

## Review

# The Role of Oxidative Stress in Autism Spectrum Disorder: A Narrative Literature Review

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**Abstract:** Autism spectrum disorder (ASD) is a multifaceted neurodevelopmental disorder that comprises a complex aetiology, where a genetic component has been suggested, together with multiple environmental risk factors. Because of its increasing incidence in the paediatric population and the lack of successful curative therapies, ASD is one of the most puzzling disorders for medicine. In the last two decades and more, the relationship between oxidative stress (OS) and ASD has been recurrently documented. For this reason, the former hypothesis, according to which reactive oxygen and nitrogen species (ROS and RNS) play an important role in ASD, is now a certainty. Thus, in this review, we will discuss many aspects of the role of OS in ASD. In addition, we will describe, in the context of the most recent literature, the possibility that free radicals promote lipid peroxidation, as well as an increase in other OS biomarkers. Finally, we will outline the possibility of novel nutritional interventions aimed at counteracting ROS production in people with ASD. In fact, new strategies have investigated the possibility that ASD symptoms, as well behavioral anomalies, may be improved after interventions using antioxidants as supplements or included in foods.

**Keywords:** autism spectrum disorder; oxidative stress; free radicals; endogenous antioxidants; exogenous antioxidants



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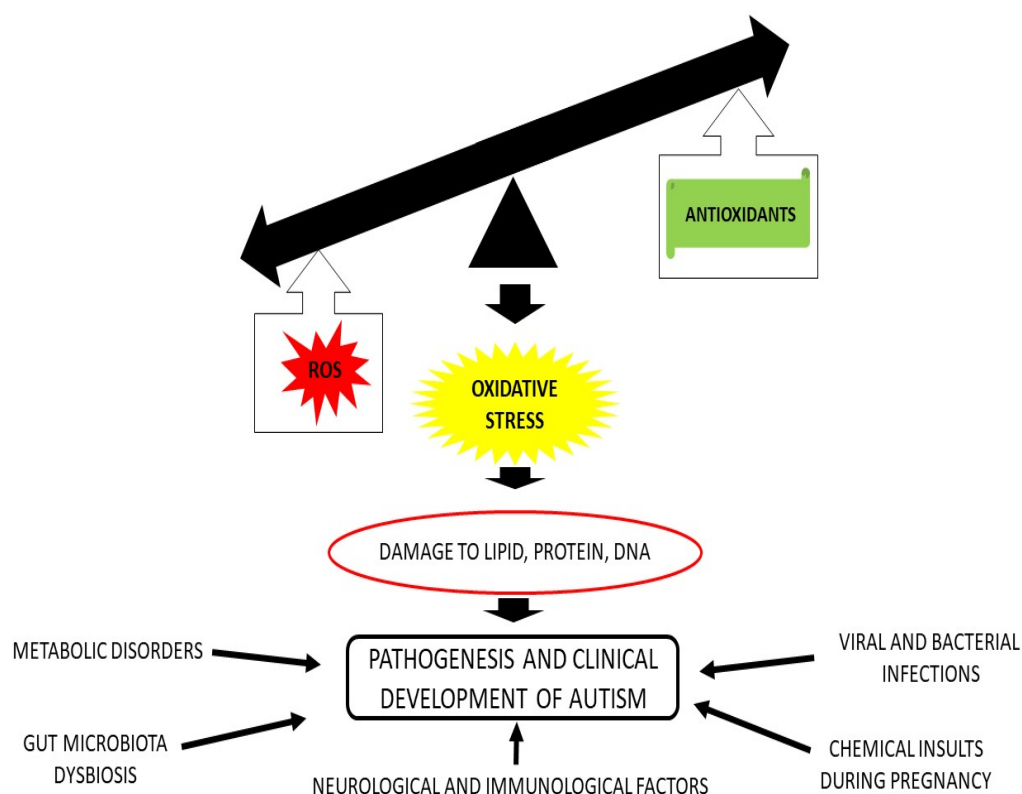
## 1. Introduction

Autism spectrum disorder (ASD) is a common neurodevelopmental disorder, which usually occurs before 3 years of age. It is characterized by the development of neuro-behavioural symptoms that can be summed up into three domains: (i) pervasive deficits in social interaction, (ii) impairment in verbal and non-verbal communication, and (iii) stereotyped patterns of interests and activities. ASD comprises a complex aetiology, where a genetic component has been suggested, together with multiple environmental risk factors. In fact, currently identified mutations affecting several genes related to processes involved in neurotransmission and neuronal circuit development, such as synapse formation and functioning, cortical organization, and maturation, which are thought to lead to transient interruptions of brain development processes [1,2].

In the last years, newly diagnosed cases of ASD have increased worldwide. The prevalence of ASD in North American children aged 8 years old has increased continuously passing from 1:150 (0.66%) in 2000 to 1:44 (2.27%) in 2018 [3]. As proposed by Barker in his theory of the developmental origins of health and disease hypothesis [4], life-long health is shaped during the embryo-fetal period and in the first two years of life (the so-called ‘First 1000 Days’) [5]. Many are the conditions that could be suboptimal and result in life-long health outcomes [6]. Thus, the most probable scenario for modeling the pathogenesis of this complex disorder is an active viewpoint embracing a multisystem pathogenetic paradigm encompassing the interaction between metabolic disorders, gut

microbiota dysbiosis, viral and bacterial infections, chemical insults during pregnancy, and neurological and immunological factors [7]. Therefore, the identification of specific biomarkers, able to correlate with autism, might increase the reliability of its diagnosis.

During the past two decades and more, the relationship between oxidative stress (OS), a particular chemical stress, and ASD has been constantly documented [8]. In fact, many recent and old studies have found a link between OS and the development of ASD, as well as the former hypothesis, according to which reactive oxygen and nitrogen species (ROS/RNS), the so-called free radicals, play an important role in ASD, which is now a certainty [9]. Although the synthesis of free radicals represents an important and necessary physiological mechanism, since they play important roles in the regulation of gene expression, in intracellular communication, in the body's defense system to fight pathogenic elements such as bacteria and viruses, as well as in the stimulation of immune functions [10], a strict regulation of their production is necessary for cell survival. OS is the result of an excessive production of free radicals when the antioxidant defenses of a cell are unable to counteract such overproduction. Over the course of evolution, each organism has developed defense systems capable of operating at different levels and with different mechanisms of action. They can be classified as endogenous, which include enzymes, antioxidant substances, and molecular mechanisms capable of repairing the molecular damage. If they comprise vitamins and other antioxidants taken by food, they are identified as exogenous (Figure 1) [11].



**Figure 1.** Relationship among the main determinants of ASD.

The specific purpose of this narrative review is to provide a critical discussion on the relationship between free radicals' production in ASD and OS defense mechanisms, as suggested by a large body of literature evidence, in an effort to elucidate how antioxidant mechanisms could positively impact on ASD pathogenesis, as well as identify putative targets for nutritional interventions. Finally, we will highlight how ASD children might benefit from tailored dietary interventions, with the purpose to alleviate their symptoms and behavioral anomalies, in order to provide new weapons to counteract OS and its related dysfunction in the best way.

## 2. ROS and RNS

As already mentioned in the Introduction, the terms ROS and RNS are used to describe a number of reactive molecules and free radicals derived from molecular oxygen and nitrogen. Among these, we include superoxide anion radical ( $O_2^{\cdot-}$ ), hydroxyl radical ( $OH^{\cdot}$ ), hydrogen peroxide ( $H_2O_2$ ), singlet oxygen ( $^1O_2$ ), nitric oxide ( $NO^{\cdot}$ ), and peroxynitrite ( $ONOO^-$ ), respectively. These compounds, when formed in high amounts overwhelming cellular buffering systems may lead to cellular injury. Different molecules are formed on the wake of uncontrolled free radicals' generation, including 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), which are able to provoke peroxidation, either to membrane lipoproteins or to polyunsaturated fatty acids (PUFAs). Therefore, the measurement of thiobarbituric acid reactive substances (TBARS) or 4-HNE is possibly the most frequently used method for the measurement of lipid peroxidation, together with the presence of nitrotyrosine, a marker of in vivo  $ONOO^-$  production, a powerful pro-oxidant and inflammatory agent [12]. Besides lipid moieties, carbohydrates, proteins, and nucleic acids can be damaged by ROS/RNS. Oxidative modification of these molecules represents a severe event that can lead to detrimental consequences, such as loss of function [13].

### *ROS and RNS Production in ASD*

Free radicals have been proposed to play an important role in different neuropsychiatric disorders, among which, we include Parkinson's disease, tardive dyskinesia, metal intoxication syndromes, Down's syndrome, schizophrenia, Huntington's disease, Alzheimer's disease, multiple sclerosis, and autism [11]. OS in ASD has been studied at several levels, with a particular focus on membrane structures and the interaction with other key cell macromolecular complexes [1].

The brain is highly exposed to OS, due to its limited antioxidant capacity, higher energy requirement, and higher amounts of lipids, in particular, PUFAs, and transition metals, such as iron [14]. Though the brain makes up about 2% of body mass, it consumes 20% of metabolic oxygen. Therefore, neurons are very susceptible to OS, since they are the first cells to be damaged by the increase in free radicals and lack of antioxidants. In addition to this, children are more vulnerable than adults to OS because, from conception through infancy, they present low antioxidant defenses, as well as lower concentration of detoxifying agents, such as glutathione.

Several studies have reported higher OS in post-mortem ASD brain samples, compared to controls by means of decreased GSH levels, oxidative damage to proteins, lipids, and nucleic acids, as well as an altered activity of enzymes involved in redox balance, such as superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, and glutamate cysteine ligase [15]. Other studies focused their attention on erythrocytes (RBC) from ASD patients [16]. Considering RBC shape, energy metabolism, and the composition of their plasma membrane-cytoskeletal network, they are thought to be sensible indicators of OS and, therefore, have been proposed as a potential biomarker in ASD. To present date, conflicting results have been reported in the literature [17–19]. In fact, while Ciccoli et al. reported multiple shape anomalies and  $\beta$ -actin alterations in RBC from ASD subjects [17], Giacometti et al. confirmed, through hyperspectral microscopy, neither the presence of shape alterations at a large extent [18] nor  $\beta$ -actin content differences between RBC from ASD children and matched controls [18]. In another paper by our group, we showed deep alterations in the erythrocyte lipid moiety, which, in turn, caused a decrease in  $Na^+/K^+$ -ATPase activity [18], as also previously described [20]. These modifications, occurring in blood cells, could be seen as a peripheral window on the events occurring in the central nervous system (CNS).

## 3. Endogenous Enzymatic Defense

In a healthy organism, in every tissue, there is a redox balance. Cells produce free radicals, but they also present an antioxidant system, made up of enzymatic and nonenzymatic antioxidants. At the end of mitochondria respiration, oxygen is the final acceptor of elec-

trons from the electron transport chain (ETC) to produce water; however, a small fraction of electrons combines with oxygen to form the superoxide anion, which can give rise to other ROS and react with NO to form reactive nitrogen species, as mentioned before.  $O_2^{\cdot -}$  is produced by mitochondrial pathways and is converted to  $H_2O_2$  by superoxide dismutase (SOD). In turn,  $H_2O_2$  is converted to  $O_2$  and  $H_2O$  by catalase (CAT) in peroxisomes or to  $H_2O$  via the activity of glutathione peroxidase (GPX) and oxidation of glutathione (GSH) to its disulfide form in the cytosol. So,  $O_2^{\cdot -}$  and  $H_2O_2$  can create hydroxyl radicals that are able to damage DNA, proteins, and lipids when present at aberrantly high levels [21].

Data present in the literature show that SOD activity in erythrocytes is higher in ASD children, in comparison with the controls [22]. An increased SOD level is considered a compensatory mechanism to counteract the cell damage caused by oxidative stress within the brain. In addition, CAT activity is reduced in erythrocytes, at variance from its plasmatic activity, which is unchanged [23].

In the last years, much attention has been devoted to glutathione peroxidase 1 (Gpx1), a seleno-enzyme that converts  $H_2O_2$  into  $H_2O$  through the oxidation of GSH to oxidized glutathione (GSSG) [24]. Gpx4 is also present in the mitochondria; its preferred substrates are lipid hydroperoxides, which are formed as a result of oxidative damage to the membrane phospholipids. In addition, mitochondria contain the peroxiredoxins (Prx) 3 and 5, along with their thioredoxin (Trx) 2 enzyme partner. Prx3 is exclusive to mitochondria, while Prx5 is also present in peroxisomes, in the cytosol, and in the nucleus. Prxs oxidize  $H_2O_2$  to cysteine-SOH, which reacts with another cysteine to form  $H_2O$  and a disulfide bond. The disulfide bonds are reduced by Trx2, which cycles from a reduced to an oxidized state. Trx2 is then reduced by thioredoxin reductase (TrxR) at the expense of NADPH oxidation. These enzymes are important for maintaining the redox environment of the mitochondria and for responding to oxidative stress imposed by external environmental factors [25]. Impairment of these antioxidant enzymes result in supraphysiological ROS production that can cause developmental neurotoxicity (DNT). The increased production of ROS both centrally (in the brain) and peripherally (in the plasma) may, in turn, result in the reduction of brain cell number, leading to autism pathology and apoptosis [26].

As already stated, oxidative stress is involved in the pathogenesis of ASD, since subjects suffering from ASD display an excessive ROS production, a decreased antioxidant capacity, and a mitochondrial dysfunction. All of these physiological abnormalities have the potential to cause oxidative stress, which, in turn, can cause epigenetic dysregulation, neuro-inflammation, cerebral injury, and neural cell dysfunction, which finally leads to aberrant development of CNS networks and, ultimately, to ASD [26].

A recent paper by our group focused the attention on GPx1 enzyme. In particular, a possible correlation between the GCG repeat polymorphism present in the GPx1 gene encoding a region of five to seven alanine residues, denominated as ALA5, ALA6, and ALA7, was investigated [27]. We showed reduced enzyme activity for GPx1 molecules carrying ALA5 sequence, followed by GPx1 expressing ALA6 and ALA7; these differences are due to the modification in the secondary and tertiary structures present in the three enzyme variants. It is of note that the ALA5 variant is not only the most frequent polymorphism among ASD subjects and unaffected family members, but also revealed the lower enzymatic activity, compared to that of the other variants tested [27]. In their paper, Ming et coll. showed that, among all recruited subjects (proband and parents), there was a transmission disequilibrium of the three ALA alleles. In particular, GPX1 expressing ALA6 was significantly under-transmitted from parents to probands diagnosed with ASD, thus suggesting that owning this allele may be protecting for autistic disorder [28]. Former studies, on the ALA6 allele, described a link with different human diseases, even though it was with controversial results. Specifically, it was found that ALA6 variant has an increased frequency in prostate cancer, without any association with prostate cancer risk [29]. In another paper, in subjects experiencing lung tumors, the presence of ALA6 polymorphism may be protective against oxidative stress [30], in accordance with our study. Later on, a significant association between the GPX1, Ala6, and Ala7 alleles and squamous cell

carcinoma development was proposed [31]. In addition, in another paper, having one or two ALA6 alleles seemed to be related to a modest increased risk of coronary artery disease [32]. In this regard, further insightful evidence and clinical reports are necessary, since GPx1 activity is only a part of a multifaceted pathway in which other proteins and enzymes are involved [27].

### 3.1. Polymorphism in Endogenous Enzymes

As described in the previous paragraph, in addition to GPX1, SOD is a powerful enzyme devoted to the conversion of  $O_2^{\cdot-}$ , generated inside the cell, into  $H_2O_2$ . Few contradicting reports on the involvement of the SOD enzymes in ASD are present in the literature. Some of them describe an increased activity [33], others show decreased activity [34], and some show no significant change [35] in the SOD enzyme activity. Kovač et coll. [36] first hypothesized that the connection between the variation in SOD activity and the etiology of ASD might be due to the presence of SOD genetic variants that, in turn, could influence enzyme activity. Such an assumption was confirmed by the discovery of a significant association between the noncoding genetic variants in the SOD1 gene (rs2234694 and rs36233090) and ASD etiology.

Another important class of isoenzymes, responsible for the formation of the free radical NO, is the nitric oxide synthase (NOS) family. Three NOS isoforms, named NOS1 (neuronal NOS), NOS2 (inducible NOS), and NOS3 (endothelial NOS) have been well-defined [37]. Since their discovery, many single nucleotide polymorphism (SNPs) of the NOS gene family have been described, some of which are associated with mental disorders and neurological diseases/conditions [38]. Regarding ASD, in their family-based association study of ASD in a Korean cohort, Kim and coll. [39] identified a significant, even though weak, association between SNPs in NOS2 gene and ASD. In their conclusions, the authors stated that many other research studies, in a larger population or other ethnic groups, are warranted.

Taken together, all these data indicate that variations in the expression of genes involved in the generation of ROS and diminution of antioxidant defense might have a significant impact on the development of the clinical symptoms of ASD. In fact, as stated elsewhere, OS may be responsible of different undesirable processes, ranging from oxidation of lipids, proteins, and DNA [21], up to increase of inflammation, which, in turn, can cause brain tissue damage and alteration of the immune response [40], leading to the clinical symptoms of ASD.

### 3.2. Endogenous Non Enzymatic Antioxidants

Besides antioxidant enzymes, eukaryotic cells possess many endogenous antioxidants acting against ROS outbreak. Among all, GSH is the main intracellular thiol used for defense against ROS, electrophiles, and xenobiotics. In fact, it can be oxidized by ROS, with the formation of the GSSG dimer, which, in turn, can be reduced by the action of glutathione reductase (GR), keeping the redox state of the cell [25]. Genetic variations in glutathione pathways have been observed in ASD, and they have been correlated to ASD behaviors [28,41]. Therefore, GSH has been indicated as a biomarker of ASD oxidative stress, since its levels in ASD patients have been reported to be variable, as some studies have reported higher levels of GSH than healthy controls, while other studies have shown lower levels [42]. Nevertheless, a very recent meta-analysis highlighted that blood GSH and total glutathione (tGSH) levels are lower in ASD subjects, compared to controls [43]. In another paper, Rose et al. described a negative association between the GSH/GSSG ratio and oxidative protein and DNA damage. This might suggest that diminished glutathione redox capacity in the autistic brain may have significant consequences, in terms of augmented mitochondrial superoxide production and a chronic inflammatory condition [44].

Another important endogenous antioxidant is the coenzyme Q10 (CoQ10), a lipophilic molecule consisting of a benzoquinone ring with 10 isoprene units in the side chain, also known as ubiquinone. It is a very fascinating compound because, although similar in structure to some vitamins (eg, vitamin K), CoQ10 is not a vitamin, since it is synthesized



in the body, whereas vitamins must be obtained from the diet. CoQ10 is an electron mobile carrier for mitochondrial enzyme complexes involved in oxidative phosphorylation in the production of adenosine triphosphate (ATP), but it also serves as a potent antioxidant or free radical scavenger [45]. In its reduced form, ubiquinol, it is itself, is a potent lipophilic antioxidant and can recycle and regenerate other antioxidants in the body. Since CoQ10 is synthesized in all tissues, the body is not normally dependent on its exogenous supplies. However, endogenous biosynthesis tends to decline with age and following statin use. Moreover, tissue CoQ10 levels might be lowered in several CNS pathological scenarios, such as traumatic brain injury, and it has been suggested that, in these circumstances, CoQ10 could be supplemented to restore normal levels [46]. Furthermore, an early study found a role for CoQ10 in autism. In fact, given the favourable response by ubiquinol supplementation in children with autism, the authors hypothesized that a CoQ10-dependent redox system might affect porin channel function [47]. Based on these results, some authors suggested the use of CoQ10 in many brain disorders, other than ASD, comprising Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic lateral sclerosis, multiple sclerosis, Friedreich's ataxia, epilepsy, depression, and bipolar disorder, which are all associated with mitochondrial dysfunction [45].

#### 4. Exogenous Antioxidants

Exogenous antioxidants obtained from the diet are also key factors to prevent, or at least reduce, oxidative stress in humans, since particular foods contain molecules with remarkable antioxidant properties. These compounds might, therefore, protect cellular macromolecules from damage [48]. There is a growing scientific consensus about a possible role of dietary agents, mainly antioxidant-containing foods, in reducing symptom severity and improving behavioural impairment in children with autism [49]. Among exogenous antioxidants, we include ascorbic acid (vitamin C), which scavenges hydroxyl and superoxide radical anion, tocopherols, and tocotrienols (vitamin E), which is involved in limiting lipid peroxidation of cell membranes and phenolic antioxidants, such as stilbene derivatives (resveratrol, phenolic acids, and flavonoids), selenium, zinc, and many more [50].

Among all vitamins, vitamin C (VC) is considered one of the most studied and potent antioxidants, and it can be found in many plant cell types, organelles, and in the apoplast. Being the main hydrophilic ROS-detoxifying compound, VC is able to donate electrons in a wide range of enzymatic and non-enzymatic reactions in the aqueous phase. Thanks to its structure, VC can completely scavenge superoxide, hydroxyl radicals, and singlet oxygen and reduce  $H_2O_2$  to water via ascorbate peroxidase reaction. In addition, VC regenerates vitamin E (VE) from its radical (tocopheroxyl,  $TOH\cdot$ ) providing membrane protection [51].

On the other hand, VE, which exists in eight different isoforms (including  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ - tocopherol, and the corresponding tocotrienol), is synthesized only by plants and algae and represents the most important lipid phase antioxidant. All isoforms differ in their substituents, and  $\alpha$ -tocopherol, with its three methyl substituents, possesses the highest antioxidant activity among all tocopherols [52]. VE is classified as a chain-breaking antioxidant, since it can repair oxidizing radicals directly, thus preventing lipid peroxidation due to PUFA oxidation. This typical reaction occurs in the membrane–water interphase, where VE is transformed into  $TOH\cdot$ . Regeneration of the reduced form can be achieved by VC, GSH, or CoQ10. In addition to these mechanisms, tocopherols can act either as scavengers of oxygen radicals or as physical deactivators of singlet oxygen [51]. Differently from lipid-soluble VE, VC is not stored within the body, and since it is not endogenously formed in humans, it is promptly excreted in the urine, thus requiring a continuous supplementation from the diet. Hence, VC plasma concentration is related to its dietary intake, having a half-life of 12–24 h [53]. Different recent studies have shown lower VC levels in the blood of ASD children, compared to healthy controls [54,55]. In accordance with these studies, it has also been described that blood VE levels are reduced in autistic children, and they are associated with ASD-like behaviors in the same subject [56].

Although lacking antioxidant properties, various vitamins of the B group, such as vitamin B6 (pyridoxine), vitamin B9 (folic acid), and vitamin B12 (cobalamin), play important roles in the development, differentiation, and functioning of the CNS, as they are implicated in the methionine-homocysteine pathway. Metabolically active forms of vitamin B12, as well as folate, serve as essential cofactors for two key enzymes important for DNA synthesis, serine synthesis, for the regeneration of methionine for protein synthesis and methylation, and for the prevention of homocysteine accumulation. Since vitamin B12 is only synthesized by specific bacteria, humans can obtain it from animal source foods, such as dairy products, meat, eggs, fish, and shellfish [57]. Studies from the literature have pointed out that vitamin B12 levels are decreased in ASD children, compared to healthy children [58]. Such deficiency causes a decrease in homocysteine re-methylation, hence resulting in increased homocysteine levels, as well as in DNA hypomethylation, affecting the development of the CNS [59]. Vitamin B deficiency can be due to a lack of nutrients, poor absorption, or intestinal disorders, where the gut microbiota plays a crucial role, since it synthesizes essential dietary vitamins and cofactors [26]. In addition, a concomitant deficiency of vitamins, B6, B9, and B12 was found in a great number of autistic subjects that, in turn, may cause DNA methylation deficiency. This deficiency might be explained as related to intestinal dysbiosis, which is the main cause of vitamin intestinal malabsorption [60]. In order to counteract this situation, different clinical trials have been conducted, with the aim of restoring vitamin E/vitamin C/glutathione antioxidant network in ASD individuals [61]. They observed a positive correlation between changes in behavior and GSH/GSSG ratio. Thus, according to scientific evidences, ASD-like symptoms may be improved by targeting the vitamin E/vitamin C/glutathione rheostat. Although the clinical effectiveness of nutritional interventions heightening vitamin E and vitamin C levels, both present within foods, in restoring an optimal functionality of the antioxidant network has not yet been investigated on a large scale, current evidence supports a putative role [49].

Among exogenous antioxidants, found in a wide variety of cultivated plants and derived foods, polyphenols are a group of water-soluble secondary metabolites (flavonoids, tannins, hydroxycinnamate esters, and lignin). Their antioxidant properties arise from their high reactivity as hydrogen or electron donors and from their ability to stabilize and delocalize the unpaired electron, as well as to chelate transition metal ions [62]. Another mechanism related to the antioxidant properties of polyphenols is the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to modulate membrane fluidity [63]. These changes could sterically hinder the diffusion of free radicals and restrict oxidative reactions.

To date, very few studies have explored the possible benefits of dietary polyphenols in ASD patients. Some of these were performed in order to alleviate ASD symptoms, and they have demonstrated that dietary polyphenols, including resveratrol, could be promising molecules, since they are able to modulate mitochondrial activity and dysfunction, usually present in individuals with ASD [64]. In addition, resveratrol was recognized to suppress ROS production inside mitochondria in stimulated primary hippocampal astrocytes [65,66]. It is important to take into account that not all polyphenols can cross the blood–brain barrier, and for this reason, they can display a low bioavailability in the brain, despite being highly absorbed from the intestinal lumen. Therefore, they may putatively alter microbiota composition, which is of great importance for ASD, usually characterized by alterations in the microbiota–gut–brain axis [67]. On the whole, current evidence suggests that polyphenols may represent a class of natural compounds, with a potential utility in treating ASD symptoms [68].

## 5. Conclusions

In conclusion, there are many studies highlighting a crucial role for OS in the ASD disease process, since ASD subjects are characterized by high levels of free radicals and pro-inflammatory molecules, but also by nutritional deficits and decreased antioxidant capability. The evidence further indicates that OS is a fundamental part of the pathophysiol-

ogy of ASD and is, indeed, related to the severity of the distinguishing symptoms displayed by subjects having ASD. However, the number of well-designed studies that support this evidence is relatively small [9]. In fact, some studies lack control groups, other used a narrow age group or included only male children in the study, and many others used small sample sizes in their trials.

Despite this, given the current pieces of evidence, it is becoming increasingly clear how natural antioxidant and anti-inflammatory molecules may help to contrast symptoms and behavioral anomalies in people with ASD and could represent a valid strategy to tackle the disorder. Early assessment and treatment of antioxidant status may result in a better prognosis, although without changing the clinical picture of a subject diagnosed after birth, as it may decrease the overall OS before it might produce additional permanent damage within several organs, including the brain. This broadens the possibility for innovative treatments aiming at decreasing ASD-like symptoms and behaviors in the patients. Thus, the positive effects can be achieved by both the supply of selected antioxidants or after the consumption of food rich in nutraceutical molecules, which may show additive or synergistic effects.

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