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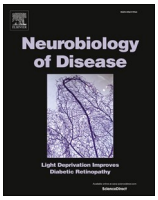

Review

Biomarkers of mitochondrial dysfunction in autism spectrum disorder: A systematic review and meta-analysis

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ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder affecting 1 in 36 children and is associated with physiological abnormalities, most notably mitochondrial dysfunction, at least in a subset of individuals. This systematic review and meta-analysis discovered 204 relevant articles which evaluated biomarkers of mitochondrial dysfunction in ASD individuals. Significant elevations (all $p < 0.01$) in the prevalence of lactate (17%), pyruvate (41%), alanine (15%) and creatine kinase (9%) were found in ASD. Individuals with ASD had significant differences (all $p < 0.01$) with moderate to large effect sizes (Cohen's d' ≥ 0.6) compared to controls in mean pyruvate, lactate-to-pyruvate ratio, ATP, and creatine kinase. Some studies found abnormal TCA cycle metabolites associated with ASD. Thirteen controlled studies reported mitochondrial DNA (mtDNA) deletions or variations in the ASD group in blood, peripheral blood mononuclear cells, lymphocytes, leucocytes, granulocytes, and brain. Meta-analyses discovered significant differences ($p < 0.01$) in copy number of mtDNA overall and in ND1, ND4 and CytB genes. Four studies linked specific mtDNA haplogroups to ASD. A series of studies found a subgroup of ASD with elevated mitochondrial respiration which was associated with increased sensitivity of the mitochondria to physiological stressors and neurodevelopmental regression. Lactate, pyruvate, lactate-to-pyruvate ratio, carnitine, and acyl-carnitines were associated with clinical features such as delays in language, social interaction, cognition, motor skills, and with repetitive behaviors and gastrointestinal symptoms, although not all studies found an association. Lactate, carnitine, acyl-carnitines, ATP, CoQ10, as well as mtDNA variants, heteroplasmy, haplogroups and copy number were associated with ASD severity. Variability was found across biomarker studies primarily due to differences in collection and processing techniques as well as the intrinsic heterogeneity of the ASD population. Several studies reported alterations in mitochondrial metabolism in mothers of children with ASD and in neonates who develop ASD. Treatments targeting mitochondria, particularly carnitine and ubiquinol, appear beneficial in ASD. The link between mitochondrial dysfunction in ASD and common physiological abnormalities in individuals with ASD including gastrointestinal disorders, oxidative

Abbreviations: DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; ABC-C, Aberrant Behavior Checklist-Community; AC, acyl-carnitine; ALT, Alanine amino transferase; ATP, adenosine triphosphate; ASD, autism spectrum disorder; CAT, catalase; CARS, Childhood Autism Rating Scale; CNVs, copy number variations; CK, Creatine kinase; DAMP, damage-associated molecular patterns; DBPC, double-blind placebo controlled; ETC, electron transport chain; ECAR, extracellular acidification rate; FCCP, Carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazon; GPx, glutathione peroxidase; GSSG, glutathione, oxidized; GI, gastrointestinal; GSH, glutathione, reduced; PM2.5, inhalable particles with diameters that are generally 2.5 μm and smaller; nIPCs, intermediate neural progenitor cells; KD, Ketogenic Diet; LDH, Lactate dehydrogenase; LCL, lymphoblastoid cell line; MRS, magnetic resonance spectroscopy; mtDNA, mitochondrial DNA; MOST, Mitochondrial Oxidative Stress Test; mtROS, mitochondrial ROS; NDR, neurodevelopmental regression; NSCs, neuroepithelial stem cells; NAD+, nicotine adenine dinucleotide +; Nrf2, nuclear factor erythroid 2-related factor 2; OCR, oxygen consumption rate; PBMCs, peripheral blood mononuclear cells; RGCs, radial glial cells; ROS, Reactive oxygen species; SCFA, short chain fatty acid; SNPs, single nucleotide polymorphisms; TCA, tricarboxylic acid; TMLHE, Trimethyllysine dioxygenase; UCP, Uncoupling Protein.

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stress, and immune dysfunction is outlined. Several subtypes of mitochondrial dysfunction in ASD are discussed, including one related to neurodevelopmental regression, another related to alterations in microbiome metabolites, and another related to elevations in acyl-carnitines. Mechanisms linking abnormal mitochondrial function with alterations in prenatal brain development and postnatal brain function are outlined. Given the multisystem complexity of some individuals with ASD, this review presents evidence for the mitochondria being central to ASD by contributing to abnormalities in brain development, cognition, and comorbidities such as immune and gastrointestinal dysfunction as well as neurodevelopmental regression. A diagnostic approach to identify mitochondrial dysfunction in ASD is outlined. From this evidence, it is clear that many individuals with ASD have alterations in mitochondrial function which may need to be addressed in order to achieve optimal clinical outcomes. The fact that alterations in mitochondrial metabolism may be found during pregnancy and early in the life of individuals who eventually develop ASD provides promise for early life predictive biomarkers of ASD. Further studies may improve the understanding of the role of the mitochondria in ASD by better defining subgroups and understanding the molecular mechanisms driving some of the unique changes found in mitochondrial function in those with ASD.

1. Introduction

Autism spectrum disorder (ASD) now affects 1 in 36 children in the United States according to the recent Autism and Developmental Disabilities Monitoring Network estimates (Maenner et al., 2023). Although ASD is defined by behavioral features, many individuals with ASD demonstrate brain-based and/or systematic physiological abnormalities as well as genetic underpinnings, suggesting that ASD is not a purely psychological disorder but has biological underpinnings that can be leveraged to develop targeted medical treatments (Frye, 2022).

Abnormalities of the central nervous system that have been repeatedly associated with ASD include imbalances in the excitatory to inhibitory ratio (Frye et al., 2016a), and monoamine neurotransmitter production (Frye et al., 2010) as well as epilepsy (Frye et al., 2013a). However, despite the fact that ASD is believed to mostly be driven by abnormalities in the brain, many individuals with ASD manifest systemic abnormalities such as metabolic and mitochondrial disorders, immune system abnormalities and oxidative stress (Rossignol and Frye, 2012a). Metabolic disorders are particularly important since many of these disorders can be mitigated with safe, well tolerated treatments (Rose et al., 2018a; Niyazov et al., 2016).

Disorders of mitochondrial metabolism are relatively compelling since the mitochondria are central to metabolism and can metabolically and epigenetically influence cellular functions (Kopinski et al., 2019; Wallace and Fan, 2010). The notion that mitochondrial dysfunction is associated with ASD is not new. Mary Coleman at Georgetown University Medical School suspected a defect in pyruvate dehydrogenase complex when she found that 5% of children with ASD had lactic acidosis (Coleman and Blass, 1985). In 2007, the first large population-based study screened 300,000 school-age children in Portugal and the Azores and found that 20% of children diagnosed with ASD also had lactic acidosis with 46% of those children reaching criteria for a definite classic mitochondrial respiratory chain disorder (Oliveira et al., 2007).

Disorders of mitochondrial function in ASD are complicated and most are probably non-classical in nature. Indeed, a meta-analysis found that the overall prevalence of classical mitochondrial disease in ASD is 5% but about 30% or more of those with ASD manifest biomarkers of abnormal mitochondrial function (Rossignol and Frye, 2012b). Perhaps more striking, some studies find that 80% of immune cells from children with ASD have electron transport chain (ETC) complex deficits (Napoli et al., 2014; Giulivi et al., 2010). Furthermore, ASD has been linked to elevated ETC complex activity (Graf et al., 2000; Frye and Naviaux, 2011; Palmieri et al., 2010; Delhey et al., 2017a) and mitochondrial respiration (Rose et al., 2014a; Rose et al., 2014b; Rose et al., 2017a; Rose et al., 2018b; Frye et al., 2017a; Frye et al., 2016b; Bennuri et al., 2019), as well as abnormalities in fatty acid metabolism (Frye et al., 2013b). Thus, individuals with ASD appear to have unique abnormalities in mitochondrial metabolism.

This systematic review provides a broad overview of mitochondrial abnormalities associated with ASD. First, biomarkers associated with mitochondrial abnormalities in ASD will be reviewed with a goal of defining the prevalence of these biomarkers in ASD. Second, the evidence supporting the use of mitochondrial treatments in ASD will be reviewed. Third, the significance of mitochondrial abnormalities in the symptomatology of ASD will be discussed. Finally, the potential contribution of mitochondrial dysfunction to the etiology of ASD will be outlined. These findings will hopefully lead to a better understanding of the significance of mitochondrial functioning in ASD.

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 find pertinent publications. A computer-aided search of PUBMED, Google Scholar, CINAHL, EmBase, Scopus, and ERIC databases from inception through February 2024 was conducted to identify pertinent publications using the search terms “autism” OR “autistic” OR “ASD” OR “Asperger” OR “pervasive developmental disorder” OR “PDD” in all combinations with the terms “Acylcarnitine” OR “Alanine” OR “Ammonia” OR “ATP” OR “Carnitine” OR “Citrate synthase” OR “Creatine kinase” OR “Electron transport chain” OR “Coenzyme Q10” OR “Lactate” OR “Lactic acid” OR “Mitochondria” OR “Mitochondrial DNA” OR “mtDNA” OR “Pyruvate” OR “Pyruvic acid” OR “Ubiquinone” OR “Ubiquinol.” The references cited in identified publications were also searched to locate additional studies. Fig. 1 (PRISMA Flow Diagram) depicts the publications identified during the search process.

2.2. Study selection

One reviewer (DR) screened titles and abstracts of all potentially relevant publications. Studies were initially included if they (1) reported at least one potential mitochondrial marker, and (2) involved individuals with ASD. After screening all records, 204 publications met inclusion criteria; two reviewers (DR and NR) then independently reviewed these articles. Articles were excluded if they: involved animal or animal models and not humans; only presented data from Rett syndrome; did not present unique or new data (such as letters to the editor or review articles); or presented duplicate data.

2.3. Meta-analysis

MetaXL Version 5.3 (EpiGear International Pty Ltd., Sunrise Beach, Queensland, Australia) was used with Microsoft Excel Version 16.0.12827.20200 (Redmond, WA, USA) to perform the meta-analysis. The data from this meta-analysis is available upon request to the authors. Random-effects models, which assume variability in effects from both sampling error and study level differences (Lipsey and Wilson, 2001; Senn, 2007), were used to calculate prevalence across studies

while pooled Cohen's d' (a measure of effect size) was calculated from the standardized mean difference of quantitative biomarker measurements using the inverse variance heterogeneity model since it has been shown to resolve issues with underestimation of the statistical error and spuriously overconfident estimates with the random effects model when analyzing continuous outcome measures (Doi et al., 2015). Effect sizes were considered small if Cohen's d' was 0.2; medium for Cohen's d' was 0.5; and large if Cohen's d' was 0.8 or higher (Cohen, 2013). Cochran's Q was calculated to determine the heterogeneity of effects across studies, and when significant, the I^2 statistic (Heterogeneity Index) was calculated to determine the percentage of variation across studies that is due to heterogeneity rather than chance (Higgins and Thompson, 2002; Higgins et al., 2003), and the Luis Furuya-Kanamori (LFK) Index derived from Doi plots was reviewed for significant asymmetries ($> \pm 2$) (Barendregt et al., 2013; Furuya-Kanamori et al., 2018). Studies containing <10 individuals were not included in the meta-analysis. Only studies that had means with standard deviations or standard errors of the mean where numerical values were available were included.

3. Results

3.1. Biomarkers

Biomarkers are objective measures of biological or pathophysiological processes, or pharmacologic responses to therapeutic interventions. According to the Biomarkers Definitions Working Group (Biomarkers Definitions Working Group, 2001), common applications of biomarkers include biomarkers which (I) can help diagnose a disease by identifying individuals with an abnormal biological process, (II) can classify disease severity, (III) can indicate prognosis, or (IV) can predict or monitor response to therapy. Biomarkers have the potential to be utilized in several aspects of clinical care for patients with ASD, including promoting early diagnosis and selecting effective treatments.

Through our search we have identified several biomarkers that may assist in identifying mitochondrial dysfunction in ASD, particularly blood and genetic based biomarkers. In this section, we start by describing the relationship between the biomarkers identified concerning mitochondrial function to provide the reader with an overview of the significance and origin of the biomarkers discussed. We then review the blood-based biomarkers followed by the genetic based biomarkers. Further, we describe the clinical correlates of these biomarkers.

3.1.1. Metabolic biomarkers: An overview

Metabolic biomarkers are at the center of confirming suspected mitochondrial disease or dysfunction. We provide an overview of mitochondrial biochemistry (Fig. 2) to provide a better understanding of the biomarkers used to diagnose mitochondrial disease or dysfunction.

Two of the most well-known metabolic biomarkers of mitochondrial dysfunction are lactate and pyruvate (top middle of Fig. 2). Pyruvate is the product of glycolysis, the pathway that metabolizes glucose for fuel,

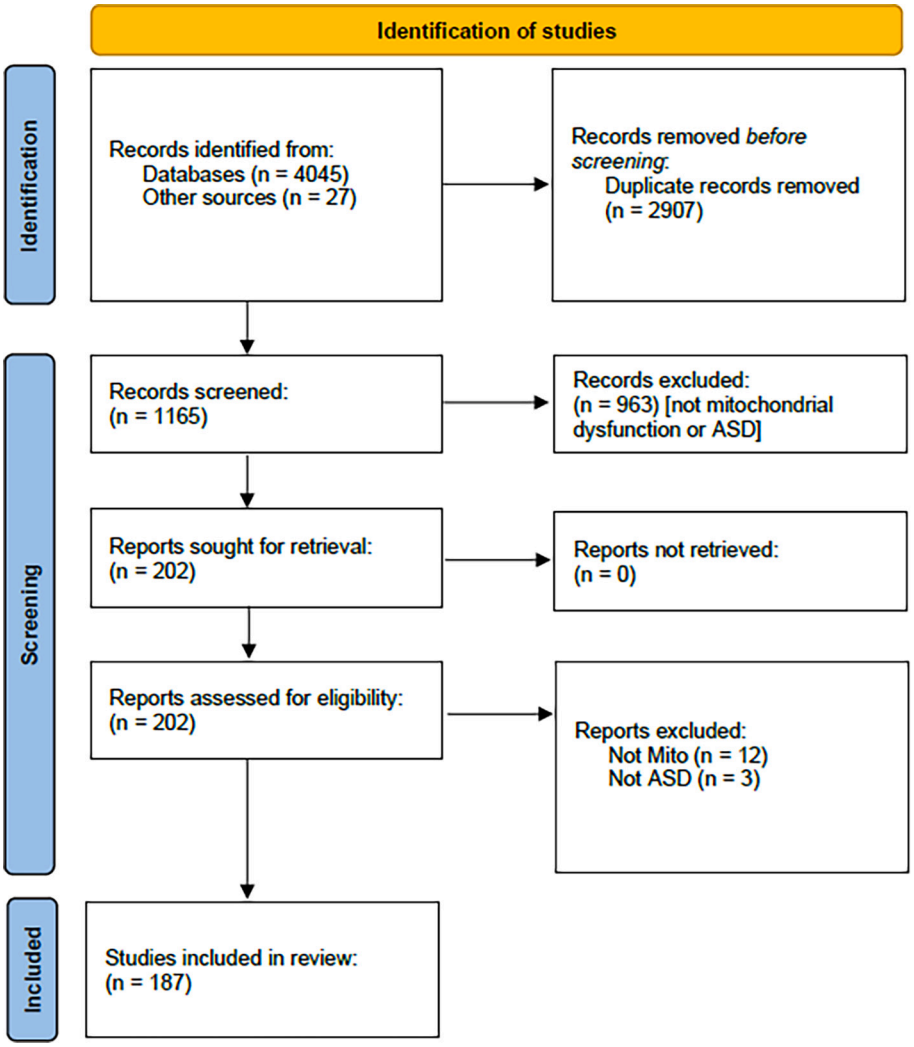

Fig. 1. PRISMA Flow Diagram for this systematic review.

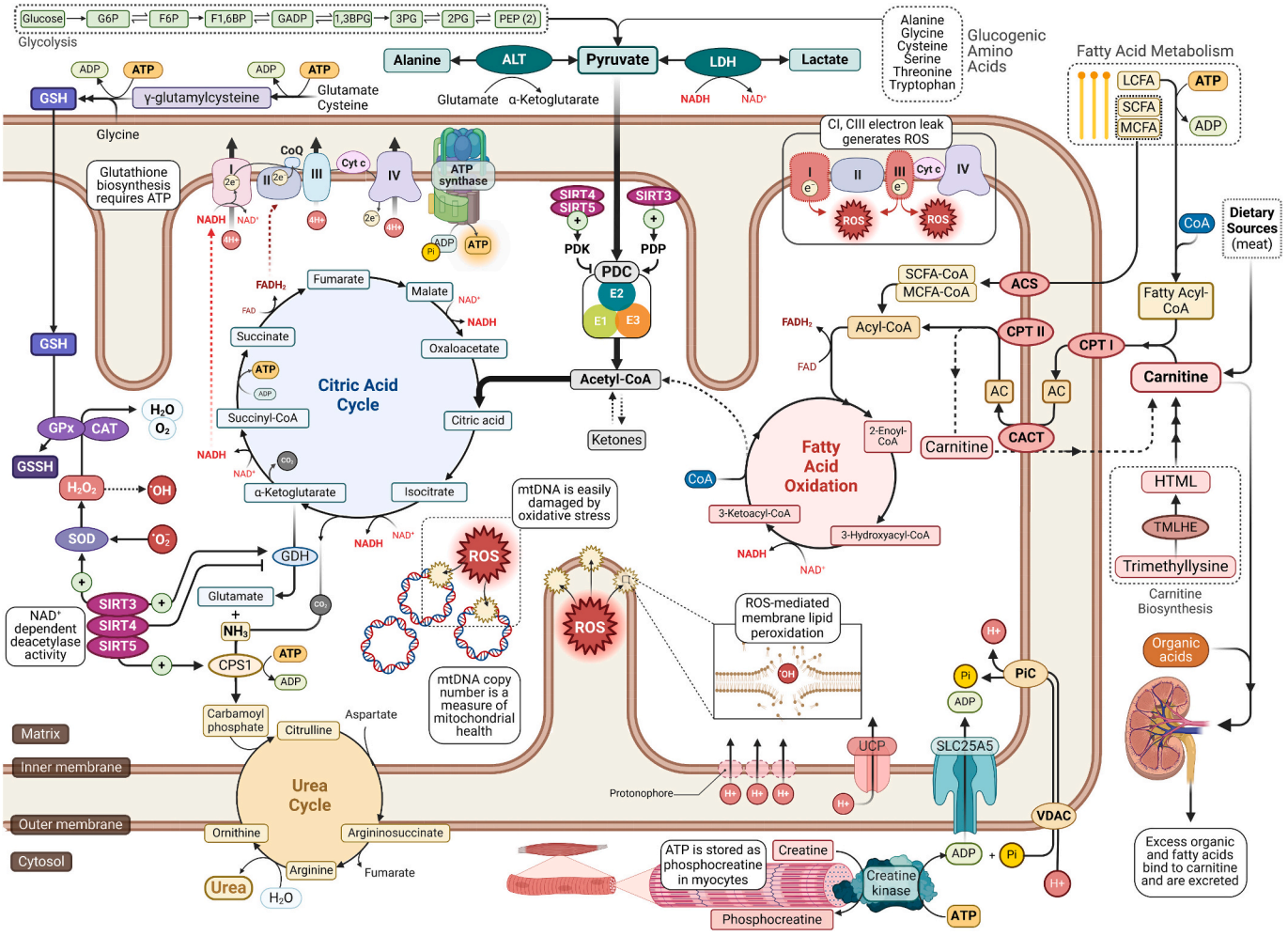

Fig. 2. Diagram of mitochondrial pathways relevant to the biomarkers discussed in this review. AC, acyl-carnitine; ACS, acyl-CoA synthetase; ALT, alanine amino transferase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BPG, bisphosphoglycerate; CI, mitochondrial complex I (NADH ubiquinone oxidoreductase); CII, mitochondrial complex II (succinate dehydrogenase); CIII, mitochondrial complex III (cytochrome *c* reductase); IV, mitochondrial complex IV (cytochrome *c* oxidase); CAT, catalase; CPT I, carnitine palmitoyl transferase I; CPT II, carnitine palmitoyl transferase II; CACT, carnitine-acylcarnitine translocase; CoA, coenzyme A; CoQ, coenzyme Q; CPS1, carbamoyl phosphate synthetase I; Cyt C, cytochrome *c*; GDH, glutamate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; GPx, glutathione peroxidase; F6P, fructose-6-phosphate; FAD, flavin adenine dinucleotide; G6P, glucose-6-phosphate; H+, proton/hydrogen ion; H_2O_2, hydrogen peroxide; HTML, Hydroxytrimethyllysine; LDH, lactate dehydrogenase; LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; NAD, nicotinamide adenine dinucleotide; NH_3, ammonia; mtDNA, mitochondrial deoxyribonucleic acid; Pi, phosphorus; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase; PEP, phosphoenolpyruvate; PG, phosphoglycerate; ROS, reactive oxygen species; SCFA, short-chain fatty acid; SLC25A5, Solute Carrier Family 25 Member 5; SOD, superoxide dismutase; SIRT, sirtuin; N-TML, *N*-trimethyllysine; TMLHE, Trimethyllysine dioxygenase; TML-OH; 3-hydroxy-Nε-trimethyllysine; UCP, uncoupling protein; VDAC, voltage-dependent anion channel. Created with BioRender.com

and is the first step in the mitochondrial metabolism of carbohydrates. Pyruvate feeds into the tricarboxylic acid (TCA) cycle, one of the main metabolic pathways of the mitochondria. If there is mitochondrial dysfunction, the TCA cycle will slow down, leading to a buildup of pyruvate. As pyruvate builds up, it becomes shunted to other pathways to be metabolized, specifically to lactate and alanine. Lactate dehydrogenase (LDH), a biomarker reported for ASD (El-Ansary et al., 2018; Khemakhem et al., 2017), converts pyruvate to lactate, consuming NADH. Alanine amino transferase (ALT), a biomarker usually associated with liver dysfunction but also reported as a biomarker in ASD, converts pyruvate to alanine, metabolizing glutamate to α − ketoglutarate, a TCA cycle metabolite. Thus, when mitochondria become dysfunctional, three metabolic biomarkers, pyruvate, lactate, and alanine may be elevated in the blood. In fact, lactate and pyruvate are used in the Morava diagnostic criterion for mitochondrial disease (Morava et al., 2006). Two ratios are also used to monitor mitochondrial function. The lactate-to-pyruvate ratio becomes elevated when lactate is overly produced from pyruvate. Another lesser-known ratio is the alanine-to-lysine ratio. Lysine is produced as a product of acetyl-CoA, the first step of the TCA cycle, by a non-TCA cycle pathway. Thus, when the TCA cycle slows down, less lysine is produced and more alanine is produced, raising the alanine-to-lysine ratio to over 2.5 (Poling et al., 2006).

The mitochondria are also responsible for metabolizing fatty acids. Fatty acids are categorized into short, medium, long chain, and very long chain fatty acids, with the latter metabolized in the peroxisomes. Short and medium chain fatty acids can enter the mitochondria directly while long chain fatty acids are bound to carnitine and transported through the carnitine shuttle (CPT I, CAC, CPT II). Fatty acids are metabolized by β-oxidation (aka fatty acid oxidation) which is a cycle that shortens the fatty acid by two carbons with each turn, producing an acetyl-CoA, which enters directly at the first step of the TCA cycle, and $FADH_2$, which directly contributes to the ETC as a substrate for ETC Complex II. The process continues until the fatty acid is either 2 carbons for even length fatty acids or 3 carbons for odd length fatty acids. Even length fatty acids produce acetyl-CoA as the last step and enter the TCA cycle at the first step, while odd length fatty acids produce propionic

acid as the last step, a short chain fatty acid (SCFA) that is also produced by gut bacteria and enters the TCA cycle at succinyl-CoA.

Carnitine is instrumental to fatty acid metabolism but also serves another purpose. Carnitine can bind to fatty acids in the blood as acyl-carnitines and can also bind to excess organic acids. This increases the amount of carnitine esters and reduces the free carnitine. Carnitine can then be excreted in the urine with these fatty acids or organic acids if they are in excess, resulting in a carnitine loss in the body, reducing total carnitine. Carnitine is obtained from meats in the diet but can also be produced in the body. Trimethyllysine dioxygenase (TMLHE) is the first step in carnitine production and is encoded by a gene on the X-chromosome. Mutations in this gene reduce carnitine biosynthesis and have been linked to ASD in male-male multiplex families (Celestino-Soper et al., 2012).

Two other biomarkers that are related to mitochondrial function but are not directly involved in the energy pathways are ammonia and creatine kinase. Ammonia is disposed of by the urea cycle, which is partially within the mitochondrial matrix and has biochemical connections to the TCA cycle. Thus, disruption of mitochondrial function can result in an elevation in ammonia. Creatine kinase is primarily found in muscles where it is essential for storing energy from adenosine triphosphate (ATP) as phosphocreatine. It is very common for mitochondrial dysfunction to cause a mild myopathy since the muscle is so dependent on mitochondrial function. Dysfunction of muscle cells such as in a mild myopathy will result in an increase in creatine kinase that is usually mild, often just outside the upper range of normal.

Many studies investigate ETC activity directly using either enzymology or respirometry. Enzymology interrogates individual complex activity by providing substrates specific to the complex and measuring the produced metabolites. There are important limitations to enzymology. Since enzymology examines ETC complexes individually, it does not provide information regarding how the ETC complexes work together, which can be very important. Also, enzymology is best measured in fresh tissue, which is often technically difficult to obtain, so fresh frozen tissue is often used. Further issues occur when tissues need to be cultured to obtain enough tissue for testing. This occurs for fibroblasts, which are commonly obtained from a skin biopsy. Fibroblasts are commonly grown for up to 6 weeks. In vitro cultivation exerts selective pressures that favor cells that survive better and grow faster. These cells are likely to have well-functioning mitochondria compared to those with relatively poor functioning mitochondria, thereby biasing the final enzymology testing.

Respirometry is a technique which measures the change in mitochondrial respiration as the mitochondria and ETC complexes are manipulated by specific reagents. Respiration refers to the use of oxygen which occurs at ETC complex IV and is a metric of the activity of the mitochondria. Respirometers have developed over the years, and currently the most advanced respirometer, the Seahorse produced by Agilent, can measure mitochondrial respiration in whole tissue samples or isolated mitochondria in a 96 well plate to allow multiple samples to be evaluated simultaneously. For example, the Agilent Seahorse XFe96 Analyzer simultaneously measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in real-time in a wide range of intact living cell types (Perez et al., 2010; Hill et al., 2010) using a 96-well plate format. The Seahorse assay is a 4-step process which monitors OCR three times during four distinct periods in response to various reagents that activate or inhibit the ETC (Fig. 3). Several key parameters are derived from this process. Three OCRs are measured over 18 min to determine mitochondrial activity for each segment of the assay. Regents are added to determine parameters of mitochondrial activity. These parameters are then used to calculate respiratory metrics. Basal Respiration is calculated 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 related to ATP-Linked Respiration and Proton-Leak Respiration. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), a protonophore, is

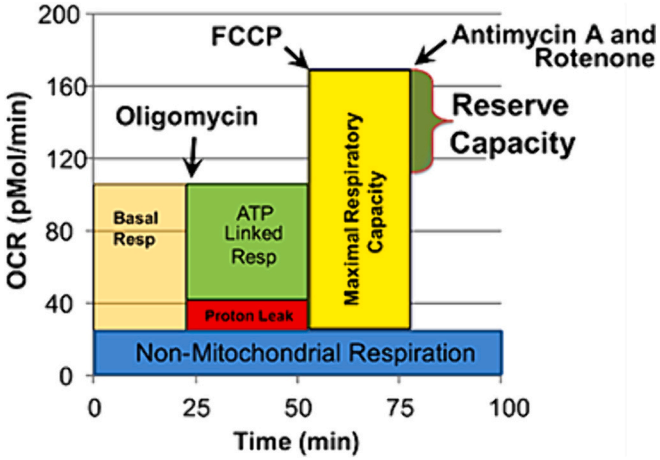

Fig. 3. Seahorse assay and various mitochondrial respiratory parameters (see text for descriptions). Reprinted with permission from Prenatal air pollution influences neurodevelopment and behavior in autism spectrum disorder by modulating mitochondrial physiology by Frye RE, Cakir J, Rose S, Delhey L, Bennuri SC, Tippett M, Melnyk S, James SJ, Palmer RF, Austin C, Curtin P, Arora M, licensed under Creative Commons Attribution 4.0 International License, *Molecular Psychiatry. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

then added to collapse the inner membrane gradient, driving the mitochondria to respire at their maximal rate. This determines Maximal Respiratory Capacity. Antimycin A and Rotenone, Complex III and I inhibitors, stop mitochondrial respiration to determine the non-mitochondrial respiration. Reserve Capacity is calculated as the difference between Basal Respiration and Maximal Respiratory Capacity.

Reactive oxygen species (ROS) are produced by metabolic processes particularly ETC Complex I and III (See Fig. 2). ROS can be destructive to many vulnerable portions of the mitochondria. Lipid membranes are particularly vulnerable to ROS. The integrity of lipid membranes is important for key reactions such as in the ETC. If the lipid membrane is damaged, protons can leak across the inner mitochondrial membrane and can reduce the proton gradient which is responsible for driving ETC Complex V to make ATP. This will diminish ATP production and reduce the inner mitochondrial membrane potential. ROS can also damage mitochondrial DNA (mtDNA), as the mtDNA is not protected like nuclear DNA and the mitochondria are not efficient at repairing mtDNA. This can lead to deleterious mtDNA mutations if the damage occurs in a critical genetic location on the mtDNA.

There are several mechanisms to control ROS. First, ROS in the form of superoxide is transformed to hydrogen peroxide by the enzyme superoxide dismutase (SOD) (See Upper Left of Fig. 2). Hydrogen peroxide can generate a hydroxyl radical which is also a ROS or can be detoxified by either catalase (CAT) or glutathione peroxidase (GPx) and converted to oxygen and water. GPx uses reduced glutathione (GSH) for this reaction, resulting in oxidized glutathione (GSSG). GSH is the most important antioxidant in the cell and the ratio of GSH to GSSH is a crucial biomarker for the cellular redox state. GSH cannot be produced inside mitochondria, so it needs to be imported. The pathway that makes more GSH requires ATP, the energy molecule produced by the mitochondria, so poor mitochondrial function will result in less GSH production and a reduced ability of the mitochondria to produce cellular energy. ROS is controlled at the inner mitochondrial membrane by leaking protons back across the membrane, a process that makes the mitochondria less efficient. This is performed by several proton channels such as the uncoupling protein (UCP) (See bottom middle of Fig. 2).

One of the important control systems of the mitochondria which have more recently been recognized are the sirtuins. First identified in humans in 1999, these conserved class III histone deacetylases all possess a nicotine adenine dinucleotide + (NAD+)binding catalytic

domain and are important in the regulation of both inflammation and metabolism (Wu et al., 2022). In mitochondria, the sirtuins use NAD+ as a sensor of the mitochondrial energy state with SIRT3 increasing the control of oxidative stress by upregulating SOD, SIRT 4 increasing the ATP/ADP transporter, and SIRT 5 increasing urea cycle function.

Mitochondria constantly undergo cycles of fission and fusion, dynamic processes which maintain mitochondrial health by eliminating dysfunctional mitochondria and repairing damaged mitochondria. Mitochondria demonstrate considerable variability in shape and size with morphology varying from long tubules to small spheres. Commonly thought of as an isolated static organelle, mitochondria are now known to form networks that may optimize their function and change subcellular positioning by moving along cytoskeletal tracks to reach sites of high-energy need like synapses (Saxton and Hollenbeck, 2012). We are beginning to understand that mitochondrial morphology is a link to mitochondrial respiration and mitochondrial health in ASD (Frye et al., 2021a) and environmental adaptations (Charrasse et al., 2023). Thus, examination of mitochondrial morphology has been adopted as a new biomarker for studying mitochondrial health.

3.1.2. *Metabolic biomarkers: Blood based biomarkers*

Table 1 reports the results of the meta-analysis concerning the prevalence of biomarkers of mitochondrial function in ASD. Table 2 lists the meta-analysis of differences in biomarker values comparing ASD to controls.

3.1.2.1. Metabolic biomarkers: Lactate. A total of 58 studies examined lactic acid levels in individuals with ASD with 29 (50%) being uncontrolled studies (Coleman and Blass, 1985; Graf et al., 2000; Poling et al., 2006; Laszlo et al., 1994; Filipek et al., 2004; Filipek et al., 2003; Oliveira et al., 2005; Pons et al., 2004; Correia et al., 2006; Germano et al., 2006; Tsao and Mendell, 2007; Castro-Gago et al., 2008; Weissman et al., 2008; Connolly et al., 2010; Ezugha et al., 2010; Guevara-Campos et al., 2010; Shoffner et al., 2010; Frye and Naviaux, 2011; Frye, 2012a; Craig et al., 2012; Spilioti et al., 2013; Esparham et al., 2015; Guevara-Campos et al., 2015; Prasad and Hussain, 2015; Kiykim et al., 2016; Burger et al., 2017; El Fotoh et al., 2019; Boterberg et al., 2022; Yang et al., 2022) (Supplementary Table 1a) and 29 (50%) being controlled (Giulivi et al., 2010; Khemakhem et al., 2017; Moreno et al., 1992; Hashimoto et al., 1998; Hashimoto et al., 1997; Chugani et al., 1999; Friedman et al., 2003; Mostafa et al., 2005; Al-Mosalem et al., 2009; El-Ansary et al., 2010; Corrigan et al., 2012; Essa et al., 2012; Kuwabara et al., 2013; Goh et al., 2014; El-Ansary et al., 2017; Lussu et al., 2017; Hassan et al., 2019; Graham et al., 2020; Oh et al., 2020; Smith et al., 2020; Sotelo-Orozco et al., 2020; Mahalaxmi et al., 2021; Gagliano et al., 2022; Esvap and Ulgen, 2023; Maier et al., 2023; Nickel et al., 2023; Smith et al., 2023; Thomson et al., 2023; Adiba et al., 2024) (Supplementary Table 1b). All studies examined serum or plasma lactate unless otherwise stated.

Uncontrolled studies. The first cases of lactic acidosis were reported in 1985 in 4 children with ASD by Coleman and Blass (Coleman and Blass, 1985). Since that publication, 20 uncontrolled studies reported elevated lactate in patients with ASD (Poling et al., 2006; Laszlo et al., 1994; Filipek et al., 2003; Oliveira et al., 2005; Pons et al., 2004; Correia et al., 2006; Germano et al., 2006; Castro-Gago et al., 2008; Weissman et al., 2008; Guevara-Campos et al., 2010; Shoffner et al., 2010; Frye and Naviaux, 2011; Frye, 2012a; Spilioti et al., 2013; Guevara-Campos et al., 2015; Prasad and Hussain, 2015; Kiykim et al., 2016; Burger et al., 2017; Boterberg et al., 2022; Frye, 2012a), including one ASD child with elevated lactate in the CSF (Pons et al., 2004). One study verified the lactate elevation in patients where it was initially found to be elevated (Frye, 2012a). In this study, 35% of children with ASD were found to have lactate elevation in the initial evaluation, but the lactate elevation could only be verified in 45% of the children when it was checked again (Frye, 2012a). Eight studies reported normal lactate levels (Graf et al., 2000; Filipek et al., 2004; Tsao and Mendell, 2007; Connolly et al., 2010; Ezugha et al., 2010; Craig et al., 2012; Esparham et al., 2015; El Fotoh et al., 2019) including one reporting normal CSF lactate in a child with ASD (Graf et al., 2000). Many of these studies used laboratory reference ranges for lactate as a comparison. A meta-analysis on lactate prevalence (Supplementary Fig. 1 A) was conducted on 7 studies (Laszlo et al., 1994; Filipek et al., 2004; Oliveira et al., 2005; Correia et al., 2006; Germano et al., 2006; Spilioti et al., 2013; Frye, 2012a) resulting in an overall prevalence of abnormal lactate in ASD of 17% with a LKS index of 1.17 (Supplementary Fig. 2 A, 3 A) suggesting minor asymmetries, and a significant Cochran's Q demonstrating considerable heterogeneity due to one study showing zero prevalence and two studies showing higher prevalence. However, the I^2 of 93% suggests that the variation was due to heterogeneity and not chance.

Controlled studies. The first prospective controlled study out of Venezuela in 1992 reported a significantly higher blood lactate in 60 patients with ASD compared to age and sex matched controls (Moreno et al., 1992). All 29 studies reported increased blood lactate compared to controls except for 4 studies which reported lower values (Khemakhem et al., 2017; Kuwabara et al., 2013; Lussu et al., 2017; Nickel et al., 2023), including one that reported similar values between mild, moderate, and severe ASD and controls (Khemakhem et al., 2017). One study that performed a bioinformatics prediction of prefrontal lobe transcriptome data derived from post-mortem brains predicted elevations in brain lactate (Esvap and Ulgen, 2023). A meta-analysis of blood lactate concentrations (Supplementary Fig. 1B) was conducted on seven of the controlled studies which reported actual values for mean lactate levels in ASD compared to controls (Moreno et al., 1992; Al-Mosalem et al., 2009; El-Ansary et al., 2017; Hassan et al., 2019; Oh et al., 2020; Adiba et al., 2024; Mostafa and Al-Ayadhi, 2015). The meta-analysis resulted in a large effect size (Table 1) with some heterogeneity and asymmetry (Supplementary Fig. 2B, 3B).

Lactate has also been studied in the brain. Three studies reported elevated brain lactate using magnetic resonance spectroscopy (MRS) in ASD individuals (Chugani et al., 1999; Goh et al., 2014; Maier et al., 2023), while five studies reported no difference between ASD and control groups (Hashimoto et al., 1998; Hashimoto et al., 1997; Friedman et al., 2003; Corrigan et al., 2012; Graham et al., 2020). The insensitivity of standard MRS to detecting lactate, even in those with known mitochondrial disease has been discussed (Rossignol and Frye, 2012c). In one of the more advanced studies using high-resolution, multiplanar spectroscopic imaging to map the lactate distribution in the brains, lactate was most often identified in the cingulate gyrus and in adults with ASD (Goh et al., 2014). Given that standard MRS commonly examines the basal ganglia, it may very well be that some studies are missing the optimal target location to measure lactate in the brain.

3.1.2.2. Metabolic biomarkers: Pyruvate. Uncontrolled studies. Twelve uncontrolled studies (Supplemental Table 2a) reported a variety of results including elevated serum pyruvate (Laszlo et al., 1994; Weissman et al., 2008; Frye and Naviaux, 2011; Burger et al., 2017), below normal

Table 1
Meta-analysis results for the prevalence of abnormal biomarkers of mitochondrial function in children with autism spectrum disorder (ASD). Pooled prevalence with 95% confidence interval, Cochran's Q (Q), Heterogeneity Index (I^2), Luis Furuya-Kanamori (LFK) Index and number of studies involved (N). Statistics are estimated by a random-effects model. $^*p < 0.01$; ^{T} Significant Asymmetry.

Biomarker	Prevalence (95% CI)	Q	I^2	LFK	N
Lactate elevation	17% (8%, 29%)	90.5*	93%	1.17	7
Pyruvate elevation	41% (18%, 67%)	17.85*	83%	2.85^T	4
Lactate-to-Pyruvate Ratio elevation	28% (22%, 34%)				1
Alanine elevation	15% (0%, 42%)	36.17*	94%	4.23^T	3
Alanine-to-Lysine Ratio elevation	8.2% (3.4%, 14.5%)				1
Acyl-Carnitine elevation	19% (26%, 13%)	1.59			3
Creatine Kinase elevation	9% (4%, 15%)	12.37*	92%		2

Table 2
Meta-analysis results for the difference between values for biomarkers of mitochondrial function in children with autism spectrum disorder (ASD). Pooled prevalence with 95% confidence interval, Cochran's Q (Q), Heterogeneity Index (I^2), Luis Furuya-Kanamori (LFK) Index and number of studies involved (N). Statistics are estimated by a inverse variance heterogeneity model. *$p < 0.01$; ^{T} Significant Asymmetry. CNT = control individuals; WMD = Weighted Mean Difference; Ex Out = Excluding Outlier.

Biomarker	ASD	CNT	WMD	Cohen's d'	Q	I^2	LFK	N
Lactate (mmol/L)	1.47 (1.38, 1.57)	0.66 (0.63, 0.69)	0.78 (0.71,0.86)	1.4 (1.2, 1.5)	78.96*	92%	2.12^T	7
Pyruvate (mg/dL)	1.20 (1.11, 1.29)	0.76 (0.72, 0.80)	0.55 (0.48, 0.62)	1.1 (0.9, 1.3)	71.84*	94%	1.63	5
Lactate to Pyruvate Ratio	2.28 (2.08, 2.47)	1.63 (1.48, 1.77)	1.11 (1.03, 1.20)	0.6 (0.4, 0.8)	24.28*	88%	-5.65^T	4
Alanine (μmol/L)	258.4 (243.5, 273.2)	296.8 (277.7, 315.8)	−40.4 (−17.3, −63.4)	−0.4 (−0.1, −0.7)	20.45*	95%		2
Total Carnitine (μmol/L)	22.8 (21.3, 24.3)	46.8 (45.2, 48.4)	−23.8 (−26.0, −21.6)	−3.0 (−2.6, −3.9)	2.04			3
Free Carnitine (μmol/L)	23.5 (22.0, 25.1)	27.1 (24.4, 29.8)	−3.6 (−6.7, −0.5)	−0.5 (−1.0, −1.0)				2
CoQ10 (ng/mL)	5.8 (5.6, 6.1)	6.4 (6.1, 6.7)	−2.5 (−2.8, −2.1)	−0.3 (−0.60, 0.1)	53.96*	96%	-3.08^T	3
ATP (μmol/mL)	2.78 (2.6, 2.9)	0.94 (0.89, 0.99)	0.61 (0.49, 0.74)	1.02 (0.81, 1.23)	728.46*	100%	1.71	4
ATP (μmol/mL) Ex Out	1.72 (1.59, 1.86)	2.16 (2.02, 2.31)	−0.56 (−0.41, −0.72)	−0.82 (0.52, 1.11)	3.98			3
Ammonia (μg/dl)	52.9 (51.3, 54.6)	23.3 (22.4, 24.2)	29.7 (27.8, 31.5)	5.1 (4.5, 5.8)				1
Creatine Kinase (U/L)	160.9 (141.7, 180.1)	93.94 (85.7102.2)	67.2 (63.5,70.8)	1.6 (1.30, 1.95)	67.76*	96%	2.53^T	4

serum pyruvate concentration (Filipek et al., 2004; Craig et al., 2012) and normal concentrations in the urine (Esparham et al., 2015), serum (Graf et al., 2000; Ezugha et al., 2010; Guevara-Campos et al., 2010) and CSF (Graf et al., 2000; Ezugha et al., 2010). Four studies (Giulivi et al., 2010; Laszlo et al., 1994; Weissman et al., 2008; Oh et al., 2020) were included in the prevalence estimates for meta-analysis (Supplementary Fig. 1C) which demonstrated a high prevalence of elevated pyruvate in ASD (Table 1). However, there was considerable variation and asymmetry due to one study (Giulivi et al., 2010) demonstrating a higher prevalence than the others (Supplementary Fig. 2C, 3C).

Controlled studies. Elevated pyruvate blood concentrations in ASD were first reported in 1998 in a controlled study (Moreno et al., 1992). Of the 14 controlled studies (Giulivi et al., 2010; Khemakhem et al., 2017; Germano et al., 2006; Moreno et al., 1992; Lussu et al., 2017; Hassan et al., 2019; Oh et al., 2020; Smith et al., 2020; Sotelo-Orozco et al., 2020; Gagliano et al., 2022; Esvap and Ulgen, 2023; Nickel et al., 2023; Smith et al., 2023; Thomson et al., 2023) identified (Supplemental Table 2b), nine studies compared serum or plasma pyruvate in ASD individuals to controls. Five studies reported higher concentrations compared to normal controls (Giulivi et al., 2010; Khemakhem et al., 2017; Moreno et al., 1992; Hassan et al., 2019) or to children with mental retardation (Germano et al., 2006). Three studies reported similar concentrations in ASD (Oh et al., 2020; Sotelo-Orozco et al., 2020; Maier et al., 2023) and one reported lower concentrations in ASD compared to controls (Gagliano et al., 2022). A meta-analysis of five studies (Giulivi et al., 2010; Khemakhem et al., 2017; Moreno et al., 1992; Hassan et al., 2019; Oh et al., 2020) (Supplementary Fig. 1D) showed a large effect size with pyruvate being higher in ASD compared to controls with minor asymmetry (Supplementary Fig. 2D, 3D) due to one study (Giulivi et al., 2010) having high pyruvate values.

3.1.2.3. Metabolic biomarkers: Lactate-to-pyruvate ratio. Uncontrolled studies. A case report in 2000 was the first to report an elevated lactate-to-pyruvate ratio in ASD (Graf et al., 2000). Since then, six uncontrolled studies reported elevated lactate-to-pyruvate ratios in ASD (Supplemental Table 3a) (Oliveira et al., 2005; Correia et al., 2006; Weissman et al., 2008; Guevara-Campos et al., 2010; Yang et al., 2022; Guevara-Campos et al., 2013). All studies examined serum or plasma. One uncontrolled (Correia et al., 2006) study was qualified to calculate prevalence (Table 1).

Controlled studies. Seven prospective controlled studies examined the lactate-to-pyruvate ratio in individuals with ASD compared to controls using either serum or plasma (Supplemental Table 3b). Three studies reported a significantly higher ratio in the ASD group (Essa et al., 2012; Hassan et al., 2019; Oh et al., 2020), one study reported a similar ratio (Nickel et al., 2023) and two studies reported a lower ratio (El-Ansary et al., 2018; Mahalaxmi et al., 2021). One study examined 44 biomarker clusters and observed an elevated lactate-to-pyruvate ratio in 27.6% of the ASD group (Smith et al., 2023). Four studies qualified for a meta-analysis (El-Ansary et al., 2018; Essa et al., 2012; Hassan et al., 2019; Oh et al., 2020) (Supplementary Fig. 1E) which showed that ASD was associated with an elevated lactate-to-pyruvate ratio with a medium to large effect size. Significant heterogeneity and asymmetry were the result of one study (El-Ansary et al., 2018) which found a decreased ratio in the ASD group (Supplementary Fig. 2E, 3E).

3.1.2.4. Metabolic biomarkers: Alanine and alanine-to-lysine ratio. Uncontrolled studies. Alanine was first reported to be elevated compared to a laboratory reference range in ASD in 2003 (Aldred et al., 2003). Since then five other case series (Supplemental Table 4a) reported elevated alanine (Filipek et al., 2004; Weissman et al., 2008; Frye and Naviaux, 2011; Frye, 2012a) or an elevated alanine-to-lysine ratio (Poling et al., 2006; Frye and Naviaux, 2011; Frye, 2012a). One study examined the correlation of alanine with epileptiform discharges on EEG but did not find any correlation (Marcotulli et al., 2022). These studies examined serum or plasma alanine and lysine. Three studies (Weissman et al., 2008; Frye, 2012a; Adams et al., 2011a) were included in the alanine meta-analysis (Supplementary Fig. 1F, 2F, 3F) and one study (Frye, 2012a) was included in the alanine-to-lysine ratio prevalence estimates (Table 1).

Controlled studies. Fifteen studies (Supplemental Table 4b) examined alanine compared to controls (Graham et al., 2020; Sotelo-Orozco et al., 2020; Esvap and Ulgen, 2023; Thomson et al., 2023; Adams et al., 2011a; Fernell et al., 2007; Ming et al., 2012; Zaki et al., 2017; Delaye et al., 2018; Saleem et al., 2020; Usui et al., 2020; Ma et al., 2021; Yu et al., 2021; Parenti et al., 2022; Chamtouri et al., 2023). Five studies reported similar alanine between ASD and controls in plasma (Sotelo-Orozco et al., 2020; Adams et al., 2011a; Delaye et al., 2018; Saleem et al., 2020) and brain samples (Graham et al., 2020). Five studies reported lower alanine levels in ASD compared to controls in plasma (Zaki et al., 2017; Usui et al., 2020), serum (Yu et al., 2021) and urine (Ming et al., 2012; Ma et al., 2021). One study using a genome-scale metabolic model derived from transcriptome data from the prefrontal cortex predicted brain alanine to be elevated (Esvap and Ulgen, 2023). The transportation of alanine across fibroblast membranes was higher in ASD than controls in another study (Fernell et al., 2007). One study reported significantly higher fecal alanine in the ASD group compared to siblings and non-related TD controls (Chamtouri et al., 2023). Alanine was found to be lower in the placenta of children who go on to be diagnosed with ASD (Parenti et al., 2022), and one study associated the daily di-(2-ethylhexyl) phthalate exposure during pregnancy with an increase in serum alanine (Thomson et al., 2023). Two studies (Adams et al., 2011a; Yu et al., 2021) qualified for the meta-analysis (Supplementary Fig. 1G) which demonstrated that ASD individuals had lower alanine with a medium effect size (Table 2). There were no controlled studies for alanine-to-lysine ratio.

3.1.2.5. Metabolic biomarkers: Carnitine. Uncontrolled studies. One author reported low carnitine noted from personal observations (Lombard, 1998). Low carnitine was first reported in a large cohort of children with ASD but the mean reference was used to make this determination, leaving the number of children with below the normal reference range unknown (Filipek et al., 2004). Six additional case reports in ASD individuals (Supplemental Table 5a) suggested that low total or free carnitine are biomarkers for several metabolic disorders associated with ASD including glutaric aciduria type I (Kiykim et al., 2016) and II (Prasad and Hussain, 2015), TMLHE deficiency (Ziats et al., 2015; Goin-Kochel et al., 2019), and mutation in OCTN2 (SLC22A5) which is known to cause systemic primary carnitine deficiency (Guevara-Campos et al., 2019; Shi et al., 2019). Carnitine was measured in serum or plasma in these studies.

Controlled studies. Thirteen controlled studies were identified that examined carnitine levels in ASD individuals compared to controls (Mostafa et al., 2005; Hassan et al., 2019; Graham et al., 2020; Sotelo-Orozco et al., 2020; Nickel et al., 2023; Smith et al., 2023; Mostafa and Al-Ayadhi, 2015; Parenti et al., 2022; Lv et al., 2018; Canfield et al., 2019; Kim et al., 2021; Liu et al., 2022; Alzamily et al., 2024). These studies measured carnitine in serum or plasma unless otherwise noted. Four studies reported significantly lower total carnitine concentrations in ASD individuals compared to controls (Mostafa et al., 2005; Hassan et al., 2019; Sotelo-Orozco et al., 2020; Mostafa and Al-Ayadhi, 2015), while free carnitine concentrations were reported lower in plasma (Lv et al., 2018) and brain tissue (Graham et al., 2020) in ASD individuals compared to controls (Supplemental Table 5b). However, one study reported similar total and free carnitine concentrations between individuals with ASD and controls (Nickel et al., 2023). One study reported decreased carnitine in 3 biomarker clusters indicating low carnitine may occur with different metabolic states in ASD (Smith et al., 2023). One study examined 159 single nucleotide polymorphisms (SNPs) that were associated with carnitine, but these were not significantly associated with ASD risk (Liu et al., 2022). Serum carnitine was found to have an inverse relationship with plasma lactate in one study (Mostafa et al., 2005).

A meta-analysis of the two eligible studies (Mostafa et al., 2005; Hassan et al., 2019) (Supplementary Fig. 1H) found lower total carnitine concentrations in ASD with an extremely large effect size (Table 2). One study of free carnitine (Lv et al., 2018) was included in the meta-analysis (Supplementary Fig. 1I) and found lower free carnitine in ASD with a medium effect size (Lv et al., 2018). One study measuring salivary carnitine found that it was detectable in only 48% of the samples indicating saliva is not a viable measurement of carnitine levels (Ratajczak and Sothern, 2015), but the one study that compared salivary concentration between ASD and controls found similar carnitine levels (Alzamily et al., 2024).

3.1.2.6. Metabolic biomarkers: Acyl-carnitines. Eleven uncontrolled (Frye et al., 2013b; Filipek et al., 2004; Frye and Naviaux, 2011; Prasad and Hussain, 2015; Kiykim et al., 2016; Burger et al., 2017; Frye, 2012a; Clark-Taylor and Clark-Taylor, 2004; Kanavin et al., 2007; Marquez-Caraveo et al., 2021; Moravej et al., 2023) and 13 controlled studies (Graham et al., 2020; Nickel et al., 2023; Smith et al., 2023; Usui et al., 2020; Lv et al., 2018; Canfield et al., 2019; Kim et al., 2021; Barone et al., 2018; Hollowood-Jones et al., 2020; Langlois et al., 2020; Needham et al., 2021; Barone et al., 2021; Vacy et al., 2024) examined abnormalities in fatty acid metabolism in ASD, most of them using acyl-carnitine (AC) panels measured in plasma or serum to investigate disruption in fatty acid metabolism.

Uncontrolled studies. ASD was first hypothesized to be associated with long-chain hydroxyacyl-CoA dehydrogenase deficiency in a case report of an 8 year old male with ASD who showed mild elevations in unsaturated long (C14:2, C14:1) and a high normal medium (C10:1) chain acyl-carnitine (AC) (Clark-Taylor and Clark-Taylor, 2004). Several case series (Supplemental Table 6a) have reported AC elevations in individuals with ASD and metabolic disorders known to increase ACs. A boy with 2-methylbutyryl-CoA dehydrogenase deficiency was found to have elevation in C5 and C5/C3 ratio (Kanavin et al., 2007), another boy with glutaric aciduria type II was found to have elevation in short (C4, C6), medium (C8, C10, C12:1) and long (C14:1, C16:1, C18:1) chain ACs (Prasad and Hussain, 2015), a third boy with glutaric aciduria type 1 was found to have elevations in glutaryl-carnitine (C5DC) (Kiykim et al., 2016), and finally another boy diagnosed with short-chain acyl-CoA dehydrogenase deficiency was found to have elevations in a short chain AC (C4) (Kiykim et al., 2016). A case series of 51 patients found one patient with an elevation in isovalerylcarnitine (C5) along with 3-hydroxyisovaleric acid and isovalerylglycine in urinary organic acids consistent with isovaleric acidemia, and another patient had elevations in 3-hydroxy-isovalerylcarnitine (C5OH) and methylmalonylcarnitine (C4DC) along with 2-methyl-3-hydroxybutyric, 3-hydroxybutyric and 3-keto-3-methylvaleric acid consistent with beta-ketothiolase deficiency, although it does not appear that these diagnoses were confirmed molecularly (Marquez-Caraveo et al., 2021). Another study which screened 105 children with ASD with ACs for two specific metabolic disorders found two children, one with 2-methylbutyryl glycinuria and one with short-chain acyl-CoA dehydrogenase deficiency (Moravej et al., 2023). Other studies have reported elevations in ACs in disorders not known to be associated with AC elevations. One study reported that a girl with a mutation in the WDR45 gene had elevations in short (C2, C5:1), medium (C6, C8:1), and long (C14, C18) chain ACs, while her brother who was found to have a mutation in the DEPDC5 gene had elevations in medium (C6, C8:1, C10) and long (C18:2) chain ACs (Burger et al., 2017). Another study found that children with ASD and increased ETC complex IV activity demonstrated elevations in short (C4) and long chain (C12) ACs (Frye and Naviaux, 2011). Lastly, in a case series on 100 children with ASD, the average AC (C2) concentration was within the normal range (Filipek et al., 2004).

Because the AC panel contains multiple measurements, two uncontrolled studies defined an abnormal AC panel as having three or more abnormal individual ACs compared to laboratory reference values. In both studies, the AC panels were repeated on patients in whom it was abnormal to confirm the original abnormalities (Frye et al., 2013b; Frye, 2012a). Using the prevalence from these two studies, the meta-analysis (Supplementary Fig. 1 J) found that the AC panel was abnormal in 19% of ASD patients (Table 1).

Two studies compared the subgroup of children with ASD with elevated ACs to those without elevations. The first study found a consistent pattern of AC elevations with short (C4OH, C5DC, C6) and long (C14:0, C14:2,C16:0,C16:1), but not medium-chain, AC elevations (Frye et al., 2013b), while the second study found elevations in short (C5, C5DC), medium (C8:1), and long (C14:2, C16:1) chain ACs as well as an impairment in beta-oxidation in the fibroblasts in children with consistently elevated ACs (Barone et al., 2021). To better understand these abnormalities, a meta-analysis comparing the children with and without AC elevations was performed in these 2 studies. In Fig. 4, the weighted means for the two studies are graphed for individuals with and without AC elevations (Fig. 4A). The confidence interval of the Cohen's d' is provided in panel Fig. 4B. This analysis demonstrates significant differences in several short chain, mostly odd, ACs (C5:0, C4DC/C5OH, C5DC, C6:0), several medium chain ACs (C8:0, C10:0, C10:1, C12:0, C12:1) and many long chain ACs (C14:0, C14:1. C14:2. C14OH, C14:1OH, C16:1, C16OH, C18:0).

Controlled studies. These studies examined serum or plasma carnitine unless otherwise noted. One of the first controlled studies (Supplemental Table 6b) examining ACs demonstrated significant elevations in short (C2, C4DC/C5OH), medium (C10, C12) and long (C14:2, C16, C16:1, C18:1) chain ACs in Italian children with ASD (Barone et al., 2018), while another study the same year demonstrated lower short (C5DC), medium (C8:1) and very long (C24:1, C26:1) chain ACs in Chinese children with ASD (Lv et al., 2018). Adults with ASD were found to have

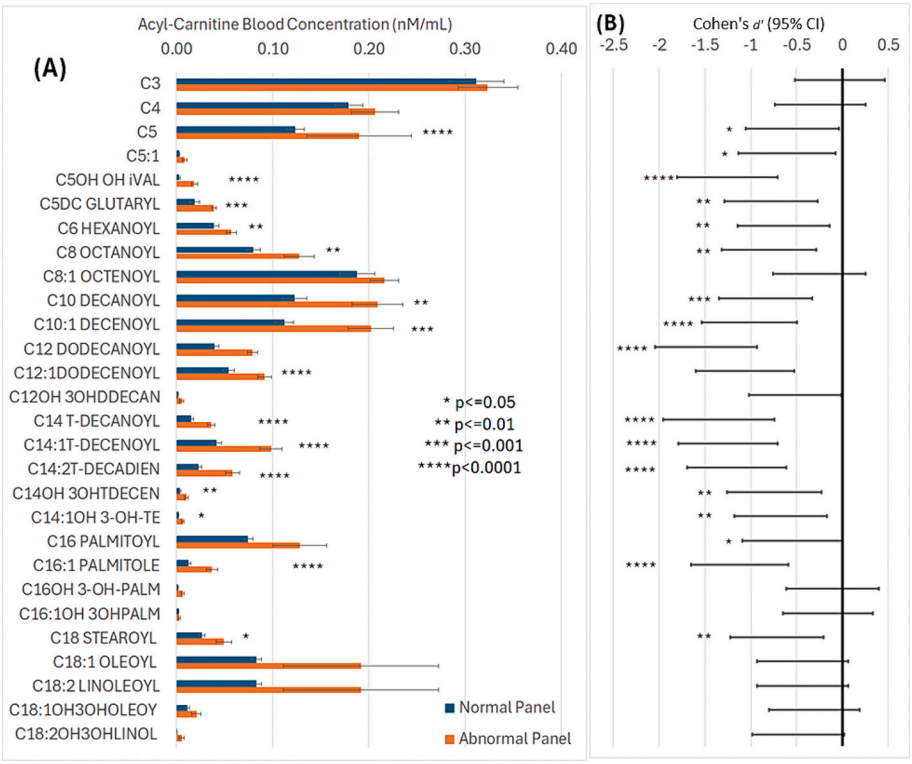

Fig. 4. Differences in acyl-carnitine concentrations in patients with and without abnormal acyl-carnitine panels. (A) Acyl-carnitine concentrations in the two groups. B) 95% confidence interval for the effect size for these differences.

significant elevations in short (C5:1) and medium (C6, C6:1, C6-OH, C10:1, C10:2) chain ACs in a recent study (Nickel et al., 2023). Another large prospective study found elevations in acetyl-carnitine (C2) in the stool from children with ASD compared to controls (Needham et al., 2021). ACs were found to be important in four of the 30 metabolic biomarker clusters identified in a diagnostic algorithm for ASD (Smith et al., 2023). Another large study using lipidomic analysis reported elevations in long chain ACs (C13:1, C14:0, C14:1, C14:2, C14:3, C16:1, C16:2, C16:1, C18:2) and very long chain ACs (20:1) (Usui et al., 2020). Elevations in medium (C12) and long (C14:1, C14:2, C16, C16:1, C16:2, C18:1, C18:2) chain ACs were found in post-mortem cerebellum from individuals with ASD (Graham et al., 2020). Another study examined mothers of children with ASD who were between 2 and 6 years of age and found lower AC concentrations in the mothers (Hollowood-Jones et al., 2020). The three controlled studies examining blood AC were analyzed in a meta-analysis for the ACs that all studies had in common (Graham et al., 2020; Lv et al., 2018; Barone et al., 2018). None of the differences were statistically significant but in almost every case, ACs in the ASD group were higher than in the control group (Table 3). These results are consistent with the notion that a subgroup of individuals with ASD have AC elevations.

3.1.2.7. Metabolic biomarkers: Coenzyme Q10. One case series of three children (Supplemental Table 7a) (Guevara-Campos et al., 2015) and seven prospective controlled studies (Supplemental Table 7b) (El-Ansary et al., 2018; Khemakhem et al., 2017; Adams et al., 2011a; Kurup and Kurup, 2003; Mousavinejad et al., 2018; El-Ansary et al., 2020; Hirayama et al., 2020) examined Coenzyme Q10 levels in serum or plasma (unless otherwise noted) in individuals with ASD.

Uncontrolled studies. A case series found that the CoQ10 concentration in muscle homogenate in one child was 12% reduced compared to the laboratory reference range and two patients became ataxic with a strong hand tremor when CoQ10 supplementation was discontinued from their treatment (Guevara-Campos et al., 2015).

Table 3
Meta-analysis of Acyl-Carnitines differences between children with autism spectrum disorder (ASD) and controls. Because of the variation in the acyl-carnitines measured or reported, only a subset of acyl-carnitines could be analyzed. Pooled prevalence with 95% confidence interval, Cochran's Q (Q). Statistics are estimated by a random-effects model. *$p < 0.01$.

	ASD	CNT	Q	N
C2	5.2 (4.6, 5.7)	4.4 (3.7, 5.0)	1.47	3
C4-DC/C5-OH	0.6 (0.5,0.7)	0.5 (0.3, 0.6)	2.20	3
C12	0.05 (0.02, 0.09)	0.04 (0.0, 0.08)	1.52	3
C16	0.87 (0.65, 1.10)	0.83 (0.56, 1.10)	4.72	3
C16:1	0.07 (0.04, 0.09)	0.07 (0.03, 0.09)	2.28	3
C18:1	0.98 (0.64, 1.32)	0.85 (0.50,1.19)	0.15	3

Controlled studies. Mean Coenzyme Q10 levels were higher in two studies (Khemakhem et al., 2017; El-Ansary et al., 2020), similar in one study (Adams et al., 2011a), and lower in two studies in children with ASD compared to controls (El-Ansary et al., 2018; Kurup and Kurup, 2003). Another study reported that the oxidation rate of Coenzyme Q10 (a marker of oxidative stress) was higher in the ASD group compared to controls (Hirayama et al., 2020), while one study could separate ASD from controls using an ROC analysis (Mousavinejad et al., 2018). Meta-analysis of the three eligible studies (Adams et al., 2011a; Kurup and Kurup, 2003; El-Ansary et al., 2020) (Supplementary Fig. 1 K) demonstrated a lower CoQ10 in ASD with a small to medium non-significant effect size (Table 2). Cochran's Q demonstrated heterogeneity, with one study (El-Ansary et al., 2020) weighted very little because of the large variation in its CoQ10 measurements (Supplementary Fig. 2G,3G).

3.1.2.8. Metabolic biomarkers: Tricarboxylic acid (TCA) cycle metabolites. Uncontrolled studies. Two uncontrolled studies examined TCA cycle metabolites in ASD (Supplemental Table 8a). The first study, published in 1995, reported a marked increase in urinary TCA cycle metabolite analogs in two brothers with ASD features including citramalic, tartaric

(3-OH-malic), and 3-oxoglutaric acids as well as citric acid analogs (Shaw et al., 1995). A case series of 25 patients with ASD and mitochondrial disease found that elevated urinary TCA cycle intermediates or 3-methylglutaconate were present in 42% (10/24) patients (Weissman et al., 2008).

Controlled studies. Six controlled studies examined TCA cycle metabolites in individuals with ASD (Supplemental Table 8b). Three studies examined urine (Kaluzna-Czaplinska, 2011; Yehia et al., 2019; Harutyunyan et al., 2021), one plasma (Sotelo-Orozco et al., 2020), and two brain samples (Graham et al., 2020; Esvap and Ulgen, 2023). The first study reported higher urine citric acid and lower urine isocitric acid in ASD (Kaluzna-Czaplinska, 2011). Another study reported that urinary cis-aconitate, isocitrate, alpha-ketoglutarate, and hydroxymethylglutarate were elevated by 55–76% in ASD (Harutyunyan et al., 2021). One study found that a combined group of individuals with ASD and developmental delays had higher urinary citrate and decreased urinary isocitrate, succinate and fumarate; however, the two groups were not examined separately (Yehia et al., 2019). Higher plasma cis-aconitate, fumarate, and succinate were observed in another study in ASD (Sotelo-Orozco et al., 2020). One study examining brain tissue found similar succinate concentrations between ASD and controls (Graham et al., 2020), while another study examining the prefrontal cortex transcriptome data using a genome-scale metabolic model predicted that the production of citrate, cis-aconitate, and succinate were faster, and the production of isocitrate, alpha-ketoglutarate, malate, and oxaloacetate were slower in the ASD model (Esvap and Ulgen, 2023). Meta-analysis could not be performed as there were no eligible studies.

3.1.2.9. Metabolic biomarkers: ATP. Uncontrolled studies. One case report found higher ATP levels in fibroblasts from a child with ASD compared to his father (Park et al., 2018) (Supplemental Table 9a).

Controlled studies. Six controlled studies examined ATP levels in ASD individuals (Supplemental Table 9b). Four studies which examined plasma ATP found it to be lower (El-Ansary et al., 2017; Adams et al., 2011a), similar (Al-Mosalem et al., 2009) and higher (Vellingiri et al., 2023). Other studies found ATP similar to controls in lymphocytes (Giulivi et al., 2010) and brain samples (Graham et al., 2020). The meta-analysis (Supplementary Fig. 1 L) of the four plasma studies demonstrated a large effect size for ATP being higher in ASD as compared to controls (Table 2), but these results were driven by one large study (Vellingiri et al., 2023) that demonstrated opposite effects of the others (Al-Mosalem et al., 2009; El-Ansary et al., 2017; Adams et al., 2011a) (Supplementary Fig. 2H, 3H). Removing the outlier results in significantly less variation in the studies (Supplementary Fig. 1 M, 2I, 3I) and a large effect size in the opposite direction, with ASD individuals demonstrating a lower ATP than controls (Table 2).

3.1.2.10. Metabolic biomarkers: Ammonia. Uncontrolled studies. Hyperammonemia was first reported in a child with ASD in 2002 (Cohen, 2002). Three additional case series (Supplementary Table 10a) reported high ammonia in children with ASD (Filipek et al., 2004; Germano et al., 2006; Gorker and Tuzun, 2005), while 4 studies reported normal serum or plasma levels (Guevara-Campos et al., 2010), including in some ASD individuals with metabolic disorders (Ezugha et al., 2010; Kiykim et al., 2016; El Fotoh et al., 2019).

Controlled studies. Eight controlled studies (Supplementary Table 10b) measured ammonia in children with ASD (Hassan et al., 2019; Esvap and Ulgen, 2023; Adiba et al., 2024; Zaki et al., 2017; Saleem et al., 2020; Chamtouri et al., 2023; Abu Shmais et al., 2012; Wang et al., 2012). Five studies reported significantly higher mean blood (Hassan et al., 2019; Adiba et al., 2024; Zaki et al., 2017; Saleem et al., 2020) or fecal (Wang et al., 2012) ammonia levels in ASD compared to controls, while two studies reported similar concentrations in blood (Abu Shmais et al., 2012) and stool (Chamtouri et al., 2023). One study that performed a bioinformatics prediction of prefrontal lobe transcriptome data derived from post-mortem brains predicted elevations in brain ammonia (Esvap and Ulgen, 2023). Analysis of one study (Supplementary Fig. 1 N) found higher blood ammonia in ASD with a very large effect size (Hassan et al., 2019) (Table 2).

3.1.2.11. Metabolic biomarkers: Creatine kinase. Uncontrolled studies. Eleven case series (Supplemental Table 11a) reported elevated creatine kinase (CK) levels in patients with ASD (Germano et al., 2006; Weissman et al., 2008; Frye and Naviaux, 2011; Prasad and Hussain, 2015; Zwaigenbaum and Tarnopolsky, 2003; Karakaya et al., 2010; Schweitzer et al., 2016; Xu et al., 2017; Loo et al., 2022; Rong et al., 2022; Yoshimura et al., 1989) including two studies in patients with mitochondrial disorders (Weissman et al., 2008; Frye and Naviaux, 2011), three studies with 4 muscular dystrophy patients (Zwaigenbaum and Tarnopolsky, 2003; Loo et al., 2022; Yoshimura et al., 1989), one with progressive encephalomyelitis with rigidity and myoclonus (Xu et al., 2017), and two with rhabdomyolysis (Prasad and Hussain, 2015) with the rhabdomyolysis due to olanzapine use in one case (Karakaya et al., 2010). These studies examined serum or plasma levels. Meta-analysis on 2 studies (Germano et al., 2006; Weissman et al., 2008) showed that CK was elevated in 9% of individuals with ASD (Supplementary Fig. 1O).

CK was first reported to be similar in ASD and controls in a 1976 study (Cohen et al., 1976). Eight controlled studies examined CK in ASD (Supplemental Table 11b) with 6 studies reporting elevated CK in ASD (Khemakhem et al., 2017; Al-Mosalem et al., 2009; El-Ansary et al., 2017; Hassan et al., 2019; El-Ansary et al., 2020; Lv et al., 2016), one study reporting similar CK levels between ASD and controls (Cohen et al., 1976), and another reporting lower mean CK in male ASD individuals compared to controls (Nickel et al., 2023). One study used units of measurement that precluded inclusion in the meta-analysis (El-Ansary et al., 2020). These studies used serum or plasma levels. Meta-analysis (Supplementary Fig. 1P) of the four eligible studies (Al-Mosalem et al., 2009; El-Ansary et al., 2017; Hassan et al., 2019; Cohen et al., 1976) demonstrated a higher CK in ASD with a very large effect size. Cochran's Q demonstrated considerable heterogeneity with the LFK index showing significant asymmetry (Supplementary Fig. 2 J and 3 J) due to one study (Cohen et al., 1976) demonstrating low CK for both ASD and controls, resulting in a low absolute mean difference.

3.1.3. Biomarkers: Enzymology

Thirty-eight studies examined mitochondrial ETC activity in ASD individuals (Supplemental Table 12a).

Uncontrolled studies. The first study to report mitochondrial disease in ASD was from a publication in 1994 which reported a 5 year old child with complex IV deficiency (Laszlo et al., 1994). Since then, 17 uncontrolled studies have reported abnormal ETC complex activity in ASD (Graf et al., 2000; Poling et al., 2006; Filipek et al., 2003; Oliveira et al., 2005; Correia et al., 2006; Tsao and Mendell, 2007; Weissman et al., 2008; Ezugha et al., 2010; Guevara-Campos et al., 2010; Shoffner et al., 2010; Frye and Naviaux, 2011; Craig et al., 2012; Burger et al., 2017; Guevara-Campos et al., 2013; Fillano et al., 2002; Holtzman, 2008; Patowary et al., 2017). One study (Correia et al., 2006) reported on the same children as a previous study (Oliveira et al., 2005). Most studies used muscle or skin biopsies to diagnose the ETC complex defects, although one used a buccal swab analysis (Ezugha et al., 2010). A previous study by this same group reported a high correlation (over 82%) between buccal and muscle tissue in diagnosing ETC complex deficiencies (Goldenthal et al., 2012). Using the buccal swab technique to examine ETC complex I and IV and citrate synthase activity, two studies suggested that 65% (Goldenthal et al., 2015) and 62% (Delhey et al., 2017a) of individuals with ASD demonstrated mitochondrial enzyme activity outside the normal range. One study reported 2 patients improved with carnitine, Coenzyme Q10 and folic acid supplementation (Guevara-Campos et al., 2013).

Controlled studies. Twenty-five controlled studies (Supplemental

Table 12b) examined ETC complex activity (Napoli et al., 2014; Giulivi et al., 2010; Delhey et al., 2017a; El-Ansary et al., 2018; Khemakhem et al., 2017; Frye et al., 2021a; Mahalaxmi et al., 2021; Esvap and Ulgen, 2023; Barone et al., 2021; Goldenthal et al., 2015; Taurines et al., 2010; Chauhan et al., 2011; Marui et al., 2011; Anitha et al., 2013; Gu et al., 2013; Tang et al., 2013; Delhey et al., 2017b; Rose et al., 2017b; Pecorelli et al., 2020; Hassan et al., 2021; Hassan et al., 2022; Kato et al., 2023; Ginsberg et al., 2012; Smith et al., 2012; Bu et al., 2017). One study used buccal swabs (Goldenthal et al., 2015) and five studies utilized brain tissue (Chauhan et al., 2011; Anitha et al., 2013; Gu et al., 2013; Tang et al., 2013; Ginsberg et al., 2012) while the rest used muscle tissue, blood cells or skin tissue. Fourteen studies reported lower ETC complex activity in ASD compared to controls including 13 studies of Complex I (Giulivi et al., 2010; Delhey et al., 2017a; Khemakhem et al., 2017; Frye et al., 2021a; Mahalaxmi et al., 2021; Goldenthal et al., 2015; Chauhan et al., 2011; Gu et al., 2013; Tang et al., 2013; Hassan et al., 2022; Kato et al., 2023; Smith et al., 2012; Bu et al., 2017), 3 studies of Complex II (Frye et al., 2021a; Barone et al., 2021; Chauhan et al., 2011), 5 studies of Complex III (Frye et al., 2021a; Barone et al., 2021; Chauhan et al., 2011; Tang et al., 2013; Bu et al., 2017), 5 studies of Complex IV (Giulivi et al., 2010; Delhey et al., 2017a; Goldenthal et al., 2015; Chauhan et al., 2011; Tang et al., 2013) and 5 studies of Complex V (Napoli et al., 2014; Giulivi et al., 2010; Chauhan et al., 2011; Gu et al., 2013; Tang et al., 2013). One study reported lower ratios of Complex I/caspase-7, Complex I/CK, and Complex I/COQ10 in ASD (El-Ansary et al., 2018). In two studies of immune cells, abnormal ETC complex activity was found in 80% of lymphocytes (Giulivi et al., 2010) and ETC complex activity was 31% of that of typically developing children in granulocytes (Napoli et al., 2014). A study examining children with ASD and elevated blood ACs found a significant decrease in mitochondrial fatty acid β-oxidation, muscle ETC complex II activity, and fibroblast ETC Complex II/III activity (Barone et al., 2021). Six of these studies also examined genes involved in the production of ETC complexes with 4 studies reporting lower gene expression in the ASD group compared to controls (Taurines et al., 2010; Marui et al., 2011; Anitha et al., 2013; Ginsberg et al., 2012), one study where findings were similar in both groups (Taurines et al., 2010), and one that showed higher protein expression (Pecorelli et al., 2020). One study that performed a bioinformatics prediction of prefrontal lobe transcriptome data predicted decreases in brain ETC complex III and IV but also increases in complex I, II, and V (Esvap and Ulgen, 2023).

Interestingly, rather than decreased ETC complex activity typically seen in classic mitochondrial disease cases, children with ASD and symptoms of mitochondrial abnormalities sometimes show elevated ETC complex activity. The first case to report this was in the ASD brother of a girl with Leigh syndrome. The girl demonstrated decreased ETC activity due to a heteroplasmic (blood 82%; muscle 86%) G8363A $tRNA_{Lys}$ mutation but the boy who did not have Leigh syndrome harbored the same mutation with lower heteroplasmy (blood 60%; muscle 61%). Instead of demonstrating lower ETC complex activity, the boy with ASD demonstrated markedly higher than normal activity in ETC Complex I (Graf et al., 2000). The next case series reported five patents with ASD who had marked elevations in ETC Complex IV activity (~200% normal) in muscle (Frye and Naviaux, 2011). Subsequently, this association between significant elevations in ETC Complex IV activity and ASD has been reported in fresh frozen superior temporal gyrus (Palmieri et al., 2010), buccal swab enzymology (Delhey et al., 2017a), gastrointestinal (GI) tissue (Rose et al., 2017b) and fibroblasts (Frye et al., 2021a). Interestingly, the fibroblast study reported abnormal mitochondrial morphology associated with alterations in complex activity. This pattern of ETC complex abnormalities has been suggested to be a compensatory effect (Hassan et al., 2021).

3.1.4. Biomarkers: Metabolic biomarkers: Respirometry

Mitochondrial respiration has been investigated in individuals with ASD in cellular models, specifically two studies using fibroblasts (Pecorelli et al., 2020; Oyarzabal et al., 2016), nine studies using lymphoblastoid cell lines (LCLs) (Rose et al., 2014a; Rose et al., 2014b; Rose et al., 2017a; Rose et al., 2018b; Frye et al., 2017a; Frye et al., 2016b; Bennuri et al., 2019; Hassan et al., 2021; Rose et al., 2015a), nine studies examining peripheral blood mononuclear cells (PBMCs) (Burger et al., 2017; Frye et al., 2021b; Frye et al., 2020; Gevezova et al., 2021; Gevezova et al., 2022; Jyonouchi et al., 2019; Singh et al., 2020; Zimmerman et al., 2021; Frye et al., 2024), one study using platelets (Abdel-Rahman et al., 2021), and one study using olfactory ecto-mesenchymal stem cells (Féron et al., 2024).

Of the LCL studies, one study used the Oxygraph-2 k high-resolution respirometer (Hassan et al., 2021), while the remainder was by the same group using the Seahorse high throughput respirometer. Using the Oxygraph, LCLs derived from a boy with ASD and his sibling, as well as an unrelated healthy control, were compared. The LCLs from the ASD individual were found to have a high oxidative phosphorylation capacity with high ETC Complex IV activity and an elevated membrane potential (Hassan et al., 2019). This elevation in respiration in LCLs has been characterized by our group for the last decade. After noticing this overall elevation in mitochondrial respiratory rates in ASD LCLs, initial studies demonstrated that these overall differences were driven by a subgroup of one-third of ASD patients whose LCLs repeated showed respiratory rates approximately 200% of controls for respiratory parameters associated with ATP production (Rose et al., 2014a). This subset of LCLs were named AD-A for children with ASD and abnormal mitochondrial function, while the LCLs from children with ASD and normal mitochondrial function were called AD-N.

To better understand mitochondrial dysfunction in ASD, an assay was developed to examine the resilience of mitochondrial function in ASD. This assay, called the Mitochondrial Oxidative Stress Test (MOST), systematically increased physiological stress in vitro by exposing the LCLs to increasing concentrations of 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) one hour before the Seahorse assay. Fig. 5 provides an example of this assay in PBMCs, showing an increase in mitochondrial superoxide production with increasing DMNQ concentration.

Besides having elevated respiratory rates, the MOST assay demonstrated that the AD-A subgroup of LCLs was more sensitive to physiological stress (See Fig. 6). For example, while reserve capacity, a measure of mitochondrial health, was significantly higher in the AD-A LCLs compared to controls and AD-N in the absence of physiological stress (i.e., DMNQ 0 μM), when physiological stress was introduced, reserve capacity quickly plummeted in the AD-A LCLs to become significantly below the control and AD-N LCLs (See Fig. 6D). These findings have been replicated in seven studies (Rose et al., 2014b; Rose et al., 2017a; Rose et al., 2018b; Frye et al., 2017a; Frye et al., 2016b; Bennuri et al., 2019; Rose et al., 2015b). Raw data was available for these seven studies and the data was combined to produce Fig. 6. These additional studies demonstrated that the AD-A LCLs were different in their mitochondrial response to environmental agents associated with ASD including trichloroacetaldehyde hydrate (Frye et al., 2017a) and the microbiome associated short-chain fatty-acids propionate (Frye et al., 2016b) and butyrate (Rose et al., 2018b). Given the relation to environmental agents, it was hypothesized that changes in AD-A represented an adaptive response to previous environmental exposures, a phenomenon known as mitoplasticity (Bennuri et al., 2019). To support this hypothesis, it was shown that these changes in mitochondrial function could be induced in LCLs with prolonged exposure (96 h) to mild ROS, a microenvironment that is believed to simulate the effects of environmental toxicants on mitochondria (Bennuri et al., 2019).

Studies examining PBMCs have also demonstrated elevations in mitochondrial respiration in ASD, similar to the LCL findings (Gevezova et al., 2021; Gevezova et al., 2022; Frye et al., 2024). Given the LCLs studies, it was hypothesized that the AD-A subset of LCLs represents a cellular model of neurodevelopmental regression (NDR), as the mitochondria in AD-A LCLs lose reserve capacity with physiological stressors, similar to children with NDR who lose skills, usually following

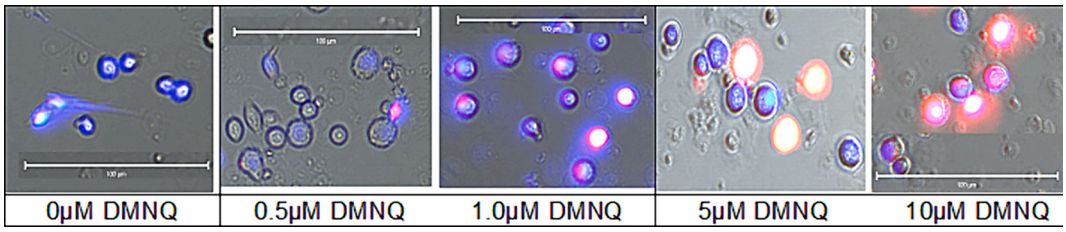

Fig. 5. Mitochondrial superoxide production increases as DMNQ concentration increases in PBMCs. Reprinted from (Frye et al., 2024) licensed under Creative Commons Attribution 4.0 International License, Frontiers in Physiology.

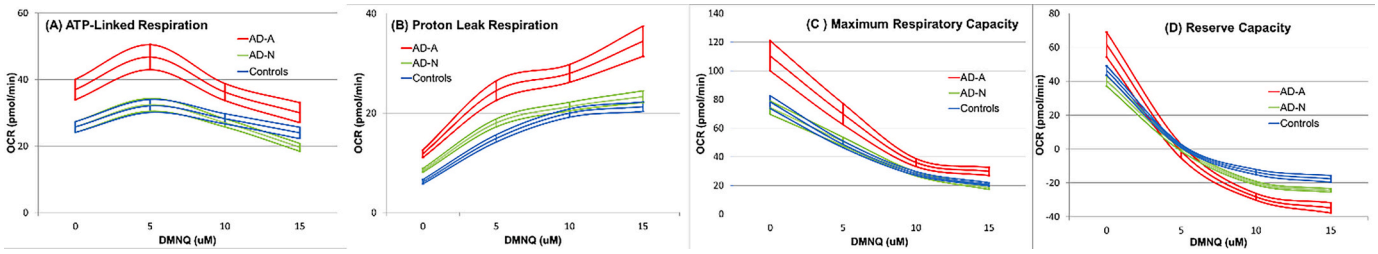

Fig. 6. Four key measures of mitochondrial respiration in three types of lymphoblastoid cell lines (LCLs), those derived from healthy controls, those derived from children with autism spectrum disorder (ASD) without mitochondrial dysfunction (AD-N) and those derived from children with ASD with a novel type of mitochondrial dysfunction (AD-A, see text). Mitochondrial respiration is measured by the oxygen consumption rate (OCR) with and without physiological stress. Physiological stress is induced by incubating the LCLs in 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) for 1 h prior to mitochondrial measurements in the Seahorse 96 XF analyzer. These curves represent the mean and standard error from data combined by seven published experiments where LCLs were stressed with DMNQ (Rose et al., 2014b; Rose et al., 2017a; Rose et al., 2018b; Frye et al., 2017a; Frye et al., 2016b; Bennuri et al., 2019; Rose et al., 2015b).

physiological stressors. To investigate this further, PBMCs were examined from those with and without NDR. In the first study, individuals with NDR were found to have higher respiratory rates in their PBMCs, similar to the AD-A LCLs (Singh et al., 2020). A more recent study using the MOST assay demonstrated that individuals with NDR also demonstrated a similar decrease in maximal respiration and reserve capacity with physiological stress (Frye et al., 2024). Interestingly, these mitochondrial abnormalities appear also to be shared by the parents of those with NDR (Frye et al., 2024). Further studies have linked these mitochondrial abnormalities with prenatal air pollution (Frye et al., 2021b) and nutritional metal exposure (Frye et al., 2020) in children with ASD. Using PBMCs, other studies have demonstrated that mitochondrial dysfunction in ASD is associated with DEPDC5 and WDR45 genetic mutations (Burger et al., 2017) and immune dysregulation (Jyonouchi et al., 2019), and that treatment with sulforaphane modulates mitochondrial function in clinical trial patients (Zimmerman et al., 2021). In contrast to PBMCs and LCLs, a depression in mitochondrial respiration was found in platelets (Abdel-Rahman et al., 2021) and olfactory ecto-mesenchymal stem cells (Féron et al., 2024) from individuals with ASD.

3.1.5. *Biomarkers: Mitochondrial morphology*

Three studies have examined changes in mitochondrial morphology: two studies performed on fibroblasts which showed high respiratory rates (Frye et al., 2021a; Pecorelli et al., 2020), and one study using olfactory ecto-mesenchymal stem cells which demonstrated depressed respiratory rates (Féron et al., 2024). Using Seahorse measurements, the first study demonstrated elevations in maximal respiration, reserve capacity and proton leak respiration as well as basal glycolysis and glycolytic reserve capacity in 12 individuals with ASD compared to 12 sex- and age-matched controls (Pecorelli et al., 2020). Upregulation of both ETC genes and genes involved in mitochondrial fission and fusion dynamics and quality control was also found. Electron microscopy demonstrated that the mitochondria in ASD fibroblasts appeared as large, interconnected structures with a compact and condensed matrix and densely packed and swollen cristae (Pecorelli et al., 2020).

The second study examined the relationship between ETC complex activity in fibroblasts to morphological parameters measured with a high-resolution confocal microscope processed using MITOTOUCH® proprietary software, which derives 31 geometrical and non-geometrical features of mitochondrial morphology (Lionnard et al., 2018; Chajra et al., 2019). Morphology was found to be associated with ETC complex I + III and IV activity, with a strong relationship with the disparity of the activity of these ETC measures such that the ASD fibroblasts could be divided into those with and without elevations in ETC complex IV activity as compared to ETC complex I + III. Interestingly, like the other morphological study, mitochondrial morphology in ASD fibroblasts with relative increases in ETC complex IV activity were more elongated with complex interconnected structures (Fig. 7A), whereas fibroblasts with more typical mitochondrial function were more isolated with normal appearing morphology (Fig. 7B). Interestingly, morphological parameters were correlated with ASD social functioning and behaviors (Frye et al., 2021a). In contrast to the above studies, the olfactory ecto-mesenchymal stem cells which demonstrated decreased respiration also demonstrated punctate mitochondria rather than mitochondria in complex networks (Féron et al., 2024).

3.1.5.1. *Genetic biomarkers: Nuclear gene mutations. Uncontrolled*

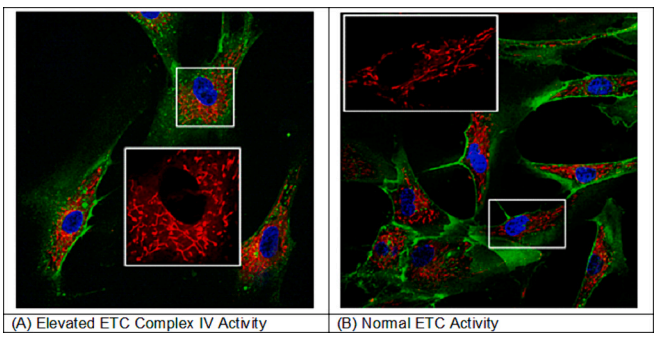

Fig. 7. Mitochondrial morphology in fibroblasts from two groups of patients with ASD. Adapted from (Frye et al., 2021a) licensed under Creative Commons Attribution 4.0 International License, *Translational Psychiatry*.

studies. Four case series reported chromosomal deletions (Laszlo et al., 1994; Ezugha et al., 2010; Frye and Naviaux, 2011) or duplications (Filipek et al., 2003; Frye and Naviaux, 2011) were related to mitochondrial dysfunction in ASD (Supplemental Table 13a). Two case studies reported mitochondrial dysfunction in ASD related to three non-mitochondrial genes, one reporting the SCN1A mutation (Craig et al., 2012), and one study reporting a girl with a novel c.795delT mutation in the WDR45 gene and her brother who harbored a novel heterozygous Y1546H variant in the DEP domain-containing protein 5 gene (Burger et al., 2017).

Other studies have identified variations in mitochondrial genes in case series or studies. Five uncontrolled studies reported variations in SLC25A12 (Correia et al., 2006; Ramoz et al., 2004; Segurado et al., 2005; Rabionet et al., 2006; Silverman et al., 2008), while one case series found a rare homozygous c.790C > T (His264Tyr) variation in the TFB2M gene in Korean siblings (Park et al., 2018). Interestingly, the fibroblasts from one sibling demonstrated higher respiratory rates compared to the father (Park et al., 2018). UQCRC1, NDUFS3, NDUFA9, NDUFA6, NDUFB7, NDUFA1, and COX4I1 were nuclear mitochondrial genes that displayed the most significant changes in their expression (Zeidan-Chulia et al., 2014).

Of note, other studies have reported mutations associated with carnitine deficiency, including mutations in TMLHE, the gene encoding the enzyme for the first step in carnitine synthesis (Celestino-Soper et al., 2012; Ziats et al., 2015; Goin-Kochel et al., 2019; Celestino-Soper et al., 2011; Nava et al., 2012), and OCTN2 (SLC22A5), the organic cation transporter type 2 which is a high affinity carnitine transporter. Defects in this are known to cause systemic primary carnitine deficiency (Guevara-Campos et al., 2019).

Controlled studies. Eighteen controlled studies (Marui et al., 2011; Anitha et al., 2013; Ginsberg et al., 2012; Smith et al., 2012; Blasi et al., 2006; Lepagnol-Bestel et al., 2008; Anitha et al., 2012; Kong et al., 2013; Durdiakova et al., 2014; Mahfouz et al., 2015; Schwede et al., 2018; Varga et al., 2018; Carrasco et al., 2019; Stathopoulos et al., 2020; Bam et al., 2021; Wang et al., 2021; Luo et al., 2024; Pourtavakoli et al., 2024) and three meta-analyses (Liu et al., 2015; Aoki and Cortese, 2016; Gevezova et al., 2023) (Supplemental Table 13b) examined the association between genetic variations in mitochondrial-related nuclear genes and ASD. Seven studies examined SLC25A12 including five controlled studies (Blasi et al., 2006; Lepagnol-Bestel et al., 2008; Anitha et al., 2012; Durdiakova et al., 2014; Pourtavakoli et al., 2024) and two meta-analyses (Liu et al., 2015; Aoki and Cortese, 2016). One study reported that NDUFA5 rs12666974 and rs3779262 SNPs were associated with ASD, particularly the A-A haplotype containing both the two SNPs. Using the transmission disequilibrium test, the global and A-A haplotype were significantly associated with ASD (Marui et al., 2011). Another study found that copy number variations (CNVs) in UBE3A, NDUFA4, NDUFA10, PPCDC, COX5, CYP11A, SLC25A15, MTRF, ETFDH, and CBR4 were more common in ASD (Smith et al., 2012). One study found that two ASD children with mtDNA deletions also had nuclear DNA mutations involved in mitochondrial function: MGME1 (likely pathogenic) and SUCLG1 (variant of uncertain significance) (Varga et al., 2018). One exceptionally large study found an excess of protein-truncating variants and deleterious missense variants in 1421 nuclear-encoded mitochondrial genes from 41,376 cases (31,058 undiagnosed developmental disorders and 10,318 ASD). Overall, 3.23% of de novo deleterious mutations and 3.20% of de novo protein-truncating variants contributed to 1.1% and 0.39% of ASD cases, respectively (Luo et al., 2024).

3.1.5.2. Genetic biomarkers: Nuclear gene expression. Studies have also examined changes in nuclear mitochondrial gene expression associated with ASD. One controlled study examining oral mucosal samples found a non-significant downward trend in HIGD2A and SOD2 expression and an upward trend in DRP1, FIS1, MFN1, MFN2, and OPA1 expression in ASD (Carrasco et al., 2019). Another study examined differences in gene expression between ASD individuals and healthy controls and found upregulation of 104 genes involved in oxidative phosphorylation (Kong et al., 2013). One study found that expression of ATP5A1, the gene that encodes for ATP synthase F1 subunit alpha, was lower in ASD (Wang et al., 2021).

Eight studies examined gene expression in brain tissue including one examining SLC25A12 (Lepagnol-Bestel et al., 2008). Down-regulation of mitochondrial ETC genes, especially those associated with complexes I, III, and ATP synthase (Complex V), were found in ASD brain tissue (Ginsberg et al., 2012). Another study found brain region-specific expression alterations in ASD: MTX2 and SLC25A27 showed consistently reduced expression in the anterior cingulate gyrus, motor cortex and thalamus. SLC25A27 was also associated with ASD in Japanese samples. DNAJC19, DNM1L, LRPPRC, SLC25A12, SLC25A14, SLC25A24 and TOMM20 were reduced in expression in at least two of the brain regions in ASD (Anitha et al., 2012). Brain region-specific reduced expression of several ETC genes, including eleven complex I genes, five complex III and IV genes, and seven complex V genes was associated with ASD. ATP5A1 (Complex V), ATP5G3 (Complex V) and NDUFA5 (Complex I) showed consistently reduced expression in all the brain regions of ASD patients (Anitha et al., 2013). Another study found that genes related to mitochondrial function were downregulated in the cerebral cortex of ASD patients, with expression of these genes correlated with the expression of genes related to synaptic function (Schwede et al., 2018). A meta-analysis reported downregulated transcripts in the brain of ASD patients in mitochondrial genes: NDUFB7, UQCRQ and NDUFS4 (Gevezova et al., 2023). One study reported that 31% of 374 mitochondrial genes analyzed were differentially expressed in ASD cerebellum, most significantly UQCRC1, NDUFS3, NDUFA9, NDUFA6, NDUFB7, NDUFA1, and COX4I1 (Zeidan-Chulia et al., 2014). Finally, ASD candidate genes in the brain were enriched in modules related to mitochondrial function, protein translation, and ubiquitination in another study (Mahfouz et al., 2015).

3.1.5.3. Genetic biomarkers: Nuclear gene methylation. Two studies examined differentially methylated mitochondrial genes in ASD. One study reported differentially methylated genes in complex I (NDUFA4, NDUFB2, NDUFB4, NDUFB6), complex III (UQCRC2) and complex IV (COX7B), as well as STOML2 which regulates mitochondrial fusion, and PCCB, which is involved in the catabolism of propionyl-CoA (Stathopoulos et al., 2020). The second study found differential methylation in genes involved with mitochondrial biogenesis, fission, and fusion in ASD, specifically PGC-1α, STOML2, MFN2, FIS1, OPA1, and GABPA (Bam et al., 2021).

3.1.5.4. Genetic biomarkers: Mitochondrial DNA mutations. Uncontrolled studies. The first study reporting a mtDNA mutation in ASD was reported in one child in 2000 (Graf et al., 2000). Since then, 14 other uncontrolled studies (Supplemental Table 14a) have examined mtDNA mutations in ASD. Two of these studies found that mtDNA mutations were not associated with ASD (Burger et al., 2017; Kent et al., 2006). The remaining 12 studies reporting mtDNA mutations in ASD were case series involving 1–15 affected individuals (Pons et al., 2004; Weissman et al., 2008; Connolly et al., 2010; Frye and Naviaux, 2011; Guevara-Campos et al., 2013; Park et al., 2018; Patowary et al., 2017; Avdjieva-Tzavella et al., 2012; Piryaei et al., 2012; Abdi et al., 2023; Akouchekian et al., 2019; Cameli et al., 2021). Three studies examined heteroplasmy of mtDNA and found likely deleterious heteroplasmic mutations (Wang et al., 2016). mtDNA variants with 15%–5% heteroplasmy were associated with severe ASD phenotypes (Caporali et al., 2022), and predicted pathogenic heteroplasmic mutations in mtDNA were increased in ASD children compared to their siblings and parents (Wang et al., 2022).

Controlled studies. Thirteen controlled studies (Supplemental Table 14b) examined mtDNA deletions or variations in blood (Mahalaxmi

et al., 2021; Vellingiri et al., 2023; Varga et al., 2018; Wang et al., 2022; Mousavizadeh et al., 2013), peripheral blood mononuclear cells (Wong et al., 2016; Napoli et al., 2013), lymphocytes (Giulivi et al., 2010), leucocytes (Valiente-Palleja et al., 2018), granulocytes (Napoli et al., 2014), brain (Gu et al., 2013; Tang et al., 2013) and samples from a biobank (Chang et al., 2023). One study found mtDNA deletions in 16.7% of ASD patients, with 90% of these patients also having rare variations in ASD associated nuclear genes. Interestingly, nuclear genes responsible for mtDNA maintenance were more frequently associated with mtDNA mutations in the mitochondrial disease control group than those with ASD (Varga et al., 2018). In another study, mtDNA deletions were 1.9-fold higher in fathers of children with ASD compared to fathers of TD children, and an increased copy number of P53 was also found suggesting upregulation of DNA mechanisms to promote DNA stability (Wong et al., 2016). One study found that mtDNA deletions occurred in CytB and ND4 more often in children with ASD and their fathers, while variations in mtDNA of children with ASD and their mothers contained more GC transitions, which is a preferred site for oxidative damage (Napoli et al., 2013). Another study found that the mtDNA contained deletions in ND4 and CytB genes in 44% and 33% of ASD frontal cortex, respectively (Gu et al., 2013). One study found deletions in CytB in 20% of ASD lymphocytes (Giulivi et al., 2010), and significant increases in granulocytes (Napoli et al., 2014) without any increased difference in ND4 deletions. Another study found missense mutations in ND1 and ND4 were more common in those with ASD in whole blood (Mahalaxmi et al., 2021). One study examined mtDNA tRNA genes and found three mutations that were more frequent in ASD in the non-coding regions near tRNA genes (Mousavizadeh et al., 2013). A study that examined MT-ATP6 in whole blood found variants in >10% of ASD patients with most variants being tolerated but several being deleterious (Vellingiri et al., 2023). Another study found that 28.6% of individuals with ASD carried at least one putative pathogenic mtDNA mutation in leukocytes (Valiente-Palleja et al., 2018). In a large cohort study, variants in ND1, ND4L and RNR1 were found to be associated with ASD. Even though these variants were supposedly synonymous, they were found to be associated with changes in the expression of mitochondrial ETC genes (Chang et al., 2023). Another large study found mutations in mtDNA genes encoding ETC complex I subunits (Wang et al., 2022). Finally, a small study examining the temporal lobe did not find any mtDNA variants in ASD brains (Tang et al., 2013).

3.1.5.5. Genetic biomarkers: Mitochondrial DNA copy number. mtDNA copy number in ASD was examined (Supplemental Table 14a and 14b) in one meta-analysis (Al-Kafaji et al., 2023) and 30 controlled studies (Napoli et al., 2014; Giulivi et al., 2010; Oh et al., 2020; Mahalaxmi et al., 2021; Adiba et al., 2024; Vellingiri et al., 2023; Gu et al., 2013; Tang et al., 2013; Singh et al., 2020; Varga et al., 2018; Carrasco et al., 2019; Bam et al., 2021; Wang et al., 2016; Caporali et al., 2022; Wang et al., 2022; Mousavizadeh et al., 2013; Wong et al., 2016; Napoli et al., 2013; Valiente-Palleja et al., 2018; Chang et al., 2023; Kent et al., 2008; Zhang et al., 2010; Alvarez-Iglesias et al., 2011; Hadjixenofontos et al., 2013; Chen et al., 2015; Chalkia et al., 2017; Yoo et al., 2017; Budd et al., 2018; Tsilioni and Theoharides, 2018; Wei et al., 2023). Only 5 studies reported no significant differences in mtDNA findings in ASD individuals compared to controls (Oh et al., 2020; Adiba et al., 2024; Kent et al., 2008; Alvarez-Iglesias et al., 2011; Hadjixenofontos et al., 2013). Two studies found significantly lower mtDNA content in peripheral blood mononuclear cells (Valiente-Palleja et al., 2018) and blood (Caporali et al., 2022), and one study reported lower ND1, ND4, and CYTB mtDNA copy numbers compared to controls (Singh et al., 2020). Seven studies reported increased mtDNA copy number in ASD compared to controls in blood (Chen et al., 2015; Wei et al., 2023), peripheral blood cells (Napoli et al., 2014; Wong et al., 2016; Yoo et al., 2017), buccal swabs (Bam et al., 2021) and brain (Gu et al., 2013). Interestingly, one study linked three CYTB variants (15049C > T, 15341 T > C, 15796 T>) to increased mtDNA copy number (Adiba et al., 2024). Five studies reported higher amounts of mtDNA in serum (Zhang et al., 2010), lymphocytes (Giulivi et al., 2010), leucocytes (Budd et al., 2018), oral mucosa (Carrasco et al., 2019), and extracellular vesicles (Tsilioni and Theoharides, 2018) in ASD. Interestingly, buccal mtDNA copy number was found to correlate with PGC-1α methylation and concentrations of mitochondria, oxidative stress, and neuroendocrinology urinary organic acids (Bam et al., 2021).

Studies of mtDNA content are very heterogeneous in their approach which makes it difficult to assay this metric. While many use a standard approach of averaging the ratio of ND1, ND4 and CYTB gene copy number to the nuclear PK gene, other investigators use alternative approaches such as the ratio of examining mtDNA L394 region to B-globulin nuclear gene (Chen et al., 2015), examining the ratio of the mtDNA D-Loop to the nuclear albumin gene (Budd et al., 2018), or comparing the tRNA-Leu gene to the nuclear PPIA gene (Carrasco et al., 2019), as well as only using the ND1 gene to PK ratio (Wong et al., 2016). Overall, the meta-analysis could include seven studies for overall mtDNA copy number (Giulivi et al., 2010; Oh et al., 2020; Gu et al., 2013; Bam et al., 2021; Wong et al., 2016; Chen et al., 2015; Yoo et al., 2017) (Supplementary Fig. 1Q, 2 K, and 3 K), three studies for ND1 copy number (Gu et al., 2013; Wong et al., 2016; Yoo et al., 2017) (Supplementary Fig. 1R, 2 L, and 3 L), two studies for ND4 and CytB copy number (Gu et al., 2013; Yoo et al., 2017) (Supplementary Fig. 1S and T) and two studies for ND4/ND1 and CYTB/ND1 ratios (Giulivi et al., 2010; Gu et al., 2013) (Supplementary Fig. 1 U and V). Overall mtDNA copy number was higher in ASD with a moderate effect size (Table 4). ND1, ND4 and CytB all demonstrated higher copy numbers in ASD with a medium effect size, even when outlier studies were removed from the ND1 analysis. The ratio of ND4 and CytB to ND1 was not different across the two groups, suggesting that the increase found in overall mtDNA and individual mtDNA genes is most likely due to an increase in the copy number of the entire mtDNA genome. Since overall mtDNA and ND1 demonstrated significant variation and asymmetry in studies, the meta-analyses were calculated removing the outlier studies with very similar results.

3.1.5.6. Genetic biomarkers: Mitochondrial DNA Haplogroups. mtDNA haplogroups represent evolutionary changes in mtDNA which are believed to be linked to the spread of humans from their origins in Africa to the remainder of the world. Haplogroups are defined by mtDNA polymorphism which are associated with regional areas of the world. Although such polymorphisms by themselves do not cause mitochondrial disease, such gene alterations could predispose a certain culture to specific diseases. Four studies have examined mtDNA haplogroups in ASD. One study reported European haplogroups I, J, K, O-X, T, and U were associated with an increased risk of ASD, as were Asian and Native American haplogroups A and M (Chalkia et al., 2017). In another study, mtDNA haplogroup K was significantly associated with a decreased risk of ASD (Chang et al., 2023), and one study observed the paternal superhaplogroups H and JT were both associated with milder ASD phenotypes (Caporali et al., 2022). Finally, one study of 162 ASD individuals demonstrated no difference in haplogroup compared to two control groups (Kent et al., 2008).

3.1.6. Biomarker correlates: Clinical symptoms

The relationship between biomarkers and ASD symptoms has been examined in a number of studies. Lactate elevation has been associated with more severe ASD symptoms including more severe language, social and adaptive function deficits (Sotelo-Orozco et al., 2020), more severe repetitive behavior (Oh et al., 2020), lower cognitive skills (Smith et al., 2023), and motor delay (Frye, 2012a). However, one study did not find a relationship between lactate and ASD severity (Khemakhem et al., 2017). Pyruvate elevation has been associated with poorer language, cognitive development, and adaptive behavior in TD children (Sotelo-

Table 4
Meta-analysis results for the difference between values for mtDNA copy number metrics in children with autism spectrum disorder (ASD). Pooled prevalence with 95% confidence interval, Cochran's Q (Q), Heterogeneity Index (I2), Luis Furuya-Kanamori (LFK) Index and number of studies involved (N). Statistics are estimated by a random-effects model. *$p < 0.01$; [T]Significant Asymmetry.

Biomarker	ASD	CNT	WMD	Cohen's d'	Q	I^2	LFK	N
mtDNA Overall	241.5 (221.1, 261.9)	179.6 (161.0, 198.1)	6.4 (3.8, 9.0)	0.4 (0.2, 0.5)	27.2*	78%	6.83[T]	7
ND1	275.4 (242.1, 308.8)	230.8 (187.8, 273.7)	12.7 (4.7, 20.8)	0.4 (0.2, 0.6)	8.95*	78%	7.79[T]	3
ND4	259.9 (222.5, 297.4)	220.0 (173.9, 266.2)	13.0 (1.1, 24.9)	0.4 (0.1, 0.6)	4.40			2
CytB	537.5 (417.0, 658.0)	411.3 (311.3, 511.2)	19.3 (4.6, 34.0)	0.4 (0.2, 0.7)	5.36			2
ND4/ND1	0.84 (0.81, 0.87)	0.85 (0.76, 0.93)	−0.04 (−0.10, 0.03)	−0.3 (−0.9, 0.3)	0.05			2
CytB/ND1	1.31 (1.27, 1.35)	1.37 (1.28,1.45)	−0.01 (−0.69, 0.55)	−0.1 (−0.7, 0.6)	1.16			2

Orozco et al., 2020). However, other studies have reported no association between pyruvate and clinical phenotypes (Oh et al., 2020) or odor identification (Yang et al., 2022) in ASD. One study found a negative relationship between lactate-to-pyruvate ratio and odor identification (Yang et al., 2022), while three studies did not find an association between lactate-to-pyruvate ratio and ASD severity (El-Ansary et al., 2018; Hassan et al., 2019; Mahalaxmi et al., 2021). One study found a potential relationship between alanine-to-lysine ratio and epilepsy (Frye, 2012a). Serum carnitine has been associated with ASD severity (Mostafa et al., 2005) and GI symptoms (Mostafa and Al-Ayadhi, 2015), but another study found no association with ASD symptoms (Hassan et al., 2019). AC abnormalities have been associated with neurodevelopmental regression in one study of children with ASD (Frye, 2012a), and with metrics of core ASD symptoms, empathy and depression in adults with ASD (Nickel et al., 2023). Another study found that elevated short and decreased long chain ACs were associated with more GI symptoms (Needham et al., 2021). A study examining TCA cycle metabolites found that higher aconitate correlated with poorer visual reception skills, and higher cis-aconitate, succinate and fumarate were correlated with poorer receptive and overall language skills, while higher succinate was correlated with poorer daily living skills (Sotelo-Orozco et al., 2020). One study found that plasma ATP was related to the severity of ASD symptoms (Adams et al., 2011a). Coenzyme Q10 concentration has been associated with ASD severity (Prasad and Hussain, 2015). One study reported that ammonia concentrations were not associated with ASD severity (Hassan et al., 2019). Another metabolomics study found that sleep and neurodevelopment were associated with energy metabolism metabolites (Brister et al., 2022).

Two studies reported the severity of ASD was associated with a high Complex I/IV ratio (Goldenthal et al., 2015) and lower complex I activity (Khemakhem et al., 2017), although one study found that complex I activity was similar in severely affected children with ASD compared to those less affected (Mahalaxmi et al., 2021). Another study found poorer adaptive skills on Vineland Adaptive Behavior Scale were associated with Complex IV activity either lower or higher than average and lower Complex I activity (Delhey et al., 2017a). Complex I-dependent mitochondrial respiration in platelets was found to moderately correlate with ASD behavior (Abdel-Rahman et al., 2021). Using structural equation modeling to simultaneously account for the effect of prenatal exposure to air pollution and redox metabolism, Seahorse respiratory methods associated with ATP production were found to be related to neurodevelopment, while behavior and ASD symptoms appeared to be related to mitochondrial function indirectly through neurodevelopment (Frye et al., 2021b).

mtDNA variants with 15%–5% heteroplasmy have been associated with more severe ASD, while paternal super-haplogroups H and JT have been associated with milder ASD (Caporali et al., 2022). Predicted pathogenic heteroplasmies in ASD individuals were associated with lower verbal and non-verbal IQ and an increased risk of intellectual disability. However, in ASD individuals with predicted pathogenic heteroplasmies, increased mtDNA content was associated with better cognition, communication, and behaviors (Wang et al., 2022). Increased CYTB copy number was associated with better social language and communication (Yoo et al., 2017), and increased ND1 copy number was associated with less repetitive behavior (Singh et al., 2020). However, one study did not find associations between mtDNA copy number and ASD symptomatology (Chen et al., 2015). Finally, one study identified a relationship between four CytB gene variants (14,793 A > G, 14893 A > G, 14971 T > C, 15218 A > G) and ASD severity (Adiba et al., 2024).

3.1.7. Biomarker correlates: Environmental exposures

Third trimester maternal lactate was related to material urinary phthalate metabolites, and a doubling of phthalate levels in pregnancy was associated with an increase in serum pyruvate, with these biomarkers associated with more severe ASD symptoms at 2 and 4 years of age (Thomson et al., 2023). In addition, doubling of daily phthalate exposure during pregnancy was associated with an increase in alanine and alanine was associated with ASD symptoms at 2 years of age (Thomson et al., 2023). Long-term respiratory changes in mitochondrial function in children with ASD have been found to be related to prenatal environmental exposures such as air pollution (Frye et al., 2021b) and prenatal concentrations of the essential nutrient metals Zn and Mn, particularly in those who experienced neurodevelopmental regression (Frye et al., 2020; Curtin et al., 2018).

3.1.8. Diagnostic potential of biomarkers: Confirming ASD

One study of metabotypes examined six metabolic clusters based on ratios of either lactate or pyruvate, succinate, glycine, ornithine, 4-hydroxyproline, or alpha-ketoglutarate with other metabolites in 1102 children from 8 centers (the Children's Autism Metabolome Project) across the United States; these metabotypes predicted ASD risk with 53% sensitivity and 91% specificity (Smith et al., 2020). A follow up study of the same children found biomarker clusters in ASD with increased lactate, pyruvate, alanine, and sometimes tricarboxylic acid cycle intermediates (Smith et al., 2023). Another study of 41 children with ASD and 41 age- and sex-matched controls reported that neither lactate nor pyruvate could discriminate between ASD and control groups (Khemakhem et al., 2017).

Three studies have found that fatty acid metabolism may be fruitful in diagnosing those with ASD from controls. In the first study of 60 children with ASD and 30 controls, serum carnitine concentrations have been found to distinguish those with ASD from controls (Lv et al., 2018). In the second study examining postmortem cerebellar tissue from 11 individuals with ASD and 11 controls, machine-based learning algorithms found brain concentrations of 9-hexadecenylcarnitine and phosphatidylcholine could discriminate those with ASD from controls with a sensitivity and specificity of 80% (Graham et al., 2020). Finally, in the last study of 83 children with ASD and 57 controls, AC metabolites could accurately classify children with ASD younger than 5 years of age with a sensitivity and specificity of 72%, accuracy of 73% and a diagnostic odds ratio of 11.25 (Barone et al., 2018).

3.1.9. Diagnostic potential of biomarkers: Prenatal period

Some studies examined mitochondrial biomarkers during the prenatal period. A controlled study of 30 mothers of a child with ASD and 29 control mothers found that levels of vitamin B12, the ratio of S-adenosylmethionine/S-adenosylhomocysteine (a marker of methylation), and 8 metabolites ratios of carnitine-conjugated molecules were

significantly lower in mothers with an ASD child (all $p < 0.05$) (Hollowood-Jones et al., 2020). Another study of 166 mothers who had an eventual ASD child and 98 control mothers who lived in California's Central Valley and were exposed to high levels of traffic related air-pollution demonstrated that higher alpha-linolenyl carnitine and tetracosapentanoyl carnitine serum concentrations during pregnancy were associated with the offspring developing ASD (Kim et al., 2021). However, another study did not find that third trimester serum (from 106 mothers) or placenta (from 132 mothers) carnitine concentrations were associated with ASD after adjustment for covariates (Parenti et al., 2022).

3.1.10. Diagnostic potential of biomarkers: Presymptomatic period

Two very large case-control studies have suggested that AC abnormalities found during newborn screening can predict the later development of ASD. The first study of 3296 ASD children and 7460 controls showed that elevations in free carnitine, isovalerylcarnitine (C5) and octanoylcarnitine (C8) and decreased methylmalonylcarnitine (C4DC) and adipylcarnitine (C6DC) were associated with an increased risk for developing ASD (Canfield et al., 2019), while the second study of 3005 ASD cases and 6212 controls found that an increase in isovalerylcarnitine (C5) and a decrease in methylmalonylcarnitine (C4DC) on the first screen and an increase in adipylcarnitine (C6DC), octanoylcarnitine (C8) and the octanoylcarnitine (C8) to acetylcarnitine (C2) ratio on the second screen were associated with an increased risk of ASD (Langlois et al., 2020). In another large prospective study of 1074 mother-child pairs examining lipid metabolites in cord blood, ACs, phosphatidylethanolamine, triglycerides and phosphatidylserine were found to be related to ASD and attention deficit hyperactivity problems at 2 years of age (Vacy et al., 2024).

3.2. Treatment for mitochondrial dysfunction in ASD

Treatment for mitochondrial disorders originally began with supplementation of specific cofactors, mostly B-vitamins. The advantages of these treatments are that there are typically few adverse effects and, for the most part, no serious or long-term adverse effects. In addition, B-vitamins are water soluble and eliminated by the kidneys and therefore possess a low risk of developing toxic levels. Regardless, with any treatment it is important to monitor the outcomes of treatments on a regular basis.

Table 5 outlines some of the commonly used cofactors to treat mitochondrial disease and the pathways they support. Interestingly, some of the mainstays for the treatment of mitochondrial disease have been studied in children with ASD but not specifically in children with identified mitochondrial dysfunction. The best studied mitochondrial supplement is L-Carnitine with three double-blind, placebo controlled (DBPC) studies: two studies (30 ASD children in each study) found that it improved the Childhood Autism Rating Scale (CARS) (Fahmy et al., 2013; Geier et al., 2011) with improvements correlating with increases in blood carnitine levels in one study (Geier et al., 2011), while the third study of 68 ASD children reported improvements in the Aberrant Behavior Checklist-Community (ABC-C) (Nasiri et al., 2023). Another open-label trial of 10 boys with ASD used high doses of carnitine (up to 400 mg/kg/day in three divided doses, maximum of 6000 mg/day) with odorous loose stools being the only major adverse effect. Improvements in language correlated with post-treatment blood carnitine concentrations (Goin-Kochel et al., 2019). In a DBPC study of 78 individuals with ASD, Ubiquinol 30-60 mg per day was found to improve oxidative stress and CARS scores (Mousavinejad et al., 2018). Small studies have also found favorable effects in children with ASD using reduced NAD (open label study of 8 ASD children) (Adams et al., 2011b), thiamine tetrahydrofurfuryl disulfide (open label study of 10 ASD children) (Lonsdale et al., 2002) and biotin (one girl with ASD) (Benke et al., 2018). Three systematic reviews have reported improvements with cofactors important for redox and methylation abnormalities, such as methylcobalamin

Table 5
Cofactors for treatment of mitochondrial disease. Adapted from (Niyazov et al., 2016) with permission.

Vitamin	Dose	Adverse Effects	Function
Electron Transport Chain Support			
Coenzyme Q10 (Reduced): Ubiquinol	5-30 mg/kg/day, 1–2×/day	Appetite loss, nausea, diarrhea at high doses	Energy Carrier between Complex I and III and Complex II and III
Coenzyme Q10 (Oxidized): Ubiquinone	10-30 mg/kg/day; 1–2×/day		
Electron Carrier Support			
Niacin (B3)	50-100 mg given daily	Flushing Reaction	Nicotinamide adenine dinucleotide (NAD) precursor
Riboflavin (B2)	100-400 mg given daily	Nausea at High Doses	Flavin adenine dinucleotide (FAD) precursor
Energy Storage			
Creatine monohydrate	100 mg/kg/day; 1–2×/day	Increased urination	High-energy phosphate buffer. Precursor to phosphocreatine
Fatty Acid Oxidation Support			
L-carnitine or Acetyl-L-carnitine	30-120 mg/kg/day,1–2×/day	Stool loose/fishy smell	Carrier of long chain fatty acids
Biotin (B7)	5-10 mg/day given daily	None	Cofactor for carboxylase enzymes
Mitochondrial Enzyme Co-Factors			
Thiamine (B1)	50-100 mg given daily	None	Cofactor for citric acid cycle enzymes
Pantothenic Acid (B5)	5-1200 mg/day, 1–3×/day	Diarrhea at high doses	Precursor to Coenzyme A
Pyridoxine (B6)	200 mg given daily		Cofactor for over 100 enzymes
Biotin (B7)	5-10 mg/day given daily	None	Cofactor for carboxylase enzymes
Alpha-lipoic acid	50–200 mg given daily	Headache, paresthesia, rash, muscle cramps	Cofactor for citric acid cycle enzymes
Antioxidants			
Coenzyme Q10: Ubiquinol	5-30 mg/kg/day, 1–2×/day	Appetite loss, nausea,	Targets ETC oxidative stress
L-carnitine	30-120 mg/kg/day,1–2×/day	Stool loose/fishy smell	Scavenger of organic acids
Vitamin E	200–400 IU given daily	Bleeding at high doses	Protects Cell Membranes
Vitamin C	100-500 mg given daily	Diarrhea at high doses	Protects Iron and Copper
Redox Metabolism Support			
Methylcobalamin (B12)	5-2000μg every 1–3 days	Hyperactivity, Sleep Disruption	Supports methylation and folate cycles and glutathione production
Reduced folate (B9)	400-800μg daily	Hyperactivity	Supports methylation and folate cycles
N-acetyl-L-cysteine (NAC)	10-70 mg/kg/day, 1–3×/day	Diarrhea at high doses	Precursor to Glutathione

(*continued on next page*)

Table 5 (*continued*)

Vitamin	Dose	Adverse Effects	Function
Zinc	10-40 mg daily	Suppresses iron and copper absorption	Supports superoxide dismutase
Central Folate Support			
Folinic Acid / Leucovorin Calcium (B9)	0.5-4 mg/kg/day, 1–3×/day	Hyperactivity	Supports adequate folate levels in the brain

(Rossignol and Frye, 2021a), N-Acetyl-Cysteine (et al., 2015) and leucovorin (Rossignol and Frye, 2021b).

The Ketogenic Diet (KD), a diet traditionally used for drug-resistant epilepsy, has been studied in ASD. The KD has been found to have a therapeutic effect on many mitochondrial disorders including pyruvate dehydrogenase complex deficiency (Sofou et al., 2017; Wexler et al., 1997), ETC complex deficiencies (Seo et al., 2010; O'Byrne et al., 2018), PLOG1 mutations (Martikainen et al., 2012; Cardenas and Amato, 2010), and MELAS syndrome (Steriade et al., 2014). In a retrospective parent survey of 1023 individuals with ASD, the KD had a favorable effect on epilepsy and core and related ASD symptoms (Frye et al., 2011). The KD has also been studied in ASD in a prospective controlled study of 45 children with ASD (El-Rashidy et al., 2017) and in a retrospective parental survey of 818 individuals with ASD (Matthews and Adams, 2023). Of the patients who could tolerate the KD, the majority had favorable effects. One animal model reported that the KD improved mitochondrial function and morphology (Ahn et al., 2020). The safety of the KD has already been studied in epilepsy, so it is a potential alternative therapeutic treatment for children with ASD and mitochondrial dysfunction, especially if they have epilepsy.

Several studies have measured changes in biomarkers of mitochondrial function as a result of treatments in the general ASD population. A combination nutritional vitamin/mineral supplement that included L-Carnitine, Coenzyme Q10 as well other mitochondrial targeted supplements demonstrated improvements in ASD symptoms as reported by the Parental Global Impressions-Revised in two controlled studies (141 individuals with ASD in one study and 67 in the other), and also found improvements in ATP, NADH and NADPH blood concentrations (Adams et al., 2018; Adams et al., 2011c). In a DBPC clinical trial of 57 children with ASD, Sulforaphane was found to increase ATP linked respiration and maximum reserve capacity in those without NDR and decrease these respiratory parameters in those with NDR (Zimmerman et al., 2021). This study also showed that improvements in aberrant behavior were associated with an increase in ATP linked respiration and a decrease in proton leak respiration.

Several studies have directly examined the effect of mitochondrial supplements on mitochondrial function in individuals with ASD and mitochondrial defects. A case series of two children with ASD and ETC dysfunction noted improvement with carnitine, CoQ10 and folic acid supplementation (Guevara-Campos et al., 2013). A large natural history study of 127 individuals with ASD and 68 controls examined the effect of supplements on citrate synthase and ETC Complex I and IV in children with ASD with and without identified mitochondrial disease (Delhey et al., 2017b). Folate and antioxidants increased ETC Complex I and citrate synthase activity, respectively, in those with mitochondrial disease, while fatty acid supplementation increased ETC Complex I activity for all children with ASD. Interestingly, the correlation between ETC Complex I and IV was positively modulated by folate, and the correlation between Complex I and citrate synthase was positively modulated by cobalamin and folate (Delhey et al., 2017b). Only one study has examined the response to treatment in children with ASD and mitochondrial dysfunction. In a small open-label cross-over study of 11 children with ASD, three months of treatment with L-Carnitine, Coenzyme Q10 and alpha-lipoic acid improved both mitochondrial enzyme activity and behavior while discontinuation of the supplement resulted in a return of the aberrant behavior (Legido et al., 2018).

Treatment of children with ASD and inborn errors of metabolism have been studied, primarily with L-Carnitine treatment. For example, clinical improvements with carnitine were found in two children with ASD and a TMLHE gene mutation causing a deficiency in carnitine biosynthesis (Ziats et al., 2015; Goin-Kochel et al., 2019), a girl with a mutation in SCL22A5, the organic cation transport type 2, resulting in a systemic primary carnitine deficiency (Guevara-Campos et al., 2019), and a boy with glutaric aciduria type 1 (Kiykim et al., 2016).

4. Discussion

This systematic review has provided an overview of the abnormalities related to mitochondrial function in individuals with ASD through the investigation of biomarkers. Several biomarkers of mitochondrial dysfunction such as pyruvate and lactate-to-pyruvate ratio have been estimated to be abnormal in more than one-quarter of children with ASD, while other biomarkers such as lactate, alanine and ACs have been estimated to be abnormal in 15% or more of children with ASD. In controlled studies, lactate, pyruvate, lactate-to-pyruvate ratio, ammonia, and creatine kinase have been shown to be elevated in individuals with ASD, with most of these differences showing large effect sizes, while total and free carnitine appear to be depressed in individuals with ASD, with large to medium effect sizes. ND1, ND4, CytB and overall mtDNA copy number were found to be elevated in ASD with medium effect sizes. Studies have also frequently found abnormal TCA cycle metabolites associated with ASD. Repeated studies have demonstrated depressed ETC activity in several tissues, including immune cells, fibroblasts, muscle, and post-mortem brain. All these findings are consistent with classic abnormalities associated with mitochondrial disease.

In contrast to these findings consistent with classic mitochondrial abnormalities, other studies have demonstrated elevations in ETC activity with two studies demonstrating changes in mitochondrial morphology linked to these abnormalities. Additionally, studies using respirometry have demonstrated elevated respiratory rates in LCLs and PBMCs with a series of studies suggesting that this phenomenon is being driven by a subset of patients and may be linked to a NDR developmental phenotype.

Mutations in nuclear metabolic genes have been reported, but few consistencies have been observed except for variations in the SLC25A12 gene and a mutation in the TMLHE gene. Reports have suggested that mutations in mtDNA genes are more common in ASD as is mtDNA damage. Mutations in CytB, ND1, ND4 and tRNA mtDNA genes and mtDNA haplogroups have been associated with ASD. Interestingly, studies have reported that synonymous and non-coding mtDNA variations are associated with changes in mtDNA gene expression and ASD. Gene expression studies have shown increased expression of genes related to mitochondrial fission-fusion and ETC activity in peripheral tissues, but down regulation of the ETC and other mitochondrial genes in brain tissue. Other studies have demonstrated changes in methylation in ETC and fission-fusion genes.

Biomarkers of mitochondrial dysfunction have been linked to clinical symptoms in ASD. For example, lactate, carnitine, acyl-carnitines, ATP, CoQ10, as well as mtDNA variants, heteroplasmy, haplogroups and copy number have all been associated with ASD severity. Lactate and ND1 copy number have been associated with repetitive behavior. Lactate, TCA cycle metabolites, and mtDNA copy number have been linked to language. Lactate, TCA cycle metabolites, ETC complex I and IV activity in buccal samples, and PBMC respiration are associated with overall neurodevelopment. AC elevations and increased PBMC respiration are associated with NDR, while AC elevations and carnitine deficits have been linked to worsening GI symptomatology. Several studies have also demonstrated that mitochondrial biomarkers, particularly those linked to fatty acid metabolism, could distinguish individuals with ASD from controls with good accuracy, while fatty acid metabolites appear to be useful for predicting ASD in the presymptomatic and prenatal periods.

Several studies have examined the therapeutic effects of mitochondrial targeted treatments in children with ASD with favorable effects found on behaviors and ASD symptoms. A number of ASD treatments have been associated with favorable changes in mitochondrial function, and mitochondrial targeted supplements appear to improve mitochondrial activity and behavior in children with ASD and mitochondrial dysfunction.

Thus, there is evidence that mitochondrial function is disrupted in individuals with ASD. Below, we will discuss further evidence linking mitochondrial dysfunction to ASD, potential subtypes of mitochondrial dysfunction, and specific biological mechanisms which link mitochondrial dysfunction to neurodevelopmental symptoms associated with ASD.

4.1. Multisystem medical complexity of ASD suggests a mitochondrial etiology

Although ASD is traditionally thought to only involve the brain, over the last few decades it has been appreciated that 95% or more of individuals with ASD have at least one comorbid medical diagnosis (Soke et al., 2018). One study identified three patterns of comorbid conditions affecting individuals with ASD (Vargason et al., 2019). About half were found to have a low number of comorbid conditions, with these comorbid conditions occurring with a similar prevalence to the general population. About a quarter of ASD individuals demonstrated a medium number of co-morbid conditions, particularly developmental delays and auditory conditions. Lastly, a quarter manifested many co-morbid conditions, with immune, GI, and psychiatric conditions being most prevalent. This suggests that there are at least a quarter of individuals with ASD who have a multisystem presentation.

Mitochondrial disorders are characterized by multisystem dysfunction (Parikh et al., 2017), particularly affecting the brain (Clemente-Suarez et al., 2023) and gastrointestinal system (Frye et al., 2015), two systems that are commonly affected in ASD *(*Frye, *2022)*. In addition, it is becoming clearer that those with mitochondrial disorders also have functional immune disorders, making them susceptible to infections and sepsis (Walker et al., 2014; Edmonds et al., 2002). Many children with ASD demonstrate immune dysfunction, including early recurrent treatment resistant infections as well as autoimmunity (Hughes et al., 2018). Of note, two of the major criteria for diagnosing mitochondrial disease, the modified Walker (Bernier et al., 2002) and the Morava (Morava et al., 2006) criteria, heavily weigh multisystemic presentation in the diagnosis of mitochondrial disease.

4.2. Mitochondrial dysfunction and ASD symptomatology: Gastrointestinal abnormalities

GI disorders are rather prevalent in ASD, but a meta-analysis comparing those with ASD with and without mitochondrial disease found that having concurrent mitochondrial disease increased the prevalence of GI disorders significantly. Specifically, the prevalence of GI disorders was 74% in those with ASD and mitochondrial disease while the prevalence of GI disorders in the general ASD populations and in those with mitochondrial disease without ASD were significantly lower at 20% and 39%, respectively (Rossignol and Frye, 2012b). This is not surprising as GI abnormalities are strongly associated with several well-defined mitochondrial diseases with genetic underpinnings. For example, TYMP mutations cause pseudo-obstruction, MELAS, and Kearns-Sayre syndrome; other mtDNA mutations and polymorphisms cause cyclic vomiting syndrome; and hepatocerebral mtDNA depletion syndromes (caused by C10orf2, DGUOK, MPV17, PEO1, SUCLG1 mutations) cause liver failure (Frye et al., 2015). Other studies have linked mitochondrial disorders and GI function. For example, a case series of neonates with GI dysmotility within 2 weeks of life found significant ETC abnormalities (Chitkara et al., 2003). Another case series found that 56% of children with mitochondrial disease were also affected by GI disorders (Nissenkorn et al., 1999). Lastly, delayed gastric emptying and/or prolonged intestinal transit time were found in another case series of children with mitochondrial disease (Bhardwaj et al., 2012).

One study has examined mitochondrial function in GI tissue in children with ASD. ETC complex I and IV and citrate synthase activities and complex I-V protein abundance were compared between children with ASD, Crohn's disease, and neurotypical children with nonspecific GI complaints in rectal and cecum mucosal using a single-blind case-control design (Rose et al., 2017b). The cecum mitochondrial parameters were compared to the rectal mitochondrial parameters to understand if mitochondrial activity was specifically changed in the colon as the authors hypothesized that SFCAs produced by the microbiome could be modulating mitochondrial activity in the colon, the site of the majority of fermentative bacteria (Fig. 8A). Indeed, in the colon mucosa relative to the rectum mucosa, complex I, III, IV and V protein abundance was higher, and complex I activity was higher in the cecum of children with ASD compared to the other groups.

The two major SCFAs produced by the microbiome which are imbalanced in ASD are butyrate and propionate, which are also the terminal fatty acids in fatty acid oxidation for even and odd chain fatty acids, respectively, particularly for the TCA cycle (Fig. 8B). Propionic acid is produced by *Clostridia* spp. which is overrepresented in the microbiome of individuals with ASD (Finegold et al., 2012; Finegold et al., 2002). A rodent model of ASD demonstrated that intraventricular propionic acid causes ASD behavior as well as inhibition of lipid metabolism in the brain resulting in reactive astrocytosis and activated microglia along with a distinct pattern of AC elevations (Thomas et al., 2012; MacFabe et al., 2007; Thomas et al., 2010; MacFabe et al., 2008) which is also observed in children with ASD (Frye et al., 2013b). Studies on LCLs have demonstrated that propionic acid has a detrimental effect (Frye et al., 2016b), while butyrate has a protective effect (Rose et al., 2018b) on mitochondrial respiration in LCLs derived from children with ASD.

Several lines of evidence suggest that a subtype of mitochondrial dysfunction might be driven by GI microbiome imbalance. The propionic acid rodent model of ASD shows distinct AC and glutathione redox abnormalities (Thomas et al., 2012; MacFabe et al., 2007; Thomas et al., 2010; MacFabe et al., 2008) which have also been reported in a subgroup of children with ASD (Frye et al., 2013b). *Clostridia* spp. which produce propionic acid are particularly overrepresented in the gut of children with ASD and NDR (Finegold et al., 2012; Finegold et al., 2002), as well as those with GI symptoms preceding the onset of ASD symptoms (Williams et al., 2011). Interestingly, elevations in ACs in ASD have been also linked to NDR (Frye, 2012a) and GI symptoms (Needham et al., 2021), as well as core ASD symptoms (Nickel et al., 2023), and propionic acid has detrimental metabolic (Frye et al., 2016b) and immunologic (Frye et al., 2017b) effects on ASD LCLs.

Propionate is the terminal fatty acid in fatty acid oxidation for odd chain fatty acids and enters the TCA cycle at succinyl-CoA (Fig. 8B). This can have a profound effect on the TCA cycle which is consistent with other mitochondrial biomarker data. Studies have shown consistent elevations in citrate, cis-aconitate, isocitrate, and alpha-ketoglutarate in ASD (Sotelo-Orozco et al., 2020; Esvap and Ulgen, 2023; Kaluzna-Czaplinska, 2011; Yehia et al., 2019; Harutyunyan et al., 2021), consistent with slow metabolism of these intermediates in the TCA cycle prior to succinyl-CoA, potentially by excess propionic acid feeding into the TCA cycle (See Fig. 8B). The consequence of this alteration can be seen in Fig. 9 where the alternative pathways for metabolism of these elevated intermediates can be considered.

Elevation in alpha-ketoglutarate, the intermediate before succinyl-CoA, can result in several consequences. Glutamate, an amino acid and neurotransmitter well known to be elevated in ASD, can be overproduced potentially exacerbating cortical hyperexcitability associated with ASD. The production of glutamate also results in two other metabolic changes. First, branched chain amino acids will be metabolized into branched chain ketoacids. This is significant as a metabolic subset of

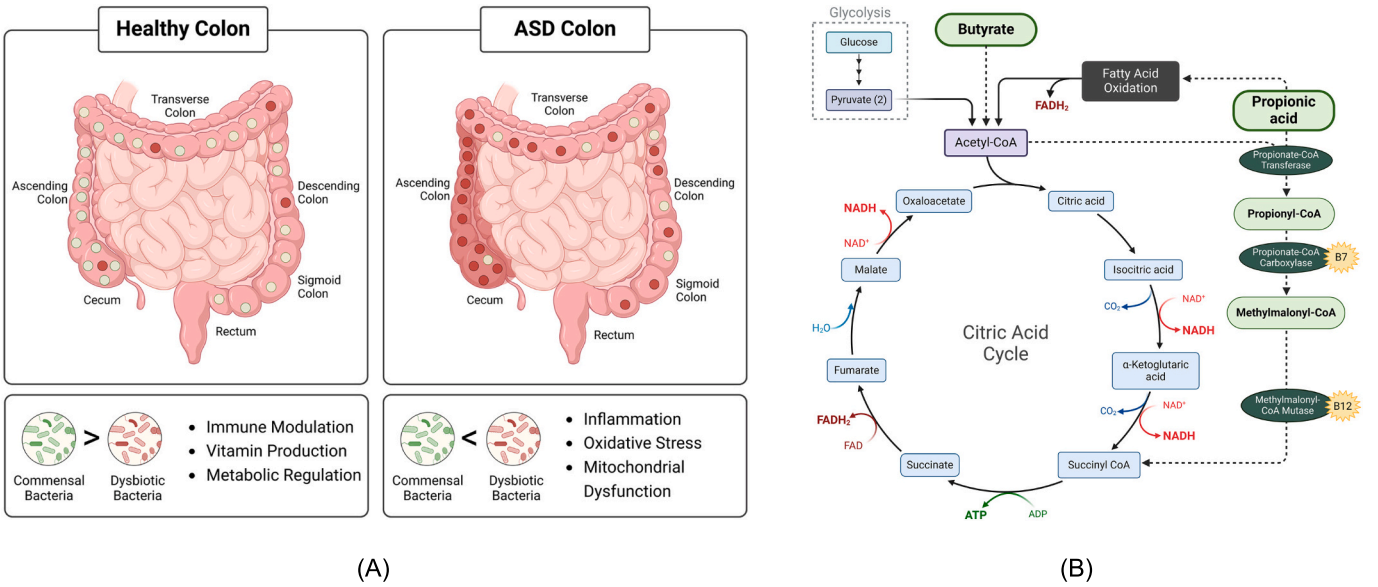

Fig. 8. Variations in colonic microbiome content can disrupt mitochondrial function. (A) More dysbiotic flora is found in the ascending colon of individuals with autism spectrum disorder resulting in inflammation, oxidative stress, and mitochondrial dysfunction. (B) Two major short chain fatty acids are produced by gut bacteria, specifically butyrate and propionate. These enter the citric acid system at different intermediates. Propionate is particularly disruptive to the citric acid cycle since it "short circuits" the proximal portion of the cycle and overloads the cycle at succinyl-CoA. Created with BioRender.com

ASD has been described with decreased branched chain amino acids (Smith et al., 2019), and branched chain amino acids have been found to improve speech, cooperation, stereotypy and hyperactivity in children with ASD in a recent open labeled trial (Aspragkathou et al., 2024). The production of branched chain ketoacids will feed back to acetyl-CoA, the beginning of the TCA cycle and the step before citrate, resulting in a further exacerbation of the buildup of initial TCA cycle intermediates. The second pathway to produce glutamate consumes glycine, an amino acid that is needed to produce GSH which is known to be deficient in ASD.

An inability of isocitrate to be metabolized into alpha-ketoglutarate can result in it being shunted to make succinate and/or malate. A decrease in the conversion of isocitrate into alpha-ketoglutarate will reduce the production of NADH by isocitrate dehydrogenase and the activity of Complex I. Shunting isocitrate to malate, rather than succinate, will reduce the production of $FADH_2$ by succinic dehydrogenase, resulting in a decrease in complex II activity. Thus, in ASD it is very possible that changes in the metabolic structure of the TCA cycle can result in alterations to neurotransmitter production as well as stoichiometric changes to ETC complex activity. Clearly there are complex interactions between the microbiome and mitochondrial function which could contribute to GI and ASD symptoms, although future research will need to dissect these complexities to better understand these relationships. Most importantly, the relationship between the microbiome and common alterations in the diet seen in individuals with ASD is complex, making the origins of the changes in the microbiome uncertain and confounding which changes to the gut environment are due to microbiome, diet, or the combination of these factors. However, these patterns of metabolic alterations are a good candidate for a distinct subtype of a mitochondrial disorder in ASD.

4.3. Mitochondrial dysfunction and ASD etiology: The role of oxidative stress

Increased ROS, particularly mitochondrial ROS (mtROS) (James et al., 2009), are so prevalent in ASD that biomarkers of the associated transmethylation and transsulfuration alterations may aid in the diagnosis of ASD (Howsmon et al., 2017; Howsmon et al., 2018; Vargason et al., 2018). Several studies link mitochondrial dysfunction with oxidative stress in ASD (Naviaux, 2014; Naviaux et al., 2013; Boccuto et al., 2013; Schwartz, 2014; Frye et al., 2013c); the origin of the increased ROS and its effect on the mitochondria are still under study.

Several lines of research suggest that mitochondrial dysfunction may not be due to the mtROS itself but related to the manner in which the mitochondria can regulate the mtROS. For example, in one sibling study of mitochondrial respiration in LCLs, both the children with ASD and their siblings demonstrated equally poor glutathione redox ratios, but only the LCLs from the children with ASD manifested mitochondrial dysfunction (Rose et al., 2017a). mtROS at the inner mitochondrial membrane is regulated by leak proteins. Increased proton leak is consistently shown in LCLs from ASD individuals with mitochondrial dysfunction (Fig. 6B) (Rose et al., 2014b; Rose et al., 2017a; Rose et al., 2018b; Frye et al., 2017a; Frye et al., 2016b; Rose et al., 2015a; Rose et al., 2015b; Rose and Bennuri, 2019). Uncoupling Protein 2 (UCP2), the main leak protein, demonstrated increased gene expression (Rose et al., 2017a) and protein concentration (Rose et al., 2014b; Bennuri et al., 2019) in LCLs derived from children with ASD and mitochondrial dysfunction. Abnormalities in another proton leak regulator, the Adenine Nucleotide Translocator, are associated with non-syndromic intellectual disability with ASD (Vandewalle et al., 2013) and in a mouse model of ASD with mitochondrial disease (Picard et al., 2014). Interestingly, increased proton leak may be central to synaptic growth in the Fragile X syndrome mouse model as correcting increased proton leak restores synaptic growth in this mouse model (Licznerski et al., 2020). Thus, regulation of mtROS by significantly increasing proton leak could prevent ROS damage but lead to consequences for brain development.

Another major mechanism of mtROS dysregulation may be linked to abnormalities in superoxide dismutase (SOD). SOD is essential for controlling ROS and has abnormally low activity in both genetic (Bai et al., 2021) and environmental exposure (Rigobello et al., 2021) animal models of ASD as well as LCLs derived from children with ASD (Nayan et al., 2020). In addition, it is epigenetically suppressed in a gestational diabetes animal model of ASD (Lu et al., 2020). Furthermore, therapeutic treatments increasing SOD activity in LCLs (Nayan et al., 2020)

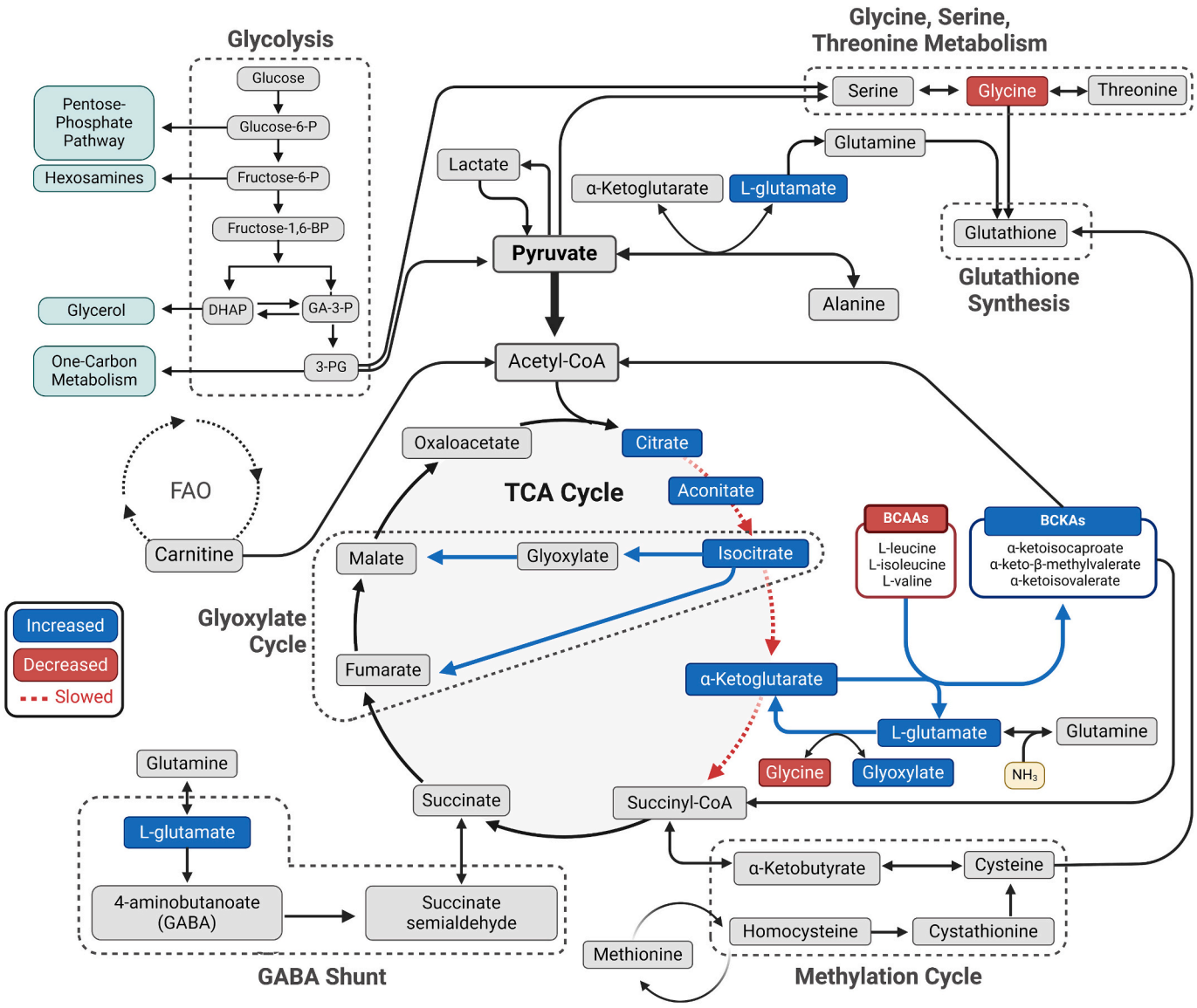

Fig. 9. The Tricarboxylic Acid cycle is associated with several interconnected pathways which can influence amino acid neurotransmitter balance, particularly glutamate and glycine. Patterns of abnormalities reported in the Tricarboxylic Acid cycle associated with autism spectrum disorder have the potential to increase glutamate production and consume branched chain amino acids, consistent with findings in individuals with autism spectrum disorder. BKAAs: Branched-chain amino acids; BCKAs: Branched-chain α-ketoacids. Created with BioRender.com

and genetic models of ASD improve behavior and/or SOD levels (Eissa et al., 2021). What is very compelling is that the nutritional metal co-factors, Zn, Mn, and Cu, have all been found to be dysregulated in ASD. For example, one twin study demonstrated that the difference between the twin who developed ASD and the one who did not was characterized by distinct deficiencies in prenatal Zn and Mn concentrations in the fetus (Arora et al., 2017), while another study demonstrated that prenatal and postnatal differences in zinc-copper metabolic cycles can predict the development of ASD (Curtin et al., 2018). Furthermore, a study of PBMCs demonstrated that mitochondrial respiration as a child was dependent on fetal Zn and Mn concentrations in those with ASD and NDR (Frye et al., 2020).

4.4. *Mitochondrial dysfunction and ASD symptomatology: Immune dysfunction*

The immune system is intimately dependent on the mitochondria. One retrospective study which examined 97 patients with mitochondrial disease found that 42% of them had a history of serious or recurrent infections, with the majority (21%) being bacterial and lesser ones being bacterial and fungal (7%), bacterial and viral (7%) or all three (6%). Furthermore, 13% of patients with mitochondrial disease had more than one episode of sepsis (Walker et al., 2014). This is not so surprising when we consider that mitochondria are necessary for inflammasome activation.

The mitochondria are predominantly utilized by regulator T cell (Liu and Ho, 2018) and healing macrophages (Galli and Saleh, 2020), while inflammatory macrophages turn off the mitochondria, primarily using a non- mitochondrial pathway (Galli and Saleh, 2020). Bacteria try to turn off the immune response by disrupting mitochondrial function. For example, *L. pneumophila* impairs oxidative phosphorylation and triggers mitochondrial fission, while L. *monocytogenes* induces mitochondrial fragmentation (Galli and Saleh, 2020). The mitochondria are also involved directly in combating bacteria. Lipopolysaccharide stimulated macrophages reprogram the ETC so that complex I essentially runs backwards to produce more mtROS, while *E. coli* results in macrophages turning off complex I to decrease cellular ROS (Sancho et al., 2017). Furthermore, TCA cycle intermediates such as fumarate are used for

their antibacterial effect (Sancho et al., 2017).

Children with ASD have several forms of immune dysfunction. ASD is associated with chronic infections in early life (Jyonouchi et al., 2008) and recurrent infections later in childhood that is commonly linked to behavioral exacerbations (Jyonouchi et al., 2008). Children with ASD also have dysregulated microbiomes (Taniya et al., 2022), suggesting that the gut-associated lymphoid tissue cannot regulate bacteria well in the gut (Hosie et al., 2022). In addition, ASD appears to be associated with autoimmune disorders (Spann et al., 2019). ASD individuals have been found to have a particular dysfunction in innate immunity which is highly dependent on mitochondrial function (West et al., 2011). This suggests non-specific dysfunction of the immune system, potentially implicating the mitochondria in the etiology of these immune abnormalities in ASD.

4.5. Mitochondrial dysfunction and ASD etiology: The "terrible trio"

One of the reasons that the underlying biology of ASD may be difficult to understand is the fact that it involves the interaction between multiple biological systems, rather than abnormalities in one well-defined system or biological mechanism (Rossignol and Frye, 2012a; Rossignol and Frye, 2014). Above, we have outlined the major systemic abnormalities in ASD, specifically mitochondrial and immune dysfunctions and oxidative stress. One systematic review found that studies have linked these three abnormalities in the ASD brain (Rossignol and Frye, 2014). Other reviews point to the fact that these three abnormalities are related to the effects of the prenatal environment in increasing the risk of developing ASD (Frye et al., 2021c).

Interactions between mitochondria, redox metabolism, and the immune system can be mutually detrimental; such detrimental interactions have been documented in ASD (Fig. 9) (Rossignol and Frye, 2014). Mitochondria are both a major producer and target of ROS. Dysfunctional mitochondria, particularly Complex I and III, produce high amounts of ROS which can cause ETC dysfunction and damage to the mitochondria (Fig. 1). Control of mtROS can result in inefficient mitochondrial function since the inner mitochondrial membrane proton gradient will be lost to proton leak and complex V will therefore have no driving force. There are also redox sensitive enzymes such as aconitase, the first enzyme in the TCA cycle. To compound this problem, GSH, the main intracellular and mitochondrial antioxidant, requires ATP for its de novo production. As such, a decrease in ATP production resulting from reduced mitochondrial function will result in less GSH production, resulting in poorer control of ROS (Fig. 10). In fact, a lower GSH redox ratio has been correlated with lower aconitase activity in post-mortem brain from individuals with ASD (Rose et al., 2012). Oxidative damage to cellular lipids, proteins and nucleic acids (Frustaci et al., 2012) have been associated with ASD; this is especially important since mtDNA is vulnerable to oxidative damage, and studies have shown that children with ASD demonstrate mtDNA damage in a pattern consistent with oxidative damage (Napoli et al., 2013).

Mitochondrial dysfunction can drive immune activation. Production of ROS from dysfunctional mitochondria can result in damage to lipids and proteins which can activate inflammatory pathways (Rossignol and Frye, 2014; Rose et al., 2012). Dysfunctional mitochondria can release damage-associated molecular patterns (DAMP), including cardiolipin, n-formyl peptides, ROS and mtDNA, which can activate the inflammasome (Banoth and Cassel, 2018). As regulatory immune cells are highly dependent on oxidative phosphorylation, once an immune response has been started, it may be difficult to regulate this release (O'Neill et al., 2016). Once activated, the immune system produces ROS which can result in a further detrimental effect on already dysfunctional mitochondria. In ASD, studies have linked proinflammatory cytokine production with mitochondrial dysfunction (Jyonouchi et al., 2019).

4.6. Mitochondrial dysfunction and ASD etiology: Environmental influences linked to ASD

Many environmental toxicants have been linked to ASD and other neurodevelopmental disorders (Rossignol et al., 2014). These same toxicants can induce mitochondrial dysfunction and produce excess ROS. For example, pesticides such as chlorpyrifos (and its active metabolite), monocrotophos, and dichlorvos induce mitochondrial

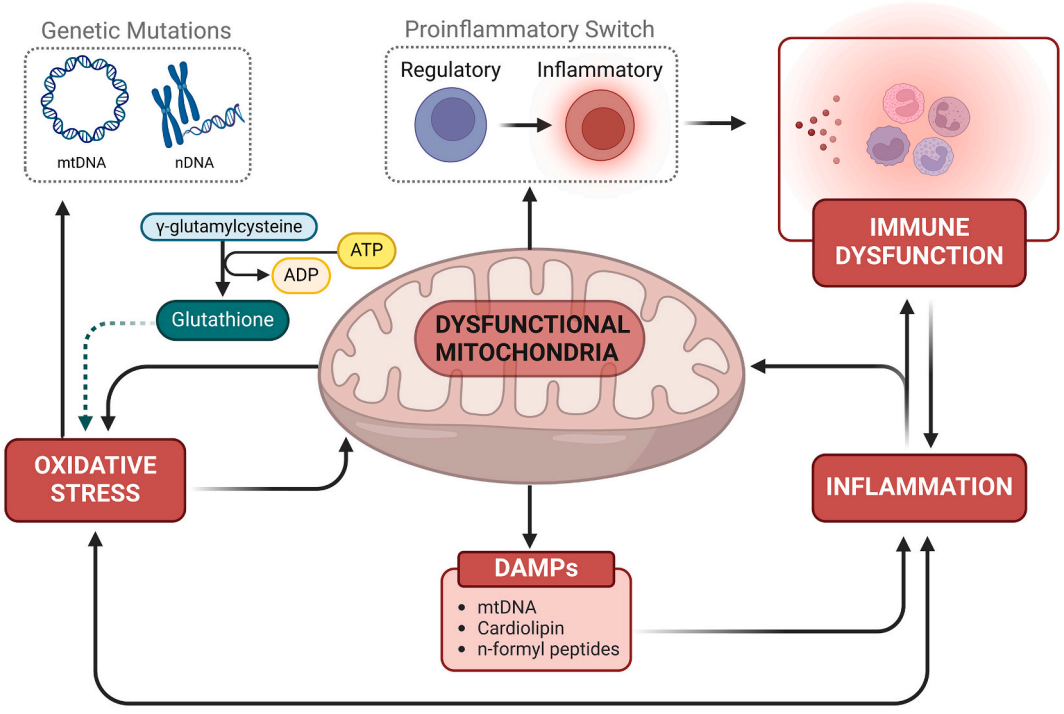

Fig. 10. The terrible trio of oxidative stress, mitochondrial dysfunction and inflammation mutually negatively reinforce each other to result in chronic physiologic dysfunction. DAMP = damage-associated molecular patterns; mtDNA = mitochondrial DNA; nDNA = nuclear DNA. Created with BioRender.com

structural and functional damage and increase ROS (Middlemore-Risher et al., 2011; Basha and Poojary, 2014; Lee et al., 2012; Masoud et al., 2009). Phthalates increase mitochondrial respiration and fatty-acid metabolism (Posnack et al., 2012), detrimentally affecting the mitochondrial membrane potential (Pant et al., 2008) and increasing ROS (Pant et al., 2008). For mothers whose pregnancy results in a child with ASD, exposure to these toxicants is associated with biomarkers of mitochondrial dysfunction. For example, higher concentrations of maternal urinary phthalates measured during the second and third trimester have been associated with lower placental carnitine levels (Parenti et al., 2022). Another study reported that urinary phthalate metabolites measured in the third trimester were associated with elevated pyruvate in maternal serum at 28 weeks of pregnancy. Higher phthalate concentrations were associated with increases in serum lactate. Higher lactate levels were associated with more severe ASD symptoms at 2 and 4 years old (Thomson et al., 2023).

Several recent studies have demonstrated an association between air pollution and ASD (Chun et al., 2020). Recent studies have shown that prenatal air pollution exposure is associated with mitochondrial-derived peptides (Breton et al., 2019) and abnormal oxidative phosphorylation from mitochondria derived from cord blood (You et al., 2024). One study examined the relationship between prenatal air pollution exposure (as indexed by inhalable particles with diameters that are generally 2.5 μm and smaller, $PM_{2.5}$) and the long-term changes in mitochondrial function by measuring mitochondrial respiration in PBMCs from children with ASD (Frye et al., 2021b). Interestingly, prenatal $PM_{2.5}$ exposure was related to mitochondrial respiration in children, but the relationship was different for those with and without a history of NDR. For those with a history of NDR, higher prenatal $PM_{2.5}$ exposure was related to higher mitochondrial respiration, while for those without a history of NDR, higher prenatal $PM_{2.5}$ exposure was related to lower mitochondrial respiration. Structural equation modeling found that the mitochondria mediated (accounted for) between 25%–50% of the effect of prenatal air pollution exposure on neurodevelopment (Frye et al., 2021b).

Many environmental metals connected to ASD can also be linked to causing mitochondrial dysfunction. Pb can cause behavioral and learning problems in ASD (EPA, 2018), damages mitochondrial structure (Perkins et al., 2012; Navarro-Moreno et al., 2009; Wu et al., 2012) and function (Geier et al., 2009; Yin et al., 2008), and depletes mitochondrial GSH (Baranowska-Bosiacka et al., 2012). Ethylmercury has been linked to ASD and has been shown to cause mitochondrial dysfunction in ASD LCLs (Rose et al., 2015a). As previously mentioned, prenatal Zn and Mn concentrations differentiate twins who developed ASD and those who do not (Arora et al., 2017). Mitochondrial respiration in PBMCs derived from children with ASD and NDR have been found to be related to prenatal exposure to Zn and Mn (Frye et al., 2020).

The long-term effects of prenatal inflammation may be mediated by changes in mitochondrial respiration. For example, mitochondrial dysfunction in the brain (Naviaux et al., 2013) and leukocytes (Napoli et al., 2013) has been found in the maternal immune activation mouse model of ASD, a model of an inflammatory environmental trigger during pregnancy causing ASD behavior in offspring.

Meta-analysis suggests that mtDNA copy number is increased in ASD. mtDNA copy number is believed to be a biomarker that reflects cumulative environmental toxicant exposure (Cheng et al., 2024). Increased mtDNA copy number is induced by bisphenol A exposure, a toxicant linked to ASD (Kaur et al., 2014). Prenatal arsenic (Qiu et al., 2023) and essential metal (Bi et al., 2023) exposure are associated with changes in cord blood and newborn mtDNA copy number.

4.7. Mitochondrial dysfunction and ASD etiology: Neurodevelopmental regression

Individuals with mitochondrial disease are susceptible to NDR, especially with illness (Edmonds et al., 2002). One study showed that most children with ASD and mitochondrial disease developed ASD symptoms after a sudden and rapid NDR associated with a fever (Shoffner et al., 2010), and a meta-analysis found that NDR was more common in children with ASD and mitochondrial disease than in the general ASD population (Rossignol and Frye, 2012b).

Recent studies have demonstrated that NDR is associated with a specific type of mitochondrial dysfunction. Mitochondria derived from the PBMCs of children with ASD and NDR have elevated respiratory rates and are more sensitive to physiological stress (Gevezova et al., 2021; Gevezova et al., 2022). These findings parallel the AD-A subset of the in vitro LCL cellular model of ASD which also have elevated respiratory rates and are more sensitive to physiological stressors (Rose et al., 2014b; Rose et al., 2017a; Rose et al., 2018b; Frye et al., 2017a; Frye et al., 2016b; Bennuri et al., 2019; Rose et al., 2015b). This pattern of sensitivity to physiological triggers parallels the group of children with ASD and NDR who typically regress into ASD with physiological stressful triggers such as fever (Shoffner et al., 2010), seizure (Frye et al., 2011), or rapid extremes in air pollution or temperature (Frye et al., 2022).

High respiratory rates have been associated with NDR in individuals with ASD in other studies. In the first case report of its kind examining the G8363A $tRNA_{Lys}$ mutation in siblings, the girl with high heteroplasmy (blood 82%; muscle 86%) had Leigh syndrome and decreased ETC activity, while the brother who had ASD with NDR but not Leigh syndrome had lower heteroplasmy (blood 60%; muscle 61%) and demonstrated markedly higher than normal ETC Complex I activity (Graf et al., 2000). The next case series reported five patents with ASD and NDR who had elevations in ETC Complex IV activity (~200% normal) in muscle (Frye and Naviaux, 2011). Subsequently elevations in ETC Complex IV activity and ASD have been reported in fresh frozen superior temporal gyrus (Palmieri et al., 2010), buccal swab enzymology (Delhey et al., 2017a), and LCLs using high-resolution respirometry (Hassan et al., 2021), but these reports did not note whether or not the cases had NDR. This pattern of mitochondrial dysfunction is also seen in genetic syndromes associated with ASD such as Phelan-McDermid Syndrome (Frye et al., 2016c), 22q13 duplication (Frye, 2012b), and Rett syndrome (Condie et al., 2010) as well as the PTEN haploinsufficient mouse (Napoli et al., 2012).

Studies suggest that mitochondrial dysfunction may originate from prenatal exposures. Two studies directly linked prenatal environmental exposures to elevated respiratory rates during childhood for those that have ASD with NDR (Frye et al., 2021b; Frye et al., 2020). These elevated respiratory rates are believed to be linked to cellular compensatory mechanisms for handling oxidative insults. In one study, LCLs from individuals with ASD and controls were exposed to low levels of oxidative stress for 96 h in vitro (Bennuri et al., 2019). This exposure resulted in elevations in ATP linked respiration, proton linked respiration, maximal respiratory capacity and reserve capacity, and demonstrated that prolonged exposure to oxidative stress could induce these elevations in respiratory rates. In addition, the changes in these metrics of respiration were accentuated in a manner similar to the changes found in the AD-A LCLs. These changes were not different across the LCLs groups except for reserve capacity, in which the loss of reserve capacity with physiological stress was more accentuated in the ASD LCLs compared to the CNT LCLs. Thus, these data suggest that these changes in mitochondrial function seen in the AD-A LCLs can be induced by exposure to physiological stress, consistent with the notion that prenatal stressors, including environmental exposures, could cause mitoplasticity resulting in elevated respiratory rates and greater sensitivity to physiological stressors.

In addition to these studies, many prenatal exposures to toxicants such as air pollution, organophosphates, phthalates, cigarette smoke, inflammation, exposure to acetaminophen, and antibiotics as well as maternal microbiome alterations and nutritional deficiencies in zinc, copper, manganese, iron, folate and carnitine, are associated with an increased risk of ASD and mitochondrial dysfunction (Frye et al.,

2021d). As these prenatal exposures can change mitochondrial function which lasts into the post-natal period, it is possible that these prenatal exposures influence the mitochondria to be vulnerable to post-natal triggers which result in NDR (Fig. 11). Specifically, elevated mitochondrial respiration resulting from prenatal exposure may be able to support cellular and neuronal function until a stressor causes a rapid fall in respiration resulting in mitochondrial, cellular, and neuronal dysfunctions causing the clinical symptoms which are seen as NDR.

Overall, it appears that individuals with elevated respiratory rates and NDR might represent a second emerging subtype of mitochondrial dysfunction in ASD which might be associated with prenatal exposures. Further research will need to investigate this further, however this is a promising subtype that might be treatable by proactive reduction of environmental exposures or by supporting mitochondrial function to prevent the NDR.

4.8. Mitochondrial dysfunction and ASD symptomatology: Brain development

The molecular mechanisms of embryonic neurodevelopment are complex (Gotz and Huttner, 2005). In the embryonic period, neuroepithelial stem cells (NSCs) in the ventricular zone undergo symmetric, proliferative expansion via self-renewal (Silva-Vargas et al., 2018), followed by a transition towards asymmetric neurogenic division, resulting in the production of intermediate neural progenitor cells (nIPCs) and radial glial cells (RGCs). Both RGCs and NSCs have the capacity for self-renewal and asymmetric division and they expand and differentiate in a spatiotemporally distinct manner (Silbereis et al., 2016) (Fig. 12A). Following the embryonic period, the fetal period is characterized by continued growth and proliferation of RGCs and nIPCs (Silbereis et al., 2016) along with migration of non-cortical interneuron precursors derived from the ganglionic eminences towards the cortex (Hansen et al., 2013). Throughout the early postnatal period, glial cell populations continue to differentiate and neurons undergo substantial myelination, dendritic maturation, and synaptogenesis (Iwata, 2022). Further refinement and reorganization of the neural circuitry continues throughout late childhood and adolescence.

Thus, mitochondria function as central signaling platforms at virtually all stages of developmental neurogenesis (Kelley and Pasca, 2022). Recently, mitochondrial dynamics have been described as an upstream regulator of early neurogenesis, wherein NSC fate is regulated by morphologically-mediated increases in mROS, which then activate developmental gene expression via a nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent retrograde pathway (Fig. 12A) (Khacho et al., 2016; Khacho et al., 2019). In this framework, uncommitted NSCs are characterized by their predominantly anaerobic metabolism and elongated mitochondria, whereas committed progenitor cells are characterized by small, fragmented mitochondria and activation of aerobic metabolism and oxidative phosphorylation (OXPHOS) mechanisms. This morphological and bioenergetic switch towards aerobic OXPHOS metabolism leads to a physiologic increase in mtROS (Khacho et al., 2016) and downstream activation of Nrf2-mediated antioxidant and metabolic stress response mechanisms (Gureev et al., 2019). Together, these mechanisms result in conditions that inhibit self-renewal and activate neurogenic differentiation (Khacho et al., 2016), highlighting the fundamental role of mitochondria in cell fate decisions throughout neurogenesis and neurodevelopment.

Mitochondria are also essential for synaptic transmission (Mironov, 2009), the glutamate-GABA-glutamine cycle, neurotransmitter homeostasis (Tani et al., 2014) and calcium regulation (Voorsluijs et al., 2024) (Fig. 12D). Disturbances of neurotransmitter homeostasis during prenatal development can lead to underdeveloped neurotransmitter networks, and impaired neuroplasticity and network-level information integration during crucial developmental periods. Calcium flux has been

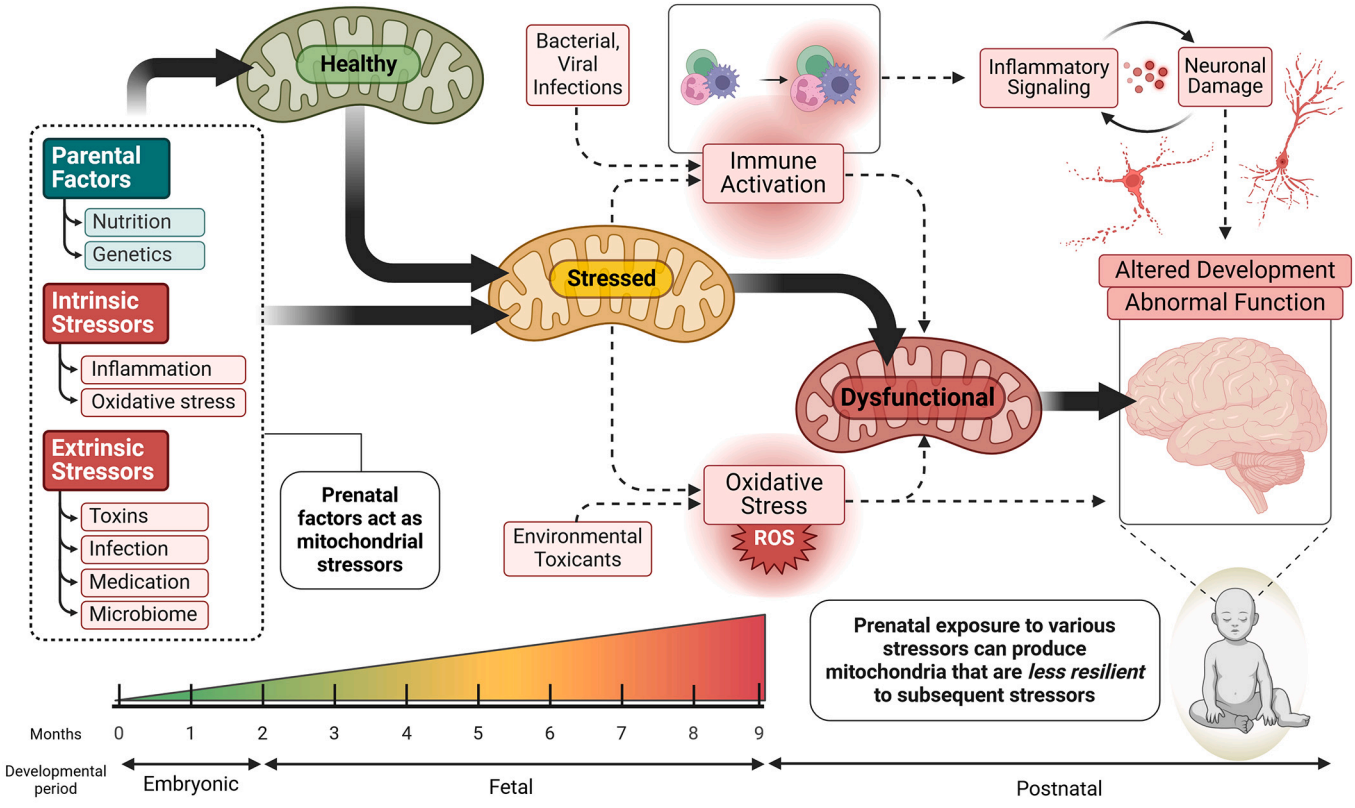

Fig. 11. Prenatal factors can cause the mitochondria to enter a stressed state in which they are less resilient to post-natal stressors. Post-natal stressors then can push the mitochondria into mitochondrial dysfunction, resulting in what is recognized clinically as neurodevelopmental regression. Created with BioRender.com

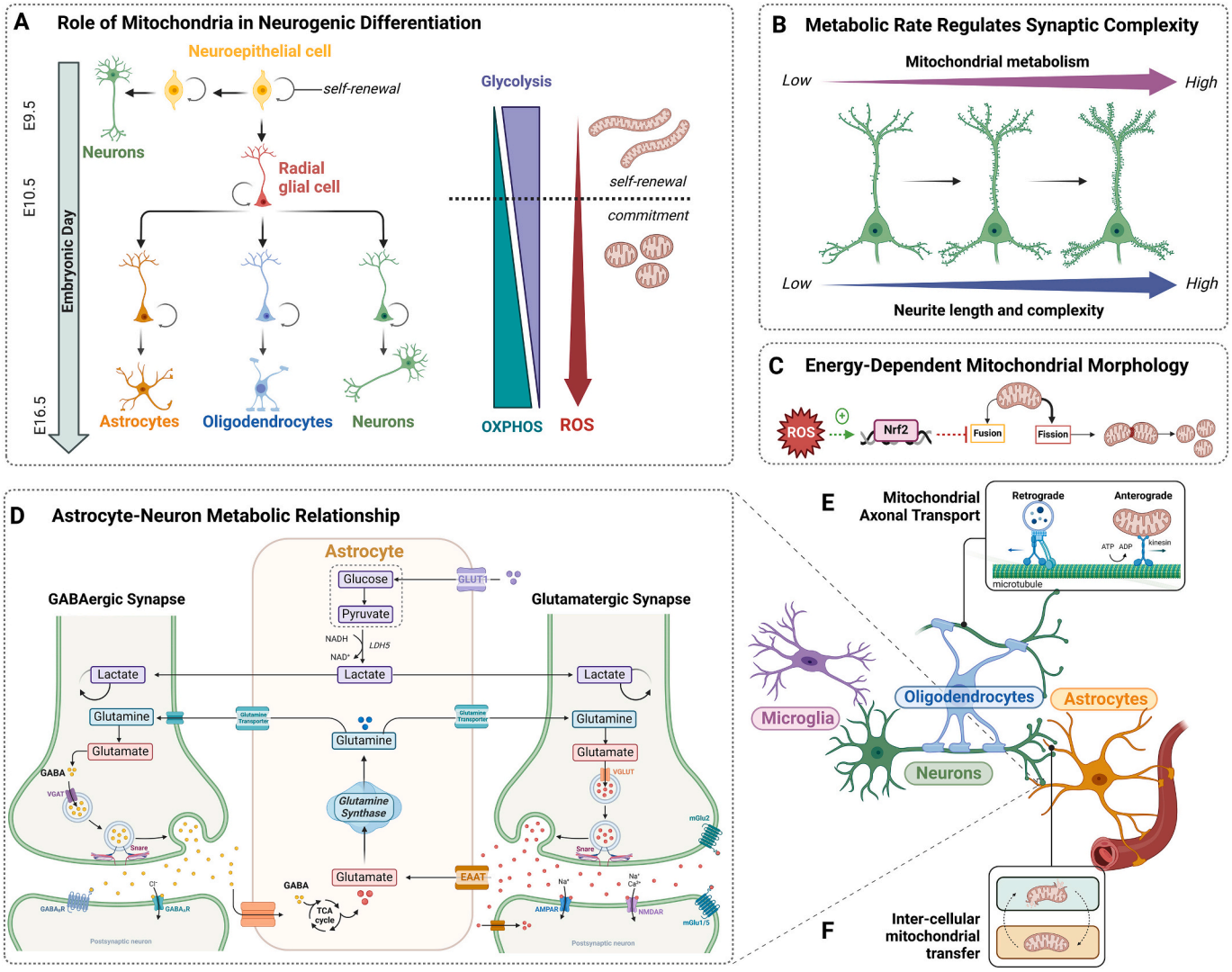

Fig. 12. Mitochondria are involved in both brain development and brain function. (A) Mitochondria are needed for neurogenic differentiation; (B) The metabolic rate of mitochondria regulate synaptic length and complexity such that a low metabolic rate is associated with short neurons with less complex synaptic structure and a high metabolic rate is associated with longer neurons with more complex syntactic structure; (C) Mitochondria change morphology depending on the energy needs and undergo repair through processes which eliminate dysfunctional portions of the mitochondria; (D) Mitochondria are essential for maintaining membrane potentials by providing energy to ion exchangers and are essential for production and maintenance of synaptic vesicles. In GABAergic synapses, mitochondria are essential in the glutamate-GABA cycle while in glutamatergic synapses mitochondria are essential for regulating glutamate-glutamine cycle.; (E) Mitochondria are essential for providing energy to transport organelles and other important factors between the neuronal cell body and the synapse through the axon; (F) Mitochondria can be transferred between cells to restore damaged mitochondria. NRF2: Nuclear factor erythroid 2-related factor 2. Created with BioRender.com

demonstrated to mediate some of the morphogenetic signaling mechanisms that direct cortical expansion and organization during embryonic neurodevelopment (Rash et al., 2016). Interestingly, altered calcium flux has been linked to mitochondrial dysfunction in brain tissue from individuals with ASD (Palmieri et al., 2010). These mechanisms, known as mitoplasticity, permit real time bioenergetic adaptations that integrate stress signals and adjust for energy requirements (Yun and Finkel, 2014) (Fig. 12C). The state transition between stem cell self-renewal, differentiation, and reprogramming is characterized by distinct changes in mitochondrial dynamics, including mitochondrial biogenesis, fission/fusion, and mitophagy (Khacho et al., 2016).

Studies have reported the consequences of mitochondrial dysfunction on brain development in several mouse models. Other studies have shown that the mitochondrial metabolic rate is critical for structural development. Both elevated and decreased mitochondrial metabolism result in different developmental abnormalties (Fig. 12B). Low mitochondrial respiration will cause decreased neuronal length and complexity, while elevations in mitochondrial metabolism result in increased neuronal length and complexity as well as increased synaptic function and hyperexcitability (Iwata et al., 2023). A mouse model of the DiGeorge/22q11 deletion syndrome demonstrated that decreased mitochondrial activity was linked to underconnectivity of the white matter and cognitive impairment (Fernandez et al., 2019). Interestingly, the mitochondrial dysfunction was found to be linked to oxidative stress and prenatal treatment with *N*-acetyl-cysteine normalized brain development.

4.9. Mitochondrial dysfunction and ASD symptomatology: cognitive dysfunction

Various cell-cell interactions in the central nervous system rely on mitochondrially-mediated mechanisms, such as synaptic transmission (Fig. 12D) (Mironov, 2009), the glutamate-glutamine cycle (Fig. 12D), neurotransmitter homeostasis (Fig. 12D) (Tani et al., 2014), the glutamate-GABA cycle (Fig. 12D) (Waagepetersen et al., 1999), neuron-astrocyte shuttling of metabolic substrates like fatty acids and lactate

(Fig. 12D) (Bonvento and Bolanos, 2021), intercellular mitochondrial trafficking (Fig. 12E) (Norkett et al., 2020), and mitophagy (Fig. 12C) (Youle and Narendra, 2011; Palikaras and Tavernarakis, 2020). Thus, mitochondria serve as central signaling platforms for numerous aspects of intra- and intercellular communication. Disturbance of neurotransmitter homeostasis can occur due to perturbations in energy dependent cellular mechanisms like neurotransmitter reuptake (Jackson et al., 2014) and re-packaging into secretory vesicles (Rizzoli and Betz, 2005), leading to underdeveloped neurotransmitter networks, impaired neuroplasticity and network-level information integration during crucial developmental periods as well as into childhood and adulthood. Evidence from genetic studies suggests that mitochondrial genes are downregulatred in synapses in ASD (Schwede et al., 2018). Although incompletely characterized, these mitochondrially-mediated signaling pathways are myriad and have become increasingly implicated in many underlying mechanisms of developmental neurogenesis (Iwata and Vanderhaeghen, 2021).

Mitochondria are morphologically dynamic organelles that can respond to, or induce, cellular processes via fusion and fission (Fig. 12C). These mechanisms, known as mitophagy, permit real time bioenergetic adaptations that integrate stress signals and adjust for energy requirements (Yun and Finkel, 2014). Additionally, studies have uncovered the ability for mitochondria to be transferred between cells; for example, astrocytes can replace damaged mitochondria in neurons (Fig. 12F) (Geng et al., 2023). Unsurprisingly, mitochondria continue to play a vital role in normal brain functioning throughout adulthood through their involvement in synaptic plasticity (Khacho and Slack, 2018) and adult neurogenesis (Traxler et al., 2021).

4.10. Mitochondrial dysfunction and ASD etiology: Contribution of genetics

While the high heritability rate of ASD has driven the idea that ASD is genetic, studies fail to find genetic mutations in a majority of children with ASD (Tammimies et al., 2015) and when genetic mutations are found, the majority of them are de novo *(*Hamdan et al., *2017;* Sanders et al., *2012)*. In fact, the majority of siblings with genetic etiologies of ASD have different genetic mutations (Yuen et al., 2015). The reasons for this are unclear. A study of multiplex ASD families suggested that recurrent cases of ASD in families was due to rare variants in known ASD risk genes combined with common genetic variants which result in a sufficient genetic load that exceeds a threshold, suggesting a complex genetic landscape (Cirnigliaro et al., 2023).

Other studies suggest that the etiology of ASD is likely driven by genetic-environmental interactions with each contributing about equally (Hallmayer et al., 2011). This is remarkably interesting since mitochondria are very vulnerable to environmental factors, potentially making them the key mediators of environmental-genetic interactions. Several lines of evidence suggest that this may be the process at work in ASD. For example, mtDNA copy number, a metric that is believed to be linked to environmental toxicant exposures, is significantly elevated in individuals with ASD. Other studies have found high rates of mtDNA mutations in individuals with ASD with some studies also finding this elevated rate in their fathers. As mtDNA is inherited from mothers, these data might suggest common environmental exposures for both the child and father which result in mtDNA mutations.

Various nuclear genes have been associated with mitochondrial dysfunction and ASD with few consistent findings. The SLC25A12 is perhaps the best studied gene with both uncontrolled and controlled studies but with mixed results. Three case series (Ramoz et al., 2004; Segurado et al., 2005; Silverman et al., 2008), three controlled studies (Anitha et al., 2012; Durdiakova et al., 2014) including one examining brain tissue (Lepagnol-Bestel et al., 2008), and one meta-analysis (Aoki and Cortese, 2016) reported an association between ASD and SLC25A12. One of the uncontrolled studies reported that the SLC25A12 rs2056202 genotype was significantly associated with routines and rituals in ASD (Silverman et al., 2008). However, two case series (Correia et al., 2006; Rabionet et al., 2006), two controlled studies (Blasi et al., 2006; Pourtavakoli et al., 2024), and one meta-analysis (Liu et al., 2015) found no association with ASD. Perhaps the second most well studied gene is the TMLHE gene which is the first step in carnitine synthesis. One study found an association of a TMLHE mutation with ASD male-male multiplex families (Celestino-Soper et al., 2012) and 4 other reports have observed TMLHE mutations in children with ASD (Ziats et al., 2015; Goin-Kochel et al., 2019; Celestino-Soper et al., 2011; Nava et al., 2012). A case of a mutation in the organic cation transporter 2 (SLC22A5) which causes systemic primary carnitine deficiency (Guevara-Campos et al., 2019) has also been reported in ASD. Of note, the TMLHE mutation is treatable with L-Carnitine.

Additionally, both the environment and the mitochondria have influences on epigenetic regulations which have also been linked to ASD (LaSalle, 2023). Interestingly, epigenetic changes have been linked to ETC complex genes and genes associated with mitochondrial biogenesis and maintenance.

Clearly, like ASD itself, the genetics of mitochondrial dysfunction in ASD is highly complex and does not converge on specific genetic changes. ASD is associated with hundreds of risk genes and is clearly not a simple Mendelian disorder. Further research into interactions between the environment, mtDNA, nuclear gene mutations, and epigenetics will provide more insight into this complexity.

4.11. Mitochondria as central to the etiology of ASD

The discussion above provides some insight into how mitochondria may be a central driving force resulting in ASD, at least in some of the cases, particularly those that are medically complex. Mitochondrial dysfunction can explain the common immune and GI dysfunction seen in many children with ASD as well as the physiological abnormalities of oxidative stress and redox and methylation abnormalities. Mitochondria are highly influenced by the environment, potentially explaining the strong environmental-genetic interaction associated with the etiology of ASD, and evidence suggest that adverse prenatal environmental influences may result in long term mitochondrial dysfunction associated with ASD. Mitochondrial dysfunction can also explain the NDR phenotype of ASD which is experienced by approximately one-third of children with ASD. Finally, abnormalities in mitochondrial function can explain the prenatal developmental brain disorders and postnatal central nervous system dysfunction associated with ASD.

4.12. Clinical implications: An approach to diagnosing mitochondrial dysfunction in ASD

This lack of simple Mendelian genetic changes associated with ASD has implications for the diagnosis of mitochondrial disease, particularly in the usefulness of the Modified Walker Criteria which heavily relies on known genetic mutations. A previous review has pointed out that using Morava et al. criterion (Morava et al., 2006), a criterion which is based on symptoms and biomarkers, may be more appropriate to assess the presence of mitochondrial disease in ASD given the lack of a straightforward genetic etiology (Frye and Rossignol, 2011). The Morava et al. criterion (Morava et al., 2006) is based on multisystem symptomatology with an emphasis on the muscular and central nervous systems. As can be seen in Table 6, individuals with ASD share clinical abnormalities commonly seen in mitochondrial disease such as developmental delays, ataxia, muscle weakness, peripheral neuropathy, endocrinology abnormalities, and failure to thrive. The prevalences of these clinical abnormalities listed in Table 6 are based on recent studies in the literature. The table also provides metabolic imaging criteria for additional evidence for the diagnosis of mitochondrial disease. For these metabolic criteria, the prevalences found in this meta-analysis are provided. Of course, the exact prevalences have not been fully established in studies that describe abnormalities such as TCA cycle intermediate elevations

Table 6
Clinical findings used to define the Morava criteria and their overlap with findings in children with autism spectrum disorder.

		Probably associated with ASD (% in ASD)	Might be associated with ASD	Probably not associated with ASD
I. Clinical signs and symptoms, 1 point/symptom (max. 4 points)	A. Muscular presentation (max. 2 points)	Muscle weakness (myopathies)	Abnormal EMG Exercise intolerance Rhabdomyolysis	Ophthalmoplegia† Facies myopathica
	B. CNS presentation (max. 2 points)	Developmental delay (67%) Loss of skills (33%) Seizures (25%–46%) (Gundogdu et al., 2023)	Extrapyramidal signs Myoclonus Pyramidal signs	Stroke-like episode Migraine Cortical Blindness Brainstem involvement
	C. Multisystem disease (max. 3 points)	GI tract (39%) (Lasheras et al., 2023) Endocrine/growth Recurrent/familial (10.9%) Neuropathy	Heart Kidney	Vision Hearing (4%) (Lasheras et al., 2023) Hematology
II. Metabolic/imaging studies (max. 4 points)		Elevated lactate† (17%) Elevated L/P ratio (28%) Elevated alanine† (15%) Elevated lactate/MRS Urinary TCA excretion† (42%) (Weissman et al., 2008)	Elevated CSF lactate†, protein and alanine Stroke-like picture/MRI	Ethylmalonic aciduria Leigh syndrome/MRI†
III. Morphology (max. 4 points)		Abnormal mitochondria/EM† Reduced COX staining‡ Ragged red/blue fibers‡		COX-negative fibers‡ Reduced SDH staining SDH positive blood vessels†

GI = gastrointestinal; L/P = lactate/pyruvate; COX = cytochrome *c* oxidase; SDH = succinate dehydrogenase; EM = electron microscopy.
EMG = electromyography; TCA = tricarboxylic acid.; CSF = Cerebrospinal fluid.
† Scores 2 points.
‡ Scores 4 points.

and abnormal mitochondrial morphology.

Like the modified Walker criteria, the Morava criteria divides patients into those with possible (2–4 points), probable (5–7 points), and definite (8–12 points) mitochondrial disease based on the number of clinical abnormalities with a score of 3 or more suggesting a muscle biopsy should be considered. Children with ASD have either developmental delay and/or loss of skills, so they already start out with one or two points. Having comorbidities such as GI abnormalities or seizures and/or a biochemical abnormality easily puts them in the range for a possible mitochondrial disorder with a consideration of a muscle biopsy or further testing such as buccal swab mitochondrial enzymology.

There is no standard for the work-up of mitochondrial dysfunction in ASD, although previous publications have outlined some guidance (Rossignol and Frye, 2012b; Frye et al., 2013b). This review provides suggestions as to the most useful biomarkers and an algorithm is outlined in Fig. 13 for a proposed workup. One of the most common sources of variation is in the collection manner of biomarker samples. It is well known that lactate can be elevated from prolong tourniquet application and that arterial blood lactate may be a superior method for collection of lactate. However, arterial collection is rather impractical, so some studies have suggested repeating abnormal biomarkers to verify the abnormalities (Frye et al., 2013b; Frye, 2012a). It is also established that metabolic abnormalities are uncovered during episodes of physiological stress such as illness and some investigators repeat metabolic labs during illness if there is a high index of suspicion. One way of obtaining labs under physiological stress is to collect them before breakfast in the

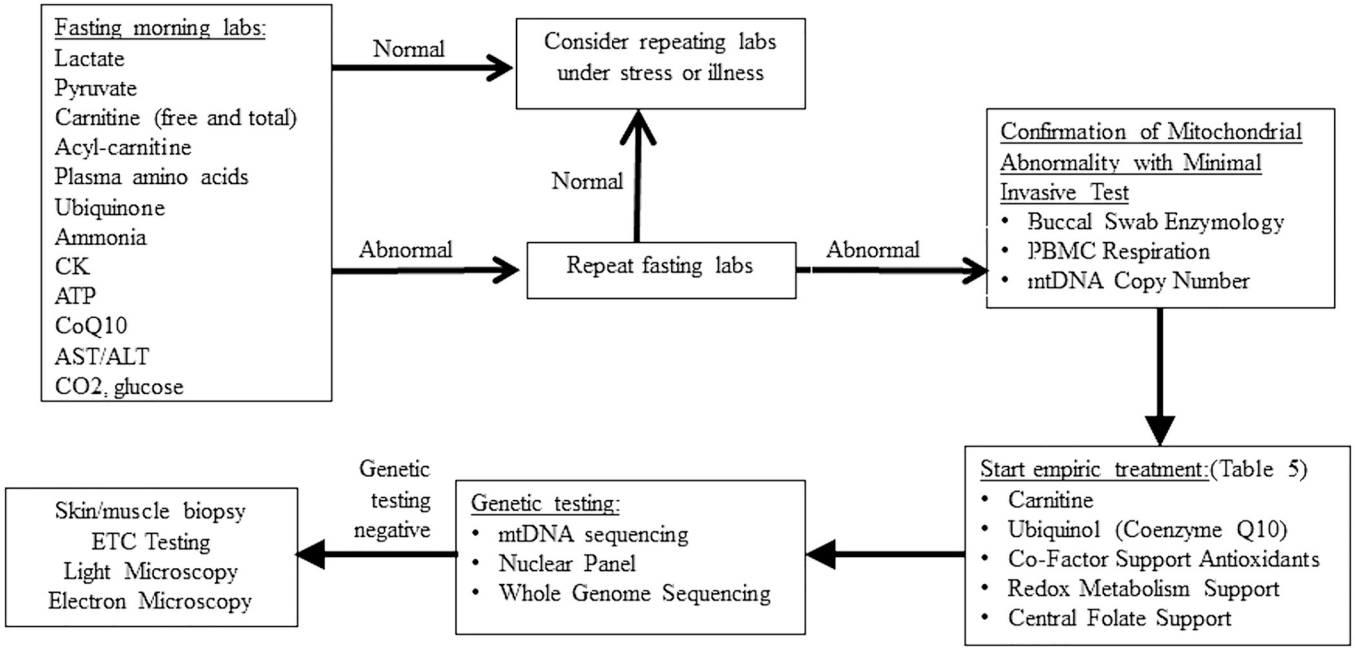

Fig. 13. Proposed flow chart for diagnosing and managing children with autism and suspected mitochondrial dysfunction.

morning as overnight fasting can provide a natural physiological stressor. This type of collection also eliminates the confounding factors of dietary intake before the biomarker measurement. Once biomarkers of mitochondrial dysfunction have been found, it is best to confirm mitochondrial dysfunction with a functional non-invasive test such as buccal enzymology, PBMC respiratory, or perhaps mtDNA copy number. However, only buccal enzymology is available clinically with normative values, and the buccal swab technique to measure enzymology has aided in the work-up of mitochondrial dysfunction (Delhey et al., 2017a; Ezugha et al., 2010; Goldenthal et al., 2015; Frye et al., 2016c; Delhey et al., 2017b; Legido et al., 2013). Since this technique is new with limited validation studies, it is important not to use it as a diagnostic tool but rather a part of the workup to confirm mitochondrial abnormalities. If a mitochondrial disorder is suspected, it is important to consider initiation of a trial of treatments and determine the response as children with ASD have few treatment options and some children have been found to make significant advancements with treatments of mitochondrial support. Testing for genetic causes should be pursued, including mtDNA sequencing. Our personal preference at this time is whole genome sequencing. If questions still arise regarding whether the patient has mitochondrial dysfunction or disease, muscle or skin biopsy can be performed to clarify the diagnosis and treatment course.

The larger consideration is that many individuals with ASD have symptoms consistent with mitochondrial disease as discussed above when considering the Morava criteria symptomatology. Thus, the symptoms used for the diagnosis of mitochondrial disease and/or dysfunction may not be sensitive to differentiate those with and without mitochondrial abnormalities in the ASD population. As such, it is important to consider the unique symptoms of those with ASD with and without mitochondrial disorders. For example, in our previous meta-analysis we found that individuals with ASD and mitochondrial disease were more likely to have GI abnormalities, seizures and NDR as compared to individuals with ASD without mitochondrial disease (Rossignol and Frye, 2012b). Further research differentiating these groups may be helpful to determine other unique symptoms that may assist in diagnosing mitochondrial disorders in those with ASD. Furthermore, as discussed above, there appears to be several patterns of alterations in mitochondrial function associated with ASD that do not fit into the classic paradigm of mitochondrial disease. Thus, a diagnostic criterion for individuals with ASD with alteration in mitochondrial function will have to consider the many potential subtypes associated with ASD.

4.13. Clinical implications: Mitochondrial disease vs dysfunction

The review above provides evidence that mitochondrial dysfunction is associated with ASD in many forms which may not be consistent with the classic definition of mitochondrial disease as outlined by the modified Walkers criteria. For example, clear well-known genetic changes are not strongly associated with ASD, with only case studies finding such associations. In addition, there are novel changes in ETC activity such as elevated, rather than depressed, respiratory rates. Thus, the biomarkers and methods for diagnosing mitochondrial dysfunction in ASD are evolving, as are the particular patterns of mitochondrial dysfunction, which may define specific ASD related metabolic syndromes. However, one thing is clear, finding evidence of mitochondrial dysfunction suggests that treatment should be considered for the abnormalities found. Specifically, the idea that an exact diagnosis needs to be made in order to start treatment should be abandoned for the good of the patient. ASD has few treatments, so evidence for a physiological abnormality that may be helped by treatment, especially safe, low risk treatments, need to be highly considered. It may very well be that many children have mitochondrial dysfunction secondary to other genetic or non-genetic abnormalities. Disorders such as muscular dystrophy, which is clearly not a primary mitochondrial disorder but causes secondary mitochondrial dysfunction, respond to mitochondrial treatments which slow progression of the disease (Niyazov et al., 2016). Several genetic abnormalities associated with mitochondrial dysfunction in ASD, such as WDR45, SCN1A, DEPDC5, are clearly not mitochondrial genes. Thus, the findings of mitochondrial dysfunction should drive the clinician to consider a trial of targeted treatments.

Furthermore, genetic disorders which are associated with ASD but not classically associated with mitochondrial disease, including Down syndrome, Rett syndrome, Phelan-McDermid Syndrome (Frye et al., 2016c), 15q11–13 duplication (Frye, 2009), and others (Burger et al., 2017) sometimes involve mitochondrial dysfunction and respond to mitochondrial interventions (Niyazov et al., 2016). Thus, in our previous publication we recommended a trial of mitochondrial targeted treatments when mitochondrial dysfunction is identified and while the search for the genetic causes of the mitochondrial abnormality is ongoing (Niyazov et al., 2016). For children with ASD, it is important to realize that finding a genetic explanation is unlikely and withholding treatment for those where a disorder of mitochondrial function is evident just because a gene abnormality cannot be found may deprive a child with a severe neurodevelopmental disability a treatment that may be life changing. This is especially true for treatments which are safe and have a low incidence of adverse effects. Of course, it is important to recommend all treatments based on the precise overall clinical picture and to ensure that response to treatment is monitored, preferably with standardized validated tools, in order to determine if the specific treatment is useful for a particular child.

As more specific criteria for the diagnosis of specific subtypes of alterations in mitochondrial function are developed, it will be possible to study the targeted treatments which best address specific alterations in mitochondrial function. Given that biomarkers for determining mitochondrial dysfunction in ASD are still undergoing development, diagnostic criteria will most likely not be developed in the near term. Thus, the decision regarding treatment is particularly challenging in the ASD population. In this way, a personalized medicine approach is important to adopt to ensure that the best treatment is chosen for each child.

4.14. Limitations of biomarkers and this study

This systematic review and meta-analysis has several limitations, mostly due to the variability in the studies reviewed. This variation can be seen in the asymmetry of study results as represented by the LFK index in Tables 1 and 2 as well as the DOI plots in Supplementary Fig. 2. Several biomarkers, including lactate, pyruvate, lactate, lactate-to-pyruvate ratio, alanine and CoQ10 demonstrated significantly LFK values suggesting outlier studies might be contributing to the results and suggesting publication bias. However, there are several sources of variation besides publication bias that might account for the variability in the results. First, biomarkers are derived from a wide variation of tissues, ranging from blood to brain tissue to neuroimaging to genetics. Second, even for common biomarkers, the techniques used to measure these biomarkers, including collection and laboratory techniques, are not standardized. For example, some insist on biomarkers measured in the fasting state while others may measure biomarkers after a meal. As food intake changes the metabolic state, these two methods of measuring biomarkers might not be completely comparable. Some studies did not provide the results of the biomarkers in tabular form so the values could not be incorporated into the meta-analysis. Although many studies were performed in the United States, many studies were performed in the Middle East and some in the Far East. Differences in the characteristics of children in these different areas may bias individual study samples. Some findings were unexpected. For example, the mean alanine was lower in ASD compared to controls yet 8.2% of those with ASD showed a consistent elevation in the alanine-to-lysine ratio even when it was repeated (Frye, 2012a). This could be due to the fact that many children with ASD have low amino acid concentrations in general, presumably due to feeding or digestive abnormalities (Zaki et al., 2017; Yu et al., 2021). As such, the relative ratio of alanine-to-lysine may be a

superior biomarker than the alanine concentration alone. Finally, from reviewing the patterns of abnormalities associated with mitochondrial dysfunction related to ASD, it is clear that there are several patterns that emerge. These will need to be better defined in the future so a clearer understanding of the various metabolic abnormalities related to ASD can be developed.

4.15. Future studies

Future studies will need to address many gaps in our knowledge of mitochondrial physiology in ASD. Clearly, biomarkers need to be better developed. Additional research is needed to investigate traditional mitochondrial biomarkers using standardized techniques in order to decrease the large variability seen in these studies. Novel biomarkers, such as mtDNA copy number, lipid profiles, respirometry and morphological measurements, which have been investigated in ASD appear to be promising but also need systematic investigation. Most significantly, linking these biomarkers to subtypes of mitochondrial abnormalities related to ASD as well as targeted treatments would be a great advance to the field.

Aside from biomarkers of mitochondrial alterations, it is critical to understand why these changes in mitochondrial physiology exist and whether they are adaptive or maladaptive changes. For example, there is evidence that the increase in mitochondrial respiratory rates seen in a subset of individuals with ASD may be an adaptive change to a chronic oxidative microenvironment in the context of a failure to activate typical molecular pathways to enhance cellular resilience to physiological stress (Bennuri et al., 2019). In this way, this may be a functional adaptation mechanism that protects the cell in the short term but makes the mitochondria more vulnerable to physiological stress. Likewise, changes in the utilization of specific TCA cycle enzymes due to increased propionic acid (or other intermediates) may also be an adaptive change to use the abundant fuels available and thus reduce this neurotoxic compound (Frye et al., 2015).

Lastly, there is emerging evidence that mitochondrial metabolic abnormalities are found during pregnancy and during the neonatal period, raising the possibility of the development of early biomarkers that can flag children who are at high risk for developing ASD. This could also lead to interventions that are useful during the prenatal or neonatal periods to correct these metabolic alterations. Studies have demonstrated that prenatal environmental exposures to toxicants such as air pollution (Frye et al., 2021b) and a reduction in prenatal nutritional metals (Frye et al., 2020) are related to mitochondrial respiration during childhood. This provides some clues to potential environmental changes that can be made to improve long-term mitochondrial function.

5. Conclusion

This systematic review has provided an overview of the abnormalities related to mitochondrial function in individuals with ASD through the investigation of biomarkers and provides evidence that at least a subset of children with ASD manifest abnormal biomarkers of mitochondrial dysfunction. The underlying origin of the abnormalities in mitochondrial function in ASD is not clear as ETC enzymology shows significant depression and elevation in complex activity, and respirometry finds increased respiratory rates in LCLs, PBMCs and fibroblasts from children with ASD. There does not appear to be a common nuclear gene association with mitochondrial dysfunction in ASD and several studies have suggested that an increase in mtDNA mutations and copy number is associated with ASD.

Biomarkers of mitochondrial dysfunction are linked to clinical features, including language and cognitive development, ASD severity, gastrointestinal symptoms, and neurodevelopmental regression. Several biomarkers, particularly fatty acid metabolites, may be useful in confirming the diagnosis of ASD, while carnitine metabolites during the prenatal (in the mothers) and neonatal (in the offspring) period were associated with an increased risk of ASD.

The fact that the mitochondria can be contribute to many abnormalities associated with ASD, including brain development and cognition as well as comorbidities such as immune and gastrointestinal dysfunction, neurodevelopmental regression, and environmental exposures, lend support for the notion that mitochondrial dysfunction may be central to ASD, at least in some cases. Interestingly, several patterns of mitochondrial dysfunction have been uncovered that may be specific to the mitochondrial dysfunction subtype. A clearer understanding of the various patterns of mitochondrial dysfunction can lead to the development of predictive biomarkers and treatments that target specific metabolic abnormalities.

A diagnostic approach to identifying mitochondrial dysfunction in children with ASD is provided given the lack of clear genetic markers. Given that preliminary evidence suggests that treatments targeting the mitochondria, particularly carnitine, may be beneficial in children with ASD, considering the possibility of mitochondrial dysfunction in patients with ASD and providing treatment if there is evidence of mitochondrial dysfunction may improve the lives of many children with ASD.

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Richard E. Frye: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nicole Rincon:** Writing – review & editing, Writing – original draft, Data curation. **Patrick J. McCarty:** Writing – review & editing, Writing – original draft, Visualization. **Danielle Brister:** Writing – review & editing, Visualization. **Adrienne C. Scheck:** Writing – review & editing. **Daniel A. Rossignol:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Data availability

The data is provided in the research article

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Appendix A. Supplementary data

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