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Review Article

Current Progress and Challenges in the Search for Autism Biomarkers

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Autism spectrum disorders (ASD) encompass a range of neurodevelopmental conditions that are clinically and etiologically very heterogeneous. ASD is currently diagnosed entirely on behavioral criteria, but intensive research efforts are focused on identifying biological markers for disease risk and early diagnosis. Here, we discuss recent progress toward identifying biological markers for ASD and highlight specific challenges as well as ethical aspects of translating ASD biomarker research into the clinic.

1. Introduction

Autism is a neurodevelopmental disorder diagnosed primarily on clinical criteria. The core clinical manifestations of autism consist of deficits of social communication, language impairments, and repetitive-restrictive behaviors. Comorbid conditions such as intellectual disability, epilepsy, anxiety, and depression are frequently associated with autism [1]. The hallmark of autism's clinical picture is its marked heterogeneity: no two autism patients are alike. Each autistic individual presents with a unique combination of symptom severity in the core domains and a variable mix of comorbid conditions. The clinical heterogeneity of autism, encompassing large variations in disease severity from markedly impaired individuals who need permanent care, to highly functioning patients who fulfill higher education and are entirely self-sufficient, has led to the concept that autism is in fact a spectrum of conditions, rather than a single disease [2]. It is worth noting that mild impairments in language abilities and social communication are also observed as normal variation in the general population and are more frequent among relatives of autistic individuals [2], further supporting the concept that autism encompasses a spectrum of phenotypic variation.

The current Diagnostic and Statistical Manual of Mental Disorders (DSM-IV TR [3]) defines several distinct pervasive developmental disorders: Asperger syndrome, autistic disorder, pervasive developmental disorder not otherwise specified (PDD-NOS), childhood disintegrative disorder (CDD), and Rett syndrome. The first three conditions are generally included under the definition of autism spectrum disorders (ASD). While autistic disorder consists of deficits in all three core domains: language, social interaction, and repetitive-restrictive behaviors, and is often associated with cognitive deficits, Asperger syndrome patients have normal language development and normal cognition. Patients with PDD-NOS have deficits in at least one of the core domains but do not meet the clinical criteria for autism or any of the other pervasive developmental disorders. ASD are four times more prevalent among males than among females, and the overall prevalence of ASD has increased in recent years to a current estimate of 0.5%–1% depending on the study and world area [4].

Recent research has shown that although different clinicians assess the symptoms of a given patient in a very similar manner, the diagnostic ascertainment of these symptoms as autistic disorder, Asperger syndrome, or PDD-NOS varies from clinician to clinician [5]. In addition, the recent increase in ASD prevalence might partially reflect insufficient specificity of current diagnostic criteria [6]. In order to increase the specificity and reduce the variability of ASD diagnosis, the

updated DSM-V manual [6] proposes significant revisions of autism classification and diagnostic criteria. A detailed discussion of the updated DSM-V criteria is beyond the scope of this review and has been addressed by several recent papers [6, 7]. In brief, instead of defining three distinct conditions: Asperger syndrome, autistic disorder, and PDD-NOS, DSM-V proposes a single diagnosis of ASD. The DSM-V ASD diagnosis is based on two core domains rather than three: social communication (which includes language and social behavior) and repetitive-restrictive behaviors. ASD is then further subclassified in three levels of severity (levels 1–3). In addition, a novel category of Social Communication Disorder will be added, describing individuals with significant social and communication difficulties similar to those observed in ASD, but without repetitive or restricted behaviors [3].

Several aspects of the updated DSM-V diagnostic criteria are relevant to our discussion of ASD biomarkers, given that the specificity of novel biomarkers can only be as good as the standard diagnostic criteria, used for selecting the research cohorts [8]. First, the need for majorly revised ASD diagnostic criteria highlights the fact that conceptual understanding of autistic symptomatology is still developing in the clinical community. Thus, significant variability in the composition of ASD case cohorts from various studies should be taken into account when interpreting the ASD biomarker data. Second, the inclusion of all three ASD disorders under a single diagnosis might lead to future research cohorts being phenotypically less homogeneous, unless the study design includes careful examination of the symptomatology in all core domains. Asperger patients and patients with deficits restricted to one of the core domains will receive the same diagnosis as patients with full-blown autism and may be included in the same research cohort. On the other hand, the DSM-V criteria are stricter than the previous DSM-IV criteria, and thus, patients diagnosed with ASD by DSM-V may be more homogeneous in terms of severity, regardless of the specific symptomatology that contributes to the disease. A recent study noted that many of the milder Asperger and PDD-NOS cases diagnosed using DSM-IV criteria would not be diagnosed with ASD by DSM-V [7], but this observation remains to be confirmed after DSM-V criteria become widely used.

Identifying biomarkers for ASD has been the focus of intense research since the first description of the disease in the early 1940s [9], but no ASD biological marker has yet demonstrated enough sensitivity and specificity to be translated into the clinic. This review begins with an overview of the specific needs and challenges of identifying biomarkers for ASD and then discusses recent advances toward biomarker development for this complex disorder.

2. General Considerations on ASD Biomarkers

Broadly defined, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [10]. Thus, disease biomarkers include any measurable characteristic, such as DNA sequence variation, MRI imaging, and blood and urine metabolites, can be used as an indicator of disease risk, diagnosis, or prognostic (Figure 1).

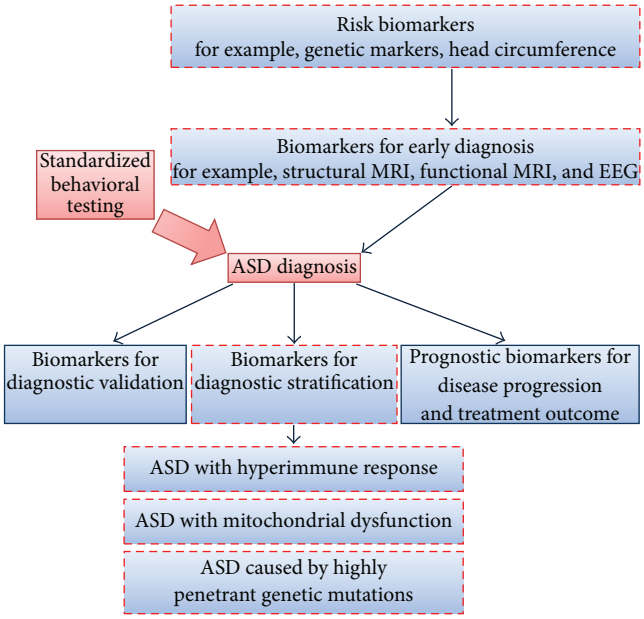

Figure 1: Schematic classification of ASD biomarkers and their stage of development. Biomarkers shown with a solid red border (genetic markers for syndromes with high incidence of ASD) are already being used in the clinic; biomarker classes shown with a dashed red border are the subject of intensive research and show preliminary encouraging results; biomarker classes shown with a dark blue border are yet under development. The red arrow highlights the fact that at present the ASD diagnosis is established solely on standardized behavioral criteria. Risk biomarkers and biomarkers for early diagnosis may be applied before standard behavioral testing, while biomarkers for diagnostic validation and stratification and prognostic biomarkers would be employed after establishing an ASD diagnosis based on behavioral testing.

(i) *Risk biomarkers* are used for identifying individuals at risk for developing the disease and often include genetic markers.

(ii) *Diagnostic biomarkers* are aimed toward *early diagnosis* (by screening the general population or a selected group in order to diagnose the disease before symptomatology occurs), *diagnostic validation*, and *diagnostic stratification*.

(iii) *Prognostic biomarkers* are biological markers that aid in predicting the disease progression and treatment outcome [11].

Given the lack of specific pharmacological therapy for ASD and the clinical heterogeneity of the disease, the current research efforts are geared mainly toward identification of *risk biomarkers* and *markers for early diagnosis*. The most effective therapeutic intervention currently available for ASD is early behavioral therapy [12–14]. While the symptomatology of ASD is usually not apparent until 2 years of age, an additional lag time occurs between the moment when parents become worried about their child and the time the child receives a diagnosis and is enrolled in behavioral therapy [15].

Therefore, the short-term goals of ASD biomarker research are (a) to identify risk biomarkers, which can then define a population pool to be screened for early diagnosis, and (b) to develop effective biological and/or behavioral measures that allow diagnosis and early intervention before the full clinical picture develops. Identification of biomarkers for early diagnosis would also aid in eliminating the time lag between referral and diagnosis. In the long term, it would be very valuable to validate ASD diagnosis on an objective biological marker, much like cancer diagnosis is suspected on clinical grounds and validated on biological tests. However, this goal has not yet been achieved for any of the psychiatric disorders, and biological markers are unlikely to replace behavioral testing for standard ASD diagnosis in the next few years. The current research focus is rather on defining strong behavioral diagnostic criteria for ASD, which in turn inform effective biomarker studies.

One of the puzzles faced by ASD research in general, which certainly applies to biomarker research as well, results from the marked disease heterogeneity [16]. As mentioned previously, it has been recognized that there are wide differences in the clinical manifestations of ASD patients, and it is reasonable to assume that the biology underlying the disease in a patient with language and social impairment may be entirely different from the biological mechanisms leading to a clinical picture of intellectual disability, seizures, and repetitive behaviors. Thus, ASD researchers often face the question of whether to investigate a narrow subset of ASD cases, all of whom share similar clinical manifestations, or aim at obtaining more generalizable results by investigating the wider ASD spectrum.

Subphenotyping, that is, selecting a subgroup of ASD patients based on their common clinical manifestations, has proven to be a fruitful research avenue in ASD genetics [17] and may also prove valuable in the search for ASD biomarkers [18] by reducing the cohort sizes necessary for obtaining statistically significant results. It has also been proposed that due to the much higher incidence of ASD in males than in females, the underlying disease mechanisms may be at least to some extent gender specific [19]. Some studies thus subgroup the study cohort by gender in order to increase the likelihood of identifying ASD biomarkers [20]. Conversely, other studies aim to identify biomarkers that can subgroup ASD patients in a manner relevant for prognosis and therapeutics, which is not obvious from the clinical picture. For instance, multiple studies have demonstrated altered immune responses in ASD patients (see below), opening an active area of research on immune biomarkers that can distinguish ASD patients who might benefit from specifically targeted therapy.

The second approach, which aims to identify commonalities among ASD patients is more challenging. Current research from genetic studies as well as research on structural and functional brain MRI indicate that identifying biological changes common to the majority of ASD patients requires large cohort sizes and often a panel of markers, rather than a single marker. As a result, multivariate analyses [21] (such as support vector machine algorithms) are progressively replacing univariate analyses in many areas of ASD biomarker research, from genomics to structural and functional brain imaging. Instead of comparing measurements of a single biological marker between disease and control groups, multivariate analyses are based on the notion that several variables of a certain type (such as the expression level of multiple genes) may be necessary in order to discriminate between disease and normal states. Multivariate analyses thus aim to identify a pattern of data variation in a complex dataset, that best discriminates between disease and control groups. Typically, such a "classifier" pattern is obtained by training an algorithm on data generated from ASD and control individuals, and its ability to discriminate between disease and normal states is then tested on a second, independent set of ASD and controls [21].

Many biological markers for ASD have been proposed to date [22], but with the exception of highly penetrant genetic changes, none have yet advanced to clinic or been consistently reproduced. Conceivably some of the reasons behind the variability of ASD biomarker results are (a) small cohort sizes, (b) small differences between disease and control groups that do not stand replication, (c) lack of replication of results in an independent test group, (d) clinical heterogeneity, and (e) disease variability along developmental trajectory, leading to biological markers being specific for certain age groups or developmental windows. In the following sections, we discuss the main directions taken by research on ASD biomarkers and highlight studies that have attempted to evaluate the sensitivity and specificity of the proposed biological markers.

3. Brain Imaging Biomarkers

Studies using structural magnetic resonance imaging (MRI) have attempted to identify differences in brain structure associated with ASD. These studies have been applied to adult ASD cases, aiming to identify diagnostic markers, as well as children with ASD, and infants with a family history of ASD, in a search for markers of early diagnosis. Structural brain changes observed in ASD patients (reviewed in [23]) include increased total brain volume in young ASD children [24], increased frontal lobe volume [25, 26], increased cortical thickness in temporal and parietal lobes in ASD children, and decreased cortical thickness in ASD adolescents and adults, as well as structural changes of corpus callosum, basal ganglia, amygdala, and cerebellum [23]. However, none of these changes have been reliably replicated in order to become valuable as ASD biomarkers.

Research focus has lately shifted from comparing individual brain regions between ASD cases and controls to performing multivariate analyses, using structural brain imaging data from multiple brain regions. This approach is supported by the notion that ASD likely affects more than one brain region in any individual and affects the same brain region to a different extent in different ASD patients. Ecker and colleagues [27] have used an SVM approach to analyze whole brain data from 22 adult ASD cases and 22 matched controls in order to identify spatially distributed networks of brain regions with structural properties that could discriminate between ASD cases and controls. This study identified brain networks including limbic, frontal-striatal, frontotemporal, frontoparietal, and cerebellar systems, which could correctly

classify 86% of ASD cases using grey matter scans and 68% of ASD cases using white matter scans. This study used a leave-two-out approach for cross validation. More recently, Uddin et al. [28] used multivariate pattern analysis applied to structural MRI data from children and adolescents with ASD. This study built a classifier based on grey matter in the posterior cingulate cortex, medial prefrontal cortex, and bilateral medial temporal lobes, which reached an accuracy around 90% in discriminating between the ASD and control groups.

Diffusion tensor imaging (DTI), an MRI method that analyzes white matter microstructure, also demonstrated measurable changes in ASD subjects: decreased fractional anisotropy, reflecting reduced coherence in fiber tract directionality, was observed in several brain regions including ventromedial prefrontal cortex, orbitofrontal cortex, and superior temporal gyrus [23, 29, 30]. A diagnostic classifier built using SVM applied to DTI data demonstrated a prediction accuracy of 80% [29].

Functional MRI (fMRI) is an imaging method that captures patterns of brain activation and has contributed greatly to the overall understanding of functional brain abnormalities that underlie autism and related disorders (reviewed in [23, 31]). Studies of ASD brain activation during social cognition tasks have demonstrated brain activation changes in the fusiform face area (FFA) in response to face processing [32–34], decreased FFA and amygdala activation during emotional face tasks [35, 36], and impaired activation of the mirror neuron system [37]. Studies of neural correlates of language development in ASD children have shown an abnormal right hemisphere lateralization of temporal cortex activation during language tasks [23, 38]. fMRI studies of ASD patients also demonstrated decreased long-distance connectivity between brain regions during resting state [39, 40].

Anderson and colleagues used pairwise functional connectivity data from 7266 brain regions across the entire grey matter to build a diagnostic classifier for a set of 40 ASD subjects and 40 matched controls [41]. This classifier had 83% sensitivity and 75% specificity (79% accuracy) on the initial dataset and 71% accuracy in a replication dataset of 21 individuals. Interestingly, the classification accuracy was 89% and 91% for the two datasets, respectively, when the classifier was applied only to individuals younger than 20 years, suggesting that functional connectivity differences between ASD and controls diminish after 20 years of age [41].

While these results are very exciting and offer hope for an objective measure that could help ASD diagnosis, the structural and functional imaging biomarkers await replication in larger cohorts and multicenter studies, before translation to clinic becomes feasible.

4. Electrophysiological Biomarkers

MRI-based methods and fMRI in particular are expensive and laborious investigations. By comparison, electrophysiological methods are less costly and would be easier to implement in the clinic. Thus, several studies focused on identifying electrophysiological changes associated with ASD [42–45].

Event-related potentials (e.g., electrical brain response to faces) were shown to be delayed in children with ASD, and this measure of brain activity was reposted to be normalized in response to early behavioral interventions [43, 45]. Delays in auditory evoked responses in superior temporal gyrus were also proposed as a potential biomarker for autism with a sensitivity of 75% and a specificity of 81% for discriminating between 25 children with ASD and 17 controls [44].

Recently, Bosl and colleagues [42] proposed that EEG complexity could be used as a biomarker for ASD risk. This study used resting state EEG data from normally developing children and children at high risk for autism, defined as children having an older sibling diagnosed with autism. By calculating a modified multiscale entropy (mMSE) measure and applying an SVM algorithm, the authors were able to discriminate between the high-risk and control groups with 80% accuracy. It has been objected that this study did not demonstrate that children at high risk for ASD eventually do develop the disease [46]. Moreover, the observed EEG differences might in fact reflect brain adaptive responses to genetic vulnerability. While studies attempting to determine early brain changes in ASD children are very valuable, careful interpretation of these results is necessary given their ethical implications [46].

5. Genetic Markers

Initial autism twin studies demonstrated that ASD is highly heritable, with disease concordance rates of 70–90% among monozygotic twins and 6–10% among dizygotic twins [47, 48]. More recent estimates show somewhat lower heritability rates than the initial studies (77% for ASD male monozygotic twins and 50% for female ASD monozygotic twins [49]) but still support the notion that ASD is highly heritable. Given the high heritability of ASD, intense research efforts have been aimed at uncovering the genetic basis of ASD, and identifying genetic markers that estimate the disease risk. Current research on ASD genetics has been reviewed elsewhere [2, 16, 17], and here, we focus on the data relevant for potential translation of genetic research into disease markers.

The discovery of several single gene mutations and cytogenetic abnormalities with high ASD prevalence has led to the possibility of identifying a genetic cause of ASD in as many as 20% of patients [2]. Chromosomal microarray analysis (CMA) using either comparative genomic hybridization (CGH) arrays or single nucleotide polymorphism (SNP) arrays can identify copy number variations (CNVs) such as microdeletions and microduplications in 5–10% of ASD patients [50]. Thus, it has been proposed that CMA should be used as a first-tier clinical diagnostic test for ASD [51, 52]. While CMA is not intended for diagnosing ASD, it can be used to investigate the genetic cause of the disease, once the ASD clinical diagnosis has been established. Heil and Schaaf [51] proposed an algorithm for ASD clinical genetic diagnosis, which employs CMA as a screening tool. The interpretation of a positive CMA result (i.e., the identification

of one or more CNVs in an ASD patient) is complicated by the fact that not all CNVs are pathogenic. Several CNVs have been associated with ASD as being more frequent in the ASD population than in the general population [16]. If a CNV previously associated with ASD is identified by CMA analysis, it should be considered as contributing to the disease and taken into account for genetic counseling for the family [51]. If the CNV identified by CMA has not been previously associated with ASD, it is recommended that the parents of the ASD patient be tested by CMA as well, in order to determine whether the CNV is inherited or *de novo*. A *de novo* CNV or a CNV inherited from a parent with a clinical psychiatric disorder is more likely to be causal for ASD. Finally, if a copy number variant (CNV) is not identified by CMA, screening of specific genes for point mutations or other types of genetic changes not detectable by CMA is recommended in patients with a clinical picture suggestive of syndromic forms of ASD.

Syndromic forms of ASD are recognizable clinical syndromes that have high ASD prevalence and often a known genetic cause. Cytogenetic abnormalities such as dup(15q), as well as single gene mutations affecting *CNTNAP2* (cortical dysplasia focal epilepsy syndrome), *CACNA1C* (Timothy syndrome), *MECP2* (Rett syndrome), and *FMR1* (fragile X syndrome), are associated with ASD in more than 50% of the cases [2]. These highly penetrant genetic changes are valuable markers for subclassifying ASD. Animal models have been successfully generated for several of the single gene disorders [53–56] and can be used for effective drug screening. Thus, specific therapy is likely to emerge sooner for these genetically defined forms of ASD [54].

Collectively, known genetic causes of ASD are observed in around 20% of ASD patients [2]. Yet each of these genetic changes is rare and only accounts for less than 2% of cases [2]. What are the genetic changes underlying the high heritability in the rest of the 80% of ASD patients? This question has been the focus of ASD genetics research over the last decade, which demonstrated that the clinical heterogeneity of ASD is mirrored by an equally daunting genetic heterogeneity [2, 16]. A combination of common genetic variants and rare mutations is currently believed to underlie ASD heritability. It has been proposed that genetic changes in many genes, estimated in the hundreds, are necessary in order for the disease to occur [16, 57–62]. Thus, to advance toward estimating the genetic risk of ASD before symptomatology occurs, it appears necessary to develop genetic tests that simultaneously take into account multiple genetic markers, and perhaps multiple types of biological markers.

In concordance with this model, Skafidas and colleagues used genome-wide SNP data in order to build a diagnostic classifier [63]. In this study, the genotyping data for 975 ASD cases and their unaffected relatives were used to identify pathways associated with the disease using a set enrichment analysis. The genes included in the 13 significant pathways identified contained 775 unique SNPs. Of these, 237 SNPs were determined to be highly significant and were used as a diagnostic classifier, applied on a training cohort and two independent validation cohorts. The classifier reached 84% diagnostic prediction accuracy in a cohort ethnically similar to the one used to build the classifier but was suboptimal for a cohort of ethnically dissimilar individuals (prediction accuracy of 56%).

A recent study [64] attempted to use pattern classification based on SNP markers and brain imaging markers (regional thickness and regional volume) in order to discriminate between Asperger syndrome and high-functioning autism patients. It would be conceptually interesting to try to incorporate these distinct types of measurements into a single classifier. However, the study only genotyped SNPs in 8 ASD susceptibility genes rather than genome-wide and analyzed the SNP and imaging data separately, comparing their performance for diagnostic classification. In this particular study, SNP genotyping was superior to brain imaging in terms of classification accuracy, but the number of subjects (15 high-functioning autism and 3 Asperger syndrome) was too small for the results to be generalized.

Future studies combining common sequence variants and rare genetic variation are warranted for identifying panels of genetic markers with high ASD predictive value.

6. Gene Expression Biomarkers

Unlike genetic markers, which are variations in DNA sequence and are largely invariable across tissues and during an individual's life, the amount of RNA transcribed from each gene is tissue specific and varies in response to environmental changes. Thus, gene expression levels represent a functional readout of DNA sequence. In a search for biomarkers for ASD, several groups have investigated gene expression profiles of readily available peripheral tissues (i.e., blood and lymphoblasts) from ASD patients [65–70]. Notably, the selection criteria for the ASD group varied markedly between studies and consequently so did the gene expression signatures identified (reviewed in [70, 71]). However, a common theme of these studies was the upregulation of genes involved in immune and inflammatory responses, consistent with gene expression studies on postmortem brain [72, 73] and neuropathological studies [74, 75].

A recent study attempted to build a diagnostic classifier using microarray expression profiling of peripheral blood from infants and toddlers with ASD [76]. Out of an initial set of differentially expressed probes, 48 were selected as an optimal classifier by applying a support vector machine (SVM) algorithm to half of the microarray dataset. The accuracy of this classifier in correctly diagnosing ASD cases from the second half of the dataset was 91%. However, the validation dataset was not independently generated; so, the performance of this classifier remains to be replicated.

The largest study on blood gene expression profiling in ASD patients to date [77] used two independently generated datasets: one consisting of genome-wide expression profiles from 66 ASD males and 33 male controls and another set of data from 104 ASD cases and 82 controls. The mean age for subjects in this study was 8-9 years. Based on genes differentially expressed in the first dataset, the authors generated a set of 55 probes that had the highest accuracy in discriminating between cases and controls. Using this set

of 55 genes, the accuracy of diagnostic classification in the second dataset was 67.7%. As expected from the fact that the first dataset contained only males, the classifier accuracy in the second dataset was higher for males than females. It is worth noting that the two classifiers described previously are entirely distinct in their composition of genes. This may be explained at least partially by the fact that the two studies looked at different age groups.

Overall, gene expression measurements in peripheral tissues from ASD patients are still far from achieving diagnostic accuracy. Subphenotyping, detailed clinical characterization of study subjects, and combining genome-wide expression profiles with data on DNA sequence variation and/or epigenetic modifications would be needed in order to increase the power of detecting disease-relevant gene expression changes in peripheral tissues.

7. Biomarkers of Altered Immune Responses

Mounting evidence suggests that ASD is associated with abnormalities in the innate and adaptive immune responses (reviewed in [78]). Increased levels of plasma interleukins IL-1, IL-6, IL-8, and IL-12, interferon γ, and macrophage migration inhibitory factor, as well as decreased levels of TGF-β, have been described in ASD patients [79–82]. In addition, ASD patients were reported to have increased levels of plasma IgG immunoglobulin and abnormal activation of natural killer cells [65, 83, 84]. Autoantibodies against neural cells and brain tissue were also detected in the plasma of ASD children [85–89]. Postmortem brain studies have demonstrated an increase in microglial activation in ASD patients, and this observation has recently been confirmed by positron emission tomography in adults with ASD using a radiotracer for microglia [74, 75, 90]. Suzuki et al. [91] observed an increased signal for activated microglia in cerebellum, midbrain, pons, fusiform gyri, anterior cingulate, and orbitofrontal cortex in ASD subjects. Recently, Momeni et al. [92] used surface-enhanced laser desorption/ionization time-of-flight (SELDI TOF) mass spectrometry to identify potential peptide biomarkers in the plasma of ASD children. The study identified three related complement C3 peptides differentially expressed between ASD and control children. Although no validation dataset was available, the study highlighted the potential of proteomic approaches to detect immune biomarkers for ASD.

It is not entirely clear if the immunological changes observed in ASD patients are causally implicated in the disease, but at least some of the abnormal immune changes appear to contribute to behavioral changes. For example, mouse studies have shown that maternal immune activation during pregnancy leads to ASD-like behaviors in offspring [78], and the increase in inactivated immune cells in ASD brain may lead to altered synaptic plasticity [93].

The immune mechanisms in ASD are a promising research avenue that may result in targeted therapy, and thus, further studies are needed to identify reliable immune markers that can define the group of ASD patients likely to benefit from treatments targeting immune responses.

8. Other Types of Biomarkers

8.1. Head Circumference. Head circumference is one of the most extensively investigated early biological markers of autism, used as a proxy for brain size. The increased head circumference in autistic children was one of the clinical characteristics described by Kanner [94] and was further assessed by multiple studies (reviewed in [95]).

Although there was some variability between the results of various groups, the overall conclusion of earlier studies, based on small samples of less than 100 individuals, was that at birth, the head circumference of children who are later diagnosed with ASD is normal or smaller than normal [95]. During the first three years of life, however, autism appeared to be associated with an accelerated rate of head growth leading to macrocephaly, that is, a head circumference more than two standard deviations above the population mean [95]. The exact time window when the increased head growth occurs is still debated. For example, one study observed that the increased head growth rate was limited to the first year of life [96], while another group found no increase in head growth rate during the first year of life but did observe an increase in the rate of overall body growth [97]. Rommelse and colleagues noted that although the head circumference of autistic children was higher than the population norm, the same was true for children with other psychiatric disorders [98]. This study thus concluded that increased head growth is a characteristic of psychiatric disorders in general, rather than being specific for ASD.

A recent multicenter study examined the head circumference in a sample of 9000 children, 1% of whom were diagnosed with autism [99]. Barnard-Brak et al. observed no difference in head circumference between autistic and nonautistic children and no difference in the incidence of macrocephaly between the autism and control groups. The authors suggested that the difference between their results and previous studies may result from selection bias affecting clinic-based, small cohort studies. Thus, it is possible that a subgroup of autistic children may be characterized by macrocephaly, but the observation does not appear to be generalizable to the wide ASD spectrum. Notably, head circumference is a heritable trait in the general population, and Froehlich and colleagues recently reported an increased incidence of macrocephaly in the group of autistic children studied, as well as in their unaffected twins [100].

8.2. Serotonin. Hyperserotonemia is one of the first blood biomarkers to have been implicated in ASD. Increased levels of serotonin in whole blood are consistently observed in 25–35% of ASD patients [101, 102]. Serotonin levels have been shown to be heritable and regulated by genetic variants in the serotonin receptor gene *SLC6A4* and the integrin beta gene *ITGB3* [103, 104]. Interestingly, a recent study describing a mouse model carrying an *SLC6A4* variant [105] showed hyperserotonemia and behavioral changes including social deficits and repetitive behaviors, suggesting that the serotonin imbalance may causally contribute to ASD, and offering hope that therapies targeting the serotonin pathway may prove beneficial for a subset of ASD patients.

A number of other soluble brain biomarkers such as VIP, substance-P, NGF, BDNF, and secretin have been reported in ASD (reviewed in [22]) and await replication in independent studies.

8.3. Mitochondrial and Metabolic Markers. Mitochondrial disease (MD) has been reported to occur with higher frequency among ASD patients than in the general population [106, 107]. In addition to mitochondrial disease being diagnosed more frequently among ASD cases, biochemical markers of mitochondrial function are also altered in ASD patients without MD [108, 109]. Thus, it has been proposed that ASD with mitochondrial dysfunction may represent a phenotypically distinct subgroup of ASD. A meta-analysis of mitochondrial dysfunction in ASD noted that many of the studies implicating MD in ASD are based on small cohorts or single case reports [110], and thus, further research is warranted for establishing the value of biomarkers of mitochondrial dysfunction in ASD.

Changes in porphyrin metabolism have been associated with ASD [111, 112] and are believed to reflect exposure to environmental toxins. Heyer et al. [113] investigated the urine levels of pentapophyrin and coproporphyrin as potential markers for ASD risk by comparing a group of 30 male children with autistic disorder, 14 with PDD-NOS, and 32 neurotypical controls. ASD children (including PDD-NOS and autism) had higher urinary levels of pentaporphyrin and coproporphyrin compared to age matched controls. The sensitivity of urinary pentaporphyrin was 30% for autism and 36% for PDD-NOS, and the sensitivity of coproporphyrin was 33% for autism and 14% for PDD-NOS; both makers reached 94% specificity for ASD in this study.

Multivariate analyses of blood and urine proteins and metabolites have also been attempted in search for ASD biomarkers. Yap and colleagues [114] used NMR spectroscopy to measure urine metabolites in 39 ASD children and their unaffected siblings. This study reported higher levels of urinary taurine and lower levels of urinary glutamate in ASD children, as well as metabolic changes consistent with abnormalities in gut microbiota. Schwarz et al. [20] performed immunoassays of 147 analytes using blood serum from 45 adult subjects with Asperger syndrome (AS) and 50 controls. The AS and control groups were divided into a discovery and validation group for the male and female subjects, respectively. A panel of 9 analytes were found to be significantly different between male AS cases and male controls in the male discovery group. Applying this panel as classifier to the male validation group resulted in correct classification of 70% of AS males in the validation group but was inefficient at discriminating between female AS and female controls. Similarly, the panel of 14 biomarkers identified as significantly different between female AS and female controls was able to correctly classify 90% of the AS females in the validation group but did not discriminate between male AS and male controls.

8.4. Biomarkers of Oxidative Stress. Oxidative stress (OS) results from insufficient counteracting of endogenous and exogenous reactive oxygen species (ROS), as a result of either ineffective antioxidant mechanisms, excessive production of ROS, or both. Evidence of OS in ASD has been reported by numerous studies of blood and brain OS biomarkers. Decreased plasma levels of reduced glutathione, glutathione peroxidase, methionine, and cysteine and increased plasma levels of oxidized glutathione have been reported in ASD subjects [115–117]. Measurements of OS biomarkers in postmortem brain tissue from ASD cases [118] demonstrated changes in reduced and oxidized glutathione and increased levels of 3-nitrotyrosine and 8-oxo-deoxyguanosine, which are markers of oxidative protein damage. A meta-analysis of OS biomarkers in ASD [115] showed that the strongest differences between ASD cases and controls in the mean OS biomarker levels were observed for reduced glutathione (decreased by 27%) and oxidized glutathione (increased by 45%). This meta-analysis study also highlighted the fact that OS biomarker changes associated with ASD tend to be heterogeneous, and the observations are based on small sample sizes and moderate effects, thus cautioning on the need for further standardized studies.

9. Conclusions and Ethical Considerations

Although many avenues have been tried for identifying biological markers for ASD, a clinically valuable ASD biomarker is not yet in sight. For a biomarker to become clinically valuable, it would need to be highly sensitive and specific (even if limited to a well-defined subgroup of ASD patients or to a developmental window), be feasible for use in the clinic, and not be cost prohibitive. The majority of studies currently available suffer from small cohort sizes and lack of replication in independent datasets, which make the estimation of biomarker reliability hard to evaluate. The difficulty of putting together a large ASD research cohort may be balanced out in the near future by more open data sharing, allowing investigators to replicate their results using published data.

As the development of ASD biomarkers is still in its infancy, there are valid concerns among clinicians that premature translation of research data into commercially available tests may be harmful rather than beneficial for ASD patients and their families [119]. The majority of genetic risk factors identified thus far have small effect sizes (i.e., they only marginally increase the disease risk over the population standard). Thus, it is questionable whether disclosing the result of such an ASD risk marker to the family is in fact beneficial. The results of genetic biomarker tests are likely to have a huge impact on parental decision making for reproduction, and thus, more research may be needed for better understanding parental needs and attitudes [11]. In addition, communicating the results of a risk biomarker should ensure that children do not receive a disease label that will affect their future options and potential.

To properly control the translation of research results to the clinic, it has been proposed [120] that assessing ASD genetic research should follow a similar process to the ACCE Model Project established by the Office of Public Health

Genomics (OPHG) of Center for Disease Control, which provides an analytic framework to evaluate the *analytic validity, clinical validity, clinical utility*, *and associated ethical, legal*, *and social implications* of genetic tests [121]. Similar analytical frameworks would be very valuable for all classes of ASD biomarkers.

The lack of effective biomarkers for ASD despite progressive accumulation of research data may seem daunting, but as the field matures and incorporates a deeper understanding of disease heterogeneity into study designs and analytical methods, the translation of biomarker research to clinic may eventually become within reach.

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