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Diagnostic and Severity-Tracking Biomarkers for Autism Spectrum Disorder

Geir Bjørklund[1] · Nagwa A. Meguid[2,3] · Afaf El-Ansary[4,5] · Mona A. El-Bana[3,6] · Maryam Dadar[7] · Jan Aaseth[8,9] · Maha Hemimi[2,3] · Joško Osredkar[10] · Salvatore Chirumbolo[11,12]

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder afflicting about one in every 68 children. It is behaviorally diagnosed based on a triad of symptoms, including impairment in communication, impairment in sociability and abnormal and stereotypic behavior. The subjectivity of behavioral diagnosis urges the need for clinical biomarker tests to improve and complement ASD diagnosis and treatment. Over the past two decades, researchers garnered a broad range of biomarkers associated with ASD and often correlating with the severity of ASD, which includes metabolic and genetic biomarkers or neuroimaging abnormalities. Metabolic biomarkers are either involved in key pathways such as a trans-sulfuration pathway or produced due to the derangement of these pathways in the case of oxidative stress. Recent studies reported several genetic abnormalities related to ASD, encompassing various mechanisms, from copy number variations (CNVs) and single nucleotide polymorphisms (SNPs) to chromosomal anomalies. However, it is still premature to consider these genetic variants as true biomarkers for ASD, due to their low reproducibility and regional-specific nature. Herein, we comprehensively review state of the art about major biomarkers reported in ASD and the association of some biomarkers with ASD symptoms and severity. It is important to establish those biomarkers to be able to help in the diagnosis and to optimize the treatment of ASD.

Keywords Autism · Biomarkers · Diagnosis · Severity

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder affecting between 1 and 2% of children worldwide (CDC - Centers for Disease Control and Prevention, 2018) and is characterized by impairment in social communication and abnormality in the relationship with external inputs, leading to a stereotypic behavior (Saad et al., 2015a, b).

Epidemiological studies have pointed out that diverse prenatal, perinatal, and childhood environmental exposures increase the risk for ASD (Atladóttir et al. 2010; Stoltenberg et al. 2010; Roberts et al. 2013; Surén et al. 2013; Zerbo et al. 2017). Continued pollution exposure from the

✉ Geir Bjørklund
bjorklund@conem.org

[1] Council for Nutritional and Environmental Medicine (CONEM), Toften 24, 8610 Mo i Rana, Norway

[2] Research on Children with Special Needs Department, National Research Centre, Giza, Egypt

[3] CONEM Egypt Child Brain Research Group, National Research Center, Giza, Egypt

[4] Central Laboratory, King Saud University, Riyadh, Saudi Arabia

[5] CONEM Saudi Autism Research Group, King Saud University, Riyadh, Saudi Arabia

[6] Medical Biochemistry Department, National Research Centre, Giza, Egypt

[7] Agricultural Research, Education and Extension Organization (AREEO), Razi Vaccine and Serum Research Institute, Karaj, Iran

[8] Faculty of Public Health, Inland Norway University of Applied Sciences, Elverum, Norway

[9] Department of Research, Innlandet Hospital Trust, Brumunddal, Norway

[10] Institute of Clinical Chemistry and Biochemistry (KIKKB), Ljubljana University Medical Centre, Ljubljana, Slovenia

[11] Department of Neuroscience, Biomedicine and Movement Sciences, University of Verona, Verona, Italy

[12] CONEM Scientific Secretary, Verona, Italy

environment (toxic metals, strong oxidizing agents, pesticides, herbicides, photosensitizers, etc.) (Hepel and Stobiecka 2011; Stobiecka et al. 2011; Hepel et al. 2012), ionizing radiation or UV light, besides to the consequent generation of reactive oxygen species (ROS) in many metabolic and biochemical processes, have a major role in damaging DNA, modifiy lipids, and affect protein functions, all factors that may elevate the ASD pathogenetic risk, despite the observation that de novo mutations make up only about 7% of the ASD population (Shen et al. 2010). Although there is no general agreement on the ASD pathogenesis, well-documented data has proposed multiple risk factors associated with ASD pathogenesis. Some potential etiology agents are composed of fetal hypoxia, bleeding during pregnancy, diet and medication used during the prenatal period, gestational diabetes, obstetric complications, and maternal or paternal age (Meguid et al. 2017; Bjørklund et al. 2018b). Also, mutations in related genes of fetal neurodevelopment, as well as an increase in the chromosomal abnormalities which are associated with the paternal or maternal age, could be related with ASD pathogenesis (Goddard et al. 2016; Kourtian et al. 2017; Bjørklund et al. 2018b). Chromosomal mutations could occur through the spontaneous or induced by environmental agents such as exposures to heavy metal-derived toxicants (Roberts et al. 2013; Pietropaolo et al. 2017). Furthermore, a recent study revealed that an imbalance between glutamaergic and GABAergic neurotransmission and GABAergic play an important role in prevalent in ASD cases (Al-Otaish et al. 2018).

Several recent studies also indicate that some combination of gastrointestinal (GI) factors (Horvath et al. 1999; Fung et al. 2017; Kang et al. 2017), immunological factors (Careaga and Ashwood 2012), and heavy metal toxicity (Grandjean and Landrigan 2006; Kern and Jones 2006; Fujiwara et al. 2016), as well as metabolic abnormalities including dysfunctional neurotransmitter systems (McDougle et al. 2005; Zafeiriou et al. 2009) and oxidative stress (Main et al. 2010), all play an etiological role in ASD and in each individual's ASD diagnosis and prognosis. Evidence supporting that oxidative stress plays an etiological role in ASD, including (a) increased lipid peroxidation (Ming et al. 2005; Yui et al. 2016), (b) altered antioxidant enzymes in the plasma (Yorbik et al. 2002) and mitochondrial dysfunction (Oliveira et al. 2005), and (c) genetic factors (Cohen et al. 2003; Hovatta et al. 2005; Rahbar et al. 2016), has been recently reported. The brain is especially sensitive to oxidative stress because of its (1) higher energy requirements, (2) higher levels of lipids and iron, (3) significant levels of autooxidized catecholamines, and (4) lower concentrations of specific endogenous antioxidant molecules. Mitochondrial function plays a critical role in ASD progression and pathogenesis. Therefore, mitochondrial dysfunction has been highlighted in ASD individuals because of an abnormality in carbohydrate metabolism (Endreffy et al., 2016) and neurobiological subtype (Rossignol and Frye 2012; Goh et al. 2014; El-Ansary et al. 2018a, b). According to numerous studies, mitochondria are critical for many basic cellular activities throughout the body and its dysfunction known as an important candidate for a main cellular abnormality that could induce disturbances in different organ and physiological systems (Goh et al. 2014). This would suggest that biomarkers, being able to shed light on brain function, might give insightful information about ASD pathogenesis and progression.

Changes in Cerebral Perfusion as a Biomarker

Cerebral imaging techniques have disclosed hypoperfusion in many areas of the brain in patients diagnosed with ASD (Bjørklund et al. 2018a). Reduced fusion has been found both by using positron emission tomography (PET) or single-photon emission computed tomography (SPECT). The hypoperfused areas include prefrontal, frontal, temporal, occipital, and parietal cortices and also other brain regions (Bjørklund et al. 2018a). Correlations between symptom scores and hypoperfusion have indicated that the greater the autism symptom pathology, the more significant is the cerebral hypoperfusion or vascular pathology in the brain (Zilbovicius et al. 2006; Bjørklund et al. 2018a). It has been proposed that brain inflammation and vascular inflammation may explain a part of the hypoperfusion.

Inflammatory Biomarkers

Numerous studies have been reported a wide range of evidence of inflammation and/or immune dysregulation in ASD individuals (Reichelt et al. 2012; Rossignol and Frye 2012; Depino 2013; Bjørklund et al. 2016), including lipid impairment, which have been associated with ASD severity (Rossignol and Frye 2012; Qasem et al. 2018). Furthermore, recent studies have indicated a role for gestational maternal infection and innate immune responses to infection in the pathogenesis of at least some cases of ASD (Hornig et al. 2018). It has also been presumed that intolerances for gluten and casein act as triggers for inflammations and thus contribute to the pathogenesis (Whiteley et al. 2013). Also, an increase of pro-inflammatory cytokines (Xu et al. 2015) and expression of genes regulating inflammatory pathways in brain regions (Vargas et al. 2005; Li et al. 2009; Wei et al. 2012) and in cerebrospinal fluid (CSF) have been reported in ASD individuals (Chez et al. 2007; Li et al. 2009). Below, some of these inflammatory biomarkers are discussed.

TNF-α

Tumor necrosis factor-α (TNF-α) is one of the cytokines that is produced by interactions between immune system cells and the

CNS (Ren and Dubner 2010). TNF-alpha is a polypeptide that plays a significant role in brain immune and inflammatory activities (Feuerstein et al. 1994). It is known to affect hormone release, neural activity, and normal autonomic function as well as to modify patients' behaviors. TNF-α plays an important role in synaptic pruning and it modulates cell death and neural cell proliferation (Schmidt et al. 2011). Studies find that TNF-α is elevated in the plasma, in the cerebral spinal fluid, and in the brains of children with ASD (Chez et al. 2007; Li et al. 2009). Increased serum levels of TNF-α have also been reported in ASD children to correlate with serum levels of adipokines such as visfatin and resistin (Ghaffari et al. 2016). A relationship between ASD and obesity has also been reported (Hill et al. 2015). Recent reports have outlined that children with ASD had significantly higher odds of overweight and obesity than control subjects (Broder-Fingert et al. 2014).

IL-6

Interleukin-6 (IL-6) is a neuropoietic cytokine that exerts different effects on neural proliferation, survival, synapse formation, differentiation, and migration. The pathway in the maternal immune system activation, which may be associated with a subsequent diagnosis of ASD in a child, is affected by IL-6 (Woods et al. 2010). Also, IL-6 plays a critical role in elevation and modulating autism-like behaviors via impairments in neuronal circuit balance, synapse formation and dendritic spine development (Hegazy et al. 2015). The brain is inherently sensitive to oxidative stress because of its (1) higher energy requirements, (2) higher levels of lipids and iron, (3) significant levels of auto-oxidized catecholamines, and (4) lower concentrations of specific endogenous antioxidant molecules as compared to other organs and tissues. Clinical and laboratory findings suggest that those with ASD diagnosis have a BBB that is more permeable than the BBB in neurotypical individuals. The high autoimmune titers to CNS proteins that have been found in those diagnosed with ASD (Vojdani et al. 2002) suggest abnormal exposure of their immune system to brain antigens via a "leaky" (more permeable) BBB (or by chemicals present in injected drugs that increase BBB permeability). The role of IL-6 in brain development is crucial (Gumusoglu et al. 2017), and this could be a sufficient motive to further investigation of the fundamental role of this cytokine in ASD (Wei et al. 2012).

Oxidative Stress Biomarkers

A hypothesis explaining ASD onset and progression involves increased oxidative stress (Deth et al. 2008; Yui et al. 2016), which might also be associated with neuroinflammation and hypoperfusion. Oxidative stress markers may be of inorganic or organic nature. 8-Oxo-deoxyguanosine, a marker of DNA insult, and malondialdehyde, a byproduct of lipid peroxidation, are the most common organic markers of oxidative stress (Rose et al. 2012; Bjørklund and Chirumbolo 2017). There are also many other DNA adducts and lipid derivatives that are considered potential biomarkers. Among the inorganic biomarkers is a high copper/zinc ratio, which appears to be a useful indicator of oxidative stress (Brack et al. 2013; Brack et al. 2016). Possible biomarkers of lipid peroxidation, such as the 4-hydroxy-2-nonenal (HNE), have been identified in experimental animals, and the largest amount of them originates from the ω-oxidation of 4-hydroxy-2-nonenoic acid (HNA) and 9-hydroxy-HNA (Alary et al. 1998). Much more often, urinary derivatives of NO metabolism can be associated with oxidative stress-mediated lipid peroxidation (Ciancarelli et al. 2003). Finding robust lipid peroxidation urinary biomarkers whose levels are uniquely linked to the diagnosis of ASD still presents a fundamental research goal (Ming et al. 2005; Damodaran and Arumugam 2011).

Vitamins

Even metabolites of certain vitamins and dietary digestive catabolites can be possible biomarkers of oxidative response for ASD diagnosis. Increased vulnerability to oxidative stress could impair vitamin D metabolism (Saad et al., 2015a, b; Saad et al. 2016). Vitamin D deficiency has been recently related to ASD (Saad et al. 2016; Chirumbolo et al. 2017; Saad et al. 2018) focusing on mutations in the vitamin D receptor gene (Li et al. 2009). The metabolism of vitamin B12 may be a potential cause for severe and irreversible damage, particularly in the nervous system, and, thus, it should exert a major action in the development of ASD and its clinics (James et al. 2004; Meguid et al. 2011). Inborn errors of metabolism or, more recently, propionic acidemia (also known as propionic aciduria, propionyl-CoA carboxylase deficiency, and ketotic glycinemia), an autosomal recessive metabolic disorder that poisons the liver, seem to be found in about 5% of patients with ASD diagnosis (Manzi et al. 2008). Screening for the levels of urinary creatine, guanidinoacetate, and creatinine as biomarkers for guanidinoacetate methyltransferase or creatine transporter deficiencies should be considered for early dietary intervention in those diagnosed with ASD (Wang et al. 2010; Witters et al. 2016). Also, recently, it has been reported that vitamin D could be applied as a promising biomarker for the early diagnosis of ASD (Saad et al. 2016; El-Ansary et al. 2018; Saad et al. 2018).

Thioredoxins

Thioredoxins (TRXs) are multifunctional and ubiquitous proteins having a redox (reduction/oxidation)-active disulfide/dithiol within their metabolically conserved active site (Jikimoto et al. 2002). TRXs have been reported to possess

multiple biological functions (Tinkov et al. 2018) and to regulate various cellular functions via thiol redox control (Nakamura et al. 1996). The gene that encodes for TRXs has a cysteine-regulatory element (Taniguchi et al. 1996) that strongly can be induced by oxidative stress due to various oxidative agents, ultraviolet irradiation, and ischemic reperfusion. The most important biological activities of TRXs, which rely on human diseases, include inflammation modulating, anti-apoptotic growth promoting, and antioxidant functions. The system using TRXs for redox control is also involved in many cellular processes, including DNA synthesis, transcriptional regulation, cell-cell communication (Oliveira and Laurindo 2018; Tinkov et al. 2018), and cell signaling (Lillig and Holmgren 2007). Overexpression of thioredoxin reductase (TrxR) indicates that oxidative stress may be an etiological factor in ASD (Al-Gadani et al. 2009). Zhang et al. (2015) reported that elevated serum concentrations of TRXs aggregates have potential as an independent diagnostic biomarker for ASD.

Prooxidants and Antioxidants

Nitrous oxide (NO) is a potentially poisonous free radical that can react with superoxide anion and release cytotoxic peroxynitrite anions (ONOO−). Chauhan and Chauhan (2006) reported that NO affects the development and function of the CNS. Past papers reported that its roles include involvement in (a) neurite growth, memory, and learning (Hölscher and Rose 1992); (b) neurotransmitter release (Lonart et al. 1992); (c) macrophage-mediated cytotoxicity (Hibbs Jr. et al. 1988); and (d) synaptogenesis (Truman et al. 1996). Some years later, it has been discussed the connection of nitrogen species, including ammonia, in ASD (Nasrat et al. 2017). This relationship has suggested some author about a possible relationship with neuromodulators, due to the altered oxidative stress response (Tostes et al. 2012). The release of NO, as well as the expression of inducible nitric oxide synthase (iNOS) is known to induce inflammatory processes. Inflammatory cytokines like interferon (IFN)-γ, TNF-α, and IL-1 mediated induction of iNOS (Zoroglu et al. 2004). Söğüt et al. (2003) suggested that the activation of NOS and the elevation NO levels in red blood cells may be found in patients diagnosed with ASD. Also, Sweeten et al. (2004) reported increased plasma levels of nitrite and nitrate in subjects having ASD diagnosis. Those researchers also observed a positive correlation between nitrates and IFN-γ levels in patients with ASD, indicating that increased NO level might be correlated with IFN-γ activity in those with ASD. Elevated oxidative stress and lowered activity of receptors sensitive to NO have been reported in patients diagnosed with ASD.

Additionally, NO toxicity decreased the level of cholinergic receptors found in the cortex of patients with ASD diagnosis (Perry et al. 2001). Treatment with cholinergic agonists was reported to diminish behavioral abnormalities in those having ASD diagnosis (Hardan and Handen 2002). In other studies, oxidative stress was found to reduce the level of gamma-aminobutyric acid receptors in the hippocampus of patients with an ASD diagnosis (Blatt et al. 2001). Xanthine oxidase (XO) is an endogenous pro-oxidant that generate superoxide radicals through the transformation of xanthine to uric acid (Chauhan and Chauhan 2006). Elevated XO activity has been found in the erythrocytes of subjects diagnosed with ASD (Zoroglu et al. 2004). Ceruloplasmin (a copper-transporting protein) is a major antioxidant protein that is synthesized in the brain. It prevents the peroxidation of membrane lipids stimulated by metal ions, such as copper and iron (Menezo et al. 2016). Ceruloplasmin also acts like superoxide dismutase and ferroxidase. In red blood cell membranes, it protects polyunsaturated fatty acids from active oxygen species (Prandota 2010). Transferrin (an iron-transporting protein) has an antioxidant activity through the reduction of the concentration of free ferrous ion (Fe^{2+}) by oxidizing it to ferric ion (Fe^{3+}) (Prandota 2010). Ferrous ion contributes to oxidative stress via the Fenton reaction, which catalyzes the transformation of hydrogen peroxide into extremely toxic hydroxyl radicals. Also, Fe^{3+}protoporphyrin (heme) is also found in the subunits of the catalase enzyme (Chauhan and Chauhan 2006). Recent studies have reported that children with ASD diagnosis have low levels of ceruloplasmin and transferrin in their serum compared to their neurotypical siblings. The transferrin levels were decreased in 84% of children with ASD diagnosis when their levels were compared to those concentrations in their neurotypical siblings. Additionally, ceruloplasmin levels were decreased in 68% of children with ASD diagnosis compared to its level in their unaffected siblings. Moreover, the levels of transferrin and ceruloplasmin were further reduced in children having ASD diagnosis who had also lost acquired language skills (Chauhan et al. 2004).

Lipofuscin

Lipofuscin is a term indicating the yellow to brown-pigmented granules of oxidized lipid-containing residues from the lysosomal degradation of cross-linked protein, which normally forms in tissue due to age-related oxidative damage and is another important biomarker in ASD (Wegiel et al. 2012). In the CNS, lipofuscin forms in the hippocampus and the pyramidal and non-pyramidal neurons of the cortical brain (Kim et al. 2002). It can be induced experimentally by strong oxidants such as kainic acid and iron III. The existence of lipofuscin with injurious agents and specific subcellular components may provide an indicator of neuropathogenesis that has been associated with oxidized mitochondrial DNA in Alzheimer's disease (Hirai et al. 2001). Lipofuscin was greater in areas of the autistic cortical brain related with language and communication (McGinnis 2004).

Plasma F2t-Isoprostanes

The marker that is considered most practical to indicate redox dysfunction is plasma F2t-isoprostanes (F2-IsoPs). In ASD patients, F2t-isoprostanes may increase and have even found to be higher in those with gastrointestinal dysfunction (Gorrindo et al. 2013). The level of F2t-isoprostanes (F2-IsoPs) can also be measured in the urine (Goldani et al. 2014) and two studies have found elevated F2t-isoprostanes levels in the urine in ASD (Ming et al. 2005).

Plasma 3-Chlortyrosine

3-Chlortyrosine (3CT) in the plasma gives information about the activity of myeloperoxidase in the presence of reactive nitrogen species and is a recognized biomarker of the patients' response to chronic inflammatory conditions. Reportedly, the plasma 3CT levels are elevated as those diagnosed with ASD mitochondrial dysfunction grow older but are not elevated in those diagnosed with ASD who do not have mitochondrial dysfunction (Frye et al. 2013b).

3-Nitrotyrosine

3-Nitrotyrosine (3NT) is an indicator of chronic immune system activation and neuron death caused by oxidative protein damage. Research has shown that the plasma levels of 3NT in ASD patients with mitochondrial dysfunction correlate with behavior, cognitive function, and development of the disorder. However, this is not the case for those without mitochondrial dysfunction (Frye et al. 2013a; Goldani et al. 2014).

Neopterin

Neopterin is a urine marker for immune system activation and dysfunction. There have been found a correlation between the urine neopterin level and excess production of ROS, and that concentration has been suggested as a measurement of the oxidative stress level of the immune system. Some studies have shown that ASD children have significantly higher urine neopterin concentrations than neurotypical controls (Sweeten et al. 2004; Zhao et al. 2015). The severity of the patient's ASD diagnosis, behavioral symptoms, and regressive onset have been correlated with microglial cell activation and chronic inflammation caused by oxidative stress. The diagnosis of ASD has also been correlated with altered pro-inflammatory cytokines, chemokines, complement proteins, growth factors, and adhesion molecules (Streit 2000).

Heavy Metals

In the physiology runtime of daily life, fundamental macroelements are calcium (Ca), magnesium (Mg), sodium (Na), and potassium (K) while about 22 other elements are in trace quantities (microelements). Usually, trace elements play a role in enzymes, catalytic processes, or different complex molecules, as the function of cobalt (Co) in vitamin B12. Generally, a trace element is considered as such if the human body needs less than 200 mg/day of that element (Schofield 2016). Past reports have shown the existence of at least 40 case-control studies that investigated the level of potentially toxic metals in a total of 2089 subjects with ASD versus 1821 healthy controls, by measuring their levels in peripheral blood, urine, hair, nails, teeth, and even brain samples (Rossignol et al. 2014). Nineteen of these studies reported higher levels of toxic metals (Adams et al. 2006; Yasuda and Tsutsui 2013; Rossignol et al. 2014; Vasquez 2017). Heavy metals can cause birth (postnatal) neurological defects, abnormal fetal development, behavioral abnormalities, and immune dysfunctions (El-Ansary et al. 2011a; Karri et al. 2016). Many heavy metals have their typical pattern in ASD (Skalny et al. 2017a, b). For instance, some trace elements like zinc, manganese, molybdenum, aluminum, and selenium were found to be deficient, while it can be found an excess of some elements like copper, lead, mercury, and cadmium (Mostafa et al. 2016a, b; El-Ansary et al., 2017; Geier et al., 2014; Skalny et al. 2017a, b). Manganese is for humans an essential trace element. However, it is also a neurotoxin of concern for industrial workers, pregnant women, and children (Schofield 2016; Bjørklund et al. 2017).

There are some recently suggested theories grounding the mechanisms underlying these changed concentrations. One of the most crucial is the inadequate maternal intake and malabsorption in mothers before pregnancy and during pregnancy, as well as an inadequate intake and malabsorption of newborns and infants. One of the possible mechanism is harmful toxin exposures in mothers (employment-related hazards, smoking, alcohol, and illicit drug abuse). Use of certain medicines during pregnancy can also be a source of toxicity. On the other hand, there might be a defective excretion what leads to accumulation of certain heavy metals (Saldanha Tschinkel et al., 2018). Abnormalities in gastrointestinal permeability, disturbances of the blood-brain barrier, and placenta are also possible pathogenetic mechanisms of ASD. Some heavy metals such as mercury, lead, and arsenic can destruct cells through the biochemical process by the production of adverse effects such as depleting glutathione, increasing oxidative stress, impairing cellular signaling, and neurodevelopmental disorders (Li et al. 2007; Hassan et al. 2018).

Many physiologists in recent years have highlighted the link between ASD symptoms and plasma concentrations of trace elements such as copper, selenium, and zinc (El-Ansary et al., 2017a, b), and because of the evidence that the impaired

homeostatic regulation of trace elements, their potential neurotoxicity and their levels in the bloodstream are involve in the etiology of persons who are diagnosed with ASD (Grabrucker et al. 2013; Tschinkel et al. 2018). Moreover, several studies have suggested a disturbance in the copper and zinc metabolism in ASD (Bjørklund 2013; Li et al. 2014; Crăciun et al. 2016). Zinc has an important role in the immune system. Also, it is crucial in enzyme function, the metabolism of nucleic acid, growth, and finally cellular repair, most importantly in newborns and pregnant women. Deficiency of zinc is linked to delays in the development, malabsorption, and immune dysregulation (Walker and Black 2004). Copper plays many important roles in mechanisms of cell propagation and growth (Leary et al. 2009). Copper and zinc are also functional antagonists. The normal zinc to copper ratio in children and adults is close to 1:1 (Van Weyenbergh et al. 2004). Previous studies showed that zinc deficiency, elevated copper levels, and, therefore, low zinc/copper ratio are common in ASD children (Faber et al. 2009; Bjørklund 2013; Li et al. 2014; Macedoni-Lukšič et al. 2015; Crăciun et al. 2016). Low zinc/copper ratio can also cause neurological impairment and liver dysfunction in ASD children. Also, a study revealed that ASD children did not show a significant difference in the micro-nutrient intake as associated to their metabolic state, dietary habit, and resident geographical area, although a slight difference in the phosphorus and magnesium levels was recovered because of sex difference (Tschinkel et al. 2018). Furthermore, it is well-known that high mercury levels cause toxicity (Mostafa et al., 2016b, Saldanha et al., 2018) and this could be reflected in the zinc/copper ratio (Bjørklund, 2013). Low zinc/copper ratios can be associated with total body zinc deficiency or accumulation of toxic metals.

Trans-sulfuration Biomarkers

The methionine cycle and trans-sulfuration pathway are interdependent, where cystathionine β-synthase enzyme catalyzes the irreversible conversion of homocysteine—the metabolite with re-methylation potential in methionine cycle—into cystathionine, thus, initiating the trans-sulfuration pathway. Under optimal conditions and oxidative stress, the trans-sulfuration pathway provides the cells with sulfur and cysteine, the availability of which determines the rate of glutathione synthesis. The levels of trans-sulfuration metabolites are altered in ASD (Geier et al. 2009; Belalcázar et al. 2013).

Homocysteine and Cysteine

Plasma and urine levels of homocysteine were significantly elevated presumably due to the deficiencies of folate and vitamins B12 and B6 in ASD (Kałużna-Czaplińska et al. 2013; Han et al. 2015). Such abnormal high levels of homocysteine were positively correlated with the severity of ASD especially the impaired communication domain. The high levels of homocysteine can contribute to ASD symptomatology (Ménézo et al. 2011). Also, low methionine or low S-adenosylmethionine could induce DNA hypomethylation, which causes brain dysfunction (Puig-Alcaraz et al. 2015).

By contrast, cysteine levels were decreased in individuals with ASD especially in patients with severe autistic features (ElBaz et al. 2014). The homocysteinemia reported in ASD may arise from (a) inadequate dietary intake/absorption of cysteine amino acid (Kałużna-Czaplińska et al. 2017a, b), (b) higher consumption of sulfate, and/or (c) lower activity of cystathionine lyase (Main et al. 2012). Melnyk et al. (2012) found that the extracellular redox ratio between free cysteine and its oxidized form cystine (Cys/Cys-S) is significantly lower in ASD due to increased oxidation of cysteine concomitant with oxidative damage of DNA and proteins in the studied ASD patients. They concluded that under chronic oxidative stress, the trans-sulfuration pathway is unable to support its extracellular (cysteine/cystine) and intracellular (GSH/GSSG) redox balance (Melnyk et al. 2012). Reportedly, the administration of N-acetylcysteine provides sufficient levels of the amino acid cysteine and enhanced glutathione synthesis (Wink et al. 2016). Although the available data for its therapeutic potency is spoiled by the low statistical power of the N-acetylcysteine in the improvement of symptoms of irritability, different formulations of N-acetylcysteine, small sample sizes, different dosage regimens, and short duration (Naveed et al. 2017). Taurine is another sulfur-containing amino acid involved in the trans-sulfuration pathway; it is considered a biomarker for ASD due to its altered levels in the urine and plasma (Tu et al. 2012; An and Gao 2015). Furthermore, it has been reported that amino acid dysregulation metabotypes could be used as promising biomarkers for early diagnosis and individualized treatment for subtypes of ASD patients (Kałużna-Czaplińska et al. 2017a; Kałużna-Czaplińska et al. 2017b). The combination of glycine, glutamine, and ornithine amino acid dysregulation metabotypes (AADM) showed a dysregulation in amino acid/branch chain amino acids metabolism (leucine, isoleucine, and valine) that is seen in 16.7% of the ASD patients of Children's Autism Metabolome Project with a specificity of 96.3% and a positive predictive value of 93.5%. This may present disruption of the mTORC1 system which may be an underlying reason for decreased levels of free plasma branch chain amino acids metabolism.

Oxidized and Reduced Glutathione

The redox ratios of glutathione (GSSG/GSH) in ASD showed alteration in many metabolic and postmortem brain studies due to the abnormal elevation of oxidized glutathione and concomitant decrease of reduced glutathione (GSH) levels;

this redox imbalance can represent a biomarker for ASD pathophysiology (Castejon and Spaw 2014). NADPH deficiency may increase the oxidized glutathione and thus induce glutathione redox imbalance (Adams et al. 2006; Adams et al. 2011) The abnormally high levels of 3-nitrotyrosine, a biomarker of protein oxidative damage, was also correlated with the increased percentage of oxidized glutathione in ASD. As mentioned before, a dual shift toward oxidized state was reported in the major extracellular and intracellular redox buffers (cysteine and glutathione respectively) reflecting the poor redox homeostasis in ASD (Melnyk et al. 2012). Transdermal and oral glutathione supplementation enhanced the plasma levels of four trans-sulfuration metabolites in ASD: sulfate, cysteine, reduced glutathione, and taurine (Kern et al. 2011). Vargason et al. (2017) developed a mathematical model for the metabolites and reactions involved in trans-sulfuration pathway in ASD and found that the first step in glutathione synthesis, that is catalyzed by glutamate-cysteine ligase (GCL), is a critical parameter in the whole pathway because it determines the stability of metabolites' concentrations (Vargason et al. 2017). The cerebella of ASD individuals have compromised GCL activity (Gu et al. 2013a). Moreover, Meguid et al. (2017) reported a significant low gene expression of the catalytic and modifier subunits of the enzyme (GCLC and GCLM respectively) in the peripheral blood of ASD subjects.

Sulfate

Sulfur-containing amino acids such as cysteine are the major source of sulfates in the human diet, which represent essential minerals that are notoriously deficient in ASD. Presumably, low ATP levels contribute to the deficiency of free and total plasma sulfate in ASD. Correlation analysis showed that severe ASD cases had the lowest levels of sulfate suggesting that essential minerals deficiency may underlie ASD severe manifestation (Adams et al. 2011). Sulfate deficiency and the subsequent reduction in heparin sulfate levels impair neurodevelopment and cause brain structural abnormalities in ASD (Pérez et al. 2016). Hartzell and Seneff (2012) hypothesized that the prenatal and postnatal exposure to xenobiotics depletes sulfate and other sulfur metabolites, thus, contributing to neurological damage and ASD. They also recommended the administration of sulfur-rich diets and dietary supplements to alleviate autistic symptoms (Hartzell and Seneff 2012).

Hormonal Biomarkers and Obesity

The imbalance in secretion and/or activity of hormones directly affects social behavior and may explain the endocrine abnormalities sometimes reported in ASD (De Luca 2016). For example, maternal obesity (BMI ≥ 30) has been found to be associated with ASD risk (Skalny et al. 2016), and paternal obesity was even more associated with increased autism risk (Surén et al. 2014). However, the role of endocrine system abnormalities in the etiopathogenesis of ASD is still unclear (Tareen and Kamboj 2012).

Cortisol or Stress Hormone

The elevation of cortisol (stress hormone) in hair and saliva samples has been recently reported in ASD both with and without a stressful stimulus. Such elevation is positively correlated with stronger ASD symptoms (Ogawa et al. 2017). Individuals diagnosed with ASD also showed higher cortisol peaks upon subjected to a stressor, and they needed a longer period than normal individuals did to recover from cortisol elevation (Spratt et al. 2012). Baron-Cohen et al. (2015) found cortisol elevated levels in amniotic fluids of pregnant women who gave birth to males diagnosed with ASD. They suggested that this stress biomarker might play an early yet unknown role during fetal development implicated with ASD (Baron-Cohen et al. 2015).

Sex Hormones

In the study mentioned above, Baron-Cohen et al. (2015) also reported the elevation of sex steroid hormones (testosterone, progesterone, and androstenedione) in amniotic fluids where the source of these elevations may be maternal, fetal, or environmental. They may act as an epigenetic factor that contributes to the development of ASD (Baron-Cohen et al. 2015). The male bias of autism prevalence can be possibly explained by the interaction between genetic factors and sex hormones (Romano et al. 2016). For instance, *RORA*—an ASD-associated gene—is differently regulated by male and females sex hormones; the deficiency of *RORA* gene product in the brain was positively correlated with higher testosterone levels in ASD (Hu et al. 2015). Pregnant women with hyperandrogenemia and polycystic ovary syndrome (PCOS), negatively affect the fetal brain development, thus, contributing to autistic symptoms in the born children (Palomba et al. 2012).

Geier et al. (2012) report that there is evidence of hyperandrogenism in a group of individuals diagnosed with ASD result that is supported by several studies in the field of the mood and behavioral framework, CNS pathology, and cell biology pre- and postnatal serum levels of androgens (Geier et al. 2012). For example, in ASD patients the relative mean levels of testosterone in serum (158%), free testosterone in serum (214%), percent free testosterone (121%), androstenedione (173%), and DHEA (192%) were significantly increased compared to the reference means (Geier and Geier 2007). In patients with ASD, levels of androgens increased,

an evidence which might be linked to the cyclic interaction between the trans-sulfuration and the androgen pathways (Geier and Geier 2007).

Oxytocin and Arginine Vasopressin

Oxytocin is a neurohormone that mediates procreation in the brain and enables the social and cognitive skills. Early defective oxytocin system, therefore, can underlie the social communication deficits peculiar to ASD (Quattrocki and Friston 2014). Alabdali et al. (2014) reported lower plasma oxytocin levels in ASD subjects, especially severe cases, as compared to neurotypical participants. Epigenetics studies revealed that oxytocin levels in ASD are associated with targeting in the promoter region of the oxytocin receptor (OXTR) gene, which is methylated in individuals diagnosed with ASD (Alabdali et al. 2014). This epigenetic modification can contribute to the autistic social and behavioral phenotypes (Kumsta et al. 2013).

Moreover, three genetic variants of OXTR have been implicated in ASD; these variants occur in three different regions of the gene: intron 3, 3′ 3′-UTR, and an intergenic region. Both intron 3 and 3′ 3′-UTR polymorphisms were also correlated with the impaired social domain of ASD (Campbell et al. 2011). Intranasal oxytocin spray can enhance the sociability of ASD diagnosed individuals (Yatawara et al. 2016). Since oxytocin indicates the social abilities in both ASD and non-ASD subjects, arginine vasopressin (AVP)—also known as antidiuretic hormone (ADH)—can be used specifically as a blood-based biomarker for ASD social interaction domain (Carson et al. 2015).

Additionally, the elevation of AVP in girls diagnosed with ASD is related to increased stress-related repetitive behaviors (Miller et al. 2013). The derangement of AVP signaling in the brain, especially in males, may be a risk factor for ASD (Carson et al. 2015). However, the mechanism of this sex-dependent dimorphism is yet unknown and needs extensive investigations (Miller et al. 2013). Based on data from previous studies, Rutigliano et al. (2016) concluded that AVP could be promising in enhancing sociability in ASD. They, however, cautiously interpreted OXT and AVP implications in ASD due to the contradictory results obtained in different studies (Rutigliano et al. 2016).

Serotonin

Serotonin hormone is involved in brain development and modulation of behavior. The high level of serotonin in the blood (hyperserotonemia) was the first biological marker in ASD (Muller et al. 2016). A common ASD-associated gain-of-function mutation in serotonin re-uptake transporter (SERT) gene led to hyperserotonemia and altered the communication and social domains in knock-in mice (Veenstra-VanderWeele et al. 2012). However, serotonin depletion in the brain of another group of rodent models induced the well-known autistic phenotype: repetitive behaviors and defective social and communication abilities (Kane et al. 2012). The level of serotonin in patients diagnosed with ASD varies spatially, where the brain and CSF show abnormally lower serotonin levels, while platelets and blood cells show hyperserotonemia (Ratajczak and Sothern 2015). For instance, Adamsen et al. (2014) found significant low levels of serotonin in CSF of ASD participants as indicated by the level of its end product—i.e., 5-hydroxindolacetic acid. Individuals with ASD have inherited and de novo gene variants associated with the serotonergic system and abnormal serotonin signaling (Adamsen et al. 2014; Chen et al. 2017). The gestational elevation of cortisol may upregulate the SERT expression and subsequently increases serotonin levels during a critical period of fetal neural development, thus contributing to ASD onset (Rose'Meyer 2013).

Mitochondrial Dysfunction Biomarkers

The implication of mitochondrial dysfunction in ASD has long been studied. A systematic review estimated that mitochondrial dysfunction was present in about 5% of the patients with ASD (Rossignol and Frye 2012). However, when the empirical evidence is examined, the percentage of ASD individuals with mitochondrial dysfunction also appears to be generated by xenobiotics and environmental pollutants. For example, (Siddiqui et al., 2016; Goldenthal et al. 2015) examined 92 children with ASD and 68 controls for skeletal muscle mitochondrial enzyme deficiencies in respiratory complex (RC) activities (I and IV). RC-I/RC-IV activity ratio was significantly increased in 64% of the entire ASD cohort including 76% of those more severely affected (Goldenthal et al. 2015). Weissman et al. (2008) examined 25 ASD patients and found that levels of lactate in the peripheral blood, plasma alanine levels, and serum concentrations of ALT and/or AST were increased in 76%, 36%, and 52% of patients, respectively. They also reported that the most common disorder in the electron transport chain was caused by deficiencies of complex I (64%) and complex III (20%) (Weissman et al. 2008).

Elevation of lactate, pyruvate, alanine, and ammonia are reported in ASD and considered by Rossignol and Frye (2012) as additional markers of ASD-associated mitochondrial dysfunction. The five complexes of electron transport chain (ETC) responsible for the production of ATP exhibited low activity in the brain of ASD subjects (Gu et al. 2013). Likewise, pyruvate dehydrogenase, a key enzyme in mitochondrial oxidative phosphorylation, showed underactivity and implication in ASD mitochondrial dysfunction (Gu et al. 2013). In addition to their low activity, the five ETC complexes have low expression levels in autistic brains

specifically in the cerebellum and the frontal and temporal cortices. These brain regions then showed abnormally high concentrations of lipid peroxides suggesting that the low expression of ETC complexes induces oxidative stress (Chauhan et al. 2011). Goldenthal et al. (2015) analyzed buccal swaps obtained from ASD subjects and reported significant deficiencies in mitochondrial respiratory complexes I and IV (RC-I and RC-IV); they suggested that these enzymes can serve as non-invasive biomarkers for ASD patients with concomitant mitochondrial dysfunction (Goldenthal et al. 2015). Oxidative stress can induce mitochondrial dysfunction in ASD, where ROS depletes the reserve capacity of mitochondria and increases both proton-leak and ATP-linked respiration. However, the supplementation of N-acetylcysteine (glutathione precursor) prevents these adverse effects of ROS on mitochondria, suggesting that glutathione-impaired metabolism can be a contributor to such abnormal mitochondrial reserve capacity in ASD (Rose et al. 2014). Many studies examined postmortem brain tissues to study mitochondrial dysfunction in ASD. Saad et al. (2016) suggested the use of neuroimaging techniques that enable the researchers to determine metabolic mitochondrial biomarkers in ASD in a non-invasively way. For instance, brain imaging revealed high lactate (a potential biomarker of mitochondrial dysfunction) in ASD especially in the cingulate gyrus region (Goh et al. 2014).

Genetic studies reported mitochondrial (mtDNA) abnormalities in 23% of individuals with ASD and comorbid mitochondrial dysfunction (Rossignol and Frye 2012). Since the mtDNA is maternally inherited, Yoo et al. (2017) quantified the mtDNA of ASD patients and their normal siblings, and they found in peripheral blood cells a significantly larger copy number of mtDNA in ASD subjects. Important throughput analysis of mitochondria can help us to comprehend the role of mitochondrial impairment in ASD (Patowary et al. 2017). These copy number variants occur in three genes encoding complex I and complex III subunits: *ND1*, *ND4*, and *Cyt b*, where the mitochondrial *Cyt b* gene copy number variant showed significant linkage to language and communication domains in ASD. These three mitochondrial genes showed higher copy numbers also in postmortem frontal cortex tissues obtained from subjects with ASD; also, these tissues showed deletions in *ND4* and *Cyt b* genes (Gu et al., 2013). The elevation of mtDNA copy number in ASD may reflect (a) overreplication of mtDNA as a compensatory mechanism or (b) decreased degradation of mtDNA (Chen et al. 2015).

In ASD, xenobiotics and environmental pollutants may also generate mitochondrial dysfunction (Wong and Giulivi 2016). The short-chain fatty acids (SCFA) formed by ASD-associated opportunistic bacteria in the gut can derange the carnitine metabolism and subsequently alter mitochondrial function (MacFabe 2015). The elevation of short and long chains of acyl-carnitines causes acyl-carnitine profile abnormalities in human and animal models with ASD and provides potential biomarkers for mitochondrial dysfunction in ASD (Frye et al. 2013a).

Cerebral Folate Receptor Autoantibodies in Autism Spectrum Disorder

A wide range of studies demonstrated that maternal hyperhomocysteinemia and the status of folate is related to early fetal loss during pregnancy (Rogers 2008; Surén et al. 2013). The incidence of infants born with open tube neural defect has been reduced via the improvement of maternal folate nutritional status by a fortified diet, natural diet, and or supplementation before and during pregnancy. In a study, the concentrations of folate receptor autoantibodies in the serum of 93 ASD children were measured and were reported an elevated prevalence of folate receptor autoantibodies (75.3%) (Frye et al. 2013b). Also, the syndrome of cerebral folate deficiency is related with a neurometabolic disorder that described by low contents of 5-methyltetrahydrofolate (5MTHF) in the CSF, although the normal levels of systemic folate were seen (Ramaekers et al. 2002). Six studies have reported ASD patients in a subset of children with cerebral folate deficiency (Ramaekers and Blau 2004; Moretti et al. 2005; Ramaekers et al. 2005; Ramaekers et al. 2007; Moretti et al. 2008; Ramaekers et al. 2008). Most of these ASD children showed decline functioning and remarkable neurological abnormalities (Moretti et al. 2005; Ramaekers et al. 2007). The deficits in folate levels in CNS of ASD patients could explain numerous findings in these patients, although the related biological pathways are not known. On the other hand, folate levels were normal in peripheral tissues, indicating cerebral folate deficiency which the treatment with folinic acid-related CSF abnormalities could improve motor skills (Moretti et al. 2005).

Porphyrin Biomarkers

Porphyrins are intermediate metabolites formed during heme synthesis through enzymatic steps. Heavy metals can hamper these enzymatic reactions, and the backlogged porphyrin derivatives are excreted in urine; therefore, porphyrinuria is an indirect indicator of heavy metal burden in tissues (Wang et al. 2011). Bjørklund (2013) attributed the persistence of heavy metals in ASD patients mainly to their poor detoxification capacity. The majority of xenobiotic researchers considered that mercury might contribute to the etiopathology of ASD (Kern et al. 2016). Specific porphyrins, particularly precoproporphyrin and coproporphyrin in urine, may indicate toxic metal poisoning. In a study by Macedoni-Lukšič et al. (2015), the coproporphyrin III in the urine was marginally lower in ASD children, compared to individuals with other neurological diseases. Children with ASD from different

ethnic populations showed high levels of urinary mercury-associated porphyrins (coproporphyrins, precoproporphyrins, and pentacarboxyporphyrins), whereas the chelation treatment lowered the urinary coproporphyrins and precoproporphyrins concentrations (Kern et al. 2014). Khaled et al. (2016) found that the higher the mercury intoxication in plasma, the higher the urinary concentrations of uroporphyrin, pentacarboxyporphyrin, hexacarboxyporphyrin, coproporphyrin, and precoproporphyrin in ASD.

Moreover, the concentrations of coproporphyrins and precoproporphyrin were linearly related to the severity of ASD (Khaled et al. 2016). The porphyrin urinary export associated with mercury toxicity is higher even in ASD than that in neurotypical individuals who live within the same residential region (Kern et al. 2011). Likewise, ASD and neurotypical children with the same history of mercury exposure through diet, vaccines, and/or dental amalgam fillings showed comparable levels of urinary mercury and different urinary porphyrin profile, where children with ASD have significantly higher concentrations of pentacarboxyporphyrin, hexacarboxyporphyrin, and coproporphyrin (Woods et al. 2010). Heyer et al. (2012) emphasized the importance of urinary pentacarboxyporphyrin and coproporphyrin measures as predictors of ASD; however, they attributed them to impaired porphyrin metabolism rather than a heavy metal burden. They assume that the perturbation of heme biosynthesis is mechanistically associated with ASD phenotype (Heyer et al. 2012). The direct estimation of heavy metal intoxication in ASD showed (a) significant high urinary levels of toxic heavy metals such as lead and tin (b) a positive correlation with ASD symptom severity (Mostafa et al. 2016a), thus, supporting the results of porphyrin measures (Adams et al. 2017). The fact that factors other than toxic heavy metals can influence the level of porphyrins in urine should be considered when taking into account the interpretation of the results (Macedoni-Lukšič et al. 2015).

Genetic Biomarkers, e.g., the Methylenetetrahydrofolate Reductase Variants

The severity and phenotype of those diagnosed with ASD are heterogeneous with significant individual differences between patients (Schaefer and Mendelsohn 2008; Eapen 2011). The heterogeneity in ASD diagnosed individuals involves both the locus and allelic heterogeneity (Chaste and Leboyer 2012). Despite extensive research and some discoveries in genetics, today there is still not identified any set of genetic differences that are collectively associated with a diagnosis of ASD. A genetic correlation with ASD diagnosis has only been established for a few genetic disorders, such as fragile X syndrome, neurofibromatosis, Bourneville-Pringlova disease, phenylketonuria, and possibly a few other chromosomal irregularities. Around 15 other genetic abnormalities have a weak correlation with ASD diagnosis (Kobal 2009).

Identified genetic associations with some groups of children with ASD diagnosis have been classified as (a) cytogenetically visible chromosomal abnormalities (~ 5%), (b) copy number variants (CNVs) (i.e., submicroscopic deletions and duplications) (10–20%), and (c) single-gene disorders (~ 5%). To date, little evidence has successfully identified the candidate genes that are responsible for about 70% of ASD (Woodbury-Smith and Scherer 2018). However, it is accepted that epigenetic modifications of genes that cannot be explained due to changes in DNA sequence are crucial for the normal development of the brain, behavior, and cognitive function. In general, the term “epigenetics” refers to stable heritable traits (or “phenotypes”) that is not possible to explain due to changes in the DNA sequence in an individual’s genes (Liu et al. 2011; Sener et al. 2014). Abnormalities in the DNA methylation may be linked to the ASD diagnosis.

Single nucleotide polymorphisms (SNPs) in the methylenetetrahydrofolate reductase (MTHFR) are known to reduce the activity of the MTHFR enzyme. Also, the MTHFR gene may through the folate metabolism play a role in the epigenetic mechanisms that modify the gene expression leading to the development of autistic symptoms (Sener et al. 2014). The MTHFR enzyme function as a catalyzer of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is essential for the metabolization of homocysteine to methionine as well as the generation of tetrahydrofolate. Common polymorphisms in the MTHFR gene can lead to the accumulation of homocysteine, which causes folate deficiency and various injuries like DNA and vascular damage (Sener et al. 2014).

Folate and MTHFR polymorphisms are related to several neural tube defects and have are linked to the pathogenesis of numerous diseases and disorders, including leukemia, colorectal disorders, cardiovascular disease, vascular disease, cancer, schizophrenia, depression, glaucoma, migraine with aura, Down syndrome, as well as other congenital abnormalities (Gilbody et al. 2006; Jamil 2014). MTHFR is highly polymorphic in the general population. More precisely, the MTHFR gene is located on chromosome 1 (cytogenetic location: 1p36.3). It is expressed in various tissues including the brain, muscle, liver, and stomach. The two most common mutations (SNPs) in the MTHFR gene lead to the production of an MTHFR enzyme that does not work as well as in normal, C677T, and A1298C enzymes. The polymorphism nucleotide 677 causes alanine to valine (C → T) substitution. MTHFR polymorphism can also lead to a change of glutamate to alanine (A → C) at position 1298, which influences the specific activity of the enzyme and results in elevated homocysteine levels, and a reduction in the plasma folate concentration but to a lesser extent than the C677T polymorphism (Jamil 2014). Evidence suggests a link between polymorphisms of the MTHFR

enzyme and the risk to get a child with ASD could be crucial for both prevention and the development of treatments of ASD. The conclusions from the studies have been contradictory in some cases, due to the multifactorial nature of the disorders and our inability to identify the multiple genetic, epigenetic, and environmental factors that interact with the MTHFR enzyme's polymorphisms (Lacasaña-Navarro et al. 2006).

Also, several other mutation in those genes that encode some functional members of protein families involved in cell signaling, cell adhesion, and synaptic function or plasticity—e.g., SHANK, neurexins, neuroligin proteins, glutamate receptors, BDNF, and KIRREL3, together TOR and FMRP signaling pathways, have been strongly associated with the causative hypotheses associated with the symptoms exhibited by those diagnosed with ASD. All these proteins play a role in the complex network of proteins related to synaptic function and have been specifically involved in those symptoms associated with ASD. Other genes, such as CHD8, TCF4, and MBD5 were also present a complex picture from both the neurobiological and clinical perspectives. Numerous biological pathways yet contribute to this disorder, and many of the putatively associated "ASD" genes possess a wider etiological role in human psychopathology (Talkowski et al. 2014). It is still unclear how these proteins might involve a final commonly shared model of ASD related to synaptic dysfunction, given that their role in gene regulation is yet not specifically linked to synapse-related proteins. The relationship between ASD and other developmental phenotypes are currently well identified in several CNV, BCR, and GWAS investigations. One field of study is to define whether clear endophenotypes have distinct genetic etiologies, which are embedded among this broader group of disease phenotypes. Another chance is to define the genetic modifiers or any further environmental effect that may epigenetically predispose a subject toward specific phenotypic outcomes. The SHANK3 gene is one of the genes that control synaptic molecules and has a specific epigenetic control mechanism (Beri et al. 2007; Talkowski et al. 2014). The survival of an organism is dependent on the ability of adaption to different environmental factors. Therefore, the influence of epigenetics is more common than alterations in the DNA sequence. More research is needed to understand the genetics and epigenetics of ASD. The novelty of the strong association between ASD and genes involved in epigenetics give the possibility to explore potential environmental influences on such regulation (Siniscalco et al. 2013; Talkowski et al. 2014).

Receiver Operating Characteristic Curves in Evaluating the Diagnostic Values of Biomarkers for Autism Spectrum Disorder

With the move toward development of biomarkers directed treatment strategies of ASD, there is a need for more specific diagnosis. Most commonly diagnostically, the accuracy measured for a biomarker is calculated for its sensitivity and specificity. Sensitivity is defined as the part of the patients who correctly are categorized to have disease among patients who truly have the disease. Specificity is similarly the part of the patients who correctly are categorized as not having the disease among all the participants who truly do not have the disease. Most of the diagnostic biomarkers give results on a continuous scale. Therefore, the specificity and sensitivity of the biomarker depending on the specific threshold that is selected (Metz 1986). Receiver operating characteristic (ROC) analysis is used in clinical research to measure how accurately diagnostic biomarkers can discriminate between two patient states, "diseased" and "non-diseased" (Swets 1986). A ROC curve is based as a separator, on which data for the diseased and non-diseased participants form a pair of overlapping distributions (Metz 1986). The complete separation of the two underlying distributions means a perfectly discriminating biomarker, while complete overlap means failed discrimination (Swets 1979; Metz 1986).

Its advantages include testing accuracy across the entire range of scores and thereby not requiring a predetermined cut-off point, also, to easily examined visual and statistical comparisons across tests or scores, and, finally, independence from outcome prevalence. Further, ROC curve analysis is a useful tool for evaluating the accuracy of a statistical model that classifies subjects into one of two categories. In the field of biomarkers in ASD, ROC curve should become a statistical tool to identify the biomarkers that are sufficiently specific and sensitive to confirm the ASD diagnosis, while further studies are needed on its usefulness regarding prognosis, evaluation of risk assessment, and therapeutic interventions. When ROC curves are appropriately used, they can help ASD researchers improving both their research on biomarkers, as well as the presentation of the results (Wieand et al. 1989; Søreide 2008).

The area under the curve (AUC) is useful for comparing various biomarkers. An AUC value that is close to one indicates that it is a very good predictive marker. If the curve is near the diagonal, this shows that it is not diagnostic useful. An AUC value that is close to 1.00 is a satisfactory value of both specificity and sensitivity of the tested biomarker (Metz 1986; Perlis 2011). With regard to the diagnostic of ASD, a high sensitivity indicates that ASD in most of the cases is present. And, when the specificity is high, only a few or none of the healthy participants will test positive for the diagnostic marker. More predictiveness values can be recorded using ROC analysis combined with two or more distinct parameters (Yang et al. 2015). This suggests that a combination of different markers should be used rather than a single marker. Among the most predictive neurotransmitter markers reported in ASD serotonin, dopamine, oxytocin, and GABA recorded high AUC with remarkably high sensitivity and specificity (AUC values of 1.00, 0.981, 0.968, and 0.881) (Alabdali

et al. 2014). Among the pro-oxidant/antioxidant markers, GSH/GSSG, total glutathione, thioredoxins, peroxiredoxin, thioredoxin reductase, and isoprostane recorded high predictive value with AUCs of almost 1 (Al-Yafee et al. 2011; El-Ansary et al. 2011a; El-Ansary and Al-Ayadhi 2012; Zhang et al. 2015). Relative concentrations of fatty acids also reported high diagnostic values using ROC analysis as a diagnostic tool. AA/DHA and (EPA)/AA, together with phospholipids PE, PS, and PC, show high predictive values while linoleic acid/AA and EPA/DHA and omega 6/omega 3 show no utility as biomarkers for the early diagnosis of ASD (Ghezzo et al. 2013; Adamsen et al. 2014; El-Ansary and Al-Ayadhi 2014). The use of ROC analysis may allow selecting proper inflammatory markers to assess satisfactory diagnostic parameters in the evaluation of AUCs values between 0.85 and 1.00. Among these are HSP-70, TGF-β, caspase-7 and caspase-3, IL-6, INF-γ, interferon-γ-induced protein-16, leukotriene, PGE2, TNF-α, neopterin, and lipoxin A4 (El-Ansary et al. 2011b, c; Yan et al. 2015; Zhao et al. 2015). Among the toxic metabolites, while urinary phthalate (MEHP, 5-OH-MEHP, and 5-oxo-MEHP) did not demonstrate good predictive values (AUCs around 0.65), zinc/copper, lead, amyloid beta (1–40), and (1–42) recorded good diagnostic values (0.8–0.9). Among the cations, Ca^{2+}, K^{+}, Ca^{2+}/Mg^{2+}, and Na^{+}/K^{+} show good diagnostic values.

Since the human brain is complex, a single marker does not have sufficient diagnostic power. Therefore, to improve the diagnostic accuracy, it is important to combine a panel of markers (Hsu and Hsueh 2013). Among different combination approaches, the multiple regressions are perfect tools to understand and interpret the relationship between different recorded markers. It is a common statistical technique to assess the relationships between two or more independent variables and their correlation with a dependent variable. Screening of this panel of markers in newborns at risk for neurodevelopmental disease (e.g., ASD) can help in the early diagnosis and intervention. In a recent data manuscript, El-Ansary (2016) was able to understand the relationship between oxidative stress and glutamate excitotoxicity as two important etiological mechanisms in ASD. Stepwise multiple regression analyses using glutamate, glutamine, and glutamate/glutamine ratio as three dependent variables and Trx1, Trx-reductase, Prx I and III, glutathione-s-transferase, mercury, and GSH/GSSG as independent variables were helpful to understand how these two signaling pathways are collectively involved in the etiology of ASD.

Yang et al. (2015) reported that the combination of Il-6 and serotonin produced the best sensitivity and specificity in the diagnosis of ASD. More recently, El-Ansary et al. (2017b) concluded that, in spite of the excellent diagnostic value of ROC analysis in the evaluation of the discriminating power of energy metabolism and pro-oxidant/antioxidant related markers, the combination of Na+/K+ (ATPase), vitamin C, glutathione, and glutathione peroxidase, and lipid peroxides produced an accurate sensitivity and specificity for the diagnosis of ASD. They suggested that the use of logistic regression and combining ROC as a simple clinical method that might help in the early diagnosis of ASD (El-Ansary et al., 2017a, b). Ogawa et al. (2017) reported that for ROC analysis to measure the predictive value of stress hormones as biomarkers to discriminate between ASD children and neurotypical children, large sample numbers in both groups is of critical importance. Although they obtained high AUC and satisfactory specificity and sensitivity, their study does not suggest the use of salivary (sCORT) and hair (hCORT) cortisol as biomarkers of diagnosis because of the small sample they used (Ogawa et al. 2017).

Concluding Remarks

The many biomarkers above are possible research items to develop and enhance the research of ASD to earn insightful diagnostic biomarkers for this complex pathology. There is no consensus on the etiopathogenetic mechanisms underlying the onset and development of ASD; however, some fundamental hypotheses would suggest that ASD is a neurodevelopmental disorder, affecting behavior, cognition, and mood, with a close relationship with the impaired gut/brain axis and the metabolic regulation of energy disposal, and dysregulation of the immune network. Neuroinflammation and the impaired astrocytes-neuron communication is a consequence of this imbalance. Some stressors, such as xenobiotics, pollutants, heavy metals, can elicit and exacerbate this mechanism. Although there is currently not a single highly predictive biomarker to diagnose and follow up ASD, some possible candidates have been put forth.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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