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Towards as Multivariate Biomarker-based Diagnosis of Autism Spectrum Disorder: Review and Discussion of Recent Advancements

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Abstract

An ever-evolving understanding of autism spectrum disorder (ASD) pathophysiology necessitates that diagnostic standards also evolve from being observation-based to include quantifiable clinical measurements. The multisystem nature of ASD motivates the use of multivariate methods of statistical analysis over common univariate approaches for discovering clinical biomarkers relevant to this goal. In addition to characterization of important behavioral patterns for improving current diagnostic instruments, multivariate analyses to date have allowed for thorough investigation of neuroimaging-based, genetic, and metabolic abnormalities in individuals with ASD. This review highlights current research using multivariate statistical analyses to quantify the value of these behavioral and physiological markers for ASD diagnosis. A detailed discussion of a blood-based diagnostic test for ASD using specific metabolite concentrations is also provided. The advancement of ASD biomarker research promises to provide earlier and more accurate diagnoses of the disorder.

1 Introduction

The diagnosis of autism spectrum disorder (ASD) is based upon its core symptoms of social/communication deficits and restricted, repetitive behaviors.[1] The Centers for Disease Control and Prevention recently estimated the prevalence of ASD among eight-year-old children in the United States to be 1 in 59 for the year 2014,[2] a prevalence that has been on a consistent upward trajectory since the 1990s and increasing significantly compared to other notable childhood disorders.[3] Similarly, work based upon the 2016 National Survey of

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Children's Health found the prevalence of parent-reported ASD to be 1 in 40 among children and adolescents aged 0–17 years.[4] The resulting economic burden of ASD in the United States, accounting for medical costs, caretaking, and lost productivity time, is estimated at $268 billion annually.[5,6] Globally, ASD has the highest burden among all mental disorders in children younger than five years of age.[7]

The current understanding of ASD is that it is caused by a combination of genetic predisposition and environmental contributors.[8–10] However, specific knowledge of the biological factors contributing to ASD etiology is generally lacking. As a result, there are no clinically accepted biomarkers for ASD diagnosis and diagnosis is instead made after clinical observation of an individual's behavior. Assessment based solely on behavior introduces substantial variation into the timing of ASD diagnosis, which is reflected in the median diagnosis age being approximately 52 months in the United States[2] despite stable diagnoses being possible by as early as 18 months.[11] The absence of biomarkers can be at least partially attributed to researchers' focus on searching for individual measurements that can identify the disorder; however, due to the heterogeneity and the multisystem nature of ASD, such univariate approaches are unlikely to provide satisfactory diagnostic results.[12] The use of multivariate analyses is likely necessary to uncover meaningful biological relationships for ASD diagnosis.

This review aims to provide a summary of recent advances towards the development of multivariate biomarkers for ASD that hold promise for achieving higher diagnostic accuracy and/or lower ages of diagnosis than the current standards for diagnosis. Towards this goal, we will present the current social and behavioral standards for ASD diagnosis and their shortcomings. We will then discuss progress made in developing multivariate biomarkers from several domains of quantitative measurements, including behavioral, neuroimaging, genetic, and metabolic measurements, and how these biomarkers can contribute to earlier and more accurate ASD diagnosis, risk prediction, and prediction of treatment outcomes. This discussion will be made in the context of differentiating individuals with ASD from typically developing (TD) individuals as most studies in the literature focus on this comparison, although differentiating individuals with ASD from those with developmental delay (DD) or siblings with ASD would also represent relevant clinical goals.

2 The Current Standard of ASD Diagnosis

The diagnostic criteria for ASD are defined in the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders* (DSM) and the World Health Organization's *International Classification of Diseases* (ICD), both of which are outlined below in further detail. In addition to these definitions, ASD screening tools exist to promote awareness and early evaluation. Many of these tools are built off the diagnostic definitions and/or documented behavioral characteristics.[13]

2.1 ASD Diagnostic Definitions

The American Psychiatric Association (APA) maintains diagnostic criteria for ASD in the DSM. The current edition of the DSM (DSM-V) was published in 2013 and defines the two primary criteria for ASD diagnosis as (1) social communication/interaction deficits and (2)

the presentation of restricted and repetitive patterns of behavior.[1] In addition, symptoms must appear during early development, cause functional impairments, and not be explained by other developmental delays/disabilities. By nature of it being a spectrum disorder, a diagnosis of ASD encompasses the symptoms of several disorders (autism, Asperger's syndrome, pervasive developmental disorders (PDDs) not otherwise specified, and childhood disintegrative disorder) that were considered separate diagnoses under the previous edition of the DSM (DSM-IV-TR).[14] All individuals previously diagnosed with any singular pervasive developmental disorder are considered diagnosed with ASD. It has been estimated that ASD prevalence was 4% higher with the DSM-IV-TR definition compared to the current definition in the DSM-V.[2]

Similarly, the World Health Organization (WHO) maintains the ICD, which is currently on its 10th revision (ICD-10) and defines/codes all diseases, disorders, and health-related issues. Contrasting with the "spectrum" designation of the DSM-V, the ICD-10 does not define ASD as its own disorder; instead, its constituent disorders are defined individually and are each coded separately.[15] However, these disorders are all still characterized by socialization and communication deficits as well as repetitive patterns in behavior, with symptoms usually presenting before three years of age.

2.2 Diagnostic Assessments for ASD

The Autism Diagnostic Interview-Revised (ADI-R)[16] and Autism Diagnostic Observation Schedule (ADOS)[17] are two gold-standard instruments used to aid with the diagnosis of ASD. Many diagnostic instruments for ASD other than the ADI-R and ADOS also exist, but a comprehensive discussion of those tools is outside the scope of this review (and we refer the reader to other articles for detailed reviews of these instruments[18–22]). Overall, the variety of tools available in addition to the diverse backgrounds of evaluators (e.g., developmental pediatrician, psychiatrist, neurologist, etc.) contributes to a highly heterogeneous process for ASD diagnosis.

2.3 Trends in Diagnosis Associated with Current Practices

The subjective nature of behavioral diagnoses as well as the heterogeneity of the disorder has contributed to significant variation in the age of ASD diagnosis. Studies have shown stable diagnoses at 36 months,[23] 24 months,[24,25] and even 18 months.[11,26] However, the median age of first ASD diagnosis in the United States is currently estimated at 52 months,[2] and is estimated at 55 months in the United Kingdom.[27] The presence of comorbid conditions introduces variation into the clinical manifestations of ASD, and may affect the age at which ASD's core symptoms are recognized depending on which conditions are present (for example, epilepsy may increase the index-of-suspicion for ASD while anxiety may lower it).[28] Earlier diagnoses are commonly found in boys, in children with an IQ of 70 or less, and in children with developmental regression.[29] Diagnosis age is also affected by a child's individual developmental trajectory[30,31] as well as sociodemographic and socioeconomic factors.[32–35] Lower maternal education and being a racial minority may contribute to a delay in age of ASD diagnosis.[34] Higher maternal age is associated with younger age of first evaluation, although dependent on other sociodemographic variables.[35]

2.4 Treatment Impact Due to an Earlier ASD Diagnosis

It is imperative that ASD diagnoses be made as early as possible to expedite the onset of treatment. Behavioral therapies[36–38] and parent-mediated intervention,[39] among other forms of intervention,[40,41] have been found to offer short- and/or long-term improvement to ASD-related symptoms. Earlier intervention also provides substantial economic savings in the long-term.[42,43] Later ASD diagnosis minimizes or eliminates these benefits, prompting the development of more reliable tests to improve the early identification of ASD. The timing of ASD diagnosis is also critical as it can affect children's eligibility for certain services such as early intervention programs and Individualized Education Plans.[44]

3 Shifting the Paradigm of ASD Diagnosis

ASD is widely acknowledged across the literature as being a multisystem disorder,[45,46] with its pathophysiology engaging the central nervous system,[47] immune system,[48] and digestive system,[49] among others. However, the current criteria for ASD diagnosis fail to consider these far-reaching effects in the body and ignore a large number of potentially valuable markers that could be used to aid in diagnosis and/or screening. Attaining more accurate tests for identifying ASD will require diagnostic standards to reflect the disorder's systemic nature and consider factors beyond its behavioral presentation. Determination of an objective, quantifiable, and biologically-based metric for assessing ASD status, which so far has not been achieved with wide agreement, would signify an important step in this direction. Biomarker-based diagnoses would also be of particular value for high-risk individuals/groups where ASD is more likely to be present, and for populations where ASD diagnoses may be missed more frequently (e.g., girls and certain ethnic groups).

3.1 The Opportunity for ASD Biomarkers

The National Institutes of Health Biomarkers Definitions Working Group define a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."[50] Examples of biomarkers already commonly used in medical practice are glycated hemoglobin levels for diagnosing diabetes,[51] blood cholesterol levels for assessing heart disease risk,[52] and mutations in the BRCA genes that signal increased risk of developing breast cancer.[53] Biomarkers have use not only for diagnosing individuals that currently have a condition or disorder, but also for assessing severity of symptoms, predicting future risk of developing the condition before onset of symptoms, evaluating outcomes of clinical treatment, and even identifying subgroups of individuals with the condition.[12] A reliable biomarker or set of biomarkers for ASD, which should be attainable given the disorder's biological origins, would help to fill in existing gaps in the biological knowledge needed to achieve these degrees of assessment.

Recent explosions in "big data" and "open data" have granted researchers access to vast amounts of information that hold great potential for identifying biomarkers for ASD diagnosis.[54–56] This has greatly accelerated the collection and sharing of genetic, metabolic, and physiological measures (to name a few) that could provide key insights into specific factors contributing to ASD's etiology. However, with these expansive volumes of data also

come large amounts of noise that may cloud the interpretation of potentially useful patterns. It is therefore necessary for data miners, in close collaboration with clinicians,[57] to determine methods for filtering out the unimportant data from those that can be useful for development of potential biomarkers.

3.2 The Role of Multivariate Statistical Modeling in Biomarker Discovery

Historically, many studies have reported biomarker efficacy of a single measurement, such as a metabolic concentration, in terms of the difference in population mean or median between a group of individuals with ASD and a control group. This difference is usually quantified statistically by a *p*-value or a measure of effect size. While such univariate comparisons are useful for identifying biological systems that may be abnormal in ASD, they do not provide information on the value that a measurement may have for diagnosing individuals with ASD; that is, the amount of separation between the groups is not reflected in the univariate statistics. A statistically significant difference between ASD and control (e.g., TD, DD, or siblings with ASD) groups does not necessarily reflect a measurement that can be used for accurately identifying individuals from the two groups (see Figure 1 for an illustration). In this regard, researchers should be cautious when reporting on potential biomarkers of ASD from univariate statistics alone.

Analyses aiming to quantify a biomarker's usefulness for diagnosing individuals with ASD should instead focus on individual-level statistics rather than differences at the population level.[12] In this regard, multivariate statistical modeling offers tremendous potential for biomarker discovery as compared to traditional univariate approaches. As the name suggests, multivariate methods incorporate the contributions of multiple variables at once and model the relationships between these variables to yield an output statistic/score for each individual. Given the complex multisystem nature of ASD, a viable biomarker for the disorder is unlikely to come from a single measurement alone. Multivariate models are more likely to be able to capture relationships, both known and unknown, between systems and variables that are abnormal in ASD pathophysiology.[58]

A subset of multivariate statistical techniques aims to maximize separation between individuals in two or more groups. These model types are useful for classification tasks in which the goal is to distinguish individuals with ASD from TD individuals (or DD individuals or siblings with ASD if desired) and are commonly applied in machine learning algorithms. Furthermore, multivariate and machine learning models can typically be categorized as either supervised or unsupervised techniques. Supervised techniques are those that make use of *a priori* group assignments (i.e., ASD or TD status) to identify patterns for classifying individuals; examples of commonly used supervised techniques include support vector machines (SVMs),[59] logistic regression,[60] discriminant analysis,[61] and classification and regression trees (CART).[62] On the other hand, unsupervised techniques use no *a priori* group information and rely solely on commonalities and differences in the data to assign individuals to groups. Principal component analysis,[63] *k*-means clustering,[64] and self-organizing maps[65] are some examples of unsupervised learning methods.

3.3 Reporting of Biomarker Efficacy

In a majority of cases, multivariate models either produce a binary decision for an individual's predicted class membership or output a continuous score or probability of class membership that can be used to decide on a threshold for classification. When an individual is evaluated by a binary classifier, four outcomes are thus possible. If the individual has ASD and is correctly identified as having ASD, the diagnosis is a true positive; however, if the same individual is incorrectly identified as being TD, the diagnosis is a false negative. Similarly, a diagnosis of a TD individual is either a true negative if they are diagnosed as TD, or a false positive if they are diagnosed with ASD. Since adjusting the classification threshold requires balancing a trade-off between true and false diagnoses, selection of the threshold is usually dependent on the distribution of classifier scores in addition to desired clinical goals.

A number of metrics exist for reporting performance of a binary classifier. Perhaps the most basic is the percentage accuracy, which is simply the number of true positives plus true negatives divided by the total number of individuals. Sensitivity can be calculated as the number of true positives divided by the total number of individuals with ASD, while specificity is the number of true negatives divided by the total number of TD individuals. Positive predictive value (PPV) and negative predictive value (NPV) are calculated as the number of true positives divided by the total number of positive diagnoses and the number of true negatives divided by the total number of negative diagnoses, respectively. A receiver operating characteristic (ROC) curve can also be constructed to graphically represent the diagnostic performance of a classifier; this curve plots the sensitivity against one minus the specificity for a number of different classification thresholds, where both axes vary from 0 to 1. The area under the ROC curve (AUROC) is a commonly reported metric to summarize the diagnostic value of a medical classifier[66] and its possible values typically range from 0.5 to 1, where a value of 0.5 indicates an uninformative classifier and a value of 1 is associated with a perfect classifier. Visual examples of these metrics are provided in Figure 2.

Clinical studies often involve a large number of measurements being recorded for relatively few participants. With these types of data, it is easy to develop a model that uses many variables to perfectly classify a small number of individuals. However, such a small sample-to-variable ratio increases the likelihood of overfitting the model to the data set such that its generalizability to new data is minimal. Overfitting is a possibility at any sample size but is a greater concern when the number of samples is small. It is thus essential when reporting results to provide some form of validation of classifier performance on a data set that was not used to develop the model. This can be done by implementing a cross-validation procedure in which a subset of samples are held out from training and reserved for model validation[67] or, in the ideal case, by evaluating the classifier on an entirely new set of validation data that was not involved in model development. Prediction accuracy on the validation set can be expected to be lower than that in the training set but should still be sufficiently high to suggest meaningful patterns for further investigation in larger clinical data sets.

4 Multivariate Approaches for Behavioral Biomarkers

Diagnostic instruments such as the ADI-R and ADOS have shown consistent reliability in terms of their sensitivity and specificity for identifying individuals with ASD. However, being that these types of tools contain a number of questions covering various domains of behavior, it is possible that a number of these evaluated behaviors provide information unnecessary for ASD diagnosis or even information that could lead to an inaccurate diagnosis in some cases.[68] There are also complex patterns in behavior that cannot be measured by behavioral interviews or observation alone that may contribute to a more accurate ASD diagnosis. With these considerations in mind, a number of recent studies in the literature have aimed to use multivariate models to reduce the number of behavioral screening items needed for ASD diagnosis and/or obtain more advanced measures of behavior that can be used to bolster the diagnostic ability of current instruments.[69]

4.1 Prediction of Future ASD Diagnosis from Early Behavioral Evaluations

One of the primary concerns associated with early ASD diagnoses is their long-term stability at older ages,[70] as a false positive diagnosis is almost as undesirable as a delayed diagnosis. In light of this, it would be of great interest to determine subsets of items from established diagnostic instruments that could be used to simplify the process of early ASD diagnosis. The potential benefits of such an approach are highlighted in a study of high-risk children from the Baby Siblings Research Consortium that reported children with early false positive diagnoses to have behavioral test scores that were overall similar to the scores of children with early false negative diagnoses, but that might have differed in individual symptoms that would have been able to differentiate these two groups.[26] Screening for ASD using only the most important behavioral items may thus provide a more precise indicator of future ASD diagnosis in addition to reducing time and costs associated with screening.

The search for early behavioral markers of ASD has prompted several studies, summarized in Table 1, to use multivariate techniques to identify subsets of behavioral symptoms that are most predictive of a later ASD diagnosis. In most cases, these studies feature high-risk children who have an older sibling with ASD, where the risk of ASD diagnosis in these high-risk children is an estimated 18.7%[71] and is substantially greater than the overall population risk of 1.7%.[2] The use of infants in some studies also introduces uncertainty as it can be difficult at this age to differentiate behaviors associated with normal development from those associated with ASD (this distinction may not become possible until nine months[30] to twelve months[72] of age). Relying on behavioral markers alone to predict a future ASD diagnosis thus requires that the child be old enough for parents and/or clinicians to make this distinction accurately, making usage of such markers somewhat counterproductive when the objective is to identify ASD as early as possible.

4.2 Reducing the Number of Behavioral Measurements Needed for ASD Diagnosis

Identification of a subset of the most important items from instruments such as the ADOS or ADI-R has the potential to streamline the ASD diagnosis process and may also increase diagnostic accuracy. Such an approach also holds promise for the development of mobile ASD screening tools that can assist with diagnosing ASD on a large scale.[73–76] Table 2 lists

recent studies that have determined the most important items from behavioral evaluations and used these items to identify individuals with ASD. The availability of data repositories containing behavioral score sheets has allowed for many of these studies to analyze larger samples of participants compared to the aforementioned studies aiming to predict future ASD diagnoses. In addition to the analyses involving greater numbers of samples, the classification results are generally more accurate, which is not unexpected given that the most important behaviors chosen for classification are likely those that originally contributed to the ASD diagnosis being made. The greater accuracy can also likely be explained by the reduced complexity of diagnosis, relatively speaking, compared to predicting a future diagnosis from current behavioral symptoms. It would be of interest to evaluate how well the behaviors identified by these ASD diagnosis studies could be used to predict future diagnoses.

The push to reduce the number of variables required for ASD diagnosis has encouraged the development of novel computational techniques for identifying the most important variables. One computational intelligence method, known as Variable Analysis, analyzes measurements from ASD screening tools and considers correlations between variables and classes (ASD or TD) while minimizing correlations between variables. This technique has been shown to maintain good accuracy in children, adolescents, and adults assessed with the Autism Spectrum Quotient.[77] Multilayer fuzzy cognitive maps are another approach that offer promise for improving ASD diagnoses based on subsets of ADOS and ADI-R measures. This type of model has shown results comparable in accuracy to other machine learning algorithms.[78] Speech and psychoeducational therapy outcomes in preschoolers with ASD have also been successfully predicted by using artificial neural networks to determine the behavioral variables most important for assessing treatment response.[79]

4.3 Motor Skills, Eye Gaze and Vocal Patterns as Diagnostic Markers

Delays in early motor skills may become apparent in children with ASD before the stereotypical deficits in behavior and socialization become recognizable,[80–83] introducing motor skill development as a potential early indicator of the disorder.[84] Similarly, quantifiable eye gaze and attention patterns in young children may serve as objective indicators of ASD[85] before the presentation of behavioral symptoms, as early as two to six months of age.[86,87] Studies recently investigating these behavioral domains for ASD diagnosis are summarized in Table 3 and show a wide range of outcomes, both in terms of the numbers/types of key features used and the classification accuracies achieved. Other studies have performed classification with eye tracking and attention metrics using only univariate methods.[88,89] Additionally, changes in eye gaze have been found to be accurate indicators of improvement in social attention resulting from behavioral intervention[90] and may be useful for clinical trials,[91] thus reinforcing the potential of these patterns as early biomarkers of ASD.

Given that the diagnostic criteria for ASD include socialization and communication deficits, it stands to reason that abnormal vocalization patterns may also serve as early biomarkers for the disorder.[92] To this end, a number of studies have aimed to use quantified vocalization patterns to differentiate individuals with ASD from TD individuals. According to a recent

review and meta-analysis,[93] however, the methods and features used for multivariate analysis vary greatly between such studies, limiting overall interpretation of the predictive value of these patterns.

4.4 Behavioral Patterns from Toy Interactions

Technology, in a broad sense, plays a central role in many modalities of potential ASD diagnosis beyond those evaluating motor, eye gaze, and vocal patterns. Using technology to quantify features that can assist with diagnosis allows for controlled and consistent interactions among multiple patients in different environments; this offers value for conditions like ASD where guided interactions may be difficult.[94,95] Sensors embedded into toys, in particular, can provide quantifiable measures of behavior, such as gesture forces and kinematic pattern data, that are not offered by parental or clinical evaluation and that are easily related to traditional ASD diagnostic criteria. For example, multivariate analysis of social and behavioral features taken from the interactions between children and a robotic parrot has distinguished ASD from TD with exceptional accuracy.[96] Evaluating children's patterns of playing with a toy car[97] and smart tablets[98] have also shown promise for ASD screening. This approach to screening would be feasible for a wide number of smart toys and opens the door for creative ways to characterize behaviors associated with ASD.

5 Medical Imaging for ASD Diagnosis

Neuroimaging may hold many avenues for ASD biomarker discovery.[99,100] Compared to their TD peers, children with ASD have been found to have different features of neuroanatomy such as brain morphometry, neurochemical components, and structural and functional connectivity, many of which correspond to brain regions associated with socio-emotional, communication, and restricted repetitive behaviors related to an ASD diagnosis.[99] Socio-emotional areas, also known as the "social brain," exhibit abnormalities in individuals with ASD and include areas for analysis of facial expression and eye gaze, emotional processing, theory of mind, and imitation and understanding.[101] Restricted repetitive behaviors are associated with abnormalities within the default mode network.[102] The advancement of pattern recognition, machine learning, and neuroimaging techniques has facilitated the development of novel image-based tools for identifying ASD.[103]

Although the advancement of neuroimaging has elucidated many mysteries of ASD pathophysiology, research is still far from obtaining a coherent and complete picture of associated brain anomalies.[101] Every neuroimaging technique uses different methodologies and relies on unique signal sources, frequency bands, spatial resolutions, neural parameters, and analysis techniques accompanied by respective limitation and noise artifacts.[101,104] Few studies have investigated the use of multimodal neuroimaging strategies, for which improved methods of integration are needed.[104] Even within the same imaging technique, inconsistent findings occur from distinct methodological variables such as field of view and type of data set.[105] Development of uniform protocols have emerged to ameliorate these disparities, which only leads to the constant push for improvement of accepted practices and ever changing "best technique."[101] There is also a question as to the cause of brain anomalies and whether they can be attributed to comorbid conditions (such as epilepsy) rather than ASD.

[106] While certain imaging and functional techniques such as magnetic resonance imaging (MRI), electroencephalography (EEG), and magnetoencephalography (MEG) are universally available, some may be restricted to scientific investigations[107] and inappropriate for clinical use.

5.1 Structural MRI

Structural MRI analysis examines brain connectivity, or the existence of abnormal anatomical connections such as axonal or synaptic arrangement. It also provides a non-invasive measurement of white matter tracts, white and grey matter regions, regional thickness, and brain volume. A review on structural MRI noted a common finding of increased growth in total cortical volume, as well as subcortical brain regions and cerebellum in children with ASD.[99] Interestingly, large brain volumes are not noted in adults with ASD compared to their TD peers; instead, brain volume growth is suggested to slow from child to adulthood.[108]

Studies using structural features for ASD classification are outlined in Table 4. A significant concern with many of these studies is the large number of features required (relative to the number of samples) to achieve higher accuracies. As a general rule, the number of samples should be several factors greater (typically by a factor of five or ten) than the number of features used; however, this ratio is far less in many of the listed studies, and in some cases the number of features comes close to exceeding the number of samples. This raises the possibility of overfitting to the available data, which is a concern not necessarily alleviated by cross-validation or validation on a small independent set, and brings into question the reproducibility of some results. Further evaluation of these feature sets on much larger cohorts of participants would help to alleviate concerns of overfitting.

Two studies listed utilize multimodal imaging, including both structural and functional MRI; for further discussion on functional MRI classifications we direct the reader to these studies.[109,110] Within their methodologies, Libero *et al.* also used ^{1}H-magnetic resonance spectroscopy, a non-invasive imaging technique that estimates specific brain metabolites through their chemical composition.[110] Another study tested multiple machine learning techniques, examining regional thickness-based and volume-based classification through SVM, multilayer perceptron, functional trees, and logistic model trees.[111] Both thickness- and volume-based classifications yielded their best results through the use of logistic model trees with overall better separation from using thickness-based classification.

For further review on using neuroimaging to predict brain disorders, we direct the reader to a recent summary of MRI-based ASD classification studies that discusses how many studies suffer from overfitting, small sample size, non-optimized classifier parameters, and other limiting factors.[112]

5.2 Functional MRI

MRI images that provide information on the functional connectivity of the brain (functional MRI, or fMRI) focus on patterns of activity via connective pathways. fMRI experiments commonly use structured cognitive tasks, such as social stimuli (faces and bodies).[113] However, these studies show varying results in part due to the many challenges they face,

including limited current diagnostic standards, small sample size, and validation results.[114] Diffusion tensor imaging studies local connectivity and white matter tracts, providing details on functional features through common methodologies of tractography and voxel-wise analysis. The two most common features measured are directionality (quantified by functional anisotropy) and diffusion (quantified by mean diffusivity). Studies commonly show decreased functional anisotropy[99,115] and increased mean diffusivity[99] in the brains of individuals with ASD compared to their TD peers. Tract-based spatial statistics is an emerging diffusion tensor imaging methodology that overcomes voxel-wise dependency on smoothing kernels.[99]

MRI studies focus heavily on individuals without intellectual disability, also referred to as high-functioning individuals, who can withstand long, motionless procedures as well as receive and follow instructions.[116] Resting state functional connectivity MRI (rs-fMRI) studies require no conscious thought-process and allow children to be scanned while asleep. rs-fMRI measures correlated and anti-correlated signals and, in children with ASD, commonly finds altered functional connectivity in the default mode network, hyper-connectivity in subcortical regions, and under-connectivity between cortical regions.[99] In addition to recruiting children with ASD able to undergo an MRI scan, sample sizes for these studies are negatively affected by the cost of the technique.

A list of fMRI (including rs-fMRI) studies employing multivariate analysis to classify ASD can be found in Table 5. The accuracy results achieved in some of these studies are notably high, even after cross-validation, while others only showed modest potential for accurate ASD diagnosis. However, like with the structural MRI studies, there are legitimate concerns of overfitting with several of these fMRI studies due to their low sample-to-feature ratios. In addition to these classification studies, the potential of using fMRI with multivariate analysis for predicting treatment outcomes in individuals with ASD has been investigated.[117,118]

5.3 ABIDE Studies

The major limitation of small sample size in neuroimaging studies has been combatted by recent research collaboratives. The Autism Brain Imaging Data Exchange (ABIDE),[119] ARIANNA,[120] and National Database for Autism Research[121] are research data repositories dedicated to collecting and sharing data to promote ASD research. Our review of these repositories will be limited to ABIDE, which collects rs-fMRI images from multiple research sites. However, these data sets are subject to between-site differences in experimental procedures,[122] and even with increasing training set sizes, many studies report accuracies of approximately 60–70%.[123–128] Various techniques, including deep learning, have been investigated to improve classification accuracy using the ABIDE data sets and are listed in Table 6. Despite the greater numbers of samples used in these studies, several rely on extremely large numbers of features that bring into question the reproducibility of their findings; reducing the sizes of these feature sets is thus essential to assess clinical applicability of these approaches.

5.4 Brain Activity Measures

Functional and effective connectivity can also be measured through EEG and MEG. Measurements from EEG have shown differentiating activity for children with ASD, specifically beta and theta waves.[129] MEG has been used to determine local and long-range functional connectivity, analyzed using a multiple regression model, and was found to correlate with age in pre-adolescent children with ASD.[130] A list of recent ASD classification studies using EEG and MEG can be found in Table 7; the classification accuracies after cross-validation in several of these studies are notable considering the small numbers of features used. Functional near-infrared spectroscopy has also been used to observe differentiating visual and auditory brain responses in children later diagnosed with ASD.[131]

While EEG and MEG are less expensive techniques compared to MRI, they have their own sets of limitations. Connectivity measures from EEG/MEG are estimated to be proportional to the physiological connectivity, and there are also differences in head and brain size between children with ASD and their TD peers that may bias results (e.g., producing dissimilar distances between sensors and electrical signal propagation); EEG and MEG are also limited to short-range connectivity and are unable to evaluate long-range functional connectivity.[132]

6 Genetic and Epigenetic Markers of ASD

Evidence points to ASD having a strong genetic component, with one meta-analysis estimating heritability of the disorder to be 52%.[8] The increased rate of recurrence in younger siblings of affected children[71] further indicates a genetic role in ASD etiology. Evaluation of genetic variants is a common approach to studying the role of specific genes in ASD,[133] but this approach only provides a statistical indicator of ASD risk[134] and yields no diagnostic information about the disorder. More physiologically dynamic measures of ASD status can be found by analyzing gene expression patterns via transcriptomics,[135–137] which measures RNA levels and is more indicative of the functional consequences of genetic variation in ASD. Potential contributions from a wide array of environmental factors in ASD[9] are also suggestive of a role for epigenetic regulation whereby the expression of certain genes is influenced by external triggers. It naturally follows that such genetic and epigenetic information may provide value for screening and diagnosis of ASD.

6.1 Gene Expression

Transcriptomics studies involve measuring levels of RNA, typically messenger RNA, to quantify gene expression in body tissues. These measurements are commonly taken from blood, providing a straightforward path for clinical translation to an ASD biomarker. Table 8 summarizes recent studies that use multivariate analyses to distinguish individuals with ASD from TD individuals on the basis of their transcriptomics patterns. Classification accuracies in these studies are generally high and the sources of study data are diverse, with some studies making use of data repositories while others take their own measurements from specific cell types. Like with the neuroimaging studies, however, there are concerns here about small sample-to-variable ratios in several studies.

6.2 Epigenetic Activity

Epigenetic mechanisms reflect environmental contributions to a disorder/disease state and may offer further potential for ASD biomarkers.[138] One particular focus of research in this area is microRNAs, which interact with messenger RNA to provide post-transcriptional regulation of gene expression.[139–141] Although microRNA is not as widely analyzed as gene expression, quantification of microRNA provides a unique aspect to studying how genetic factors can be leveraged for ASD diagnosis. Recent papers looking at the use of microRNA for diagnosis are reviewed in Table 8, with blood and saliva samples having both been used with varying levels of success. Everything else equal, a saliva-based test would be preferred as it would be less invasive than a blood test and would likely be more appealing for younger patients. The investigation by Hicks *et al.*[142] using salivary RNA levels (including, but not limited to, microRNAs) for identification of ASD obtained good accuracy in a validation set with a relatively large number of participants; these results are encouraging for the use of epigenetic markers in diagnostic testing.

7 Metabolic Markers

Abnormalities in metabolism may underlie the etiology of ASD[40] and have become a popular focal point of ASD biomarker research. Metabolite studies measured through peripheral tissue are relatively inexpensive, allow large sample sizes and time-dependent sampling, and are well-suited for a clinical setting.[143] However, a barrier to effective implementation is the need to control for environmental/external factors such as diet. The following subsections discuss metabolite studies collected from peripheral tissues and their roles in the development of ASD biomarkers.

7.1 Blood Metabolites

Analysis of blood is minimally invasive and can provide information on various types of cellular components. While DNA, RNA, and proteins have unique structural compositions, metabolite structure and physical properties vary and no single technique exists to comprehensively measure them.[144] Concentrations of blood metabolites are also closely influenced by diet, medication, metabolite solubility, and possible hemolysis during sample processing, necessitating a level of rigor in controlling for these factors. Some studies have looked at discrimination of ASD and TD individuals from blood metabolites using univariate approaches[145–147] with varying degrees of success. Studies using multivariate analysis to further characterize blood-based metabolic patterns for ASD classification are listed in Table 9. There is clearly substantial variation in the types of metabolites measured and the classification accuracies offered by analysis of these metabolites. Plasma markers from two particular metabolic pathways, the folate-dependent one-carbon metabolism and transsulfuration pathways, have shown exceptional promise as potential ASD biomarkers and are discussed in further detail later in this review.

In a retrospective immunoassay study, logistic regression was able to differentiate children diagnosed with ASD using 15 serum biomarkers measured from newborn blood spot specimens.[148] Although newborn blood spot analysis represents an opportunity at birth for predicting a future ASD diagnosis, it also faces many sources of uncertainty spanning from

the pre-analytical stage of blood collection to the actual analysis of the blood. Among these are the quality of the dried blood spots, choice of collection card, variation in sample quality due to biological factors (such as viscosity and hematocrit level), and contamination.[149] Additionally, metabolic profiles at birth may not necessarily be reflective of abnormal metabolism/pathophysiology that manifest later in life and a future ASD diagnosis may also depend upon environmental factors during early childhood that an analysis at birth cannot take into account. If all or even some of these factors can be accounted for, however, then newborn blood spots may be a promising route for early identification of ASD cases.

Plasma amino acids[150] and erythrocyte fatty acids[151] have frequently been suggested as possible biomarkers of ASD. However, methodological variation across studies raises uncertainty regarding the conclusions of these investigations. In addition, previous studies by the authors employing multivariate analyses[152,153] have reported little diagnostic value from these measures. Therefore, specific discussions of these measurements have been excluded from this review.

7.2 Excretory Metabolites

Metabolomics studies may also analyze the composition of the body's excreted waste products. Compared to other discussed approaches, the collection of urinary and fecal samples is relatively easy and noninvasive. However, the compositions of these samples are mostly reflective of compounds leaving the body, and can be difficult to interpret with respect to abnormal metabolic processes in the body. For example, it may not always be clear whether an elevated concentration of a fecal metabolite indicates increased bodily intake and/or production of this metabolite, overactive excretion of this metabolite, or a combination of factors. Similar to blood metabolites, excretory measures are also influenced by dietary and lifestyle factors that should be controlled for to aid interpretability. The results of studies using multivariate analysis to study urinary and fecal metabolites indicate a reasonable ability to classify ASD from these measurements (Table 10), albeit in small samples of participants and with a limited range of multivariate techniques used.

8 Diagnostics Involving Folate-Dependent One-Carbon Metabolism (FOCM)

Folate, not naturally synthesized in animals, activates folate-dependent one-carbon metabolism (FOCM). Folate is important for biosynthesis of purine and thymidine, maintaining amino acid homeostasis by converting glycine to serine and synthesizing methionine, maintaining epigenetics through homocysteine re-methylation, and producing and consuming redox species.[154] The transsulfuration (TS) pathway is intertwined with FOCM and is responsible for the production of glutathione, a primary antioxidant that assists with maintaining intracellular redox status and removal of toxins from the body.[155] Abnormalities within these pathways, specifically a reduced ratio of S-adenosylmethionine to S-adenosylhomocysteine (suggesting decreased capacity for DNA methylation) and an increased ratio of oxidized to reduced glutathione (indicating greater levels of intracellular oxidative stress), have been closely associated with ASD pathophysiology and also may offer value towards a diagnostic marker.[156]

8.1 Discovery of Folate-Dependent One-Carbon Metabolism (FOCM) Biomarkers

One of the most promising candidates to date for a clinical ASD biomarker is a blood-based test using FOCM/TS measurements. In a previous study by the authors, seven FOCM/TS measures from the Integrated Metabolic and Genomic Endeavor study[157] related to methylation and redox status were used to distinguish 83 children with ASD from 76 TD children (age 3–10) with 97% classification accuracy, 98% sensitivity, and 96% specificity via discriminant analysis with leave-one-out cross-validation.[158] This work also uncovered significant correlations between FOCM/TS metabolites and measures of adaptive behavior, suggesting a connection between metabolic abnormalities and observed symptoms. An independent investigation analyzed the same data set while implementing a more advanced method of selecting measurements for classification and achieved similar classification results, providing validation of the initial findings.[159]

A follow-up study using baseline FOCM/TS data for 154 children/adolescents with ASD (age 2–17) from three clinical trials[160–162] was able to validate a trained classifier with up to 88% sensitivity[163]; measures of specificity were not evaluated since this validation set did not include TD participants. Using the same sets of clinical trial data, it was further found that multivariate analysis of changes in FOCM/TS measurements could be used to characterize changes in adaptive behavior resulting from three metabolic interventions (methylcobalamin with low-dose folinic acid, tetrahydrobiopterin, or high-dose folinic acid).[164] Multivariate analysis of folate-related markers collected from a separate study in China also showed promise for diagnosing ASD.[165] Measurements from FOCM and TS describing DNA methylation and redox status may thus offer value not just as diagnostic markers, but also as indicators of treatment outcome. Despite the success of these studies, it still remains to be determined whether these measurements would be able to predict a future ASD diagnosis in infants and toddlers, which should be the true goal of any biomarker for the disorder. Furthermore, it needs to be investigated how results hold up for classifying children with ASD from children who have non-ASD related developmental delays. A current barrier to clinical translation of a FOCM/TS blood test is that certain measurements require specialized equipment and/or methods to quantify that are currently not widely available in clinics. It would be desirable to identify a subset of these measurements that yield reasonable diagnostic value while being easily measured by a standard blood panel.

8.2 Accounting for Epidemiological Prevalence

To successfully translate a classifier's performance in a study cohort to its clinical potential in the population at large, the epidemiological prevalence of the investigated disorder or disease must be accounted for.[166] In studies of psychiatric disorders, such as ASD, where the prevalence in the study population is often much greater than the true epidemiological prevalence, a classifier will likely underestimate the number of false positives and yield a relatively inflated number of false negatives. While this will have no effect on classifier sensitivity and specificity, it will provide an inaccurate representation of the positive and negative predictive values for the proposed diagnostic test. Obtaining a more accurate estimate of a classifier's clinical utility requires that the Bayes' adjusted positive and negative predictive values (PPV_{adj} and NPV_{adj}, respectively) be calculated[166] by incorporating the true population prevalence:

$$PPV_{adj} = \frac{sensitivity \times prevalence}{sensitivity \times prevalence + (1 - specificity) \times (1 - prevalence)} \quad (1)$$

$$NPV_{adj} = \frac{specificity \times (1 - prevalence)}{(1 - sensitivity) \times prevalence + specificity \times (1 - prevalence)} \quad (2)$$

Most studies do not account for epidemiological prevalence and instead limit their calculation of PPV and NPV to the study population, thus introducing the aforementioned uncertainty regarding applicability of the classifier to the overall population. Including only a population of individuals at high risk for the disorder in the study can help to alleviate this concern, as the study prevalence is likely to be more reflective of the epidemiological prevalence in a high-risk population. Furthermore, in a clinical setting it would likely be impractical, both logistically and economically, to test everyone from the overall population for a disorder; thus, limiting the classifier's scope to just high-risk individuals or individuals where there are already concerns regarding their development would improve overall translation potential. That being said, there would still be value in understanding the prevalence of the disorder in the high-risk study population compared to the high-risk population at large.

For classifiers with exceptional separation between ASD and TD study cohorts, lack of adjustment for epidemiological prevalence will likely have a less noticeable impact when translated to a clinical setting. Introducing a greater proportion of participants without the disorder when the two groups are already well-separated may yield relatively minor changes to the positive and negative predictive values. In cases where this does not hold, however, the threshold for classification can be moved to optimize the trade-off between these two values. To help demonstrate this point, Figure 3A shows the result of classification (with leave-one-out cross-validation) from FOCM/TS measurements presented by Howsmon *et al.*[158] At the original classification threshold, the sensitivity, sensitivity, PPV, and NPV are all high (Figure 3B); however, with a 1.7% overall prevalence of ASD, the PPV_{adj} and NPV_{adj} become 30.0% and 99.9%, respectively. Clearly such a low PPV_{adj} is unsatisfactory, but by a slight adjustment of the classification threshold a more optimal PPV_{adj} and NPV_{adj} can be obtained while maintaining good sensitivity and specificity (Figure 3C). At this new adjusted threshold, which correctly classifies all TD participants, the PPV_{adj} is 100% and the NPV_{adj} is 99.9% (with 95% sensitivity and 100% specificity, compared to 98% sensitivity and 96% specificity with the original threshold). As can be seen from these numbers, adjusting the threshold is a viable approach for trading off classification accuracy with desired clinical outcomes such as higher PPV_{adj}.

8.3 Heterogeneous Presentation of ASD and Biomarker Discovery

One of the points often brought up in the context of discussions of biomarkers and diagnostic tests for ASD is the very heterogeneous presentation of the disorder. It is becoming widely-acknowledged that there is no one underlying cause for all the different presentations of ASD and that a variety of different, often still unknown, gene-environmental interactions are responsible for ASD. However, identifying biomarkers does

not require identification of one or more root causes of ASD. Instead, biomarkers and diagnostic tests rely on correlations of measured quantities with the disorder and understanding causation, while highly desirable, is not required. Developing diagnostic procedures based upon biomarkers is thus a much weaker condition than determining one of the root causes underlying ASD. For example, it is conceivable that most of the children diagnosed with ASD have certain abnormalities in their metabolic function even if they show very different presentations of ASD, which may also result from different underlying causes; the data from the papers cited in this section certainly indicate that this seems to be the case.

Another point to consider for future work in this field is that children with ASD may show certain abnormalities in their metabolic function that are similar to those exhibited by children with other non-ASD-related conditions. To address this point, it is essential to not just perform comparisons of a cohort diagnosed with ASD against their TD peers, but also to perform comparison of children with ASD against children who have non-ASD-related developmental delays. Only such a comparison will be able to determine if the metabolic abnormality found when investigating ASD versus TD cohorts are indeed unique to ASD or may be indicative of a more general developmental delay (that may or may not be related to ASD).

9 Prediction of ASD Risk based on Maternal Factors

Maternal factors affect the risk of having a child with ASD. As previously discussed, the sibling recurrence rate of ASD is approximately 11-fold greater than the general population risk.[71] Therefore, already having a child with ASD is an indicator for being at higher risk of having another child with ASD (high-risk mothers) compared to their peers who have TD children (low-risk mothers). An emerging area of ASD biomarker research involves the application of multivariate analysis to maternal measures, such as metabolite concentrations and medical history, to predict the risk of the child being diagnosed with ASD. The number of studies in this area are limited, but warrant discussion. It should be noted that the timing of the maternal assessment (prenatal versus postnatal, for example) would likely affect the findings of these types of assessments.

9.1 Maternal Blood Metabolites

The Markers of Autism Risk in Babies — Learning Early Signs (MARBLES) study is a prospective study investigating environmental factors in high-risk pregnant mothers that contribute to a child being eventually diagnosed with ASD.[167] A recent study compared twenty FOCM/TS measurements of pregnant high- and low-risk mothers from blood samples at each trimester using multivariate analysis[168] with the goal of identifying stronger predictors of child ASD risk. In this study, a single set of metabolites was found to predict if a mother belonged to the high- or low-risk category with 80–90% accuracy. These results present a step towards determining a maternal blood test for ASD risk in a child, and emphasize the importance of the FOCM and TS pathways in ASD pathophysiology. However, further validation of these findings is required.

In another study, maternal serum levels of vitamin D were found to be moderately predictive of the risk of a pregnant mother having a child with ASD.[169] Among several serum measurements evaluated at the first trimester of pregnancy, decreased 25 hydroxyvitamin D3 was the strongest indicator of whether the child would later be diagnosed with ASD (on a univariate basis), yielding a sensitivity of 76% and specificity of 74%.

9.2 Maternal Medical History

The application of multivariate analysis to health surveys and interviews may allow for further probing of individuals' medical characteristics. One study interviewed mothers of children with ASD and TD children to investigate various risk factors for ASD, such as parental ages at conception or chemical exposures during pregnancy, and used these data to train an artificial neural network for differentiating ASD and TD children.[170] Employing an optimized form of two-fold cross-validation, this study attained a mean overall accuracy of 80% for classifying the two groups. Such analysis can also provide insight into the maternal factors that contribute to the risk of ASD.

10 Conclusions

Despite the many promising advances being made towards a viable multivariate biomarker for ASD, there still remain several barriers that need to be carefully considered. One of these is cost; although state-of-the-art screening tools may offer the greatest opportunity for early identification of ASD, these tools must also be affordable if they are to be successfully implemented on a large scale. Second, the heterogeneous, "spectrum" nature of ASD means that limiting classifier/biomarker designations to "having ASD" or "not having ASD" leaves a large grey area where the unique risk profiles of different children are not accurately represented[171]; different subtypes of ASD[172] may have different biomarkers, limiting the utility of a one-size-fits-all approach to diagnostic testing. Factors such as gender differences, the presence of comorbid conditions, and changes in brain function throughout life, and how these affect biomarker interpretation, also need to be eventually addressed.[142] For the majority of the reviewed studies, their findings also still require validation in independent cohorts, which would ideally come from separate research groups. Governmental approval of these approaches and assessing their feasibility in a clinical setting, not just a research setting, also pose significant challenges to successful implementation.

Although each of the approaches covered in this review have their individual advantages, they also have their individual disadvantages. For example, behavioral assessments are established, inexpensive, and the least invasive but are the most subjective and require specialized training. Neuroimaging approaches provide arguably the most complete picture of brain structure/function but are expensive and especially reliant on patient compliance; the link of brain structure to behavior will also need to be better understood. Transcriptomic and metabolic blood tests are fast and inexpensive but may require specialized laboratory tests and may be difficult to interpret without behavioral context. Implementing a multimodal approach to diagnosis may help to alleviate some of the respective concerns with these approaches while still capitalizing on their unique advantages.

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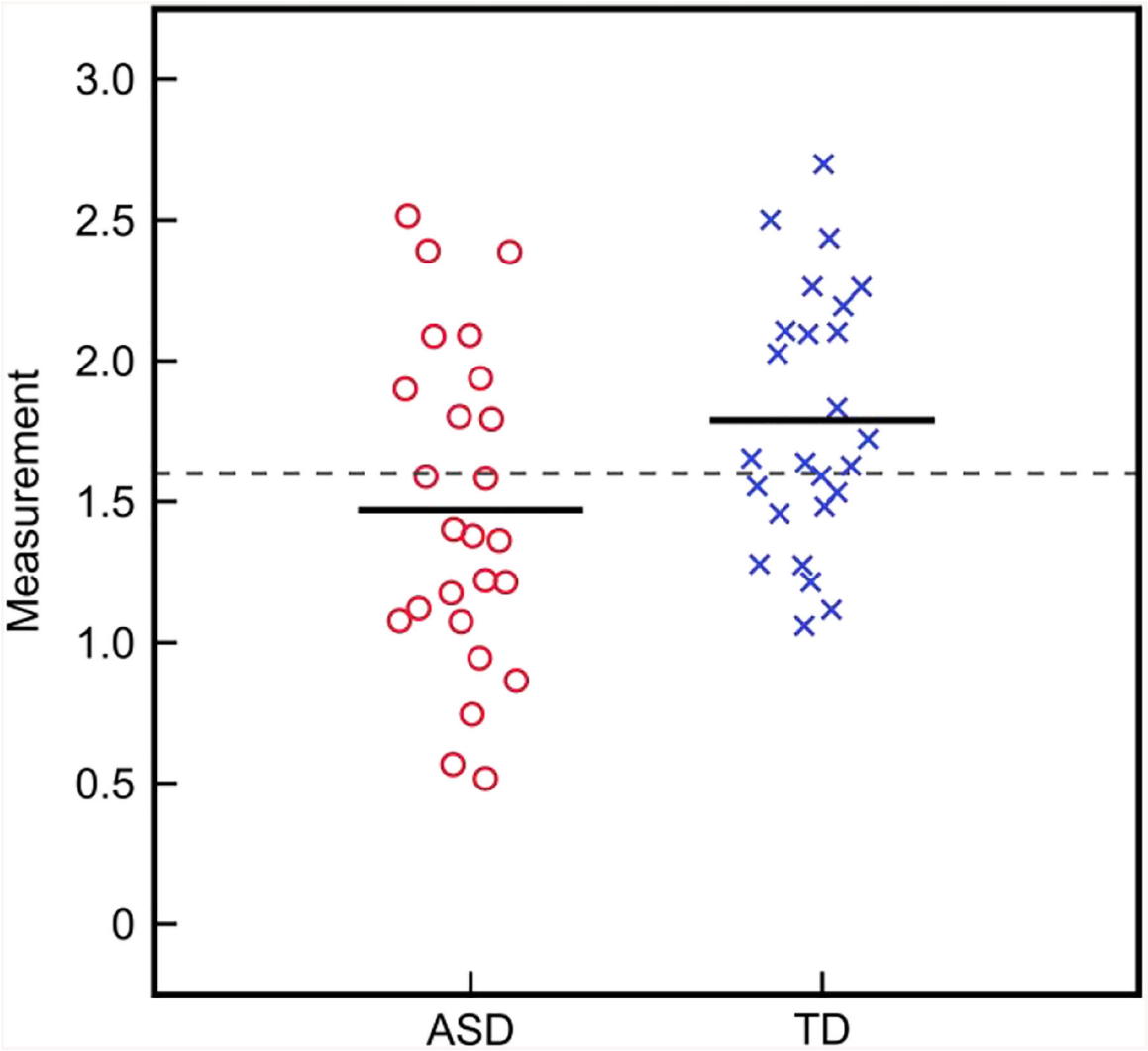

Fig 1.

Example data for an arbitrary quantity measured in 25 individuals with autism spectrum disorder (ASD) and 25 typically developing (TD) individuals. Values for both groups are sampled from normal distributions with equal variances. Comparison of group means (denoted by the solid horizontal lines) with Student's *t*-test yields a *p*-value of 0.033, indicating a statistically significant difference at a significance level of $\alpha = 0.05$. However, the difference in group means is not sufficient for meaningful classification due to the overlap in the distributions of the measurements of the ASD and TD groups.

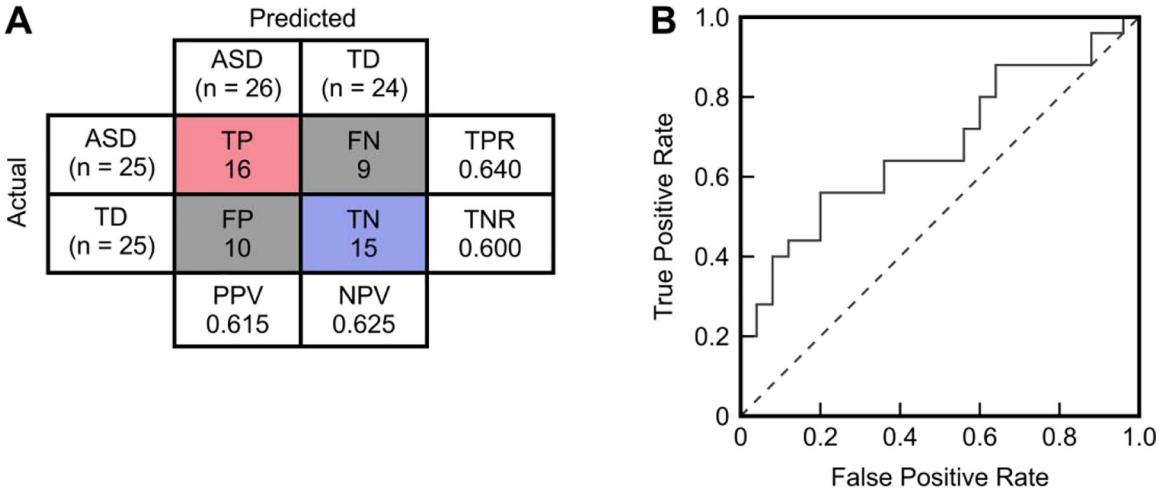

Fig 2.

Accuracy metrics for the example data provided in Figure 1. (A) Confusion matrix yielded by classifying any sample/individual with a value less than 1.6 as having autism spectrum disorder (ASD). The number of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) are used to calculate the sensitivity/true positive rate (TPR=TP/[TP+FN]), specificity/true negative rate (TNR=TN/[TN+FP]), positive predictive value (PPV=TP/[TP+FP]), and negative predictive value (NPV=TN/[TN+FN]). The overall classification accuracy is (TP+TN)/(TP+TN+FP+FN) = 62%. (B) Receiver operating characteristic (ROC) curve (solid line) plotting the false positive rate (1 – specificity) against the true positive rate (sensitivity) as the threshold for classification is varied. The area under this ROC curve (AUROC) is 0.68. An AUROC of 0.5 indicates a completely uninformative classifier and would be associated with a curve like the dashed line pictured.

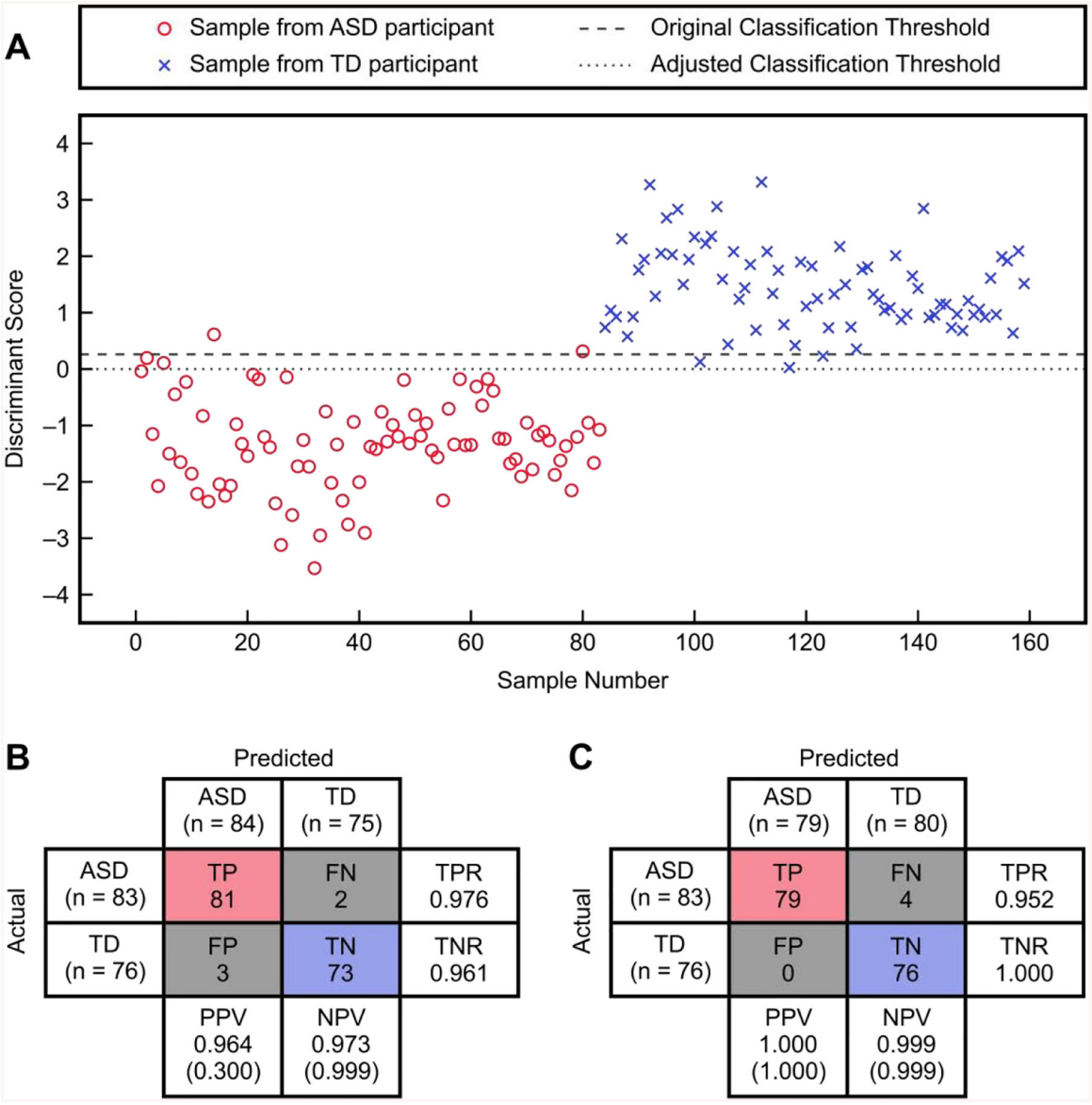

Fig 3.

Classification with leave-one-out cross-validation using seven measurements from the folate-dependent one-carbon metabolism (FOCM) and transsulfuration (TS) pathways as reported by Howsmon *et al.*[158] (A) Classifier scores for participants in the autism spectrum disorder (ASD) and typically developing (TD) cohorts. (B) Confusion matrix when individuals having a classifier score less than 0.26 (the original classification threshold) are predicted as having ASD. Assuming a 1.7% prevalence of ASD in the general population, the Bayes' adjusted positive and negative predictive values, indicated in parentheses, are 30.0% and 99.9%, respectively. (C) Confusion matrix when individuals having a classifier score less than 0 (the adjusted classification threshold) are predicted as having ASD. The Bayes' adjusted positive and negative predictive values using this threshold, indicated in parentheses, are 100% and 99.9%, respectively.

Table 1.

Summary of recent and representative studies predicting future ASD diagnosis through multivariate analysis of early behavioral evaluations. Reported sample sizes are the numbers used for classification and do not necessarily reflect the study's total sample size.

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
Macari *et al.* (2012)[173]	13 infants that were later diagnosed with ASD and 71 that were not	Evaluated behaviors at 12 months that would be predictive of ASD diagnosis at 24 months	Seven individual items from the ADOS-Toddler	Classification tree	Classified ASD versus non-ASD with 85% sensitivity and 96% specificity
Chawarska *et al.* (2014)[174]	157 high-risk infants that were later diagnosed with ASD and 562 that were not	Assessed behaviors at 18 months that would be predictive of ASD diagnosis at 36 months	Six individual items from the ADOS	CART	Classified ASD versus non-ASD with 83% training accuracy and predicted with 77% validation accuracy
Barbaro and Dissanayake (2017)[24]	77 children at risk for ASD identified from a community-based sample	At 24 months, assessed ASD status and behavior that would predict retention or loss of ASD diagnosis at 48 months	Four items total from the ADOS and Mullen Scales of Early Learning	Logistic regression	Classified the stable group 96% correctly and the crossover group 44% correctly
Bussu *et al.* (2018)[175]	32 high-risk infants that were later diagnosed with ASD and 129 that were not	Examined behavior and developmental measures at 8 and 14 months to predict ASD status at 36 months	Motor scores at 8 months and daily living score at 14 months	Least-squares SVM	Best AUROCs for classifying ASD versus non-ASD at 36 months were 0.65 and 0.71 using the 8-month and 14-month measures, respectively

Table 2.

Summary of recent and representative studies aiming to reduce the number of behavioral measures needed for ASD diagnosis through multivariate analysis. Reported sample sizes are the numbers used for classification and do not necessarily reflect the study's total sample size.

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
Wall *et al.* (2012)[176]	891 ASD and 75 non-spectrum children for training; 1976 ASD and 1000 simulated controls for validation	Used ADI-R data from three ASD data repositories	7 of 93 items from the ADI-R	Alternating decision tree, chosen from 15 algorithms	99.9% training accuracy, with both 99.9% sensitivity and specificity when predicting the validation set
Wall *et al.* (2012)[177]	612 ASD and 15 non-spectrum children for training; 446 ASD and 1000 simulated controls for validation	Used ADOS data from three ASD data repositories; pilot study for Duda *et al.* (2014)[178]	8 of 29 items from ADOS Module 1	Alternating decision tree, chosen from 16 algorithms	100% training accuracy, with 99.7% sensitivity and 94% specificity when predicting the validation set
Duda *et al.* (2014)[178]	2333 ASD and 283 non-spectrum children	Used ADOS data from five ASD data repositories	ADOS Module 1 feature set from Wall *et al.* (2012)[177]	Alternating decision tree	Validated with 98% sensitivity and 77% specificity against the original ADOS
Wilson *et al.* (2014)[179]	58 male adults with ASD and 66 TD controls	Administered three ASD evaluations and nine neuropsychological tests/tasks	Ten variables from performed tasks, plus verbal IQ and performance IQ	SVM	Achieved 81% accuracy, 78% sensitivity, and 85% specificity with leave-two-out cross-validation
Kosmicki *et al.* (2015)[180]	362 (510) ASD and 282 (93) non-spectrum individuals for training; 1089 (1924) ASD and 66 (214) non-spectrum for validation†	Used data from five ASD data repositories; evaluated score sheets separately for ADOS Module 2 and Module 3	9 of 28 behaviors from Module 2 and 12 of 28 behaviors from Module 3	Logistic regression (Module 2); radial kernel SVM (Module 3)	99% sensitivity and 89% specificity for Module 2 validation; 98% sensitivity and 97% specificity for Module 3 validation
Bone *et al.* (2016)[181]	1264 verbal individuals with ASD and 462 verbal individuals without ASD	ADI-R and Social Responsiveness Scale items taken from a data repository	Five behavioral codes total from the two assessments	SVM	Classified individuals below (above) age 10 with 89% (87%) sensitivity and 59% (53%) specificity
Cohen *et al.* (2016)[182]	535 children with ASD and 125 children without ASD	PDD Behavior Inventory forms collected from five sites	Six domain scores of PDD Behavior Inventory, parent-reported	CART	82%/83%/86% sensitivity and 88%/87%/93% specificity for training/testing/validation
Levy *et al.* (2017)[183]	1319 (2870) ASD and 70 (273) non-ASD children†	ADOS Module 2 and Module 3 score sheets from four ASD data repositories	Nine items from Module 2 and nine from Module 3	Logistic regression; SVM	Classified with 89%/95% sensitivity and 90%/87% specificity for Module 2/3
Feczko *et al.* (2018)[184]	47 children with ASD and 58 TD children	Had children perform seven tasks related to information processing	34 behavioral variables related to performed tasks	Random forest	Achieved 73% classification accuracy, 63% sensitivity, and 81% specificity

†Numbers outside (inside) parentheses indicate sample sizes for analyzing ADOS Module 2 (Module 3).

Table 3.

Summary of recent and representative studies aiming to distinguish individuals with ASD from TD individuals using multivariate analysis of motor skill development and eye gaze/tracking patterns. Reported sample sizes are the numbers used for classification and do not necessarily reflect the study's total sample size.

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
Motor Patterns					
Crippa *et al.* (2015)[185]	15 children with ASD and 15 TD children	Recorded kinematics data while children performed a reach-to-drop task	Seven kinematic features	SVM	Mean sensitivity/ specificity of 82%/89% with leave-one-out cross-validation
Dehkordi *et al.* (2015)[96]	35 children with ASD and 16 TD children	Evaluated children's social and behavioral interactions with a robotic parrot	Six behavioral features	Random forest	Classified with a maximum of 90% accuracy using seven-fold cross-validation
Anzulewicz *et al.* (2016)[98]	35 children with ASD and 45 TD children	Recorded kinematic and gesture data from children playing with tablet computers	262 motor features derived from the tablet sensor data	Regularized greedy forest, among other techniques	Achieved a maximum average AUROC of 0.93 with ten repetitions of ten-fold cross-validation
Li *et al.* (2017)[186]	14 adults with ASD and 16 TD controls	Derived kinematic parameters from a hand movement imitation task	Nine kinematic parameters (from two imitation conditions)	SVM, among others	Achieved 87% accuracy, 86% sensitivity, and 88% specificity using a two-step cross-validation method
Moradi *et al.* (2017)[97]	25 children with ASD and 25 TD children	Evaluated movement characteristics of children playing with a smart toy car	Five movement characteristics	Polynomial kernel SVM	Averaged 93% sensitivity and 76% specificity with five-fold cross-validation
Eye Gaze/Tracking					
Stahl *et al.* (2012)[187]	19 high-risk infants with a sibling with ASD, 17 control infants with no ASD in family	Recorded EEG and measured event-related potentials associated with eye gaze processing	36 event-related potential (18 direct gaze, 18 averted gaze) metrics	SVM	Classified high-risk versus control with 64% sensitivity and 64% specificity
Fujioka *et al.* (2016)[188]	21 adolescents and adults with ASD and 35 TD controls	Measured percentage of eye fixation time on objects displayed on a screen	Discrimination parameters from three visual areas of interest	Discriminant analysis	Classified with 81% sensitivity and 80% specificity
Liu *et al.* (2016)[189]	29 children with ASD and 58 TD children	Analyzed children's eye movements during a facial recognition task	Histograms of visual attention to partitioned facial regions	Radial basis function kernel SVM	With leave-one-out cross-validation, achieved 89% accuracy, 93% sensitivity, and 86% specificity
Frazier *et al.* (2018)[190]	91 youth diagnosed with ASD and 110 non-ASD youth	Recorded eye tracking patterns of participants while viewing a video containing 44 visual stimuli	Gaze metrics correlating significantly with ASD diagnosis	Multiple linear regression with ROC analysis	Achieved AUROC of 0.92 and 0.86 in the training set (75% of samples) and validation set (25%)
Wan *et al.* (2018)[191]	37 children with ASD and 37 TD children	Measured children's fixation time on ten areas of interest while watching a short video of a young female speaking	Fixation time on the body and mouth	SVM	Classified with 85% accuracy, 87% sensitivity, and 84% specificity

Table 4.

Summary of recent and representative studies aiming to distinguish individuals with ASD from TD individuals using multivariate analysis of structural MRI. Reported sample sizes are the numbers used for classification and do not necessarily reflect the study's total sample size.

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
Ecker *et al.* (2010)[192]	22 adults with ASD and 22 TD adults	Used structural MRI to obtain images of grey and white matter regions	Voxels from grey matter images	SVM	Classified with 77% sensitivity and 86% specificity using leave-two-out cross-validation
Jiao *et al.* (2010)[111]	22 children with ASD and 16 TD children	Measured regional thickness and volumetric morphometry of 66 brain structures via MRI	7 thickness-based features and, separately, 16 volume-based features	Logistic model tree, among others	Attained 95%/77% sensitivity and 75%/69% specificity for best thickness-/volume-based classification with ten-fold cross-validation
Ingalhalikar *et al.* (2011)[193]	45 children and adolescents with ASD and 30 TD controls	Computed region-based fractional anisotropy and mean diffusivity maps for diffusion tensor imaging data	18 out of 352 fractional anisotropy/mean diffusivity features	Radial basis function kernel SVM	Achieved 80% accuracy, 74% sensitivity, and 84% specificity with leave-one-out cross-validation
Ingalhalikar *et al.* (2014)[109]	75 children with ASD and 37 TD children	Evaluated two functional tasks using MEG and 74 structural white matter features using diffusion tensor imaging	Two MEG features and 12 diffusion tensor imaging features	Ensemble of classifiers fused with weighted aggregation	Averaged 73% sensitivity and 86% specificity with five-fold cross-validation; 87% accuracy on testing set
Wee *et al.* (2014)[194]	58 children and adolescents with ASD and 59 TD controls	Used structural MRI to evaluate cortical-related morphology (regional and interregional features)	Combination of regional and interregional features	Multi-kernel SVM	Achieved an average of 96% sensitivity and 97% specificity with two-fold cross-validation
Gori *et al.* (2015)[195]	21 children with ASD and 20 TD children	Calculated brain features and global volumes of brain compartments from structural MRI data	314 region of interest features from the grey matter sub-region	SVM	Averaged 0.74 AUROC with leave-pair-out cross-validation
Jin *et al.* (2015)[196]	40 infants at high risk for ASD and 40 low-risk infants	Derived connectivity features from multiscale connectivity networks measured through MRI; compared high- and low-risk participants	Multiscale regions of interest and diffusion statistics	Multi-kernel SVM	Used nested five-fold cross-validation to obtain averages of 76% accuracy and 0.80 AUROC
Libero *et al.* (2015)[110]	19 adults with ASD and 18 TD adults	Analyzed brain morphometry from structural MRI, diffusion tensor imaging, and proton magnetic resonance spectroscopy data	Fractional anisotropy, radial diffusivity, and cortical thickness	Decision tree	Classified participants with 92% accuracy after leave-one-out cross validation
Hazlett *et al.* (2017)[197]	34 (145) infants at high risk for ASD with (without) a later diagnosis of ASD	Evaluated brain volume and surface area metrics from MRI at 6 and 12 months to predict ASD at 24 months	Regional surface area, intracranial volume, cortical thickness, and sex	Three-stage deep neural network	With ten-fold cross-validation, predicted ASD with 88% sensitivity and 95% specificity
Shen *et al.* (2017)[198]	47 (174) infants at high risk for ASD with (without) a later diagnosis of ASD	Quantified cerebrospinal fluid and lateral ventricle volume from MRI data collected at 6, 12, and 24 months to predict ASD diagnosis at 24 months	Extra-axial cerebrospinal fluid volume	Balance-boosted trees ensemble algorithm	Predicted ASD with 66% sensitivity and 68% specificity after 25-fold cross-validation; similar results on a validation set

Table 5.

Summary of recent and representative studies aiming to distinguish individuals with ASD from TD individuals using multivariate analysis of functional MRI data. Reported sample sizes are the numbers used for classification and do not necessarily reflect the study's total sample size.

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
Deshpande *et al.* (2013)[199]	15 adolescents and young adults with ASD and 15 TD controls	Gathered fMRI data to study causal connectivity among different brain regions relating to Theory of Mind	19 features related to effective connectivity paths	SVM	Classified participants with maximum 96% accuracy, 97% sensitivity, and 95% specificity
Uddin *et al.* (2013)[200]	20 children with ASD and 20 TD children	Collected rs-fMRI and structural MRI data, then identified ten connectivity components associated with functional brain networks	Salience network connectivity features	Logistic regression	Achieved 75% sensitivity and 80% specificity with leave-one-out cross-validation; also validated on an independent cohort
Plitt *et al.* (2015)[201]	59 young adults with ASD and 59 TD controls; replication set with 89 ASD and 89 TD controls	Collected rs-fMRI data and defined three sets of regions of interest to create three unique correlation matrices for participants' time series	Destrieux atlas set describing 162 regions	Radial basis function kernel SVM, among others	Observed a maximum 77% accuracy with leave-one-out cross-validation (among other methods); results did not improve in replication set
Chanel *et al.* (2016)[113]	15 adults with ASD and 14 TD adults	Gathered fMRI data to study attention/emotions of participants during static faces and dynamic bodies tasks	Features from dynamic body experiment	SVM	Classified with maximum 92% sensitivity and 92% specificity with leave-one-out cross-validation
Yahata *et al.* (2016)[202]	74 adults with ASD and 107 TD adults; 44/27 individuals with ASD and 44/27 TD controls in validation sets 1/2	Evaluated functional connectivity from rs-fMRI; also examined generalizability to other disorders	16 out of 9730 functional connections	Logistic regression	Achieved 85% accuracy with leave-one-out cross-validation; validated with 75% and 70% accuracies in independent cohorts
Emerson *et al.* (2017)[203]	11 (48) infants at high risk for ASD with (without) a later diagnosis of ASD	Computed features of functional connectivity from rs-fMRI at 6 months to predict ASD diagnosis at 24 months	59 sets of features (one for each fold of leave-one-out cross-validation)	SVM	Predicted future diagnosis with 82% sensitivity and 100% specificity using leave-one-out cross-validation

Table 6.

Summary of recent and representative studies aiming to distinguish individuals with ASD from TD individuals using multivariate analysis of ABIDE imaging data. Reported sample sizes are the numbers used for classification and do not necessarily reflect the study's total sample size.

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
Zhou *et al.* (2014)[204]	127 children with ASD and 153 TD children	Obtained rs-fMRI data from ABIDE repository and used a multi-parametric analytic approach, including network analysis to study connectivity	4 of 22 quantitative imaging features	Random decision tree	Classified with 98% accuracy for the full data set and 68% accuracy when using ten-fold cross validation
lidaka (2015)[205]	312 children and adolescents with ASD and 328 TD controls	Examined rs-fMRI data taken from ABIDE to analyze functional connectivity through correlation matrices	632 cells from the correlation matrix	Probabilistic neural network	Achieved 89% accuracy, 92% sensitivity, and 87% specificity using leave-one-out cross-validation
Kam *et al.* (2017)[206]	61 individuals with ASD and 72 TD individuals, all under 20 years old	Acquired rs-fMRI data from ABIDE data site to distinguish functional networks through hierarchical clustering	Connectivity features from five clusters	Discriminative restricted Boltzmann machine	Using ten-fold cross-validation, classified with 75% sensitivity and 85% specificity
Sadeghi *et al.* (2017)[207]	29 adolescents and adults with ASD and 31 TD controls	Analyzed properties of functional networks constructed from MRI images in the ABIDE data set	17 features from nodal metrics	SVM	Averaged 92% classification accuracy with five-fold cross-validation; 68% accuracy in independent set
Syed *et al.* (2017)[208]	392 individuals with ASD and 407 age- and sex-matched TD controls	Identified reproducible independent components of functional networks from ABIDE rs-fMRI data	Regions from the default mode network	*k*-means clustering	Clustering yielded 89% sensitivity and 90% specificity
Bi *et al.* (2018)[209]	45 individuals with ASD and 39 TD individuals	Evaluated connectivity from ABIDE rs-fMRI data through application of graph theory	272 graph metrics	Random SVM cluster	Obtained accuracies as high as 96% on the testing subset (26 samples, or 30% of total)
Heinsfeld *et al.* (2018)[210]	505 individuals with ASD and 530 TD individuals	Constructed connectivity matrices using correlations for regions' time series averages using ABIDE rs-fMRI data	19900 functional connectivity features	Deep neural network	Achieved 70% accuracy, 74% sensitivity and 63% specificity with ten-fold cross-validation
Kong *et al.* (2019)[211]	78 individuals with ASD and 104 TD individuals	Analyzed brain connectivity through networks based on cortical regions constructed from ABIDE MRI data	3000 of the top cortical grey matter volume features	Deep neural network	Classified with up to 90% accuracy, 84% sensitivity, and 96% specificity using ten-fold cross-validation

Table 7.

Summary of recent and representative studies aiming to distinguish individuals with ASD from TD individuals using multivariate analysis of EEG and MEG data. Reported sample sizes are the numbers used for classification and do not necessarily reflect the study's total sample size.

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
Bosl *et al.* (2011)[212]	46 infants at high risk for ASD, and 33 low-risk controls	Collected EEG data and computed modified multiscale entropy as an indicator of normal brain development	Low, high, and mean multiscale entropy values for each of 64 channels	*k*-nearest neighbors, SVM, naive Bayes	Classified with accuracies between 72% and 77% in 9-month-olds using ten-fold cross-validation
Duffy and Als (2012)[213]	430 children with ASD and 554 TD children	Calculated spectral coherence variables from EEG measurements	40 spectral coherence factors	Discriminant analysis	Averaged 86% sensitivity and 89% specificity across ten split-half analyses and including all age groups
Khan *et al.* (2013)[214]	17 adolescents and young adults with ASD and 20 TD controls	Measured task-related local and long-range functional connectivity from MEG data	Four functional connectivity metrics	Quadratic discriminant analysis	Classified with 90% accuracy, 87% sensitivity, and 95% specificity
Jamal *et al.* (2014)[215]	12 children with ASD and 12 TD children	Extracted brain connectivity features from EEG measurements	4 of 36 brain connectivity features	Polynomial kernel SVM	With leave-one-out cross-validation, achieved 95% accuracy, 86% sensitivity, and 100% specificity
Khan *et al.* (2015)[216]	15 children and adolescents with ASD and 20 TD controls	Evaluated functional connectivity using tactile and resting state MEG recordings	Local functional connectivity index, Granger causality	Discriminant analysis	Achieved 87% sensitivity and 90% specificity using ten-fold cross-validation
Khan *et al.* (2016)[217]	15 children and adolescents with ASD and 20 TD controls	Used MEG and structural MRI to investigate abnormal functional connectivity	Three neurophysiological measures	Discriminant analysis	Averaged 90% sensitivity and 95% specificity with ten-fold cross-validation
Bosl *et al.* (2018)[171]	35 infants later diagnosed with ASD and 153 infants with no ASD diagnosis	Collected EEG measurements from 3 to 36 months of age to predict ASD diagnosis by 36 months of age	Subset of nonlinear invariant signal features selected from 1026 total	Radial basis function kernel SVM	Predicted ASD with 82–100% sensitivity and 88–99% specificity, depending on age, using leave-one-out cross-validation

Table 8.

Summary of recent and representative studies aiming to distinguish individuals with ASD from TD individuals using multivariate analysis of patterns in gene expression and epigenetic activity. Reported sample sizes are the numbers used for classification and do not necessarily reflect the study's total sample size.

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
Gene Expression					
Glatt *et al.* (2012)[218]	60 infants and toddlers at risk for ASD and 68 TD controls	Evaluated children's profiles of messenger RNA expression in peripheral blood mononuclear cells	Expression intensities of 48 probes	Radial basis function SVM	Predicted a replication sample (half of samples) with 93% sensitivity, 88% specificity, and 0.91 AUROC
Kong *et al.* (2012)[219]	66 (104) children with ASD and 33 (82) non-ASD controls for training (validation)	Profiling of blood gene expression levels in participants	55 genes	Partial least squares	Obtained 0.98 AUROC in the training set and 0.70 AUROC (68% accuracy) in the validation set
Hu and Lai (2013)[220]	87 individuals with ASD and 29 non-ASD individuals	Gene expression profiling of lymphoblastoid cell lines using DNA microarrays	74 genes	SVM	Achieved 91% sensitivity and 61% specificity with leave-one-out cross-validation
Latkowski and Osowski (2015)[221]	82 children with ASD and 64 TD children	Used gene expression data from a publicly available database	Unspecified number of genes used in ensemble classifier	Gaussian kernel SVM with ensemble of classifiers	Classified with 96% sensitivity and 83% specificity with ten-fold cross-validation
Pramparo *et al.* (2015)[222]	87 (44) toddlers with ASD and 55 (29) non-ASD toddlers for discovery (replication)	Profiling of leukocyte RNA expression in participants	Four co-expression modules containing 762 unique genes	Logistic regression	Achieved 75% accuracy, 77% sensitivity, and 72% specificity in replication set
Guan *et al.* (2016)[223]	104 children with ASD and 82 non-ASD controls	Used data on peripheral blood gene expression from Kong *et al.* (2012)	Three unique sets of five genes	Distance from multivariate centroid	In the validation set (half of samples), classified with 72%–76% accuracy
Nazeen *et al.* (2016)[224]	671 total samples from human ASD studies	Used high-throughput gene expression data from data repositories for conditions that co-occur with ASD	Genes overlapping the chemokine and Toll-like receptor signaling pathways	SVM, among others	Classified ASD versus non-ASD with average 70% classification accuracy with three-fold cross-validation
Oh *et al.* (2017)[225]	21 young adults with ASD and 21 TD controls	Used a microarray data set publicly available from a database	19 differentially expressed probes	SVM, *k*-nearest neighbors, discriminant analysis	Achieved up to 94% accuracy, 100% sensitivity, and 87.5% specificity on the validation set (16 samples)
Epigenetic Activity					
Mundalil Vasu *et al.* (2014)[226]	55 individuals with ASD and 55 TD controls	Measured microRNA profiles in serum of participants	Five differentially expressed microRNAs	ROC analysis†	Classified with AUROC up to 0.91, with associated 85% sensitivity, 87% specificity
Hicks *et al.* (2016)[227]	24 children with ASD and 21 TD children	Measured salivary microRNA levels	14 top-ranked microRNAs	Partial least squares	Classified with 100% sensitivity and 96% specificity (AUROC = 0.97).
Cirnigliaro *et al.* (2017)[228]	30 children with ASD and 25 TD children	Profiled serum expression of microRNAs	One microRNA, miR-140–3p	Logistic regression	Averaged 63% sensitivity and 68% specificity with 100-random subsampling cross-validation
Hicks *et al.* (2018)[142]	238 children with ASD and 218 non-ASD children	Measured salivary levels of five subtypes of RNA, including microRNA	32 RNAs	Radial kernel SVM	Predicted the test set (84 total samples) with 82% sensitivity and 88%

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
					specificity (AUROC = 0.88)

† Study performs classification, but only through univariate approaches.

Table 9.

Summary of recent and representative studies aiming to distinguish individuals with ASD from TD individuals using multivariate analysis of potential blood-based metabolite biomarkers. Reported sample sizes are the numbers used for classification and do not necessarily reflect the study's total sample size.

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
Momeni *et al.* (2012)[229]	22 children with ASD and 27 TD children	Analyzed plasma protein/peptide concentrations using mass spectrometry	Three differentially expressed peptides	Discriminant analysis	Classification of samples without hemolysis yielded 95% sensitivity and 85% specificity
West *et al.* (2014)[230]	52 children with ASD and 30 TD children	Measured concentrations of plasma metabolites through various mass spectrometry-based techniques	80 or 160 metabolites depending on classifier	SVM, partial least squares discriminant analysis	Predicted 21-sample validation set with AUROC of 0.84 (SVM) and 0.81 (partial least squares)
Wang *et al.* (2016)[231]	73 (100) children with ASD and 63 (100) TD children in discovery (validation) sets	Quantified serum metabolites with ultra-performance liquid chromatography and mass spectrometry	Docosahexaenoic acid and sphingosine 1-phosphate	Logistic regression	Achieved 90% sensitivity and 74% specificity for predicting the validation set
Howsmon *et al.* (2017)[158]	83 children with ASD and 76 TD children	Analyzed levels of plasma markers related to DNA methylation and oxidative stress	Seven transmethylation/transsulfuration measurements	Discriminant analysis	Classified with 98% sensitivity and 96% specificity using leave-one-out cross-validation
Anwar *et al.* (2018)[232]	38 children with ASD and 31 TD children	Investigated protein damage through quantification of glycation end-products in plasma and urine analysis	Four plasma protein adduct residues and two amino acids	SVM, among several other techniques	Observed 89% accuracy, 90% sensitivity, and 87% specificity using two-fold cross-validation
Barone *et al.* (2018)[233]	83 children with ASD and 79 TD children	Quantified acyl-carnitine and amino acid levels from dried blood spot specimens collected at time of the study	Eight acyl-carnitines	Naive Bayes	Predicted a 38-sample holdout set with 73% sensitivity and 63% specificity
Chen *et al.* (2018)[234]	32 children with ASD and 20 TD children	Profiled the serum proteome using fractionation and mass spectrometry techniques	Eight differentially expressed protein peaks	*k*-nearest neighbors	Achieved 99% sensitivity and 87% specificity using cross-validation
Shen *et al.* (2018)[235]	30 children with ASD and 30 TD children	Used isobaric tags for relative and absolute quantitation to measure medium- and low-abundance plasma proteins	Five plasma proteins	Combined ROC analysis	Classified with 0.98 AUROC, better than the AUROCs of the individual proteins
Howsmon *et al.* (2018)[163]	154 children and adolescents with ASD, compiled from three clinical trials	Validated classification with DNA methylation/oxidative stress markers presented by Howsmon *et al.* (2017)[158]	Five transmethylation/transsulfuration measurements	Discriminant analysis, among other techniques	Predicted an independent validation set of individuals with ASD with up to 88% sensitivity
Smith *et al.* (2019)[236]	253 (263) infants with ASD and 85 (79) TD infants in training (testing) set	Examined amino acid dysregulation metabotypes (AADMs) in blood plasma	Six AADMs	Ratios of AADMs to different amino acids	In the test set, sensitivities ranged from 8–14% and specificities ranged from 92100%
Zou *et al.* (2019)[165]	89 children with ASD and 89 TD children	Measured serum concentrations of folate-related metabolites	Six folate-related markers	Discriminant analysis	Correctly classified 84% of participants using leave-one-out cross-validation (87% sensitivity, 85% specificity)

Table 10.

Summary of recent and representative studies aiming to distinguish individuals with ASD from TD individuals using multivariate analysis of potential excretory (urinary and fecal) metabolite biomarkers. Reported sample sizes are the numbers used for classification and do not necessarily reflect the study's total sample size.

Reference	Study Participants	Experimental Methods	Key Features	Multivariate Technique	Key Results
Nadal-Desbarats *et al.* (2014)[237]	30 children with ASD and 28 TD children	Measured urinary metabolite profiles combined from two nuclear magnetic spectroscopy techniques	Minimum number of metabolites combined from both techniques	Partial least squares discriminant analysis	Achieved a prediction accuracy of 83% with 0.92 AUROC
Diémé *et al.* (2015)[238]	30 children with ASD and 32 TD children	Evaluated urine metabolite levels using nuclear magnetic spectroscopy and mass spectrometry techniques	46 metabolites combined across techniques	Partial least squares discriminant analysis	Predicted a 16-sample validation set with 0.91 AUROC, 100% sensitivity, and 75% specificity
Gevi *et al.* (2016)[239]	30 children with ASD and 30 TD children	Quantified urinary metabolite concentrations through liquid chromatography and mass spectrometry	25 urinary metabolites	Partial least squares discriminant analysis	Classified individuals with 0.89 AUROC
Kang *et al.* (2018)[240]	21 children with ASD and 23 TD children	Assess metabolite profiles and microbial compositions in participants' fecal samples	Five fecal metabolites	Discriminant analysis	With leave-one-out cross-validation, obtained 78% sensitivity and 81% specificity