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The Hunt for Biomarkers of Autism Spectrum Disorders

Keywords: Autism; ASD; Biomarker; Genomics; Proteomics; Metabolomics; Oxidative stress; Gene arrays; Mass spectrometry

Abstract

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders in which individuals may experience delayed or impaired language development as well as difficulties in social interactions. Diagnosis is made on the basis of a series of psychological tests and observed behavior; a clinical biochemical diagnosis does not yet exist for ASD. Advances in modern technologies in the fields of genomics, proteomics, and metabolomics are adding to our knowledge of ASD etiology. This review examines the progress in identifying potential biomarkers of ASD, particularly in the past decade; the advancements made in the areas of genomics, proteomics, and metabolomics indicate that ASD are inherently heterogeneous, yet there may yet be optimism that in spite of this heterogeneity, one or several markers may emerge to advance clinical diagnosis and eventually treatment of ASD.

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Introduction

Autism spectrum disorders (ASD) [1] are characterized by impaired social interactions, deficits in communications skills, repetitive behaviors, and other stereotypical behavioral patterns [1-6]. ASD are presumed to be etiologically and biologically heterogeneous [7]. In early onset autism (EOA), also known as "infantile" ASD, symptoms begin in infancy [2,8]; this type of ASD, first reported by Dr. Leo Kanner [1,2], has sometimes been referred to as "classical" ASD. With a second subtype dubbed "regressive" ASD, development appears to be normal until regression into ASD symptoms, typically between 18-36 months of age [9-11]. However, it should be noted that often there are some indications of minimal ASD symptoms being present in these individuals before the onset of "regressive" behaviors, based on video observations of old home movies [12,13].

ASD diagnosis is made through behavioral observation and a battery of psychological tests based on the criteria for autistic disorder as defined in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)*, and thus has a subjective element [14-16]. Diagnosis is often very difficult for children that are under the age of two, and can be complicated by the existence of additional medical problems [17,18]. ASD have become diagnosed far more frequently in recent years than in previous decades [19-22]. Recent data from the National Survey of Children's Health shows that the incidence of ASD in the United States is now 1 in 50 in 2011-12 [23]. Now, whether this is due to a true increase in the incidence of ASD, due to broadening of ASD diagnostic criteria, or increases in awareness and understanding of the diagnostic criteria for ASD, remains an open question [19]. In either case, ASD are common in the United States and are therefore a serious public medical concern. Behavioral intervention becomes increasingly effective for persons with ASD the earlier it is introduced [24-27], and consequently approaches that can accurately diagnose ASD at earlier ages are of utmost importance. The lifetime cost of ASD per capita has been estimated at $3.2 million [28].

It has long been a goal to discover and validate potential biomarkers for ASD. Identification of biomarkers would not only provide insight into the underlying mechanisms responsible for the development of ASD, but it would also generate potential therapeutic targets for treatment of ASD and provide additional *objective diagnostic criteria for ASD*, which would accelerate the introduction of behavioral therapy. The purpose of this review is to examine progress toward identifying ASD biomarkers. Naturally, such a review will examine potential ASD causative factors. While a single cause of ASD has not been identified, research has emerged that implicates genetic, epigenetic, and environmental contributions to neurodevelopment that either cause (or may confer) susceptibility to developing ASD [29,30].

Our discussion will follow the "omics cascade" shown in Figure 1 [31], starting with markers at the genomic level, followed by proteomic markers and metabolomic markers. Of course, any of the biological factors can additionally be influenced by environmental factors. In particular, several metals have emerged which may have clinically-relevant diagnostic value and a role in ASD etiology and will also be discussed.

Genetic Basis of ASD

Because of the heterogeneity of ASD, it has remained a challenge of modern medicine to decipher its etiology. In medicine, when causation of a disorder is unknown, twin and family studies are used to establish whether any genetic risk factors may be involved. Studies of monozygotic twins have revealed a concordance rate of 36-96% [32-38], while dizygotic twins showed a concordance rate of 0-30%

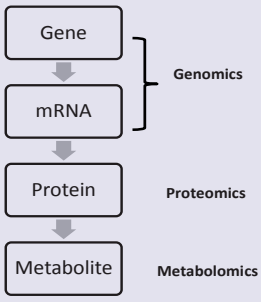

Figure 1: The Omics cascade in systems biology used in biomarker identification.

[32-38]. Thus, heritability of ASD has been estimated to be as high as >85% [39], though others posit a more conservative estimate of heritability slightly below 40% [38]. In any case, the less than 100% concordance of monozygotic twins suggests genetic factors alone do not dictate the phenotypic expression of ASD, and that environmental factors at least weakly influence the phenotype expressed.

Sibling studies of ASD incidence are useful for two reasons. First, they can have larger subject cohorts, and second, they can be compared to the studies of dizygotic twins. From 99 ASD probands, a 2.9% concordance amongst siblings has been reported [40]. A much larger study, including over 2900 children, by Constantino et al. indicated that 10.9% of families with a child diagnosed with ASD had at least one more child with ASD, and that 8.2% of all siblings of ASD children were also symptomatic [41]. A more recent study from Constantino et al. indicates that half-siblings show about one-half the incidence rate as full siblings[42]. Overall, the recurrence risk to siblings is 5-10x that of the general population [43-45]. DNA analysis of individuals with shared ancestry has also implicated individual gene loci in ASD [46]. Thus, twin and other family studies provide strong evidence of a genomic component to ASD etiology.

ASD Genomics

As noted by Geschwind, the past decade "has brought an explosion of genetic findings in ASD" [47]. As genomics methods emerged to detect single nucleotide polymorphisms (SNPs), there was some initial optimism that by collecting DNA from families containing one or more persons with ASD that genomics would rapidly indicate potential genes that might predispose individuals to developing ASD. Part of this optimism was because it had been well-established that single chromosome abnormalities including Fragile X syndrome, Tuberous Sclerosis, Down's syndrome, Smith-Lemli-Optiz syndrome, Timothy syndrome, phenyl ketonuria and dozens of others are associated with autism [48]. All of these are rare genetic variations and in total account for perhaps as much as 10% of ASD incidence.

In fact, genomics research has indicated that underlying genetic causes of ASD are highly complex [49]. Figure 2 is a pie chart representing the relative distribution of ASD causes. Miles notes that a genetic cause can only be identified in 20-25% of ASD cases; save for a small percentage of teratogenic exposures, the cause of the remaining 75-80% is unknown [50]. Thus, Mendelian (single-gene) mutations cannot account for the vast majority of instances of ASD, although they do account for approximately 5% of total incidence [50]. Another 5% of incidence is due to chromosomal abnormalities which are cytogenetically visible [50]. These single gene/single chromosome disorders represent an array of different underlying molecular processes and mechanisms. Regardless, each is relatively uncommon. This has led to speculation that autism may simply be a broad descriptor of a much larger set of conditions possessing different etiologies. One might even argue that if this is the case, that searching for an ASD genotype will be extraordinarily difficult.

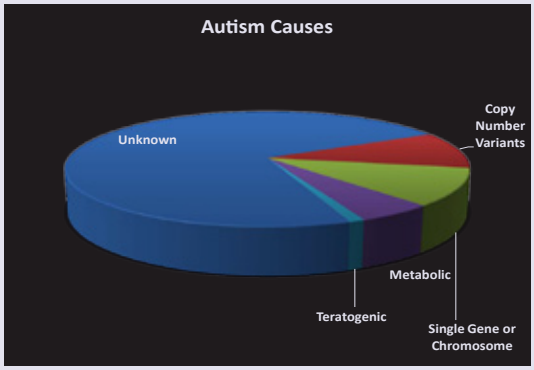

Figure 2: Relative incidence of ASD, adapted from information provided in [49] and [50].

De novo mutations tend to be paternal in origin and correlate with the father's age [51,52]. O'Roak et al. sequenced the coding regions of the genome for parent-child trios and some of the unaffected siblings, and showed that de novo point mutations are overwhelmingly paternal (4:1) in origin for ASD [51]. The paternal origin of single point mutations and small indels in ASD is also supported by the results of Iossifov et al., who additionally show that frequency of de novo mutation is associated with a parent's age, with older parents having more de novo mutations [52]. Indeed, a report by Kong et al. indicates that diversity in mutation rate of single nucleotide polymorphisms is dominated by the age of the father at conception of the child and hence implicated as a major factor in development of ASD in offspring [53].

Nevertheless, the evidence continues to mount that insertions and deletions in the genome known as copy number variants (CNVs) account for a significant fraction of ASD incidence [54], accounting for between 10 and 20% [50]. CNVs are often inconsequential because the regions duplicated or deleted contain no genes, or because of a functional compensation for the quantity of the expressed gene; however, those CNVs which occur in functionally important regions can lead to disease. A growing database containing CNVs has been in progress for the past few years [54] and can be accessed at http://projects.tcag.ca.org. Known structural variants associated with ASD exist on every chromosome. While some of these CNVs are inherited, others are *de novo* and are highly associated with ASD [55,56]. Thus, it could be argued that, ASD are heritable, but not inherited [57].

In a large scale study, the Autism Genome Project (AGP) analyzed 1000 subjects with ASD and 1300 controls, and found that individuals with ASD are much more likely to contain CNVs than their counterparts [58]. One of the first CNVs associated with ASD was the microdeletion on chromosome 16p11.2 [59]. *De novo* CNVs associated with ASD implicate genes involved in the formation and function of synapses [60] and neurons [61,62].Rare CNVs have been implicated for ASD not only at 16p11.2, but at 7q11.23, 15q11.2-13.1, and Neurexin 1[63].

Males develop ASD almost at four times the rate of females [64]. The reason for this imbalance is unclear, and it is tempting to attribute this to genetics, although a gender-linked biomarker has not yet been established. A recent European study suggests a female protective effect from developing ASD [65]. Dizygotic twin pairs were examined, and siblings of females with traits for autism showed more traits for autism than siblings of males, which suggests females may require greater familial etiologic load to manifest phenotypical expression of ASD [65].

With ASD exhibiting such genomic heterogeneity, it may be impossible to define a single or limited number of genomic markers with which to characterize and diagnose it. In spite of this, persons with ASD share phenotypic traits. Therefore, is it possible that in

spite of the genetic heterogeneity there are underlying molecular mechanisms that account for the manifestation of ASD. Inborn errors of metabolism account for less than 5% of all cases of ASD [66]. This provides the tantalizing prospect that the emerging field of metabolomics may yet play a role in defining potential ASD biomarkers, which will be discussed later in this review.

Proteins Associated with ASD

Proteomic research has proven to be a powerful tool to correlate disease detection with protein abnormalities. Even with the power of genomics, proteomics can provide additional value to clinical diagnostics because proteins translated from the genome can undergo post-translational modifications, formation of disulfide bonds, and proteolytic processing that may provide indications of disease. Potential protein biomarkers have been studied for a number of disease states including Alzheimer's, cancer, and diabetes, to name a few [67-69]. ASD has also been associated with protein research for the relation of potential biomarkers (Table 1). One study has found a potential correlation between the protein secreted amyloid precursor protein-α (sAPPα) and individuals who are afflicted with severe forms of ASD [70]. In this study, severe ASD individuals were compared to controls for sAPPα concentration. Using ELISA and Western blot techniques, blood samples were analyzed and it was shown that sAPPα levels are higher in individuals with ASD as compared to the controls. Another unique aspect of this study is that they began to attempt to use their findings as a means for early detection of ASD. Using blood samples from 150 umbilical cords, they performed the same analysis to determine the concentration of sAPPα within these samples as a means for a *potential early detection of ASD*. In this study, 7% of the umbilical cords exhibited an increased concentration of sAPPα. A follow up study with regard to the 7% increase of this protein is warranted to validate sAPPα as a potential biomarker for early detection of ASD.

In another protein analysis study, hair and nail samples from ASD and controls were analyzed by SDS-page and Western blot to assimilate a correlation between sulfur-containing proteins and the percent of nitration of keratin proteins [71]. From this study, it was demonstrated that sulfur-containing proteins in hair were decreased and nitrogen-containing proteins were increased in ASD compared to controls. With regard to sulfur-containing proteins, it is an interesting correlation to oxidative stress of GSH in ASD individuals (discussed below). The decrease in sulfur-containing proteins in hair and nails in ASD could be due to dysfunction in the regulation of sulfur-containing amino acids, such as that shown in the γ-glutamyl cycle (Figure 3). The increase in nitrogen-content in proteins with regard to ASD could also be correlated to the increase of NO species due to oxidative stress (also discussed below). In addition to these findings, there is a correlation with regard to the severity of ASD and the concentration of nitrogen-containing proteins and sulfur-containing proteins. With regard to sulfur-containing proteins, individuals with severe forms of ASD (LFA) had the lowest concentration of sulfur-containing proteins compared to less severe forms of ASD (HFA) which had a relatively higher concentration. The opposite was shown with regard to nitrogen-containing proteins where LFA individuals had the higher concentration and HFA had a lower concentration.

Another potential protein of interest is the high mobility box group 1 (HMBG1) protein, a ubiquitous protein that can act as an activator for the immune response or signal for induced inflammation [72]. More importantly, it has also been shown that an increase of [HMBG1] in the cytosol may be due to glutamate excitotoxicity, which correlates with the oxidative stress theory previously discussed [73].With these correlations, Emanuele et al. have shown that there is an increase in [HMBG1] with ASD [74]. They have also found a potential correlation with regard to the higher [HMBG1] protein with respect to the severity of ASD phenotype symptoms.

One protein that has received considerable recent attention as an ASD biomarker is engrailed-2 (EN2). EN2is a homeobox transcription factor located on chromosome 7q36 that is important in the development of the central nervous system (CNS). Several studies have reported that transgenic mice in which EN2has been overexpressed show a decrease in the number Purkinje cells, a

Table 1: Potential protein biomarkers for ASD diagnosis.

Protein Studied	Specimen Type	Instrumental Techniques	# ASD	# Controls	Results	Reference
sAPPα	Blood	ELISA and Western Blot	21	18	sAPPα is significantly increased in ASD individuals	[70]
sAPPα	Umbilical cord blood	ELISA and Western Blot	n/a	150 (no diagnosed ASD)	7% of samples demonstrated increase in sAPPα	[70]
Sulfur -containing proteins	Hair and nails	SDS-Page and Western blot	45	45	Sulfur -containing proteins concentration decreased in ASD	[71]
Nitrogenated Keratin	Hair and nails	SDS-Page and Western blot	45	45	Nitrogen-containing proteins increased in ASD	[71]
HMGB1	Blood	ELISA	22	28	Higher [HMGB1] with ASD as compared to controls	[74]
Apo B-100	Blood	LC-ESI-MS	69	35	Lower in ASD compared to controls	[79]
FHR1	Blood	LC-ESI-MS	69	35	Higher in ASD compared to controls	[79]
Complement C1q	Blood	LC-ESI-MS	69	35	Higher in ASD compared to controls	[79]
FN1	Blood	LC-ESI-MS	69	35	Higher in ASD compared to controls	[79]

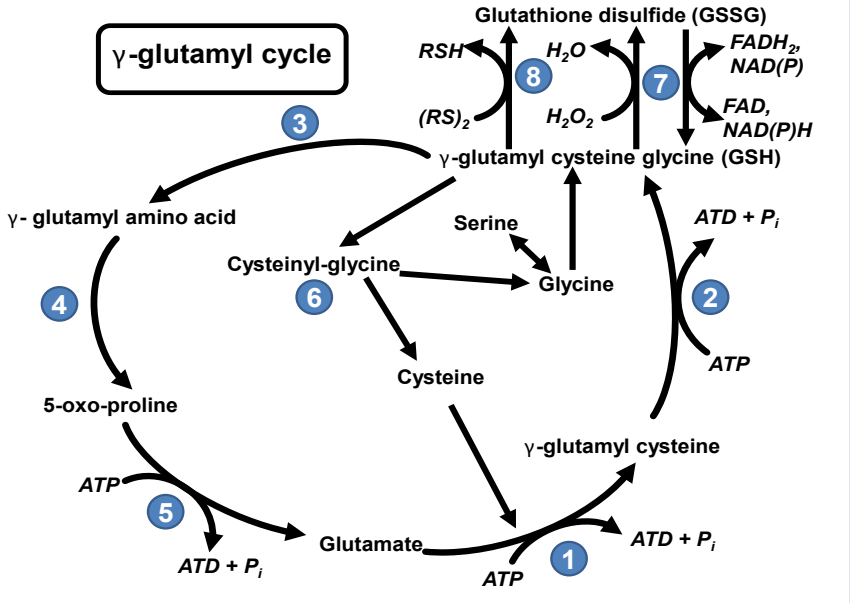

Figure 3: γ-glutamyl cycle (see text for full explanation). 1 Glutamate and Cysteine synthesis; 2 glutathione synthase; 3 γ -glutamyl transpeptidase; 4 cyclotransferase; 5 5-oxo-prolinase; 6 Dipeptidase; 7 Glutathione peroxidase and Glutathione reductase; 8 transhydrogenase.

smaller cerebellum, and hypoplasia [75-78]. Two intronic EN2 single nucleotide polymorphism (SNP) have been found to be associated with ASD (*rs1861972* and*rs1861973*). These SNP alleles have found to be over expressed in ASD individuals and under representative in their paired siblings [77]. In addition to sibling genetic comparisons, it has also been shown that these alleles are overexpressed in ASD individuals compared to controls [76,78]. Table 2 is a brief summary of the relationship of alleles on EN2 and their correlation to ASD.

A final study that is discussed here is based on the work performed by Corbett et al. that uses liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) for identification of possible proteins as biomarkers in ASD [79]. From their work, four potential protein biomarkers were found: apolipoprotein (apo) B-100, complement factor H related protein (FHR1), Complement C1q, and Fibronectin 1 (FN1). These proteins serve a variety of different functions including but not limited to lipid transportation, phagocytosis and cell mediation. The results from this study are discussed in Table 1 and demonstrate a negative correlation of ASD to the [apo B-100] and a positive correlation of ASD with respect to [FHR1], [Complement C1q] and [FN1]. This study also demonstrated that concentrations of the studied proteins are closer to controls with HFA subjects compared to LFA subjects.

Metabolic Indicators of ASD

Because the underlying genomics of ASD is heterogeneous while there are expressed similarities in ASD symptoms makes it natural to suspect that the genomic influence may be expressed as a metabolic imbalance. In the past decade, significant progress has been made toward developing phenotypic metabolic panels for ASD. Much of this research has revealed systemic oxidative stress in individuals with ASD. This will now be discussed.

Oxidative stress

Several studies have been performed studying the role of oxidative stress on cells and tissues with respect to ASD. Oxidative stress is the process by which reactive oxygen species (ROS) oxidize key components of cells and tissues. These reactions include, but are not limited to, oxidation of polyunsaturated fatty acids, molecular species containing thiols, and in some cases the reduction of Ferric-containing proteins and nitric oxide [80]. Several of these oxidative species and processes have been examined with respect to the onset of ASD.

Glutathione

Glutathione (GSH) is a tripeptide consisting of L-γ-gluamyl-L-cysteinyl-glycine with a mol. wt. of 307 g/mol [81]. Glutathione acts as an antioxidant in cell systems, helping regulate the oxidized form of GSH, glutathione disulfide (GSSG) or a glutathione-S-conjugate (Figure 3). The first reaction involved with the formation of GSH is the ATP dependent reaction between glutamate and cysteine to form γ-glutamyl cysteine (Figure 3, Reaction 1) which occurs intracellularly. This γ-glutamyl cysteine then undergoes another ATP dependent reaction where glycine binds to γ-glutamyl cysteine via glutathione synthase to form GSH (Figure 3, Reaction 2). GSH can either be oxidized or it can undergo degradation via γ -glutamyl transpeptidase where it is exported out of the cell and cleaves to form cysteine-glycine and γ-glutamyl amino acid moiety (Figure 3, Reaction 3). The γ-glutamyl amino acid moiety can then travel back into the cell via cyclotransferase which cleaves the amino acid to form 5-oxo-proline (Figure 3, Reaction 4). ATP then catalyzes the reaction of 5-oxo-proline to form glutamate with 5-oxo-prolinase (Figure 3, Reaction 5), which is the starting precursor for the γ -glutamyl cycle [82,83]. The cysteine-glycine that was cleaved during the degradation of GSH (Figure 3, Reaction 3) undergoes further degradation into cysteine and glycine by dipeptidase (Figure 3, Reaction 6). Once GSH is formed, it can undergo oxidative stress via two different pathways, either with transhydrogenation (Figure 3, Reaction 8) or by selenium containing GSH peroxidase (GSH-PX) (Figure 3, Reaction 7). During oxidative stress, GSH is oxidized to GSSG via GSH-Px which catalyzes

the reduction of H_2O_2 into H_2O [83]. GSSG can then be reduced to GSH by glutathione reductase (GSH-R) with NAD(P)H co-factor (Figure 3, Reaction 7) [80,82,83].

Normal cells have a higher ratio of GSH: GSSG, indicating there to be little to no oxidative stress. Autism studies have overwhelmingly shown that the balance of GSH: GSSG is reduced compared to their normal cell studies [82,84-87]. Studies have also shown that the concentration of GSH-Px in ASD specimens to be lower than that of controls, indicating that it has been used to oxidize GSH, and that there is oxidative stress occurring [88,89]. The methods to quantify the GSH: GSSG to be lower in individuals with ASD has varied from spectrometry techniques to spectrophotometric techniques. The sample types that have demonstrated these results have also varied from tissue, blood, and urine specimens [87-94]. Within these select studies that were reviewed, our focus was on the comparison on the ratio of GSH: GSSG between diagnosed ASD individuals and their paired controls (Table 3). Within these studies, ASD subjects were either compared to age/gender matched, non-ASD diagnosed individual [87,90-92,94], or a non-ASD sibling [93]. In both situations, ASD subjects showed a 30-50% decrease of GSH: GSSG compared to the non-ADS individuals. It is also prevalent that all methodologies and sample types used to quantify the GSH: GSSG demonstrate the same trend that ASD individuals have a marked decrease of GSH: GSSG.While each individual study had a small sample set of ASD and non-ASD individuals, the combined results begin to show a prevalent correlation between GSH oxidative stress and ASD diagnosis.

In addition to examining studies quantifying GSH: GSSG, we also reviewed studies measuring the concentration of GSH-Px (Table 4). As mentioned earlier, GSH-Px catalyzes the reaction that oxidizes GSH to GSSG. If oxidative stress occurs with relation to ASD, it would be expected that the overall concentration of GSH-Px would be decreased due to the fact that it is being used to oxidize GSH. From the results shown in Table 4, the overall [GSH-Px] in children with ASD is ~25% less than that of a non-ASD child. One study performed by Sogut et al. has shown a difference in this trend, that the [GSH-Px] is lower than that of the control group [95]. After comparing their methodologies with other studies that are reported, one difference is that blood is collected in a heparin-containing vial [95]as opposed to EDTA. The other difference is that Sogut et al. only used plasma to determine GSH-Px concentrations [95] where as other groups used both plasma and red blood cells for their analyses [71,88,89]. The correlating studies also measured GSH-Px using Ellman's reagent or another type of spectrophotometric assay [71,88,89], whereas Sogut et al. determined GSH-Px by the addition of H_2O_2 to a reaction mixture of GSH, NADPH and GSH-R [95]. While the majority of studies shows a correlation of GSH oxidative stress, and is comparable to the studies described above, the sample set is very small and further work needs to be done.

Table 2: *EN2* SNP correlation to ASD individuals.

EN2 Structure	Data Set	Results	P value	Reference
rs1861972	167 Families	Over expressed in ASD compared to undiagnosed siblings	narrow = 0.026 broad = 0.016	[77]
rs1861973	167 Families	Over expressed in ASD compared to undiagnosed siblings	narrow = 0.008 broad = 0.012	[77]
rs1861972	193 ASD vs 309 Controls	Over expressed in ASD compared to control	$p < 0.089$	[78]
rs1861973	193 ASD vs 309 Controls	Over expressed in ASD compared to control	$p < 0.009$	[78]

Table 3: GSH: GSSG comparison in ASD vs. Control, values obtained by different methods and samples.

Specimen Type	Instrument Technique	GSH:GSSG ASD	GSH:GSSG Control	P value	# ASD	# Controls	Reference
Blood	HPLC	8.6 ± 3.5	25.5 ± 8.9	<0.001	20	33	[91]
Blood	HPLC	14.7 ± 6.2	28.2 ± 7.0	<0.0001	80	73	[92]
Blood	HPLC	9.45 ± 4.08	17.4 ± 10.3	<0.001	40	40	[93]
Blood	Fluorescence	7.22[a]	11.3[b]	<0.0001	55	44	[90]*
Blood	Absorbance	8.03 ± 2.46	26.07 ± 5.03	<0.001	20	20	[87]
Brain tissue, cerebellum	HPLC/ MS	48.7 ± 1.7	103.4 ± 5.9	<0.0001	10	10	[94]
Brain tissue, temporal cortex	HPLC/MS	44.7 ± 3.1	113.9 ± 8.2	<0.0001	10	10	[94]

**Values reported in this paper were as GSH and GSSG and not the ratio of GSH:GSSG. The following is the actual reported values:*
a: GSH: 3.23 ± 0.48 nmol/mL and GSSG: 0.447 ± 0.13 nmol/
b.GSH: 4.09 ± 0.79 nmol/mL and GSSG: 0.362 ± 0.10 nmol/mL

Table 4: [GSH-Px] comparison in ASD and non-ASD individuals.

Specimen Type	Instrument Technique	[GSH-Px] U/g Hb ASD	[GSH-Px] U/g Hb Control	P value	#ASD	# Controls	Reference
Blood	Absorbance	19.17 ± 1.16	24.81 ± 1.19	<0.005	20	25	[88]
Blood	Absorbance	28.72 ± 2.64	38.01 ± 5.03	<0.005	45	41	[89]
Blood	Absorbance	40.9 ± 11.3	24.2 ± 6.3	<0.0001	27	30	[95]
Blood	Absorbance	418.8 ± 62.8	500.9 ± 75.1				[71]

Nitrogen oxide

Nitrogen oxide (or nitric oxide, NO) is a non-polar molecule produced from arginine by a Ca^{2+} dependent nitrogen oxide synthase (NOS) [96]. NO has many important functions including acting as a vasodilator, aiding in neurotransmission, and potentially playing a role within immune systems [97]. Although NO is vital for normal activity within the human system, there have been studies correlating an increase in [NO] in individuals who have been diagnosed with ASD [71,95,97,98]. Two proposed pathways have been suggested as to explain why [NO] is increased in ASD individuals. The first proposed mechanism is related with Gamma-amino butyric acid (GABA) and GABA transaminase (GABA-T). GABA is considered to be a major inhibitory neurotransmitter and has also been associated with other neurological diseases such as schizophrenia, depression, alcoholism, and seizure disorders [98]. A study performed by Cohen et al. has suggested that increased [NO] levels inhibit the function of GABA-T, which is responsible for the degradation of GABA. Cohen's findings demonstrate that not only is there an increase in [NO], but there is also an increase in GABA in ASD cases compared to the controls [98-100].

The second proposed mechanism associating increased [NO] levels in ASD individuals is the relationship between NO and GSH. GSH function, as discussed previously, serves to regulate oxidative stress within biological systems. In order to control [NO] in biological systems and to prevent oxidative stress, GSH can bind to NO to form S-nitrosylglutathione (GSNO) [101]. GSNO can be metabolized by GSH dependent formaldehyde dehydrogenase to form GSSG, ammonia and water while simultaneously oxidizing NADH to NAD^+. This excess ammonia production may attribute to the increased [NO] in ASD individuals, as well as correlate with the increase of GSSG in ASD biological systems [102]. Another study shows that mean nitrite (a metabolite of NO) and adrenomedullin levels are higher in plasma of individuals with ASD than controls, although there is no correlation between total nitrite and adrenomedullin levels [103].

As established in the literature, there is a positive correlation with respect to [NO] and ASD. Table 5, entitled [NOx] species comparisonin ASD individuals versus non-ASD individuals, compares the results from this literature review. All references used the Griess reaction followed by spectrophotometric techniques to measure [NO] in blood.

Lipid oxidative stress

Another potential relationship to ASD is the oxidative stress of lipids. Due to the hydrophobic nature of lipids, they are effective barriers toward polar molecules, in addition to possessing many other important properties [96]. Lipid oxidation is a multistep process in which lipids form lipid peroxide species [104]. This process can take place during oxidative stress, in which lipids react with free ROS to form lipid peroxides and hydrocarbon polymers. These lipid peroxide species can then be degraded by transition metals and metal complexes such as iron or hemoglobin. If the resulting lipid peroxides do not degrade rapidly, they can be harmful to cells due to the alteration of cell oxidation reduction balance [105].

The correlation between lipid oxidation and ASD has been an emerging area of research in recent years and has supplied some of the most promising ASD biomarker candidates. Meguid et al. have shown that malondialdehyde (MDA), which is a marker of lipid peroxidase, to be of a higher concentration in ASD individuals than the corresponding controls [88]. Chauhan et al. have also examined the effects of lipid oxidation with respect to ASD [105]. From their findings, the levels of MDA are higher in ASD individuals as compared to non ASD individuals. Another point of interest to note is that in this study the ASD individuals were the siblings of the control group, suggesting that this form of oxidative stress is correlated to the disease and is not expressed the same way in closely related family members. Al-Yafee et al. have correlating studies that support the relationship of lipid oxidative stress with ASD as well[87]. The difference between the aforementioned studies is that lipid oxidation was measured by Peroxiredoxin (Prx) I and III as opposed to MDA. Prx plays an important role in regulating cellular H_2O_2, redox signaling and apoptosis. While this potential biomarker is not exactly the same as the above mentioned in this section, it does follow the same trend that the [Prx] I and III are higher in ASD inflicted individuals in comparison to the controls. The studies which indicate lipid oxidation in ASD specimens are summarized in Table 6.

The curious case of Stercobilin

The majority of research with regard to the relationship of oxidative stress and ASD for the search for a biomarker has overwhelmingly focused on GSH, NO, and lipid peroxidase. It is also of interest that the majority of these studies have been analyzed

Table 5: [NOx] species comparison in ASD individuals versus non-ASD individuals.

Specimen Type	Instrument Technique	[NOx] ASD	[NOx] Control	P value	#ASD	# Controls	Reference
Blood	Absorbance	48.8 ± 12.1 µmol/L	40.9 ± 8.3 µmol/L	0.006	29	27	[97]
Blood	Absorbance	8.9 ± 1.3 Unit/ dL	11.4 ± 1.7 Unit/ dL		45	45	[71]
Blood	Absorbance	1.62 ± 0.49 µmol/g Hb	0.91 ± 0.22 µmol/g Hb	<0.0001	27	30	[95]
Blood	Absorbance	37.34 ± 6.95 µmol/L	27.10 ± 5.76 µmol/L	<0.001	26	22	[103]

Table 6: Lipid oxidative stress studies with relation to ASD.

Specimen Type	Instrument Technique	ASD	Control	P value	# ASD	# Controls	Reference
Blood	Absorbance	0.4969 ± 0.025 nmole/mL	0.396 ± 0.019 nmole/mL	< 0.005	15	15	[105]
Blood	Absorbance	105.7 ± 14.04U/g Hb	41.12 ± 3.81 U/g Hb	< 0.0001	20	25	[88]
Blood	ELISA	40.75 ± 17.92 ng/mL	20.25 ± 5.99 ng/mL	0.001	20	20	[87]
Blood	ELISA	43.05 ± 5.86 ng/mL	24.30 ± 2.69 ng/mL	0.001	20	20	[87]

[114,115]; final conversion to stercobilin is accomplished through the action of bacteria in the colon. Although the initial sample set was small (n = 7), it showed promise that [Stercobillin] may be useful as a putative metabolic biomarker of ASD, at least in a subset of individuals. Studies are continuing in our laboratories with larger populations to validate whether urinary stercobilin may be a general ASD biomarker. Stercobilin depletion could be related to oxidative stress, either at the level of the bilirubin (an oxidatively sensitive molecule) [116], at the level of the bacteria colonizing the colon, or due to some other factor.

Metals as Potential ASD Markers

While most of the species investigated as ASD markers fall under the Omics cascade, it is important to note that externally-derived species from environmental exposure are still likely to play some role in ASD etiology, as the concordance rate in monozygotic twins is less than unity. One class of agents that have garnered attention in the ASD field is heavy metals. Heavy metals are known to be neurotoxins, prompting research in their possible role in the development of ASD [117-121]. Adams et al. measured the levels of 39 metals and minerals in the hair of children with ASD and a subset of their mothers as well as healthy children and a subset of their mothers [121].Using inductively coupled plasma-mass spectrometry (ICP-MS), no statistical differences in heavy metal concentrations were found in children or their mothers; however, statistical differences in the following essential minerals were found in ASD children: 45% lower iodine levels (p=0.005), 12% lower phosphorus (p=0.001).Mothers of children with ASD showed lithium levels 40% lower than mothers of healthy children (p=0.05) while mothers of ASD children aged 3-8 years had lithium levels 56% lower (p=0.005).

Mercury, specifically, is widely suspected to play at least some role in the development of ASD. When looking specifically at mercury levels in the first baby haircuts of children with ASD, Holmes et al. noted a trend of lower mercury levels in the hair of autistic children (n=94) vs. controls (n=45) and potential correlations to mercury exposure in mothers. ASD child hair saw levels at 0.47 ppm compared to 3.63 ppm in controls (p<0.0000004); mercury levels varied depending on the severity of ASD and males more commonly saw lower levels (Table 7).

Mothers pregnant with a child who later developed ASD were exposed to higher levels of mercury during pregnancy than were normal controls, receiving on average 0.53 ±0.67 mercury-containing Rho D immunoglobulin shots vs. 0.09 ±29 in controls (p<0.0000004) and had 8.35 ±3.43 mercury amalgam fillings during pregnancy vs. 6.60 ±3.55 in controls (p<0.1). These results suggest that autistic infants have a reduced capacity to excrete mercury through hair, despite often being subjected to higher levels of mercury than controls before birth, and so lack much of the ability of normal children to defend against the toxicity of mercury [122].

Table 7: Variances among individuals with ASD with respect to hair mercury levels.

	Hair Mercury Level (ppm)	Percent Developmental Regression	Male: Female Ratio
Mild (n=27)	0.71[a]	100	12:15
Moderate (n=43)	0.46[b]	93	37:6
Severe (n=24)	0.21[c]	21	23:1

[a] Statistically different from severely autistic group (p<0.00000003).
[b] Statistically different from mildly autistic group (p<0.0004).
[c] Statistically different from moderately autistic group (p<0.00002).

An interesting interaction between mercury ions and bilirubin oxidase, noted by Sekia et al., is a dramatic increase in enzyme activity, while the addition of cupric, cobalt, and nickel ions resulted in strong to complete enzyme inhibition. Spectroscopic measurement exhibits bilirubin oxidase activity only increased with the addition of mercury in the presence of bilirubin and bilirubin oxidase by forming a complex with bilirubin that is a more suitable substrate for bilirubin oxidase [123]. This is of special interest to our group because bilirubin is a precursor for stercobilin, which we are investigating as a potential ASD biomarker as discussed above.

Abnormal copper and zinc levels have both been shown to have effects on neurological health [124-128]. Low levels of zinc, an anti-oxidant, has been shown to be associated with DNA damage and repair as well as oxidative stress[125]. Copper, a redox-active element, has also been linked to conditions such as Wilson's disease [126] and autism [127]. Faber et al. examined plasma levels of zinc, copper, and the zinc/copper ratio and found that the zinc to copper ratio in ASD subjects is below that of the bottom 2.5% of healthy children; they speculated that mercury toxicity might play a role in that depletion [129]. Russo et al. probed the relationship of plasma copper and zinc concentrations with ASD symptom severity, finding that autistic individuals showed lower zinc levels and significantly higher copper levels compared to controls (p=0.03); selected ASD symptom severity correlates with copper/zinc. It was hypothesized that irregular copper and zinc levels may change transmitter concentration by modulation of GABA receptors [130].

Metals may certainly provide insights into the cause and effect relationship of oxidative stress and protein regulation as related to ASD, though metal levels may be difficult to use as biomarkers because abnormal levels can be due to a number of disease states as well as environmental and nutritional factors. Despite this, when combined with other genomic, proteomic, and metabolomic information, metal measurements remain a useful tool in biomarker research.

Concluding Remarks

While the mystery of ASD etiology still remains, advances in genomics during the past decade have illuminated instances of single gene or chromosome abnormalities which lead to ASD. In addition, genomics arrays have revealed that CNVs are not uncommon in ASD. The interplay of multiple genes in the susceptibility to ASD is suspected, but specifically which particular combinations of genes are still largely unknown. ASD are therefore heterogeneous at the genomic level as they are at the phenotypic level. Thus, it appears unlikely that a small subset of genes will prove to be diagnostic for ASD. Nevertheless, genomics is already yielding clues that point to other molecular markers in the proteome and metabolome which may be far more general for ASD. In particular, markers of oxidative stress show promise as potential biomarkers of ASD. Ultimately, in spite of the heterogeneity of the disorder, protein and/or metabolite markers may eventually be found that can be useful in clinical diagnosis of ASD. Finally, the roles of environmental factors of ASD is still in its relative infancy, yet fruitful analysis in the past decade have indicated abnormalities in metal levels in ASD subjects vs. controls. How the metal levels correlate with genomic, proteomic, and metabolomics markers will certainly be an area for exploration over the next several years.

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