Molecular Diagnosis & Therapy (2022) 26:483–495
https://doi.org/10.1007/s40291-022-00600-7

CURRENT OPINION

Modern Biomarkers for Autism Spectrum Disorder: Future Directions

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Accepted: 16 May 2022 / Published online: 27 June 2022

Abstract

Autism spectrum disorder is an increasingly prevalent neurodevelopmental disorder in the world today, with an estimated 2% of the population being affected in the USA. A major complicating factor in diagnosing, treating, and understanding autism spectrum disorder is that defining the disorder is solely based on the observation of behavior. Thus, recent research has focused on identifying specific biological abnormalities in autism spectrum disorder that can provide clues to diagnosis and treatment. Biomarkers are an objective way to identify and measure biological abnormalities for diagnostic purposes as well as to measure changes resulting from treatment. This current opinion paper discusses the state of research of various biomarkers currently in development for autism spectrum disorder. The types of biomarkers identified include prenatal history, genetics, neurological including neuroimaging, neurophysiologic, and visual attention, metabolic including abnormalities in mitochondrial, folate, trans-methylation, and trans-sulfuration pathways, immune including autoantibodies and cytokine dysregulation, autonomic nervous system, and nutritional. Many of these biomarkers have promising preliminary evidence for prenatal and post-natal pre-symptomatic risk assessment, confirmation of diagnosis, subtyping, and treatment response. However, most biomarkers have not undergone validation studies and most studies do not investigate biomarkers with clinically relevant comparison groups. Although the field of biomarker research in autism spectrum disorder is promising, it appears that it is currently in the early stages of development.

1 Autism Spectrum Disorder: A Disorder in Need of Objective Quantitative Biomarkers

Autism spectrum disorder (ASD) is quickly growing to be one of the most significant neurodevelopmental disorders of our time. With an estimated 2% of US children affected by ASD [1], it is growing increasingly important to identify, diagnose, treat, and understand this disorder. Many factors make ASD difficult to understand and manage; chief among them is the fact that the diagnosis of ASD is solely based on behavioral observation. While there are many biological, physiological, and medical abnormalities associated with ASD, there is yet to be any way to utilize these abnormalities to develop an objective generalizable measurement to assist with the diagnosis or management of ASD.

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This lack of understanding of the bio-physiological processes involved in ASD not only limits diagnostic accuracy but also impacts treatment planning. The most common treatment for ASD is behavioral and educational interventions, such as applied behavior analysis and speech and/or occupational therapy. While these treatments can be beneficial, they require a large commitment of time and energy on the part of the patient and the family. Understanding the bio-physiological abnormalities of ASD can help in the development of pharmacological or other therapeutics that may augment behavior and educational therapies to accelerate habilitation. Thus, an objective validated bio-physiological measure that could represent pathophysiological processes underlying ASD may have utility for both diagnosis and monitoring treatment progress.

Biomarkers are objective measures of bio-physiological abnormalities that can have many different applications such as disease diagnosis, classification of disease severity, indication of prognosis, and measuring response to therapy [2]. While there is much research centered around biomarker development in ASD, none of these biomarkers is yet validated [3]. The heterogeneity of ASD proves to complicate biomarker development [4]. This article reviews and discusses the emerging research of various potential

Key Points

Autism spectrum disorder is a prevalent neurodevelopmental disorder worldwide with an estimated prevalence of about 2% of children in the USA. However, there is no objectively proven biological measurement to provide an indication of autism spectrum disorder risk or diagnosis or to indicate optimal treatment.

Preliminary findings on genetic, metabolic, immune, and neuroimaging diagnostic biomarkers show promise, especially in conjunction with additional measurements such as behavioral assessments. However, large validation trials and the use of appropriate control populations are lacking.

Although the field of biomarker research in autism spectrum disorder is promising, it appears it is currently in the early stages of development. Biomarkers to stratify autism spectrum disorder risk during the prenatal and postnatal pre-symptomatic period may be particularly helpful for starting interventions early when they might be most effective, and biomarkers to predict treatment response may expedite habilitation for those already diagnosed.

biomarkers that could advance the understanding and management of ASD. We discuss the current standing of each biomarker, limitations of the research, and pathways for future development.

2 Current Biomarkers

Autism spectrum disorder is a complicated disorder that spans many disciplines with the understanding of the disorder evolving over many decades. Before 1986, ASD research concentrated on mostly genetics and neuroimaging research with research into mitochondrial and immune disorders starting to increase after that time, followed by an increase in research into oxidative stress and toxicants in the 1990s [5]. As research has progressed, biomarkers for specific biological processes have been developed and translated from the laboratory into clinical research trials [3]. Given the diversity of the disciplines that are involved in evaluating children with ASD, a wide variety of biomarkers have been developed, including those associated with medical history and nutrition to those involved with more molecular processes such as microRNA (miRNA). This article reviews the more promising biomarkers, selected by the quality and replicability of the research that supports them. The authors selected original studies and systematic reviews for each potential biomarker. The team then discussed whether there was sufficient evidence and whether the published studies were of significant quality to be included in this current opinion paper. Biomarkers with little evidence or those supported by poor-quality studies were not included in this current opinion paper.

Table 1 summarizes the biomarkers discussed as well as their strengths and weaknesses and whether validation studies exist. Biomarkers are also depicted by their various types [3]: risk biomarkers can provide an estimate of disease risk but cannot necessarily be used for diagnosis; diagnostic biomarkers are aimed at making or confirming a diagnosis; subgroup biomarkers can indicate whether an individual already diagnosed may belong to a subset of diseased individuals with certain characteristics; and treatment biomarkers may indicate whether an already diagnosed individual may respond to a specific treatment. In addition, biomarkers may be used during different developmental periods, including prenatal, post-natal, pre-diagnosis, or post-diagnosis. Below, each biomarker category is discussed with evidence for the biomarker discussed.

2.1 Prenatal History

In a systematic review and meta-analysis involving over 40,000 ASD cases [6], maternal infection during pregnancy was linked to an increased ASD risk, especially in cases that required hospitalization for infection. One large prospective cohort study [7] in Norway found that the ASD risk increased significantly with three or more fevers after 12 weeks gestation. Multiple other retrospective case-controlled studies [8, 9] and individual cohort studies [10, 11] have supported these findings and this finding is consistent with a well-developed animal model of ASD known as the maternal immune activation model [12]. Maternal metabolic conditions have also been linked to an increased ASD risk [13, 14], as seen in a retrospective case-controlled study [15] in California that found children with ASD of women with metabolic conditions, such as obesity and diabetes mellitus, had poorer expressive language. A meta-analysis involving over 37,000 ASD cases linked gestational diabetes to an increased ASD risk among other factors [16]. While these risk factors currently do not provide diagnostic use or prediction of therapeutic response, they may be used in the future to identify children at a high risk for developing ASD.

2.2 Genetic

Because of the apparent high heritability of ASD, research focusing on its underlying genetics has long persisted [17]. Early research concentrated on structural DNA alterations while more modern studies have included gene expression and epigenetics. Clearly, understanding these underlying

Table 1 Biomarkers in development for autism spectrum disorder

Biomarker	Type (potential)	Period	Strength	Weakness	Validation studies
Prenatal history					
Gestational infections	Risk	Prenatal	Can obtain from medical history	Not clear If specific to specific infection or if the effects of infection treatments (eg, antibiotics) have an effect. No clear treatment consequences other than standard of care	No
Obesity/diabetes	Risk	Prenatal	Can obtain from medical history	No clear treatment consequences other than standard of care	No
Genetics					
Structural DNA alterations	Risk subgroup Treatment	All periods	High-throughput comprehensive genetic analysis is becoming clinically routine. Particularly helpful in difficult, refractory cases to provide prognostic information	Low yield for any single disorder and phenotype can be variable. Variants of unknown significance are common results leading to ambiguous information which can be hard to reconcile. Experienced geneticist is needed to interpret complex genetic information	No
Single nucleotide polymorphism	Risk Treatment	Postnatal	Single nucleotide polymorphism is generally available even on the consumer level	Lack of rigorous scientific research on risk association and treatment implications leads to dubious treatment recommendations based on results, especially at the consumer level	No
mRNA/miRNA	Diagnostic Subgroup	Post-diagnosis	Noninvasive. Some correlation with neurodevelopment outcomes	Variable performance in validation studies. Different RNA subtypes used in various studies. Utility unclear if diagnosis already established	Yes
Methylation	Risk Diagnostic	All periods	Biologically important. Important epigenetic factor than may explain inheritance and environmental exposures	Very variable results regarding direction of methylation abnormalities and specific gene target of methylations changes. Very complicated field given both inherited and environmental factors are at play	No
Neurological					
Morphology and diffusion tensor imaging	Risk Diagnostic	Pre-diagnosis Post-diagnosis	Consistent abnormalities in brain growth appear prior to diagnosis and persist into the diagnostic period. Non-invasive	Large prospective studies are needed to validate findings. MRI may require specialized centers. Limited to infants and children that can tolerate MRI scanner	No
N170	Diagnostic	Post-Diagnosis	Non-invasive. No Sedation Needed	Requires cooperation and attention to view stimuli. Utility unclear if diagnosis already established. Large prospective studies are needed to validate findings	No
MEG	Diagnostic Treatment	Post-diagnosis	Non-invasive. No Sedation Needed. Possible association with GABA signaling leading to possible prediction of treatment response	Variable protocols limit conclusions from current research. Utility unclear if diagnosis already established. Large prospective studies are needed to validate findings	No
Resting state MRI	Risk Diagnostic	Pre-diagnosis Post-diagnosis	Non-invasive	Variable protocols limit conclusions from current research. Require specialized centers. Limited to infants and children that can tolerate MRI scanner	No
Visual attention	Risk Diagnostic	Pre-diagnosis Post-diagnosis	Non-invasive	Require specialized centers. Requires cooperation and attention to view stimuli	No
Metabolic					
Transmethylation transsulphuration	Risk Diagnostic Treatment	All Periods	Only requires blood test. Possible prenatal predictive. Biologically important and possibly relevant to treatment	Requires specialized centers and equipment and not widely available clinically. May correlation with symptoms. Large prospective studies are needed to validate findings	Yes

Table 1 (continued)

Biomarker	Type (potential)	Period	Strength	Weakness	Validation studies
Mitochondrial	Subgroup Treatment	Post-diagnosis	Various biomarkers, some non-invasive. Possibly biologically important and relevant to treatment	Variable techniques and biomarkers limit conclusions from current research. Large prospective studies using a validated biomarker are needed to validate findings	No
Immune					
Maternal autism related (MAR)	Diagnostic	Prenatal	Possible prenatal prediction of ASD diagnosis with a high sensitivity for the MAR subgroup	Large prospective studies are needed to validate findings. Treatment implications unclear at this time	No
Brain autoantibodies	Treatment	Post-diagnosis	Possibly biologically important and relevant to treatment	Variable techniques and biomarkers limit conclusions from current research. Large prospective studies using a validated biomarker are needed to validate findings	No
Folate receptor alpha autoantibody	Risk Subgroup Treatment	All periods	Possible prenatal prediction of ASD diagnosis given that found also in parents May represent an ASD subgroup that is treatment responsive	May indicate distinct at-risk subgroup but lack sensitivity for ASD diagnosis. Transgenerational features require significant more investigation. Large prospective studies are needed to validate findings	No
Cytokines	Risk Subgroup Treatment	All periods	Elevated cytokines in pregnancy and neonatal period interesting as possible risk predictor. May indicate subgroup that requires specific treatment	Variable techniques and biomarkers limit conclusions from current research. Large prospective studies using a validated biomarker are needed to validate findings	No
Autonomic					
Heart rate variability	Subgroup Treatment	Post-Diagnosis	May represent an ASD subgroup that is treatment responsive	Variable techniques and biomarkers limit conclusions from current research. Large prospective studies using a validated biomarker are needed to validate findings	No
Pupillometry	Diagnostic	Post-Diagnosis	Non-invasive	Variable techniques and biomarkers limit conclusions from current research	No
Zinc	Risk Subgroup	Prenatal Post-diagnosis	Abnormal zinc level in pregnancy period is an interesting possible risk predictor with treatment implications. May indicate subgroup that requires specific treatment	Deciduous teeth typically not available until after diagnosis so fetal zinc measurements would need to be developed. Treatment implications unclear	No
Vitamin D	Risk Treatment	Prenatal Post-diagnosis	Easily obtained prenatally with routine laboratories and treatment implications straightforward. Biologically relevant	Target optimum vitamin D level widely debated. Large prospective studies are needed to validate findings	No
Folate	Risk	Pre-diagnosis	Easily obtained with routine laboratories	The complicated nature of various folate species is not captured in routine total folate levels and the interaction with other folate pathway abnormalities makes routine levels hard to interpret on their own	No

ASD autism spectrum disorder, *DNA* deoxyribonucleic acid, *GABA* gamma-aminobutyric acid, *MRI* magnetic resonance imaging, *MAR* maternal autism related, *mRNA* messenger RNA, *miRNA* microRNA, *RNA* ribonucleic acid

molecular abnormalities can have positive impacts for developing endophenotypes and response to therapies [18].

2.2.1 Structural DNA Alterations

Research into the structural DNA changes that are associated with ASD include well-known chromosomal alterations such as Down syndrome, copy number variations such as 15q11.2 microdeletion, single-gene disorders such as Phelan McDermid Syndrome, and trinucleotide repeat disorders such as Fragile X syndromes [19]. With the age of routine, clinically available, whole exome and genome sequencing upon us, the promise to identify these disorders is greeted by much enthusiasm. However, despite the high apparent heritability, empirical studies suggest that structural genetic defects account for a minority of ASD cases with empirical studies finding that a genetic disorder can be found in only about 16% of children with ASD using both a chromosomal microarray and whole exome sequencing [20]. In addition, when a variant is found, it is usually de novo rather than inherited [21–24]. In fact, there is insufficient evidence for a ASD-specific gene or a particular genetic variant with a large effect [25]. Furthermore, only a portion of individuals with prevalent genetic abnormalities such as Down or Fragile X syndrome are co-diagnosed with ASD [3], demonstrating the lack of specificity of a genetic diagnosis. The lack of good-quality outcome studies for using a structural genetic analysis in the clinical ASD field is emphasized by the need for quantitative outcome measures [26]. Although clinical improvements in management are possible with this information, this usually requires an experienced geneticist to comprehensively interpret complex genetic information [27].

Other studies have focused on common variations in the genome known as single nucleotide polymorphisms (SNPs). Attempts to develop a diagnostic classifier using large numbers (237) of SNPs have resulted in low accuracy with a range of 56–86%, potentially because of the common variation of SNPs with other factors such as ethnicity [28].

Rather than concentrating on an absolute diagnosis, other studies have identified SNPs that confer an increased risk of being diagnosed with ASD. One study of northeast Chinese Han found that synergistic interactions between SNPs on the *SHANK2* gene, a gene important for synaptogenesis and glutamate neurotransmission, increased the risk for ASD [29]. Of particular interest is the association between SNPs in folate-one carbon metabolism and ASD risk. Single nucleotide polymorphisms in *MTHFR* [30], *RFC* [31], and *MTR* [32] alone and in combination with other folate genes [33] have been associated with an increased ASD risk [31, 32]. Although still preliminary, these studies demonstrate the complicated nature of potential polygenic influences on ASD risk. Furthermore, as these genes can have downstream effects on inherited factors such as methylation, the transgenerational genomic effects need to be considered, as exemplified by a study that demonstrated that ASD risk was associated with *RFC* SNPs in the mother but not the child [34].

Other lines of research have examined the association between SNPs and ASD symptoms. Single nucleotide polymorphisms on the *OXTR* gene have been correlated with aggression, social function, and irritability [32–34]. While SNPs on the *CD38* gene, which have been translated from an animal model of ASD, have been linked to low *CD38* expression [35] and a lack of emotions [36]. Last, pharmacogenomics has promise for guiding drug therapies in ASD [37] but outcome studies provide only modest enthusiasm for predicting severe drug–drug interactions in ASD [38].

2.2.2 Messenger RNA/microRNA

Rather than focusing on structural changes in genes, other studies have focused on gene expression to better understand the molecular physiological state of the cell. While studies have examined traditional messenger RNA (mRNA) expression, more recent studies have focused on miRNA, which are important cellular regulators, and have identified high classification accuracies and correlations with neurodevelopmental measures in small cohorts [39]. Larger studies adding piwi-interacting RNA, non-coding RNA, ribosomal RNA, and oral microbial RNA appear to be the most promising. Initial studies of a large cohort established a 79% and 85% accuracy in training ($n = 372$) and validation ($n = 84$) cohorts, respectively [40]. A larger ($n = 443$) multicenter study that included individuals with developmental delays using salivary miRNA found much more modest results with 67% and 66% accuracy in training and validation cohorts, respectively [41]. Yet, a more recent, larger ($n = 898$) multicenter study examining salivary miRNAs, small nucleolar RNAs, piwi-interacting RNA, and microbial RNAs highlighted that those with ASD and gastrointestinal disorders demonstrated unique RNA profiles but did not examine diagnostic accuracy [42].

2.2.3 Methylation

Methylation patterns measured in the genome of various tissues have been investigated as another possible diagnostic tool identifying ASD. Although differences in global methylation have been inconsistent across studies in ASD, differentially methylated CpG islands have been identified that do show significant relationships [43]. Studies have suggested that differential methylated genes are enriched for ASD-associated genes across five different tissue types showing consistency across maternal and fetal blood [44]. Methylation in biological specimens from parents have been of interest to predict the likelihood of a child being born with

ASD. Distinct patterns of methylation in sperm from fathers of children with ASD have been found while global DNA hypomethylation has been found in mothers of children with ASD [34, 45]. Other studies suggest pre-conceptional multivitamins could affect cord blood and fetal DNA methylation [46, 47]. Methylation as a biomarker shows a promising future for diagnostic testing in ASD. However, the complex nature of methylation and interaction with factors such as prenatal vitamin intake and other environmental exposures, such as prenatal tobacco exposure [48], makes this a very complicated area of research.

2.3 Neurological

Given that behavior and development are centered in the brain, several approaches have used neurological testing methods to identify abnormalities associated with ASD. Some of these studies, such as magnetic resonance imaging, have been limited in scope and application because of the need for cooperation of the participant, whereas more recent approaches such as examining natural visual attention may have some more promising wide-scale applications.

2.3.1 Structural Neuroimaging

After many years, consistent morphological magnetic resonance imaging findings have converged on the overgrowth of cortical [49–51] and subcortical regions, namely the amygdala [52–54], during early infancy before ASD is diagnosable. Studies have also identified enlargement of the extra-axial fluid compartment during the postnatal presymptomatic period, which correlates with severity of the eventual ASD diagnosis [55, 56].

Studies using diffusion tensor imaging, which examines the structural development of the white matter pathways, suggest that the development trajectory of white matter organization is different between 6 and 24 months of life, typically before ASD can be diagnosed, and is associated with later severity of ASD symptoms [57, 58], with older children eventually showing brain asymmetries in white matter development of key language pathways [59] and a widespread reduction in the metrics of white matter organization [60]. Structure neuroimaging studies have not looked at the diagnostic accuracy of these evaluations, but clear differences found in early brain development at a time before ASD can be diagnosed clinically make these promising avenues to pursue.

2.3.2 Neurophysiology

Evoked response studies have identified N170 latency as a possible promising biomarker for ASD. N170 latency to facial, but not object, stimuli may be prolonged in ASD [61] with latency prolongation becoming more severe with age [62]. Other studies suggest this abnormality may be related to social skills and facial memory when comparing upright versus inverted stimuli [63] and may be specific to attending to the eyes as opposed to the nose or mouth [64]. Promising results from the Autism Biomarker Consortium for Clinical Trials (ABC-CT), a large multi-site study led by the Yale Child Study Center has leading to the acceptance of N170 latency to upright human faces as an identifier of biological subgroups of ASD into the US Food and Drug Administration's Biomarker Qualification Program [65].

Magnetoencephalography studies have used a variety of techniques and neurophysiological measurements. A recent meta-analysis found evidence for prolonged M50 and M100 latencies to pure tone stimuli [66] and a recent study suggested that the M50 latencies might be used to predict treatment response to GABA-B agonists [67]. Others have studied gamma-band coherence with various auditory stimuli [68–70].

Resting state function magnetic resonance imaging has found patterns of local hyperconnectivity associated with global hypoconnectivity [71, 72]. Studies in infants prior to diagnosis have identified connectivity pathways associated with initiation of joint attention [73, 74], severity of ritualistic behavior following diagnosis [74, 75], and progressive abnormal lateralization of language networks, which starts in infancy (prior to diagnosis) and worsens with age [74, 76]. Neurophysiological studies appear intriguing and provide some insight into biological processes, but, at this point, are based on relatively small sample sizes and have not been examined in studies that allow diagnostic accuracy to be ascertained.

2.3.3 Visual Attention

Children who are later diagnosed with ASD show reduced attention to social stimuli [77] and eyes [78, 79] after 6 months of age. Studies looking at social behavior and visual attention at a young age (under 1 year) are consistent in their findings of an early decline in social behavior and reduced preference for biological motion [80], but rely on tasks meant for very young infants and lack a long-term follow-up. While visual attention shows promise as an early biomarker for ASD because of its potential for widespread application in the clinical setting, randomized trials of young children are needed to validate the biomarker.

2.4 Metabolic

2.4.1 Trans-Methylation/Trans-Sulfuration Pathways

Prior research has shown that individuals with ASD have biomarkers of oxidative stress. One of the main

manifestations is a decrease in reduced glutathione, the body's major antioxidant, along with an increase in oxidized glutathione in plasma [81–83], brain [84], and cell lines [85]. These are accompanied by other biomarkers of abnormal trans-sulfuration [86] and oxidative damage such as 3-nitrotyrosine, 3-chlorotyrosine, and 8-oxo-deoxyguanosine [87, 88]. Trans-methylation metabolism in ASD has consistently been demonstrated to be abnormal with a decrease in S-adenosylmethionine, an increase in S-adenosylhomocysteine, and a reduced S-adenosylmethionine/S-adenosylhomocysteine ratio [86, 89]. Abnormalities in trans-methylation and trans-sulfuration pathways are so pervasive that they have been investigated as diagnostic markers for ASD. One study using the Fisher Discriminant Analysis found that these biomarkers could discriminate between ASD and typically developing individuals with a 97% accuracy with a follow-up study showing up to a 96% accuracy for the training dataset and 88–95% accuracy for the validation dataset [90, 91]. Functional variations of trans-sulfation deficits have been developed but remain rather preliminary [92].

Interestingly, maternal abnormalities in trans-methylation, including plasma homocysteine, adenosine, and S-adenosylmethionine are found in mothers who have offspring who developed ASD. Additionally, mothers who were high risk versus low risk for having a child with ASD could be determined with 90% accuracy using both trans-sulfuration and trans-methylation metabolites collected during the third trimester [34, 93]. Interestingly, glutathione was found to be a potential marker of a positive clinical response to treatment with methylcobalamin in a recent systematic review and meta-analysis [94].

2.4.2 Mitochondrial Metabolism

Biomarkers of mitochondrial dysfunction are prevalent in children with ASD. A systematic review examining 220 studies on various biomarkers [95] and a meta-analysis of high-quality studies [96] both found evidence for biomarkers of mitochondrial dysfunction associated with ASD with a prevalence range from 8 to 31% depending on the biomarker. A prospective controlled study measuring respiratory chain activity in buccal tissue found that a novel biomarker of the complex I–IV activity ratio was abnormally increased in 64% of patients with ASD, particularly the patients with more severe ASD [97], while a retrospective study of 76 children with ASD using the same technique found mitochondrial enzyme activity abnormalities in 62% of patients [98]. In a small cohort of ten patients and ten matched controls, respiratory chain abnormalities were found in 80% of lymphocytes [99]. Lymphoblastoid cell line models of mitochondrial dysfunction have consistently demonstrated that about one-third have elevated mitochondrial respiratory rates, a unique type of mitochondrial dysfunction associated with ASD [85, 100, 101], and elevated respiratory rates in peripheral blood mononuclear cells has been linked to the neurodevelopmental regression subtype of ASD [102–104]. The complex I–IV activity ratio obtained from the buccal swab technique has been used to select individuals with mitochondrial dysfunction who responded to a mitochondrial cocktail [105] and enzyme activity measured using the buccal swab technique has demonstrated the response of mitochondrial activity to specific supplement treatments [106].

Thus, estimates of the prevalence of mitochondrial dysfunction vary widely, particularly because of the various biomarkers used to measure mitochondrial function. Clearly, a subset of children with ASD manifest mitochondrial dysfunction but the non-standardization of measurements and the non-specific nature of biomarker of mitochondrial dysfunction have slowed progress in this area of ASD research.

2.5 Immune

2.5.1 Maternal Fetal Brain-Directed Autoantibodies

Over the last two decades, four case-control studies, two very large (≥ 200), have documented that 7–12% of children with ASD may be associated with the maternal autoantibody-related subtype of ASD [107]. These maternal autoantibodies are directed to the fetal brain, thus identifying them has the potential to intervene during pregnancy and prevent ASD from developing.

2.5.2 Brain-Directed Autoantibodies

A recent review outlined at least 25 both small-sized and medium-sized case-control studies identifying specific and non-specific brain-directed autoantibodies [108], while a recent systematic review outlined both case reports and case series in which brain autoantibodies predicted response to intravenous immunoglobulin [109]. A medium-sized open-label, prospective, baseline-controlled cohort study found that the anti-dopamine D2L receptor and anti-tubulin autoantibodies predicted treatment response to intravenous immunoglobulin [110].

2.5.3 Folate Receptor-Alpha Autoantibody

A recent meta-analysis found an increased prevalence of the folate receptor-alpha autoantibody (FRAA) in children with ASD (71%) as compared with parents of children with ASD (45%), typically developing children without ASD siblings (15%), and children with developmental delays without ASD (5%) but not typically developing siblings of children with ASD (61%) [111], suggesting that the FRAA might be a

strong heritable risk factor for ASD. The FRAA has been shown to predict response to leucovorin treatment in a recent double-blind placebo-controlled trial [112]. The prevalence in particular families and the utility as a treatment response predictor makes the FRAA a compelling biomarker.

2.5.4 Cytokines

Cytokine level abnormalities associated with ASD have been retrospectively investigated at different stages of development over the last decade. In a medium-sized retrospective study, women who had offspring who developed ASD were found to have mid-gestational elevations in cytokines [113], while another retrospective case-controlled study found that elevated interleukin (IL)-1β and IL-4 in neonatal blood spots was associated with an increased ASD risk [114]. A systematic review and meta-analysis of case-control studies found that elevated IL-1β, IL-6, and IL-8 was linked to ASD in childhood [115]. A series of case-control studies have identified a subgroup of children with ASD who have dysregulated IL-1β and IL-1β/IL-10 ratio in peripheral blood monocytes [116], finding that these dysregulated cytokines are linked to periodic behavioral flares [117], non-IgE-mediated food allergies [118], changes in miRNA expression [118, 119], and changes in mitochondrial respiration [119, 120]. While there is no single cytokine abnormality that has been validated in large prospective studies, abnormal cytokine profiles appear to be associated with ASD at multiple stages of development.

2.6 Autonomic

2.6.1 Heart Rate Variability

Two prospective cross-sectional study studies and two case-controlled studies [121–124] found lower heart rate variability in adults and children with ASD. One study found that heart rate variability predicted treatment response to propranolol [125].

2.6.2 Pupillometry

A systematic review and meta-analysis confirm the statistically significant differences in pupillary response latency in ASD, although the study did highlight the significant heterogeneity of pupillometry study designs, outcomes, and quality [126].

2.7 Nutritional

A meta-analysis of case-control studies suggested children with ASD demonstrated differences in copper in hair and serum, and lower zinc concentrations in the blood [127]. An innovative biomarker that examined deciduous teeth to measure prenatal nutrient metals found that prenatal fetal manganese and zinc was reduced in ASD as compared with their monozygotic and dizygotic twin discordant for ASD [128], while another study suggested that fetal zinc-copper rhythmicity was predictive of ASD [129]. One study has linked these prenatal disruptions in nutritional metal exposure to long-term bioenergetics and language development [104].

Another important nutritional biomarker is vitamin D, as lower first-trimester vitamin D levels [130] and mid-gestation vitamin D deficiency [131] are associated with severity of ASD behaviors in offspring, and lifetime maternal vitamin D deficiency increases the risk of ASD in children [132]. Thus, the maternal vitamin D level may be a biomarker for ASD risk and severity.

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 [133] while a folic acid supplement during pregnancy clearly decreases the risk of the offspring developing ASD [134]. However, two studies suggest that very high maternal folic acid blood concentrations at birth are associated with an increased risk of developing ASD [135, 136] but this notion has significant limitations when we consider that folate metabolism is more likely to be disrupted in ASD and that the type of folate within the supplement (oxidized vs reduced) is variable [137]. Indeed, studies are demonstrating that folic acid, a synthetic oxidized form of folate that is used for fortification and in most supplements, can inhibit folate metabolism, while reduced forms of folate such as leucovorin (folinic acid) or methyltetrahydrofolate do not have inhibitor effects on folate metabolism [137].

3 The Provisional State of Biomarker Development

Because of the multi-dimensional nature of ASD, biomarkers have been developed using a wide variety of techniques and approaches. Few biomarkers have undergone validation studies. For diagnostic biomarkers, salivary miRNA has, by far, undergone the largest studies, but still the particular type of miRNA used across studies and the accuracy of the biomarker varies widely across studies. Many other potentially diagnostic biomarkers have only undergone preliminary studies that are still in the optimization stages. For example, while transmethylation/trans-sulfuration pathway biomarkers appear promising, the studies remain small, lack developmentally delayed non-ASD controls, which may be the most relevant comparison population, and use a combination of measurements that still need to be optimized. Other biomarkers are very compelling but require large validation studies to be conducted. One that is extremely compelling is the maternal

autoantibody-related ASD biomarker, which has the potential to identify a subset of children who will develop ASD prenatally. Neuroimaging and visual attention studies are particularly interesting as they have the potential to provide direct biologically relevant information about brain development, which appears to start before ASD is diagnosable clinically, leading to the possibility of intervening before symptoms start. Unfortunately, these measures do require cooperation of the infant and specialized centers. Other technologies such as untargeted metabolomics [138] and proteomics [139] are emerging but are still inconsistent in their findings.

Other biomarkers such as structural genetic investigations can provide an exact diagnosis, but diagnosis of the genetic syndrome, for most syndromes, overlaps incompletely with the diagnosis of ASD, making these more of an indicator of risk, albeit high risk in most cases, for ASD. Many other biomarkers provide an indication of risk of developing ASD with many of these biomarkers available in the prenatal period with potential treatment implications. For example, verification of prenatal factors such as obesity and diabetes as risk factors for ASD may implicate more aggressive management of these maternal disorders, although studies would need to verify such an approach because it is possible that other factors related to the underlying cause of the metabolic disturbances may be the driving factor rather than the diseases themselves. While more actionable biomarkers such as vitamin D might be more directly translatable, disruptions in folate and zinc, although very compelling regarding potentially translatable treatments, need to be examined more closely to better understand the complex physiology of their pathways. For example, different types of folate (reduced vs oxidized) have different metabolisms, thus the optimal compound needs to be verified in clinical studies. Of course, not all treatment recommendations require biomarkers. Indeed, some have compiled evidence from a variety of prenatal outcomes to demonstrate the importance of specific vitamins and minerals for a healthy pregnancy [140, 141].

Biomarkers that indicate important subgroups, particularly those that lead to beneficial treatment decisions, may be very useful as we still have a limited understanding of how to identify children who require specific treatments. For example, the FRAA may provide an indication for specific treatment with leucovorin (a special type of folate) while cytokine profiles may indicate those with specific immune abnormalities who require treatment and miRNA profiles may identify those children who require an evaluation for gastrointestinal disorders.

4 Limitations

It is clear that biomarker development for ASD is only in the early stages. Aside from the lack of large validation studies, one of the major limitations of most studies is the lack of inclusion of children with developmental delays without ASD, as this is the clinically relevant comparison population in which biomarkers may be most useful in order to guide treatment and surveillance recommendations. Many studies use non-sibling typically developing children as controls, which is not ideal as they are at the lowest risk of developing ASD. While such controls may provide a good starting point for developing biomarkers, they are not particularly clinically relevant as the question that doctors are faced with in the clinic is whether a child with some symptomatology has ASD, not whether a perfectly normal child has ASD. Because of the prevalence rate of ASD, screening biomarkers will be very difficult to use as they will provide a low positive predictive rate even with a high sensitivity and specificity [142]. Furthermore, many biomarkers are measured after the child has developed ASD, thus the use of such a biomarker is limited, especially when the comparison group is a typically developing child who is past the age of ASD diagnosis. While current controls have been suitable for preliminary biomarker research, delineating between these populations in more rigorous future studies will help elucidate the biological mechanisms of those with unspecified developmental delays and ensure better controls.

5 The Promise of Future Biomarker Research

The future of ASD biomarker research lies in translating findings from the biological basis of ASD into validated clinically useful biomarkers for risk assessment, diagnosis, prediction of clinically relevant subgroups, and treatment response. Research is already moving in this direction, as evidenced by studies that have developed biomarkers such as maternal autoantibody-related autism biomarkers, which can predict ASD prenatally, and genetic biomarkers, which may link specific syndromes to ASD. Studies of metabolic biomarkers may provide insight into treatment, while neuroimaging biomarkers may provide biologically relevant indicators of abnormal brain development.

Common themes in areas of future development include the replication of studies with more diverse populations, more randomized controlled trials with larger numbers of participants, and better-defined cohorts with gold standard diagnostic instruments. Another common theme for the future is further investigation of biomarkers in subgroupings of ASD, as ASD is a very heterogeneous condition that will most likely require optimized individualized treatment. In conclusion, the current state of biomarker research is still preliminary but promising.

Declarations

Funding This research was funded, in part, by the Brain Foundation (Pleasanton, CA), the O'Sullivan foundation (Princeton, NJ), the N

of 1 Foundation (Dallas TX), The Jonty Foundation (St Paul, MN), the Gupta Family Foundation (Atherton, CA), and the Jager Family Foundation (Chicago, IL).

Conflict of interest Richard E. Frye is funded by the National Institutes of Health, Department of Defense, and Autism Speaks and receives support from the Turnabout for Autism, the Brain Foundation, the Autism Research Institute and Zynerba Pharmaceuticals. He is on the advisory boards of Iliad Neurosciences and NeuroNeeds. The remaining authors declare that they have no conflicts of interest that are directly relevant to the contents of this article.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and material All the authors are accountable for all aspects of the work, including full data access, integrity of the data, and the accuracy of the data analysis. All authors will ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Code availability Not applicable.

Author contributions All the authors contributed to the study conception and design. The first draft of the manuscript was written by all the authors. All the authors read and approved the final manuscript.

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