

Emerging biomarkers in autism spectrum disorder: a systematic review

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Abstract: Autism spectrum disorder (ASD) affects approximately 2% of children in the United States (US) yet its etiology is unclear and effective treatments are lacking. Therapeutic interventions are most effective if started early in life, yet diagnosis often remains delayed, partly because the diagnosis of ASD is based on identifying abnormal behaviors that may not emerge until the disorder is well established. Biomarkers that identify children at risk during the pre-symptomatic period, assist with early diagnosis, confirm behavioral observations, stratify patients into subgroups, and predict therapeutic response would be a great advance. Here we underwent a systematic review of the literature on ASD to identify promising biomarkers and rated the biomarkers in regards to a Level of Evidence and Grade of Recommendation using the Oxford Centre for Evidence-Based Medicine scale. Biomarkers identified by our review included physiological biomarkers that identify neuroimmune and metabolic abnormalities, neurological biomarkers including abnormalities in brain structure, function and neurophysiology, subtle behavioral biomarkers including atypical development of visual attention, genetic biomarkers and gastrointestinal biomarkers. Biomarkers of ASD may be found prior to birth and after diagnosis and some may predict response to specific treatments. Many promising biomarkers have been developed for ASD. However, many biomarkers are preliminary and need to be validated and their role in the diagnosis and treatment of ASD needs to be defined. It is likely that biomarkers will need to be combined to be effective to identify ASD early and guide treatment.

Keywords: Autism spectrum disorder (ASD); biomarkers; epigenetic; eye movement; folate; genetic; mitochondria; methylation; neuroimmune; neurophysiology; redox

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Introduction

Autism spectrum disorder (ASD) is perhaps one of the most important medical disorders of our era because of the number of people it affects. The prevalence of ASD has increased significantly over the past three decades and now is estimated to affect 2% (1) or more (2) of children in the United States (US). Even more significant is the fact that ASD does not occur in isolation. Children with ASD

require significant support from the educational, medical and social systems that results in a significant economic burden (3) which is estimated to cost the US approximately \$268 billion in 2015 (4). In addition, the disability of a child creates a spillover effect, decreasing the quality of life for the entire family (5-7).

One of the major limitations when identifying, diagnosing, treating and understanding ASD is the fact that ASD is exclusively defined based on the observation

of behaviors by trained or untrained individuals. The complications resulting from this approach are discussed in more detail below, but the fact remains that there is no proven biological measurement (e.g., blood test or radiological scan) which can identify pathophysiological processes that can aid in the diagnosis or treatment of ASD at this time. However, many promising areas of research have uncovered abnormal biological processes that are associated with ASD. Biomarkers developed to measure these biological abnormalities accurately could be important in the diagnosis and management of ASD.

Biomarkers are objective measures of biological or pathophysiological processes, or pharmacologic responses to therapeutic interventions (8). According to the Biomarkers Definitions Working Group (8), common applications of biomarkers include biomarkers which (I) can help diagnose a disease by identifying individuals with an abnormal biological process, (II) can classify disease severity, (III) can indicate prognosis or (IV) can predict or monitor response to therapy. Biomarkers have the potential to be utilized in several aspects of clinical care for patients with ASD, including promoting early diagnosis and selecting effective treatments.

This review will discuss emerging and promising biomarkers that could advance the diagnosis and treatment of ASD. The limitations of biomarkers and the current studies will be discussed along with insights into how biomarkers might be used in the future. Through this review, we hope the reader will gain a better understanding of the potential biomarkers that can be utilized in individuals with ASD and the complications and limitations of such biomarkers.

Methods

Search strategy

In order to identify studies of biomarkers in ASD, we conducted a review of the common medical literature databases PUBMED, Ovid Medline, Google Scholar, CINAHL, EmBase, Scopus, Cochrane and ERIC databases, from inception through June 2019. To identify studies, this review combined terms such as “biomarkers” and “autism”. Papers describing specific pathophysiology processes involved in ASD were also reviewed to determine if specific biomarkers had been developed for the specific research areas. Other filter terms such as “Human” or “Clinical Trials” were not uncommonly used. References in

publications identified by the search were also reviewed to identify any relevant publications.

Study selection

Studies were included if they met specific criteria such as: (I) human randomized controlled trials, non-randomized trials, case studies and/or case series, and (II) reported a direct clinical biomarker as an outcome. Some animal studies were included if they supported the clinical studies discussed. After reviewing the identified studies, studies with promising findings were included in this review if they used well-known validated techniques to measure biomarkers and used well-accepted techniques to diagnose ASD. One of the goals of this review was to select publications that provided quantitative measurements of biomarker performance or applicability to the ASD population. However, some biomarkers without such quantitative evaluation were also included if there were multiple studies with positive results.

Rating study quality

A grade of recommendation (GOR) was given to allow the estimation of biomarker quality using the Oxford Centre for Evidence-Based Medicine scale. Those biomarkers that did not have clinical data to evaluate their use in clinical practice were not included in this evaluation. For each clinical study that described a biomarker, a level of evidence (LOE) ranging from 1 to 5 (see *Table 1*) was assigned (9). From these identified studies a GOR ranging from A (solid evidence) to D (limited, inconsistent or inconclusive evidence) was assigned (see *Table 2*).

Results and conclusions

From our review, it was found that biomarkers could be grouped into five different categories that are associated with the natural history of ASD. The potential promising biomarkers will be reviewed with reference to these categories:

- (I) prenatal: starting from preconception through the gestation period, biomarkers have the potential to stratify pregnancies which may be high risk for the offspring developing ASD;
- (II) pre-symptomatic: during the pre-symptomatic stages, biomarkers can identify high-risk populations to determine who may require further diagnostic testing, early intervention or increased

Table 1 Levels of evidence (LOE)

LOE	Description
1a	SR or meta-analysis of RCTs with homogeneity or Cochrane review with favorable findings
1b	Prospective high-quality RCT (medium sized with N between 50 and 100 or large sized with N over 100 and/or higher validity trials based on adequate follow-up, intent to treat analysis, randomization, baseline similarity, equal treatment and dropout rate)
2a	SR of cohort (prospective, nonrandomized) studies with homogeneity
2b	Individual cohort (prospective, nonrandomized) study or low-quality RCT (small sized with N less than 50 and/or lower validity trials based on adequate follow-up, intent to treat analysis, randomization, baseline similarity, equal treatment and dropout rate)
3a	SR of case-control (retrospective) studies with homogeneity
3b	Individual case-control (retrospective) study
4	Open label trials, case series or reports
5	Expert opinion without critical appraisal or based on physiology or bench research

RCT, randomized controlled trial; SR, systematic review.

Table 2 Grade of recommendation

Grade	Description
A	At least one level 1a study or two level 1b studies
B	At least one level 1b, 2a, or 3a study, or two level 2b or 3b studies
C	At least one level 2b or 3b study, or two level 4 studies
D	Level 5 evidence, or troublingly inconsistent or inconclusive studies of any level, or studies reporting no improvements
N	No studies identified

surveillance;

(III) diagnostic: once symptoms are obvious, biomarkers can confirm the diagnosis;

(IV) subgrouping: biomarkers can be used to divide individuals with ASD into biological subgroups;

(V) treatment: biomarkers can be used to select the most optimal therapy by predicting treatment response or measuring a physiological index of treatment response.

Our review of the literature also found that biomarkers fall into several major types; some examples are listed below:

- ❖ Behavioral (Eye Movements);
- ❖ Genetic (mRNA, miRNA, DNA, Methylation);
- ❖ Immune (Antibodies, Interleukin);
- ❖ Medical History (Family History, Pregnancy Complications);
- ❖ Metabolic (Methylation-Redox, Mitochondria, Amino Acids, Fatty Acids);
- ❖ Neuroimaging (Functional MRI, Structural MRI);

❖ Neurophysiology (EEG, MEG);

❖ Nutritional (Folate, Vitamin D, Vitamin B12).

Biomarkers in which associated quantitative data was reported are listed in *Table 3*. Quantitative measurements included the associated risk of developing ASD, the diagnostic accuracy, or the prevalence of the subgroup. The GOR for each biomarker is also outlined.

Prenatal biomarkers

Genetic

Most centers offer routine screening for genetic birth defects, most notably Down syndrome (DS) and other trisomy disorders. DS is the only commonly screened genetic disorder associated with ASD. Several studies have examined the association between DS and ASD. One population-based cohort of 41 children found a 42% prevalence using gold-standard diagnostic tools (10). Three studies have used the social communication questionnaire,

Table 3 Summary of quantitative performance of promising biomarkers

Type	Biomarker	Risk	Accuracy (Sen, Spec)	Prevalence	GOR
Prenatal biomarker of ASD with ASD risk studied					
Genetic	Karyotype, Down syndrome	12–42%			C
Immune	Maternal fetal Ab	12–23%			B
Presymptomatic biomarkers with diagnostic accuracy studied					
Neuroimaging	Functional connectivity		97% (82%, 100%)		C
	Cortical surface area		94% (88%, 95%)		C
Diagnostic biomarkers with diagnostic accuracy for ASD reported					
Metabolic	Methylation-redox		97% (98%, 96%)		B
	Acyl-carnitine and amino acids		69% (73%, 63%)		C
DNA	Single nucleotide polymorphisms		¹ 86–56%		C
RNA	Human and microbiome RNA		85–79% (82–80%, 88–78%)		C
Neuroimaging	Brain volume		78% (84%, 65%)		C
Biomarkers defining subgroups of individuals with ASD with reported prevalence					
Genetic	Karyotype/cytogenetic			3%	B
	FMR1/fragile X			0.2–2%	B
	Chromosomal microarray			8–26%	B
	Whole exome sequencing			9–26%	B
Neuroimaging	Megalencephaly			15%	C
Metabolic	Amino acid			17%	C
	Methylation-redox			98–97%	B
	ETC by buccal swab			62–64%	B
	Lactate			27–15%	B
	Pyruvate			7–20%	B
	Lactate/pyruvate ratio			28%	C
	Alanine			2–8%	B
	Alanine/lysine ratio			16%	C
	Acyl-carnitine elevations			17%	C
Immune	FRAA			65–77%	B
Autonomic	Pupillary light reflex			87–94%	B

¹, the published manuscript did not provide sensitivities and/or specificities or enough data to calculate these metrics. Ab, antibody; BH4, tetrahydrobiopterin; DNA, deoxyribonucleic acid; ETC, electron transport chain; FA, folinic acid; FRAA, folate receptor alpha autoantibody; IVIG, intravenous immunoglobulin; mB12, methyl-B12; pNN50, proportion of successive R–R intervals differing by more than 50 ms; RMSSD, root mean square differences of adjacent R–R intervals; RNA, ribonucleic acid.

a parent reported measure, to estimate the prevalence of ASD in DS. These studies, which examined 203, 108 and 507 individuals with DS, found a prevalence of 12% (11), 19% (12) and 38% (13), respectively. Since none of these trials are prospective, randomized or controlled they are LOE 4 trials, giving the overall C GOR. Despite the fact that the genetic disorder of DS can be detected, prenatal testing is very non-specific for the development of ASD in the child. Unfortunately, some countries promote prenatal genetic testing for ASD despite a lack of evidence for such a practice (14).

Immunologic

Immune activation during pregnancy increases the risk of ASD developing in the offspring. Both viral and bacterial maternal infections during pregnancy (15), particularly if they require hospitalization (16), increase the risk for offspring developing ASD. This evidence parallels the maternal immune activation (MIA) mouse model of ASD where an inflammatory event induced during pregnancy results in offspring with ASD-like behaviors (15). Although the mouse model has identified specific immunological mediators, including cytokines such as interleukin (IL)-6 and IL-17 (17), no human biomarker has been developed, so only the medical history of such an event can be used as a biomarker for identifying an increased risk of ASD.

During pregnancy, maternal immunoglobulin G (IgG) crosses the placenta, usually to protect the offspring during fetal life and early childhood. However, specific maternal IgG can target fetal brain tissue, causing a disruption in brain development. Early studies demonstrated that sera from mothers of children with ASD could induce behavioral abnormalities in both monkeys (18) and mice (19,20) when injected into the mothers. The first human controlled study (LOE 3b) identified that plasma from 12% of 61 mothers of children with ASD had two protein bands at 73 and 37 kDa that reacted with human fetal brain tissue (21). These proteins were not found in any of the 62 mothers with typically developing (TD) offspring nor any of the 40 mothers with children who had developmental delays but not ASD. These maternal IgG were found to target seven fetal proteins involved in neurodevelopment, including lactate dehydrogenase A and B, stress-induced phosphoprotein 1, collapsin response mediator proteins 1 and 2, cypin and Y-box binding protein (22). In a larger follow-up study (LOE 3b), 23% of 246 mothers of children with ASD demonstrated at least two of these seven antibodies while only 1% of 149 mothers of TD children

demonstrated at least two of these seven antibodies. Children born from mothers with these antibodies had lower language (23,24) and cognitive ability (24), higher irritability (23), disrupted sleep-wake cycle (24) and large head circumference (24,25). With two LOE 3b studies, the GOR is B for maternal fetal brain antibodies.

Metabolic

ASD is associated with alternations in folate one-carbon metabolism (FOCM) and related pathways including methylation (26). Interestingly, maternal methylation abnormalities including DNA hypomethylation and abnormalities in metabolites integral to the methylation cycle, including plasma homocysteine, adenosine, and S-adenosylhomocysteine (SAM), were found in mothers who have offspring that developed ASD in two (LOE 3b) studies (27,28) providing a GOR B. However, these biomarkers were only able to determine whether the mother had high- or low-risk for having a child with ASD but not whether the offspring eventually was diagnosed with ASD.

Nutritional

Folate during pregnancy has a complex relationship to the development of ASD in the offspring. Folate deficiency during pregnancy increases the risk of the offspring developing ASD (29) while folate supplement during pregnancy clearly decreases the risk of the offspring developing ASD (30). However, one study suggests that very high maternal folate or vitamin B12 blood concentrations at birth is associated with an increased risk of developing ASD and that both low and high folate supplementation increase the risk of developing ASD suggesting a U-shaped relationship between folate supplementation and ASD risk (31) (LOE 3b) but this notion has significant limitations when we consider the physiology of folate transport.

The transport of folate across the placenta may be blocked in mothers of children with ASD resulting in higher folate levels in the blood (32). Indeed, mothers of children with ASD have a higher prevalence of Folate Receptor Alpha Autoantibodies (FRAAs) (32) and a single nucleotide polymorphism (SNP) in the reduced folate carrier (RFC) (27), two mechanisms that disrupt folate transport across the placenta. The binding FRAA has also been associated with higher blood levels of vitamin B12 suggesting that it may inhibit transport of vitamin B12 into tissues (33). Furthermore, a recent animal model demonstrates that ASD-like behaviors develop in offspring in which folate transport across the placenta was blocked

due to FRAAs (34). Despite this important data about folate and FRAAs prior to birth, no human studies have examined whether maternal FRAAs increase the risk of offspring developing ASD even though maternal FRAAs are linked to maternal subfertility (35) and neural tube defects in offspring (36-40). One case study (LOE 4) demonstrated that a FRAA positive woman with a history of multiple miscarriages and birth defects had a successful pregnancy after treatment targeting the FRAA abnormality (37).

Despite this evidence that folate transport across the placenta could be an explanation for changes in blood folate levels during pregnancy, other explanations for high blood folate levels are possible. For example, mothers who are at high risk for having a child with ASD may simply be ingesting higher doses of folate in order to protect their unborn child. Thus, the exact relationship between markers of folate metabolism and ASD risk is complex and it is too preliminary to provide a GOR at this time.

Other important nutritional biomarkers include vitamin D levels since lower 1st trimester vitamin D levels (41) (LOE 3b) and mid-gestation vitamin D deficiency (42) (LOE 2b) are associated with the severity of ASD behaviors in the offspring, and lifetime maternal vitamin D deficiency increases the risk of ASD in offspring (LOE 3b) (43). Thus, vitamin D has a GOR of B for predicting the diagnosis of ASD although the exact associated risk needs further study.

Given that treatment with specific folate compounds can decrease the risk of offspring developing ASD, this is clearly an important area to study (32). Nutritional interventions such as folate, vitamin D and vitamin B12 can have important health consequences, can potentially correct metabolic abnormalities noted above, and are generally regarded as safe. This area of physiology is potentially well suited for the development of safe interventions. Thus, the development of biomarkers for these nutritional abnormalities could be beneficial for preventing the development of ASD in the offspring or minimizing the severity of ASD.

Medical history

Several factors in the prenatal medical history are associated with an increased risk of ASD including advanced parental (44-46) and maternal age (45,46), birth order (44-46), and maternal prenatal factors including medications (29,44,46), smoking (29), bleeding (44), gestational diabetes (29,44,45), hypertension (44,45), proteinuria (44), pre-eclampsia (44), previous fetal loss (44), threatened abortion (45), antepartum hemorrhage (45), swelling (44),

preeclampsia (45), fetal distress (45), and pre-pregnancy body mass index and gestational weight gain (47). During the perinatal period, factors associated with ASD such as caesarian delivery (45,46), prematurity (45,46) and breech presentation (45,46) have been reported. While there is no specific model that has been developed to predict the risk of a child developing ASD due to these risk factors, future studies could incorporate these factors into such calculations. However, several systematic reviews and meta-analyses (LOE 2a) (44-46) have been conducted on these factors providing a GOR B.

Fetal stress

A recent review discusses the growing research implicating maternal psychological stress, such as, partner abuse, bereavement and other psychosocial life stressors during the 2nd and 3rd trimester of pregnancy (48). These factors were revealed in several population-based cohort studies (LOE 2b) resulting in an GOR of B. One study did demonstrate that the risk of maternal bereavement on the offspring developing ASD was accounted for by other factors such as maternal psychiatric history, demonstrating the complicated interaction of many factors which could account for some of the reported effect. Still limitations of this research include the many different types of prenatal stress and the lack of one cohesive biomarker to measure such psychological stress.

Fetal toxicant exposure

Other studies have documented that prenatal exposure to environmental stressors such as toxicants can increase the risk of developing ASD although many of the studies used estimations of exposure rather than direct biomarker measurement (49). Three studies (LOE 2b) used urine biomarkers to measure maternal exposure to organophosphate and other toxicants during pregnancy resulting in a GOR of B (50-52). Particularly compelling as a biomarker is air pollution because air pollution is being measured with increasing precision and is publicly available information (53).

Post-natal pre-symptomatic biomarkers

Neuroimaging

Both functional and anatomic neuroimaging studies have demonstrated neuroimaging features that can predict the diagnosis of ASD in the early pre-symptomatic period. Using measures of functional connectivity derived from

functional magnetic resonance imaging (MRI) scans at 6 months of age and machine learning, a diagnostic function was created which could predict the eventual diagnosis of ASD in 59 infants at high risk for developing ASD in a longitudinal non-randomized, non-controlled prospective study (LOE 2b) (54). By examining surface area obtained from MRI between 6 and 12 months of age, a deep-learning algorithm was able to predict the ASD diagnosis at 24 months in children with both high- and low-risk of developing ASD in a longitudinal non-randomized controlled prospective study (LOE 2b) (55). Although each individual technique receives a GOR of C, neuroimaging in general receives a GOR of B if both techniques are considered together.

Visual attention

Young children with ASD do not orient normally towards socially relevant point-light displays of biological motion (56) or other socially relevant stimuli (57). A prospective, longitudinal non-randomized controlled study measured visual attention in 110 infants with high- and low-risk for developing ASD from 2 to 24 months of age (LOE 2b) (58). For children that eventually developed ASD, visual attention to socially relevant stimuli started out normal at birth but then declined within the first 6-month life (58). Thus, abnormal early visual attention to social relevant stimuli is a promising early behavioral biomarker of ASD with a GOR C because of only one 2b study.

Neurophysiology

Resting state electroencephalography (EEG) was collected on 79 high- and low-risk infants at five time points from 6 to 24 months in a longitudinal, prospective non-randomized controlled study (LOE 2b). Multiscale entropy analysis was able to differentiate infants into risk groups with variable accuracy depending on gender, age and the diagnostic classifier used with the highest accuracy for boys at 9 months of age (59). Although this is a promising technique, this study did not look at ASD as an outcome measure.

Metabolic

One retrospective, population-based study (LOE 3b) assessed 3,258 3–5-year-old children with ASD and 6,838 controls without ASD and examined 36 analytes from newborn screening laboratory records. Five analytes [17-hydroxyprogesterone, free carnitine, isovalerylcarnitine (C5), octanoylcarnitine (C8), and phenylalanine/

tyrosine ratio] were positively associated, and two [methylmalonylcarnitine (C4DC) and adipylcarnitine (C6DC)] were inversely associated with a later diagnosis of ASD (60). This provides a GOR of C for this large but non-validated metabolic analysis.

Autonomic nervous system (ANS)

Several studies have investigated imbalances in the ANS in children with ASD with some suggesting chronic hyperarousal (61,62) while others suggesting underarousal (63). In a prospective controlled study (LOE 2b) pupillary reactivity was studied in siblings of children with ASD as well as low-risk controls who were 9–10 months old (64). Children with an ASD diagnosis at 36 months of age demonstrated a relatively larger pupillary light reflex constriction as infants compared to the other groups with the magnitude of this response associated with symptom severity. With only one LOE 2b study the GOR is C.

Diagnostic biomarkers

Metabolic

ASD is associated with alternations in FOCM and related pathways include methylation and redox metabolism (26,65). These abnormalities result in oxidative damage to lipids, proteins and nucleic acids (26,65). Fisher Discriminant Analysis has been used to determine the diagnostic ability of biomarkers in these connected pathways. In the first prospective, controlled non-randomized cross-sectional study (LOE 2b), 83 individuals with ASD were differentiated from 76 healthy controls with 97% accuracy (66). In a separate validation study using prospective non-randomized data from 154 individuals with ASD studied in three clinical trials (LOE 2b), a 97% sensitivity was found using a similar classification model as the first study (67). Given two LOE 2b studies, the GOR is B.

In a prospective, controlled non-randomized study (LOE 2b), amino acids and acyl-carnitines derived from dried blood spots correctly classify ASD and control participants with 69% accuracy (68). Accuracy was 69% in a validation sample and the sensitivity was 68%. Given only one LOE 2b study, the GOR is C.

Proteomics

Techniques for measuring specific proteins (targeted) and novel proteins (untargeted) have been applied to blood,

urine and saliva samples with inconsistent results across studies. Untargeted analysis of serum has demonstrated involvement of complement (69,70), cholesterol metabolism (71) and apoptosis proteins (69). Using targeted techniques, analysis of serum has identified inflammation and growth factor proteins (72). One (n=60) case-control age and gender matched, non-randomized study (LOE 2b) using isobaric tags for relative and absolute quantitation (iTRAQ) found that a combination of five proteins (C3, C5, GC, ITGA2B, and TLN1) could distinguish those with and without ASD with an impressive area under the curve of 0.982 (73). With only one clinical outcome study, the GOR is a C.

SNPs

A diagnostic classifier based of 237 SNPs was applied to three large ASD genetic databases in a retrospective, non-randomized design (LOE 2b). Individuals with ASD were distinguished from controls with an accuracy varying from 56% to 86%, depending on the ethnic similarity between the training and validation datasets (74). The sensitivity of the classifier to the ethnicity of the dataset demonstrates the potential complications of using genetic information.

Ribonucleic acid (RNA)

Cellular physiology is controlled by how genes are expressed (epigenetics), which is different from the actual coding sequence of a gene. Thus, several studies have measured various types of RNA as a biomarker to investigate cellular physiology. One intriguing, multicenter, non-randomized controlled prospective study (LOE 2b) examined both the human and microbiome RNA obtained from the oral cavity. RNA was subdivided into five types: microRNA, piwi-interacting RNA, non-coding RNA, ribosomal RNA and microbial RNA. Using machine learning and hold-out validation, 32 diagnostic features obtained a 79% accuracy in a training data (n=372). The smaller (n=84) new validation dataset performed slightly better with an accuracy of 85% (75).

Neurophysiology

The Autism Biomarkers Consortium for Clinical Trials (ABC-CT) (76) project is aimed to develop a neurophysiological biomarker of ASD. This project focuses on the N170 response to face stimuli (77). Delayed and lower amplitude N170 responses have been documented in children (78,79), adolescents (78,80) and adults (80) with

ASD and children and adults with Asperger's syndrome (81). A systematic-review and meta-analysis of the N170 (77) (LOE 2a) suggests that the latency to face stimuli are indeed delayed in individuals with ASD with the M170 amplitude affected by several factors such as age and cognitive ability. Thus, the M170 has a GOR of B as a biomarker, but its ability to classify ASD from controls has not been studied; therefore, its performance as a diagnostic biomarker for ASD is not known.

Other studies have associated neurophysiological alterations with ASD. Suppression of the mu rhythm, which is thought to reflect dysfunction in mirror neurons, is reduced in high-functioning adults with ASD while performing (82,83) or observing (83) a motor task, and in children with ASD while observing motor tasks (84) in three small controlled studies (LOE 2b; GOR B). Individuals with ASD demonstrate atypical gamma-band oscillations when viewing complex visual stimuli. The relationship between the spatial frequency of Gabor patches and gamma-band power was reduced in or near the striate or extrastriate cortex in individuals with ASD (85). However, the homogeneity of the orientation of small line-like elements did not influence the gamma-band response in high-functioning adults with ASD (86). In another study, viewing illusory Kanizsa shapes resulted in differences in the timing of gamma-band peaks between TD and ASD adolescents (87) and children (88). Finally, impaired perception of Mooney faces was related to a reduced amplitude and phase locking of gamma-band activity in adults with ASD (89). Thus, five small controlled studies (LOE 2b; GOR B) suggest that gamma-band activity could be a promising biomarker for ASD.

Magnetoencephalography (MEG) has been used to study the response of the auditory system in children with ASD. Three small controlled studies (LOE 2b) have identified delayed M100 and auditory mismatch field in adults (90) and children (91) with ASD and have correlated changes in these neurophysiological parameters with ASD severity (91,92) (GOR B). A large controlled study (LOE 2b) has pointed to abnormal oscillatory activity during the pre-stimulus period in the superior temporal gyrus during an auditory task (93) (GOR C).

Neuroimaging

Following research on anatomic brain development, one rather large (59 ASD, 77 controls) prospective, non-randomized, controlled study examined extra-axial

fluid using MRI (LOE 2b). Extra-axial fluid volume has associated with sleep disturbance and lower verbal ability (94). Machine learning data analysis of extra-axial and brain volumetric data, along with subject age and gender, resulted in a diagnostic predictive function with 78% accuracy (GOR C).

Biomarkers identifying subgroups of ASD

Genetic

ASD is associated with a 70–90% concordance rate for monozygotic twins and the risk of having a second child with ASD is increased 25-fold for families who already have a child with ASD (95). This high heritability has driven the notion that genetic factors are a major contributor to the etiology of ASD (96,97). Some studies estimate that up to 40% of cases of ASD may have a genetic underpinning (98,99). However, this high rate of genetic disorders was not confirmed in at least one large-scale study (99). Nevertheless, genetic testing is standard-of-care for children with ASD because identifying a genetic etiology might help with prescribing treatments and prognosis.

The most basic genetic biomarker is the karyotype that examines the gross chromosomal structure. Cytogenetic abnormalities are reported in 3% of individuals with ASD in two studies (both LOE 2b) (100,101). Karyotype testing has largely been eclipsed by the chromosomal microarray (CMA) which can detect duplications or deletions of small portions of chromosomes, usually down to 15,000 bp (102). Several different specific copy number variations (CNVs) have been associated with ASD (102) with an estimation of the prevalence of these CNVs varying between 8% (99) (LOE 2b) and 26% (103) (LOE 2b). As a complement to the CMA, whole exome sequencing has revolutionized the ability to identify single gene disorders. Using this technique, 9% (99) (LOE 2b) to 30% (104) of children with ASD have been estimated to have a single gene disorder.

An important genetic disorder that is not identified with the aforementioned techniques is Fragile X (98). This disorder expresses itself when there are more than 200 CGG repeats in the FMR1 gene on the X-chromosome. Testing for Fragile X is recommended as standard-of-care for ASD (98). Fragile X Syndrome is reported in 0.2% (LOE 3b) (105) to 2% (LOE 2b) (100) of individuals with ASD.

Genetic testing continues to evolve with some geneticists performing whole genome sequencing to identify disorders with involvement of non-coding regions of the genome and

examining the complex influences of polygenetic factors. One recent retrospective study of 18,381 cases of ASD and 27,969 controls (LOE 3b) reported 13 genes associated with ASD which were outside the known genome (106). Other studies have examined the patterns of SNPs associated with ASD. Of course, although the various genetic technique may identify different abnormalities, these abnormalities are not mutually exclusive, and evidence suggests that those with CNV also harbor other genetic mutations. Several reviews have discussed the complicated landscape of potential genetic causes of ASD (102).

Neuroimaging

One of the neurophenotypes identified as part of the Autism Phenome Project was boys with megalencephaly, a brain that was disproportionately large as compared to body size. The estimated 15% of boys with ASD who have this phenotype had more severe disabilities and had a poorer prognosis (107) (LOE 2b).

Metabolic

Amino acids

The Children's Autism Metabolome Project (CAMP) was a large prospective, controlled non-randomized multisite study aimed at discovering biomarkers of ASD and compared 516 children with ASD to 164 age-matched TD controls (LOE 2b) (108). This study identified a subgroup with dysregulated branched chain amino acid metabolism found in 17% of children with ASD with a 96% specificity. The CAMP method only has an accuracy of 36% and specificity of 17% for the diagnosis of ASD, so it can only identify a subgroup of children with ASD. Thus, it is not useful on its own as a diagnostic biomarker of ASD. However, the exciting aspect of this biomarker is that branched chain amino acid supplementation is a potential treatment for ASD. However, the CAMP biomarker cannot be considered a treatment biomarker since this treatment has not been studied in ASD. In the genetic form of this disorder, supplementation with branched chain amino acids improved symptoms in the mouse model but not in the human patients (109).

Methylation-redox biomarkers

ASD is associated with alternations in FOCM related to methylation and redox metabolism abnormalities (26,65). Dr. James originally described this unique ASD endophenotype (26) in three prospective, non-randomized case-control studies (LOE 2b) (26,110,111). Abnormalities

in redox metabolism as measured by alterations in glutathione redox status were also verified in case-control studies (LOE 2b) utilizing primary immune cells (112), lymphoblastoid cell lines (113) and post-mortem brain tissue (114). A meta-analysis and systematic review (LOE 2a) verified abnormal redox biomarkers related to ASD including abnormalities in glutathione, methionine and cysteine across repeated studies from several different laboratories (115). As described above, Fisher Discriminant Analysis found that biomarkers of methylation and redox metabolism and oxidative damage identified a subgroup of 98% of children with ASD in one study (LOE 2b) (66) and 97% of children with ASD in another study (LOE 2b) (67). Therefore methylation-redox markers are GOR B.

Mitochondrial biomarkers

Approximately 5% of children with ASD exhibit mitochondrial disease (116) while many more, perhaps between 30–80% exhibit some degree of mitochondrial dysfunction (117). Many biomarkers of mitochondrial dysfunction have been used in ASD, as recently reviewed (117). Using the buccal swab technique to measure electron transport chain (ETC) complex activity, abnormal mitochondrial function was identified in 64% of children with ASD in one prospective study (LOE 2b) (118) and 62% in one retrospective study (LOE 3b) (119). A previous meta-analysis estimated the prevalence of lactate (31.1%, 6 LOE 3b studies), pyruvate (13.6%, 2 LOE 3b studies) and lactate-to-pyruvate ratio (27.6%, 1 LOE 3b study) abnormalities (116). The prevalence of alanine abnormalities was reported in the meta-analysis (8.3%, LOE 2a) (116) and in a retrospective analysis of an ASD clinic (120) (1.7%, LOE 3b). The prevalence of an abnormal alanine-to-lysine ratio was reviewed in a retrospective analysis of an ASD clinic (120) (15.9%, LOE 3b). The prevalence of a unique pattern of elevations in short and long but not medium chain acyl-carnitines was discovered in a retrospective analysis of an ASD clinic (LOE 3b) as 24% (120) but refined in a larger retrospective analysis (LOE 3b) of the same clinic as 17% (121).

Other standard blood markers such as creatine kinase, ammonia and aspartate aminotransferase (AST) are commonly used biomarkers for mitochondrial dysfunction in ASD but the studies are too small to estimate the prevalence of any of these specific biomarkers being abnormal in ASD (116). Others have shown that ASD and TD children can be differentiated by novel biomarkers of mitochondrial dysfunction such as lactate dehydrogenase (122), lactate oxidase, pyruvate kinase

and hexokinase (123), Na⁺/K⁺ ATPase (124,125), Caspase-3 (126) and Caspase-7 (122). Again, these studies are not consistent in biomarkers used to differentiate the groups. Thus, while these studies do provide evidence for biomarkers of mitochondrial dysfunction being useful in identifying subgroups of individuals with ASD, the inconsistency prevents a clear prevalence to be estimated and it is unclear how to most appropriately combine these studies to judge a GOR.

The first study which examined possible carnitine deficiency used adult normative values as controls so the accuracy of the study conclusions has been questioned (127). However, other studies have verified these abnormalities in carnitine levels, particularly in individuals with ASD and GI abnormalities (128). Additionally, a genetic abnormality in carnitine metabolism has been linked to ASD (129) and primary carnitine deficiency was reported to cause ASD in one case report (130). Three clinical trials have reported that carnitine supplementation improves symptoms in ASD (131-133). Thus, the importance of carnitine is being recognized in ASD and its deficiency is being acknowledged as a potential biomarker of a metabolic subgroup of ASD. However, given the inconsistency in the approaches of the studies on carnitine it is difficult to estimate a prevalence or a GOR.

Immune

In an open-label clinical study (LOE 2b), 75% of the participants had at least one FRAA (134) while 65% of the participants had at least one FRAA in a prospective, double-blind placebo controlled (DBPC) clinical trial (LOE 1b) (135). The significance of the FRAA as a biomarker of an ASD subgroup with unique characteristics has been reported (33).

Alterations in gut permeability

Alterations in intestinal barrier integrity has been identified in patients with ASD in several studies (136) with one study demonstrating reduced expression of barrier-forming intestine “tight junction” components in 75% of patients with ASD (137). Zonulin regulates tight junctions between enterocytes and is a physiological modulator controlling intestinal permeability. This protein was increased in patients with ASD compared with healthy controls and was correlated with measures of ASD severity (138) (LOE 3b). Zonulin may be a promising biomarker for a subgroup of children with ASD with GI problems stemming from altered intestinal integrity with a GOR of C. However, it

Table 4 Biomarker that predict treatment response

Type	Biomarker	Treatment	GOR
Metabolic	Serum carnitine	Carnitine	C
	Glutathione/methylation	mB12	C
	Pterin redox ratio	BH ₄	*
Immune	FRAA	Leucovorin Ca	C
	Cunningham panel	IVIG	*
Autonomic	RMSSD, pNN50	Propranolol	C
Genetic	CD38 rs3796863 SNP	Oxytocin	C
	OXTR rs6791619 SNP	Oxytocin	C
Neuroimaging	MRS/rsfMRI	Riluzole	C

*, not enough studies to make a recommendation because the only study is open-label. BH₄, tetrahydrobiopterin; FA, folinic acid; FRAA, folate receptor alpha autoantibody; IVIG, intravenous immunoglobulin; mB12, methyl-B12; MRS, magnetic resonance spectroscopy; pNN50, proportion of successive R–R intervals differing by more than 50 ms; RMSSD, root mean square differences of adjacent R–R intervals; rsfMRI, resting state functional MRI.

should also be mentioned that not all studies support that notion of increased gut permeability (139).

Autonomic nervous system

As previously mentioned, ANS abnormalities have been noted in individuals with ASD. Many studies have concentrated on cardiac measures such as heart rate variability (61) while other studies have focused on pupillary response (140) as measures of ANS biomarkers. Heart rate variability has been studied in adults (141–144) and children (63,145–147) with ASD as well as their caregivers (148–150) and has been associated with GI symptoms (146) and used as an outcome measure in clinical treatments studies (147,151,152). Given the multiple LOE 3b and 2b studies, heart rate variability has a GOR of B. Pupillary light reflex was studied in four prospective nonrandomized controlled studies of children with ASD (LOE 2b) (153) with one study linked these abnormalities to the severity of atypical sensory behaviors (154) and heart rate variability (155), and one study demonstrating the ability of pupillary light reflex parameters to accurately select a subgroup of 86.7–93.7% of the ASD participants with a 92.5–96.7% accuracy (156). A recent review discusses some of the limitations of these measures and the questions that need to be addressed in order to develop ANS biomarkers further (157). The Guidelines for Reporting Articles on Psychiatry and Heart rate variability (GRAPH) efforts has been launched to standardize reporting of at least heart rate variability so

consistent data can be provided across studies for valid comparisons (158).

Biomarkers that predict treatment response

Three metabolic treatments have biomarkers that correlate with treatment response (*Table 4*). Core ASD symptoms improve with carnitine supplementation with one study demonstrating that the increase in blood carnitine levels were related to improvements in physical and cognitive abilities (LOE 2b) (131). Two methylcobalamin treatment studies, one open label (LOE 4) and one DBPC (LOE 2b) demonstrate a correspondence between changes in methylation and redox metabolites and functional abilities (159) and ASD symptoms (160,161). Lastly, biomarkers of tetrahydrobiopterin metabolism correlated with language, behavior and functional improvements following tetrahydrobiopterin treatment in an open-label study (LOE 4) (161,162).

Two studies demonstrate that autoantibodies can predict response to specific treatment (*Table 4*). Pretreatment FRAA titers predicted response to leucovorin calcium in children with language delay in a DBPC study (LOE 2b) (135). For patients with ASD and autoimmune encephalopathy, specific brain autoantibodies, particularly those to the D2L receptor and tubulin, predicted response to treatment with intravenous immunoglobulin in an open-label study (LOE 4) (163).

Medications that modulate the ANS, particularly the beta-adrenergic antagonist propranolol, have emerged as potential effective treatments for the core features of ASD (164). In a prospective, DBPC study (LOE 2b) of the effect of propranolol on verbal problem solving in adults and adolescents with ASD, two ANS biomarkers, the root mean square differences of adjacent R–R intervals (RMSSD) and the proportion of successive R–R intervals differing by more than 50 ms (pNN50), predicted treatment response (165) (*Table 4*).

Treatment with the hormone oxytocin is undergoing particularly active study (164). Two SNPs, one on the CD38 gene which codes for the transmembrane protein involved in oxytocin secretion (166) and another on the OXTR gene which codes with the oxytocin receptor (167) have been found to predict response to oxytocin in clinical studies (both LOE 2b).

In a medium size (n=37) DBPC cross-over study (LOE 2b) researcher used magnetic resonance spectroscopy and resting state functional MRI to demonstrate that riluzole had the predicted response on improving the cortical excitatory to inhibitory balance and improved functional connectivity in the frontal lobes (168).

Limitations of biomarker studies

One of the major limitations in biomarkers studies is the comparison groups used. Many studies compare individuals with ASD to TD unrelated controls. Although scientifically valid, this comparison has some serious limitations when considering the use of biomarkers in the real clinical world. Specifically, in the clinic, the question that needs to be answered is not whether a completely normally developing child has ASD but rather whether a child with developmental delays or an unusual behavior may have a diagnosis of ASD. Likewise, another important question is whether a sibling of a child who is already diagnosed with ASD will develop ASD since the sibling is at higher risk. Thus, although TD unrelated controls may be adequate for initially developing biomarkers, validation studies need to use more rigorous and clinically relevant controls.

The other important limitation of many biomarkers, particularly diagnostic biomarkers, is their biological validity. For example, the ability of FRAAs to predict clinical response to treatment with leucovorin is consistent with its proposed scientific physiological mechanism of action. However, if a biomarker does not represent a core biological

process of the disease, there is a danger that the biomarker represents an epiphenomenon of the disease process. Thus, even if the biomarker reliably and consistently differentiates groups of patients, the information provided about the underlying disease process may be limited.

There are many emerging biomarkers for ASD, but biomarkers need to be used in the correct context. For example, although FRAAs may be very useful in guiding therapy for those already diagnosed with ASD, FRAAs are probably not be a good test to establish a diagnosis of ASD, since they are only positive in 50–70% of children with ASD.

The promise of the use of biomarkers in the future

Even though diagnostic ASD behavior may not be clearly defined until late in the second year of life, the neuropathology associated with ASD may begin prenatally in some cases. Thus, there is the potential for biomarkers that aid in identifying individuals at risk for developing ASD well before diagnostic behaviors are clearly established. Potential biomarkers have been identified but the predictive power of most have not been studied. The most promising prenatal biomarker is maternal fetal brain autoantibodies that appear to have good specificity for an offspring developing ASD. However, while this antibody panel may appear highly translatable as a clinical tool, the lack of any proven associated treatment has reduced enthusiasm to develop a widely-used commercial test (169). Postnatal presymptomatic neuroimaging biomarkers are promising but further studies are needed to validate their performance.

Several biomarkers are being developed as diagnostic tools. Many of the studies remain preliminary and most of the biomarkers do not have adequate performance. Given the significant heterogeneity in the etiology of ASD, large samples of individuals with ASD as well as large, carefully selected clinically relevant control groups will be needed to validate such biomarkers. It is likely that the performance of these biomarkers will fail to operate adequately if considered in isolation and used as a general screening tool. Thus, any diagnostic biomarker developed needs to measure biological processes and most likely will need to be used in conjunction with other behavioral tests and clinically relevant information to perform adequately.

Research supports the notion that the etiology of ASD arises from a complex interaction between genetic susceptibility and environmental exposures (49,170).

Thus, an exciting possibility of biomarker use in ASD is in prevention by identifying those at risk during the prenatal or pre-symptomatic period so abnormal biological processes can be identified early, and potential triggers can be avoided, or biological abnormalities can be addressed. Although environmental exposures have been identified (prenatal stress, toxicants), there are no studies examining biomarkers that measure these environmental risks nor have the biological processes that may stratify risk been carefully studied. Thus, biomarkers that can stratify susceptibility risk in order to avoid exposure to environmental triggers might help avoid the development of ASD in the first place. Such studies could identify vulnerable populations that could be protected from environmental triggers.

Biomarkers may also be extremely helpful in explaining the significant heterogeneity associated with ASD (171). The underlying pathophysiology that drives ASD symptoms may be very different patient-to-patient. For example, some individuals have a well-established genetic syndrome while others demonstrate other non-genetic physiological abnormalities. Biomarkers that can subdivide patients into clear etiological subgroups may be very helpful for prescribing optimal treatments and predicting prognosis. This seems to be the most well-developed aspect of biomarkers, but studies remain in the beginning stages.

Many individuals with ASD are medically complex with multiple co-morbid medical conditions. Unfortunately, these medical conditions are not always obvious. For example, many GI disorders can lead to symptoms that are considered core ASD symptoms such as repetitive behaviors; thus, such symptoms are commonly not recognized as indicative of GI distress (172). Biomarkers that can identify these medical conditions can help target those who need specific evaluation and treatment. There is only one study examining the promising biomarker Zonulin that has the potential to identify those with GI disorders. However, several biomarkers have been developed to identify mitochondrial dysfunction and methylation-redox abnormalities. These biomarkers may be extremely helpful because metabolic disorders are potentially treatable conditions. Additionally, markers of ANS dysfunction may be very promising as such abnormalities may drive disruptive behaviors and have potential treatments.

Individual response to ASD treatment varies widely, so biomarkers that can predict treatment response could be very helpful for optimizing individualized treatment plans, leading to a personalized precision medicine approach (171).

Although several biomarkers that can predict treatment response have been described, the studies validating them are in the early stages. Many of these biomarkers have also been studied for selecting biological subgroups, which enhances their validity and clinical applicability. Subgroup and treatment biomarkers may be some of the most powerful as few guidelines are available for selecting the most appropriate treatment. Given that there are current few outcome measures to objectively monitor treatment effectiveness, treatment biomarkers may be the most promising and have the most impact.

In the future we may learn to use biomarkers together in an integrated way and learn to map them onto specific important symptoms or therapeutic actions. As an example, the National Institute of Mental Health has developed the Research Domain Criteria for studying mental health (173). This initiative divides human mental functioning into five specific domains which contains specific behavioral or emotional constructs. Various elements from genes to molecules to behaviors are mapped onto each construct in order to better understand the factors that drive the construct as well as the instruments that can measure the construct. In this way, elements of mental disease are systematically divided into understandable parts. As symptoms of ASD are mapped onto specific biological processes and biomarkers of such processes, insight into disease processes and potential therapeutics should emerge.

Conclusions

Promising biomarkers identified in this review fall into five types (*Figure 1*). Although there are many potential biomarkers for predicting the risk of developing ASD before birth, many of these biomarkers have not been systematically studied to generate estimations of the associated risk of developing ASD after birth. Several pre-symptomatic biomarkers are being developed but only two have an estimation of diagnostic accuracy based on very preliminary studies. Several diagnostic biomarkers are promising but like the pre-symptomatic biomarkers, the studies remain very preliminary. For example, one of the major efforts to develop a diagnostic biomarker, ABC-CT, does not have a study to estimate its diagnostic performance. Subgrouping biomarkers appear the most robust in regard to the number of biomarkers and the number of studies conducted that estimate the size of the subgroup. Biomarkers that predict treatment response

Prenatal	Pre-symptomatic	Subgrouping	Treatment
*Karyotype	*fMRI functional connectivity	*Karyotype	Serum carnitine
*Maternal fetal brain Ab	*Cortical surface areas	*Chromosomal microarray	Glutathione redox ratio
Maternal IL-6/IL-17	Visual attention	*Whole exome sequencing	Methylation
Methylation-redox	Resting state EEG	*CGG repeats	Pterin redox ratio
Maternal FRAA	Steroid, amino acid and carnitine metabolism	*Head circumference/brain volume	FRAA
Maternal blood folate	Pupillary light reflex	*Amino acid	Cunningham panel
Maternal blood vit B12		*Methylation-redox	Heart rate variability
Maternal RFC SNPs		*ETC by Buccal Swab technique	Oxytocin SNPs
Maternal vit D		*Lactate	
Advanced maternal age		*Pyruvate	
Advanced paternal age		*Lactate/Pyruvate ratio	
Maternal medical Hx		*Alanine	
Pregnancy complications		*Alanine/Lysine ratio	
Psychological stress		*Acyl-carnitine elevations	
Toxicants		Carnitine deficiency	
		Gut permeability - zonulin	
		*FRAA	
	Diagnostic	Heart rate variability	
	*Methylation-redox	Pupillary response	
	*Acyl-carnitine/amino acids		
	Proteomics		
	*SNPs		
	*Human and microbial RNA		
	EEG N170		
	EEG mu rhythm		
	EEG gamma band		
	MEG auditory M100/MMF		
	MEG auditory oscillations		
	*MRI extra-axial fluid		

Figure 1 Promising biomarkers for ASD. *, available studies provide quantitative estimates for biomarker. Ab, antibody; EEG, electroencephalograph; ETC, electron transport chain; fMRI, functional magnetic resonance imaging; FRAA, folate receptor alpha autoantibody; IL, interleukin; MEG, magnetoencephalography; MRI, magnetic resonance imaging; RFC, reduce folate carrier; RNA, ribonucleic acid; SNPs, single nucleotide polymorphisms.

are developing, but data supporting them are preliminary. Overall, the evidence for biomarkers in ASD is preliminary but promising.

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Footnote

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