD [164–167] and elevations in reactive nitrogen species in mitochondria from individuals with ASD [168].

A series of studies by Drs Rose and Frye using the ASD LCL model has demonstrated abnormal mitochondrial bioenergetics in a subgroup of LCLs from children with ASD as compared to LCLs from unaffected siblings as well as unaffected unrelated children and adults [169–171]. The abnormal ASD LCL subgroup exhibits increased OCR associated with all Seahorse parameters, including ALR, PLR, MRC and RC [160, 172]. The LCLs derived from children with autistic disorder (AD) are classified into two groups: those with normal mitochondrial function (AD-N) and those with atypical mitochondrial function (AD-A) [170, 171, 173]. The AD-A LCLs have respiratory rates approximately twice that of control and AD-N LCLs [170, 171, 173]. These metabolic groupings are consistent and reproducible [169–171, 174–176]. We recently demonstrated that this alteration in respiration is associated with more severe repetitive behaviors [171]. We believe that this increase in respiratory rate may be a protective adaptation designed to resist environmental stressors, perhaps because of previous exposure to environmental toxicants [170, 176].

Several other groups have also described mitochondrial dysfunction in the ASD LCL model. A metabolic profiling study of 87 ASD LCLs revealed abnormal tryptophan metabolism in ASD as compared to control LCLs [177]. Tryptophan metabolism is one of several pathways leading to the production of nicotinamide adenine dinucleotide (NAD), a critical energy carrier for the ETC. Bu et al. [178] demonstrated severe mitochondrial dysfunction in LCLs derived from ten Chinese Han male children with ASD. Specifically, compared to LCLs from unrelated, unaffected control children, ASD LCLs exhibited decreased mitochondrial membrane potential and decreased activities of C1

and C3, as well as increased intracellular and mitochondrial reactive oxygen species (ROS) generation and increased mitochondrial-mediated apoptosis. In addition, the ASD LCLs were found to have significantly reduced expression of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), a major regulator of mitochondrial function, and sirtuin-1 (SIRT1), a metabolic sensor; PGC-1α overexpression improved all mitochondrial abnormalities. In another cohort of LCLs established from Chinese Han individuals with ASD, Zhang et al. [179] examined ETC activities and reported decreased activity of C1 in two out of five ASD LCLs harboring mutations in the AMPD1 gene, which codes for adenosine monophosphate (AMP) deaminase.

3.5 Mitochondrial Dysfunction in the Brain in ASD

Since the brain has a high metabolic demand and is especially dependent on mitochondrial function, abnormalities in mitochondria function would be expected to cause brain dysfunction. Studies provide support for mitochondrial dysfunction in the brain of individuals with ASD. Magnetic resonance spectroscopy (MRS) using both ^{31}P and ^{1}H techniques have examined energy metabolites in the brain of individuals with ASD. ^{31}P-MRS has reported abnormal energy metabolites in frontal cortex [180, 181]. Some ^{1}H-MRS studies have found a reduction in N-acetylaspartate (NAA) in the global white and gray matter and the parietal, anterior cingulate and cerebellum [182] associated with ASD, while others have not been able to detect differences in NAA [183]. Another study in adults with Asperger syndrome has shown an increase in NAA and choline in the prefrontal cortex, with concentrations of these metabolites correlating with obsessive behavior and social function, respectively [184]. An interesting meta-analysis using meta-regression suggests that these discrepancies may be due to an age-related decline in brain NAA specific to those with ASD [185].

Studies using ^{1}H-MRS have found an increase in lactate in the cingulate gyrus, subcortical gray matter nuclei, corpus callosum, superior temporal gyrus, and pre- and post-central gyri, with this abnormality being more common in adults with ASD [186], while others have not found any increase in lactate in individuals with ASD [187]. MRS studies on lactate in the brain are not without controversy. Some have suggested that the fact that lactate was found more often found in adults with ASD may reflect the notion that lactate was associated with comorbidities such as anxiety rather than related to the true etiology of ASD [188]. The original authors did not agree and suggested that perhaps worsening of mitochondrial function with age or ascertainment bias in the recruitment of adults with ASD might be a more likely explanations [189]. Others have pointed out that early MRS techniques may not be powerful enough to determine lactate abnormalities, especially because such early techniques are not uncommonly negative even in individuals with known MD, thus potentially explaining the negative findings; the lack of contemporaneous controls with MD make the interpretation of negative findings even more problematic [190]. The authors of the original study pointed out that their use of propofol anesthesia could increase the sensitivity of their study by provoking mitochondrial dysfunction in the patients with an underlying MD [191].

ETC function has also been directly measured in postmortem brain tissue from individuals with ASD. ETC function has been reported to be depressed in frontal [192], temporal [154, 192] and cerebellar [192] areas from ASD-derived brain tissue. Other studies noted decreases in the activity of non-ETC mitochondrial enzymes (aconitase, pyruvate dehydrogenase) in frontal [193], temporal [173] and cerebellar [173] tissue derived from children with ASD.

3.6 Mitochondrial Dysfunction in the Gastrointestinal Tract in ASD

GI symptoms are prominent in ASD and in patients with MD, separately, and multiple GI disorders are known to be caused by MD [194]. Thus, Rose et al. [195] examined mitochondrial function in rectal and cecum biopsies from ten children with ASD and compared them to ten children with Crohn's disease and ten neurotypical children with non-specific GI complaints using a single-blind, case–control design. Except for C2, the protein quantity of all ETC complexes was found to be higher in the cecum as compared to the rectum in ASD samples when compared to other groups. For both rectal and cecum biopsies, ASD samples demonstrated higher C1 activity, but not C4 or CS activity, compared to other groups. The authors suggested that this represented a unique pathophysiology of the GI symptoms in children with ASD and proposed that because most abnormalities were localized to the cecum, there may be a role for imbalances in the microbiome in children with ASD that drives mitochondrial dysfunction in the GI tract. Another explanation is that mitochondrial dysfunction could influence the microbiome.

3.7 Evidence of Environmental Influences of Mitochondrial Function in ASD

Despite decades of research focused on the genetic basis of ASD, the minority of ASD cases can be attributed to single-gene or chromosome defects [196]. The majority of ASD cases likely result from a complex interplay of polygenetic and environmental factors [88, 89]. Mitochondrial dysfunction is one of the most compelling mechanisms of gene–environment interactions, as mitochondria are influenced directly by exogenous environmental stressors, and secondarily through intrinsic factors such as ROS,

inflammatory mediators and local metabolic modulators such as the enteric microbiome. The effect of intrinsic and extrinsic environmental factors on mitochondrial function in ASD has been examined by exposing LCLs to various agents.

3.7.1 Intrinsic Microenvironmental Stressors

3.7.1.1 The Mitochondrial Oxidative Stress Test (MOST) Two major metabolic abnormalities associated with ASD are mitochondrial dysfunction and oxidative stress. The interconnection between these two metabolic abnormalities is well known: oxidative stress causes mitochondrial dysfunction and dysfunctional mitochondria produce ROS. ROS is also a byproduct of normal mitochondrial function. Additionally, both intrinsic and extrinsic stressors can cause detrimental effects by increasing ROS and/or reducing mitochondrial function.

The interaction between mitochondrial function and oxidative stress is especially pertinent to the pathophysiology of ASD. In children with ASD, the reduced form of GSH, the major intracellular antioxidant responsible for maintaining redox homeostasis and reducing ROS in the cytosol and mitochondria, is usually deficient [163, 166, 167, 173]. Our laboratory has shown that, in ASD, oxidized glutathione disulfide (GSSG) is elevated and proteins and DNA show oxidative damage in PBMCs [164] and post-mortem brain samples [173]. Other groups have verified GSH abnormalities in post-mortem brain samples [197, 198] and have shown oxidative damage to mtDNA [127] and lipids [199] in individuals with ASD. Epigenetic changes related to redox abnormalities [200, 201] have been found in individuals with ASD, including in studies in our laboratory [201]. In addition, several studies have suggested that oxidative stress biomarkers may be diagnostic for individuals with ASD [28, 31, 76, 202], and one study has demonstrated that ASD/MD children have a different profile of redox metabolism abnormalities as compared to ASD/NoMD children [203].

Thus, to better understand the interplay between these two major metabolic abnormalities, we developed a method to systematically manipulate ROS during mitochondrial function testing. We call this the Mitochondrial Oxidative Stress Test (MOST). Excessive ROS can lead to a depletion of mitochondrial RC and can activate mechanisms designed to protect the mitochondria from oxidative damage. Hill et al. [172] showed that acute increases in ROS deplete RC and that cell viability is reduced once RC is exhausted. A decrease in RC is linked to aging [204], heart disease [205], and neurodegenerative disorders [206, 207].

Thus, because ROS is systemically increased in vitro we concentrated on the changes in RC. To increase oxidative stress in vitro we utilized 1,4-naphthoquinone (DMNQ), an agent that generates intracellular superoxide and hydrogen peroxide similar to that generated by nicotinamide adenine dinucleotide phosphate oxidase in vivo and does not directly deplete thiols [160]. To determine the change in mitochondrial response with increasing ROS, cells are exposed to one of several DMNQ concentrations (5, 10, 12.5 and 15 μM) for 1 h before the Seahorse assay. The standard mitochondrial stress test is completed and the change in RC with increasing concentrations of DMNQ is measured. This change measures the rate of loss of RC with increased physiological

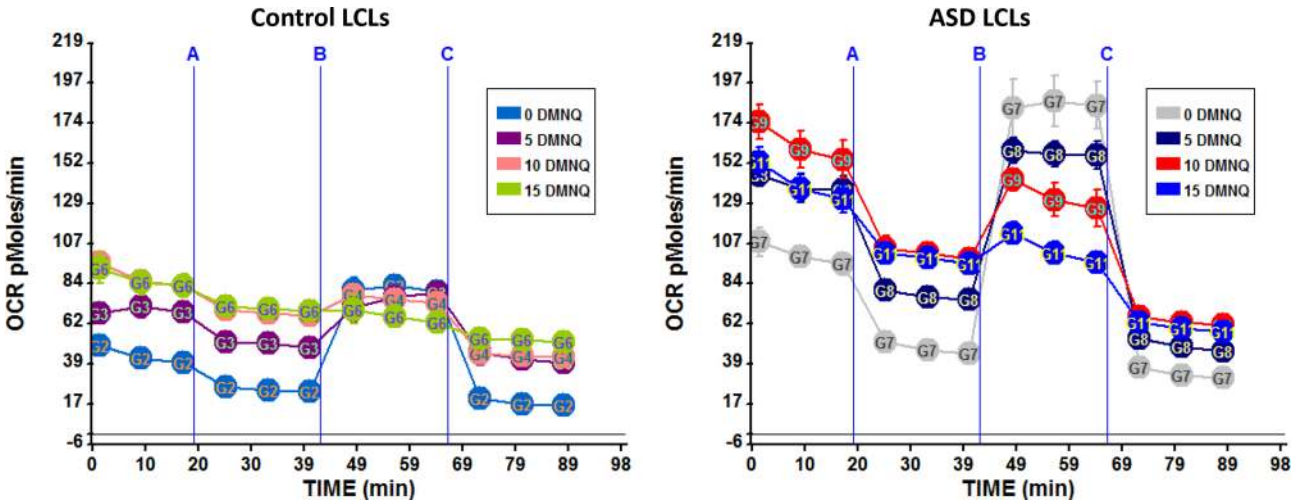

Fig. 3 The Mitochondrial Oxidative Stress Test (MOST) Seahorse assay results for lymphoblastoid cell lines (LCLs) derived from children with autism spectrum disorder (ASD) or age-matched controls. LCLs are exposed to one of three concentrations of an agent that increases oxidative stress known as 1,4-naphthoquinone (DMNQ) or not exposed to DMNQ (i.e., DMNQ 0). Reagents for the Seahorse assay are added at time points *A*, *B* and *C* as previously outlined in Fig. 2. Notice the higher overall respiratory rate of the ASD LCLs as compared to the control LCLs and the greater change in these values as DMNQ concentration is increased. *OCR* oxygen consumption rate

stress. Cells that have a greater loss of RC at lower DMNQ concentrations are considered to have mitochondria that are more vulnerable to physiological stress.

The effect of the MOST on the mitochondrial assay can be seen in Fig. 3. One of the significant findings from the MOST was found for the subset of LCLs found to have an apparent increased mitochondrial capacity to produce ATP, called AD-A LCLs in Sect. 3.4.3. The AD-A LCL subgroup exhibits an atypical mitochondrial response to increased DMNQ, with a precipitous decline in RC as ROS increases [169, 170]. In Fig. 3, we see that the AD-A LCLs have a high basal respiratory rate as well as a higher MRC as compared to the control LCLs. However, the LCLs exposed to higher concentrations of DMNQ demonstrated a smaller difference between the basal respiratory rate and the MRC such that at the highest DMNQ concentration, the MRC is below the basal respiratory rate, indicating a negative RC, which is consistent with mitochondrial exhaustion and collapse of cellular physiology.

3.7.1.2 Oxidative Stress and Mitochondrial Dysfunction Correspond in the ASD Brain Some studies have implicated an association between mitochondrial dysfunction and oxidative stress in the brain tissue of children with ASD. Mitochondrial function was found to be decreased in the temporal lobe (BA 21) of individuals with ASD, associated with an increase in oxidative DNA damage and a decrease in superoxide dismutase 2 activity [154]. In another study, increased oxidative stress and decreased ETC activity was found in the cerebellum, frontal cortex and temporal cortex of children with ASD [192]. One study found increased oxidative damage to mitochondrial proteins along with increased C4 activity in the superior temporal gyrus (BA 41/42 or 22) of individuals with ASD [208]. Lastly, another study found that aconitase activity was negatively correlated with the GSH redox ratio in the cerebellum and the temporal lobe (BA 22) of individuals with ASD [173].

3.7.2 Extrinsic Environmental Factors

Ethylmercury is an environmental toxin known to deplete GSH and induce oxidative stress and mitochondrial dysfunction. Using our LCL model, we demonstrated that acute exposure to ethylmercury induces a greater reduction in ALR, MRC and RC in the AD-A subgroup of LCLs as compared to control LCLs [174]. Pretreatment of the ethylmercury-sensitive subgroup with *N*-acetyl-cysteine to increase GSH normalized baseline respiratory parameters and blunted the exaggerated ethylmercury-induced RC depletion [174]. Similarly, Sharpe et al. [209] reported that a subset of ASD LCLs and their unaffected siblings exhibited mitochondrial hypersensitivity to ethylmercury using a surrogate assay of mitochondrial function known as 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) reduction.

Trichloroethylene (TCE) is an environmental toxicant and common environmental pollutant that has been linked to neurobehavioral and immune abnormalities as well as to Parkinson's disease and ASD [210–213]. Using our LCL model, we exposed ASD and control LCLs to trichloroacetaldehyde hydrate (TCAH), an in vivo TCE metabolite, either alone or followed by an acute exposure to DMNQ, an ROS generator [176]. TCAH exposure by itself in control and ASD LCLs resulted in a similar decline in mitochondrial respiration. However, for the AD-A subset of ASD LCLs as well as control LCLs, acute ROS exposure following TCAH exposure attenuated the decline in mitochondrial respiration, indicating that mitochondrial protective pathways may have been activated by acute ROS, and that these protective pathways had been primed for activation in an AD-A subset of LCLs by TCAH.

Bisphenol A (BPA) is a common chemical used to make plastics. Human exposure to BPA is considered widespread. Kaur et al. [214] exposed LCLs from ASD and unaffected sibling pairs to BPA. BPA induced ROS and decreased mitochondrial membrane potential in both ASD and unaffected sibling LCLs. When unaffected twin and non-twin siblings were analyzed separately, BPA-induced ROS production was greater in ASD and unaffected twin siblings as compared to non-twin siblings. BPA exposure upregulated mtDNA copy number in LCLs from unaffected twin siblings as compared to non-twin siblings. mtDNA in ASD LCLs was not examined.

3.7.3 The Enteric Microbiome Environment in ASD

ASD is also associated with environmental factors such as enteric microbiome-derived metabolites that may alter mitochondrial function by mechanisms other than oxidative stress [215]. Alterations in the gut microbiome have been associated with ASD [194, 216], and a major mechanism by which enteric bacteria influence host physiology is through the production of short-chain fatty acids. *Clostridia* spp., a major short-chain fatty acid producer, repeatedly has been found to be overrepresented in the ASD GI tract [217, 218]. Our ASD LCL model has been instrumental in gaining a better understanding of the impact of short-chain fatty acids on mitochondrial function in ASD.

PPA is a ubiquitous short-chain fatty acid which is a major fermentation product of the enteric microbiome. PPA is particularly interesting because it is a natural component of normal intermediary metabolism, being the end product of odd-chain fatty acid oxidation. PPA induces ASD-like behaviors in rodents [63, 219] and causes abnormalities in metabolic, immune and neurophysiological systems [63, 219]; PPA also regulates genes in human cell lines [220].

As mentioned above, similar redox and fatty acid metabolic abnormalities are found in both children with ASD and the PPA induced rodent model of ASD [5]. Thus, PPA is an interesting molecule as it is directly involved in metabolism as an intermediate and can modulate gene expression.

Exposing control and ASD LCLs to various concentrations of PPA for 24–48 h revealed dose and exposure time-dependent effects on mitochondrial function. ASD LCLs demonstrated an enhancement of mitochondrial function with PPA exposure as indicated by an increase in RC as compared to control LCLs [175]. However, ROS exposure negated the positive PPA effect on RC in ASD LCLs. Indeed, in the context of increased ROS, instead of enhancing RC in ASD LCLs, PPA resulted in a greater decrease in RC in ASD LCLs as compared to control LCLs exposed to PPA and ROS. Thus, it appeared that the effect of PPA on mitochondrial function was context dependent as its ability to facilitate or demean mitochondrial function was dependent on the redox microenvironment.

Butyrate is another short-chain fatty acid primarily produced by the enteric microbiome which can be a fuel source and modulate gene expression. Exposure of LCLs to butyrate for 24–48 h had differential effects on mitochondrial function in control and ASD LCLs [221]. Butyrate increased respiration in the AD-A LCLs, while decreasing mitochondrial respiration in control LCLs. When ROS was increased in vitro, butyrate prevented a diminutive effect of ROS on mitochondrial respiration in all LCLs. At the highest concentration investigated (1 mM), butyrate increased the expression of genes involved in mitochondrial fission (DRP1, FIS1), mitophagy (PINK1, LC3, PTEN), oxidative stress (UCP2, NRF2, SOD2), energy metabolism, such as mammalian target of rapamycin (mTOR), AMPK and SIRT3, as well as genes known to be involved in response to physiological stress, such as PGC1α. These findings suggest that butyrate has a protective effect on the mitochondria.

3.8 Treatments of Mitochondrial Dysfunction in ASD

There is only one study published to date that examined the behavioral effects of a customized mitochondrial supplement in ASD children with mitochondrial dysfunction. Legido et al. [55] in an open-label study treated 11 children with ASD and abnormal C1 and/or C4 activity with a mitochondrial cocktail containing carnitine, coenzyme Q10 and α-lipoic acid. Three months of treatment reduced the C1-to-C4 ratio as well as improved several behavior scales, including lethargy and inappropriate speech subscales of the Aberrant Behavior Checklist. Three months after withdrawal of the treatment, the lethargy and inappropriate speech subscales significantly worsened.

As previously mentioned, in a relatively large study, we have examined whether various treatments for mitochondrial disorders influenced enzymatic activity in children with ASD using the buccal swab technique [54]. Overall, the study demonstrated that folate, cobalamin, fatty acids and antioxidant supplementation increased mitochondrial enzymatic activity and folate and cobalamin influenced that relationship between enzyme activity, suggesting increased ETC coupling.

As we have previously reviewed, although most studies have not selected individuals with ASD and mitochondrial dysfunction for treatment with supplements that target the mitochondria, several studies have demonstrated that mitochondrial supplements may be helpful in children with ASD [73]. For example, carnitine deficiency appears to be common in children with ASD [58, 70]. Two double-blind, placebo-controlled studies demonstrated improvement in ASD symptoms with carnitine supplementation [74], with some improvements directly related to the change in blood carnitine levels [72]. In another study, reduced NAD and ribose appeared to improve metabolic biomarkers in children with ASD and symptoms of mitochondrial dysfunction [222]. Two studies have reported behavioral improvements in children with ASD using ubiquinol [223] and coenzyme Q10 [224].

4 Discussion

4.1 Defining Mitochondrial Dysfunction in ASD

Major criteria that define classic MD (i.e., PMD) include unequivocal genetic mutations, severe depressions (i.e., < 30%) of ETC function or syndromic presentation [16]. However, repeated studies have suggested that genetic defects are found only in a minority of children with ASD, including a minority of children with ASD/MD [3], and many case reports and series have described only moderate, rather than severe, deficiencies in ETC activity [5, 225]. In classic MD, the mitochondria are thought to be severely dysfunctional, with significant depression in mitochondrial respiration. Criteria such as the modified Walker's criteria reflect this notion and are commonly used to diagnose MD. Many genetic and non-genetic disorders that can cause SMD may respond to treatments that target the mitochondria [101].

Perhaps more striking is the fact that ETC activity in muscle [225, 226], skin [5], buccal epithelium [51, 52, 227] and brain [208] has been documented to be significantly increased, rather than decreased, in some individuals with ASD. This is consistent with our in vitro data showing elevated mitochondrial respiration in LCLs derived from children with ASD [169–171, 174–176, 228]. We recently

demonstrated that this alteration in respiration is associated with more severe repetitive behaviors [171]. We believe that this increase in respiratory rate may be an adaptation designed to resist chronic exposure to toxicants associated with ASD, perhaps because of previous exposure to environmental toxicants [170, 176]. We have also demonstrated that this increased respiratory rate results in an increased susceptibility of the mitochondrial to acute in vitro increases in ROS [169, 170], and we have reported that this subset of ASD LCLs respond differently to environmental exposures associated with ASD [174–176, 221]. Further research will be needed to better understand this unique alteration in mitochondrial physiology.

Thus, the current data points to possible non-genetic defects or changes causing abnormal mitochondrial function as well as unique types of mitochondrial dysfunction in individuals with ASD [5, 226]. As mitochondrial dysfunction in ASD appears to be qualitatively and quantitatively different from what occurs in classic MD, it is important to consider how to define mitochondrial dysfunction in ASD. We have suggested that other criteria, such as the Morava criteria, which is clinically based, may be more appropriate for diagnosing MD in children with ASD [16]. However, it is clear we need a better definition on what degree of mitochondrial dysfunction is abnormal in ASD and how it is linked to symptomology. We have demonstrated that changes in ETC activity are associated with changes in behavior and development on a continuum [53] and that treating mitochondrial abnormalities in children with ASD has positive benefits for ASD-related behaviors [55]. In addition, we have demonstrated that certain common supplements positively modulate mitochondrial activity in children with ASD [54]. Nevertheless, it is not clear how to define these variations in mitochondrial function as clearly abnormal or how to decide which children will most likely respond to treatment for mitochondrial dysfunction. Clearly, further research is needed in this potentially fruitful area.

4.2 Possible Links to Clinical Aspects of ASD

There are several clinical aspects of ASD that may be associated with abnormalities in mitochondrial function, including abnormalities in immune function, cerebral folate deficiency, autonomic abnormalities and prenatal brain abnormalities.

Autoimmune diseases are diagnosed significantly more often among children with ASD than among controls [229] and among their family members [230]. In addition, some children with ASD also have been documented to have immunoglobulin deficiency [231]. Several studies have shown that immune cells in children with ASD demonstrate mitochondrial dysfunction [3, 121, 169, 228, 232], and recent studies have documented that proper mitochondrial function is essential for immune regulation [233] and mitochondrial dysfunction is associated with immunodeficiencies [234]. Thus, it is very possible that mitochondrial dysfunction could lead to the immune abnormalities seen in ASD. Alternatively, autoimmunity causes inflammatory responses that result in catabolism and high rates of ATP consumption from the release of proinflammatory cytokines [235]. Thus, it is very possible that an initial autoimmune process can drive mitochondrial dysfunction. Indeed, mitochondrial dysfunction may ensue during an inflammatory process. This could result in developmental regression from typically development to an ASD phenotype, as reported by Shoffner et al. [20]. Inflammation can affect epigenetics that involve both nDNA and mtDNA. This represents yet another mechanism by which the immune system may adversely affect the mitochondria. Finally, the effect of antimitochondrial antibodies reported to be associated with ASD [80, 81] on mitochondrial function is yet to be explored in detail. This may provide an interesting connection between the immune system and the mitochondria.

About 15 years ago, Ramaekers and Blau described a case series of children with normal neurodevelopment during early infancy followed by neurodevelopmental regression. These children had low concentrations of folate in their cerebrospinal fluid, but normal concentrations in their blood [236]. This newly described neurodevelopmental disorder was named cerebral folate deficiency (CFD) syndrome to signify deficiency of folate specific to the brain. Early reports found that some children with CFD also had ASD [237], with further studies suggesting that CFD was linked to children with low-functioning ASD who also had neurological abnormalities [238]. Recent studies have suggested that CFD is found in about a quarter of children with ASD [239]. CFD is caused by dysfunction of the folate receptor α, the major transport mechanism for folate transport into the brain, which can be caused by folate receptor α autoantibodies and/or mitochondrial dysfunction. Cases of ASD with mitochondrial dysfunction have been reported in CFD [240]. Thus, the connection between mitochondrial dysfunction, CFD and ASD may be very important and has the potential to be one of the major pathophysiological mechanisms that drives cognitive and behavioral abnormalities. Most significant is the fact that CFD is treatable and potentially reversible with leucovorin calcium. Given the recent studies that have demonstrated that leucovorin calcium is beneficial to behavior and language in children with ASD [241, 242], as well as the fact that folates appear to positively modulate mitochondrial function [54, 243], this should be considered a very promising area of therapeutics in ASD.

Other abnormalities that may be connected to mitochondrial dysfunction are prenatal abnormalities and autonomic dysfunction. Patients with ASD have demonstrated abnormalities of brain growth and development including hypoplasia of the corpus callosum, cerebellar hypoplasia, failures

of synaptic development including dendritic sprouting and axonal branching, among others. These abnormalities may reflect impaired mitochondrial function, energetics of the microtubule system, and cell motility in the development of the nervous system. Weissman et al. reported prenatal abnormalities in their case series of children with ASD/MD [15]. Dysautonomia, such as excessive dilatation of the pupils and tachycardia, are commonly seen in children with ASD [244]. Autonomic dysfunction has been associated with mitochondrial problems [245] and has been reported in a child with ASD and mitochondrial myopathy [246]. Some have pointed to a potential connection between nitric oxide, mitochondrial dysfunction and dysautonomia in ASD [247]. Thus, further studies are needed to investigate the potential connection between these clinical entities and mitochondrial function in ASD.

4.3 Studying Environmental Agents Affecting Mitochondrial Function

As has been demonstrated by several groups, including ours, LCLs derived from individuals with ASD are the primary cellular model to study mitochondrial dysfunction in ASD [3, 121, 169, 228, 232]. Furthermore, LCLs from individuals with ASD paired with LCLs from unaffected twin and non-twin siblings make an excellent model to examine the mitochondrial effects of environmental factors associated with ASD and whether or not these factors can induce mitochondrial adaptations that result in vulnerabilities leading to dysfunction [174–176, 221, 248]. Given the importance of the mitochondria in environmental exposures and the fact that environmental exposures most likely have a considerable influence in the etiology of ASD, further research into the connection between environmental factors and mitochondrial dysfunction in ASD is clearly needed.

4.4 Novel Markers for Measuring Mitochondrial Function in ASD

One exciting recent development is the introduction of new biomarkers that may be quite helpful in identifying mitochondrial dysfunction. The buccal swab technique non-invasively measures mitochondrial enzymatic activity and calculates the relative activity of enzymes, by using a ratio or examining the linear relationship between enzyme activities. Other techniques such as using respirometry or enzymology on primary immune cells are other promising techniques for identifying mitochondrial dysfunction in individuals with ASD. Furthermore, novel blood-based markers have been investigated and can discriminate children with ASD from healthy controls [28, 76–79]. Abnormalities in mitochondrial function also appear to include alterations in fatty acid metabolism, including a unique pattern of acyl-carnitine elevation linked to a partial C1 deficiency that may have a connection to modulatory influences from the microbiome [5]. Clearly, these biomarkers remain a promising area, but results are still preliminary. Further development of these and other techniques are needed to help better identify and define individuals with ASD and mitochondrial abnormalities.

5 Conclusions

Mitochondrial dysfunction appears to be closely associated with at least a subset of patients with ASD. The etiology of mitochondrial dysfunction as well as how to define it in individuals with ASD is not clear at this time. Preliminary studies suggest that the mitochondria may be a fruitful target for treatment and prevention of ASD, but further research to better understand the role of the mitochondria in the pathophysiology of ASD is needed.

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Compliance with Ethical Standards

Conflict of Interest The authors (SR, DN, DR, MG, SK and RF) have no conflicts of interest to declare.

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