PERSONALIZED PRECISION MEDICINE IN AUTISM SPECTRUM RELATED DISORDERS

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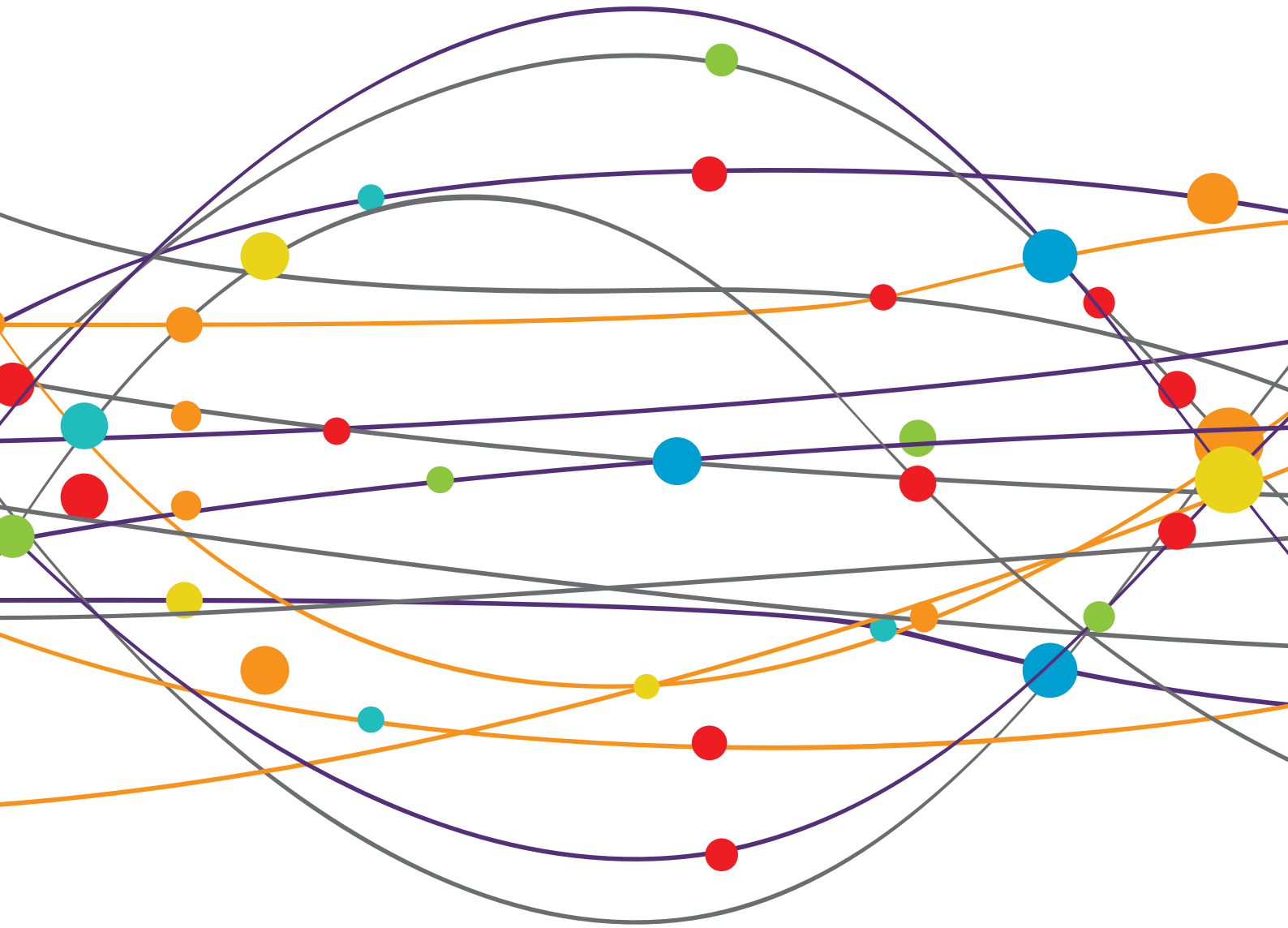

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PERSONALIZED PRECISION MEDICINE IN AUTISM SPECTRUM RELATED DISORDERS

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EDITORIAL
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Editorial: Personalized Precision Medicine in Autism Spectrum-Related Disorders

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Editorial on the Research Topic

Personalized Precision Medicine in Autism Spectrum-Related Disorders

According to the National institute of Health (NIH), the definition of Personalized Precision Medicine is to enable risk assessment, diagnosis, prevention, and therapy specifically tailored to the unique characteristics of the individual, thus enhancing quality of life and public health. This concept has been adopted in all areas of medicine, acknowledging individual differences. Despite the shared features defining the autism spectrum disorder (ASD) phenotype, as listed in Diagnostic and Statistical Manual of Mental Disorder-5 (DSM-5) (1), a more personalized and precise approach is currently recommended for individuals with ASD (2, 3).

Individual differences span from etiology and early life differences to specific developmental trajectories according to gender, age of diagnosis, and severity, and up to response to treatment (4, 5). The clinical heterogeneity of ASD in terms of etiology, symptoms, severity, and response to treatment is demonstrated in the current issue.

In terms of developmental history and trajectory, individuals with ASD may have been diagnosed in childhood, adolescence, or as adults, and clearly the clinical presentation and severity may differ according to age of diagnosis. For children experiencing developmental differences early in life, the main aim is to recognize early signs and symptoms to be able to provide early intervention, enhancing and promoting positive lifelong outcome. In the important overview of "Autism Screening in Early Childhood," Brewer et al. provide practical tools for evaluation and implementation of ASD screeners and provide insights for developing reliable screening instruments. One of the screeners covered in this special issue is seen in Barbaro et al.'s study [A Pilot Investigation of the Social Attention and Communication Surveillance (SACS) Tool for the Early Identification of Autism in Tianjin, China]. They implemented the SACS-C screening tool for a large population of children in Tianjin, which was shown to be more sensitive and reliable than the Checklist for Autism in Toddlers-23 (CHAT-23) for identification of ASD in 1–2-year-olds, after training for both instruments was implemented, and children followed after age 3. Training on SACS-C was recommended for screening large populations of young children in China.

As symptoms of autism often appear during the 1st year of life, Gabis et al. revealed an early sign that may be easily recognized, measured, and serve as a "red flag" to prompt a structured diagnostic evaluation and diagnosis of autism. This study focuses on early motor differences and points to hypotonia (low muscle tone) as a sign to prompt ASD diagnosis and enable earlier recognition in infants at risk. Gender differences were demonstrated in initial presentation, emphasizing the need for increased awareness of ASD in girls.

Early differences in language development was addressed by Oren et al. Acquisition of language in toddlers with ASD was considerably later as compared to typically developing peers. However,

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the encouraging finding was that while toddlers with ASD were late in producing first words, the accumulation pace of new words became similar.

The formal diagnosis of ASD requires clinical expertise and performance of lengthy "gold standard" tools, which can lead to a suboptimal rate of diagnosis. The significant delays in age of diagnosis and discrepancies in availability of diagnostic tools, intervention services, and in the level of professional expertise are a significant challenge in Brazil, as described by Sukiennik et al. The identification of ASD is prerequired for provision of appropriate intervention and treatment. In addition, the flourishing of clinical studies examining specific treatments and outcomes posed a new challenge of applying an objective, reliable, and reproducible measure of assessment and measure of improvement, since the current methods rely mainly on behavioral measures that are subjective, less reliable, and influenced by the "placebo effect." We hypothesize that the failure of most ASD behavioral and pharmacological therapy trials is mainly due to mixing biologically different ASD populations, as well as focusing on subjective measures of behavioral changes as endpoints for the studies, measures that are strongly influenced by placebo response. It is possible that some of the drugs or behavioral interventions could have had significant benefits in a more biologically homogenous sub-population of ASD and by using a more reliable and objective biomarker for measuring change.

Tsuchiya et al., in their paper from Japan: "Diagnosing Autism Spectrum Disorder Without Expertise: A Pilot Study of 5- to 17-Year-Old Individuals Using Gazefinder" suggest an easy to use and reliable tool that can be used as a screener for identification of ASD risk, before the recommendation of full expert assessment. This eye tracking tool is easy to use on a standard computer and monitor, the examination takes <2 min, the algorithm was accurate in identifying ASD vs. non-ASD with 78% accuracy on the 98% of participants who succeeded in completing the task. As such, this tool may serve as a screener as well as a marker to monitor improvement in ASD symptomatology (eye gaze).

ASDs are extremely variable in developmental trajectory, severity, and co-occurring conditions (across the lifespan), such as sleep difficulties in infancy, Attention Deficit Hyperactivity Disorder (ADHD) in childhood, and mood disorders in adolescence, as well as other co-occurring disorders such as dysmorphism, epilepsy, or intellectual disability. There is often also variation in how different patients are treated, as some may be treated for their core symptoms of communication difficulties and atypical behavior, and others for their comorbidities. Neurological comorbidities such as epilepsy, motor impairments, and abnormal brain growth or malformations (including macrocephaly, absence of the corpus callosum, or migration defects) may become a hint for specific genetic etiology such as PTEN (macrocephaly), SCN1A (Dravet epileptic syndrome), Tuberous Sclerosis (tumors), and Angelman's syndrome (sleep disturbance, microcephaly, and motor impairment). The extensive research into the most common inherited disorder causing ASD and Intellectual disability, Fragile X Syndrome, led to multiple research venues of treatments. One of the paths is reviewed by Rajaratnam et al. who assessed the response to Sertraline (Selective serotonin reuptake inhibitor) in Fragile X patients as compared to non-syndromic ASD, and found a specific and differential response to this treatment, emphasizing that despite of the shared ASD phenotype, only the Fragile X group with the comorbidity of anxiety responded to this treatment.

Gozes' review, "The ADNP Syndrome and CP201 (NAP) Potential and Hope," demonstrates the path leading from identification of specific clinical features including dysmorphism, motor delay, and cognitive impairments in children with ASD, and performed genetic testing to search for a specific etiology of ASD. In this case, the very rare ADNP mutation was found by exome sequencing, and research into the mechanism of action may bring hope for specific treatment and for a potential cure in ADNP syndrome patients, and possibly in additional related causes of autism, since ADNP was found to be a regulatory gene of more than 400 genes critical for brain development. A drug candidate, CP201 (NAP), for intranasal administration was developed and examined in preclinical studies for amelioration of symptoms resembling ASD in a mouse model of ADNP deficiency.

Hu and Bi approached the genetic differences in ASD from a different perspective, by analyzing phenotypic differences in view of transcriptomic data as related to specific ASD-associated genes, using Weighted Gene Correlation Network Analyses (WGCNA). Children from simplex and multiplex families were divided into phenotypic categories and differential gene expression analysis was performed, showing that phenotypic subtyping improved the ability to discern between probands and typically developing siblings in simplex families.

Susceptibility to ASD is now understood to be partially due to rare genetic variants, and partially due to environmental factors including prenatal viruses, perinatal brain insult, and premature birth. Prematurity is associated with ASD symptomatology in more than 7% of children born premature, and the risk increases with each additional week of prematurity (6). The study "A Comparison of Children Born Preterm and Full-Term on the Autism Spectrum in a Prospective Community Sample" by Luu et al., comparing children with ASD born before term to children with ASD born at term, did not reveal significant differences in visual reception, fine motor, receptive and expressive language, and autism behaviors, demonstrating that ASD impact is similar regardless of prematurity risk factors, which differed to that of most previous research; however, a large proportion of the sample was in the moderate to late preterm group, which is less impacted by disability than extreme prematurity.

Language, communication, and behavioral brain pathways are complex and vulnerable. There are many factors that cause similar features, signs, and symptoms presenting as an ASD phenotype. Some pathways may have a common response to treatment and intervention, especially during early development where there is increased brain plasticity, driving the research toward earlier identification of atypical trajectories. Other treatments in clinical research focus on individual differences and attempt to tackle specific autism-related pathways to circumvent

the shared features and address the specific underlying disorder. Challenges and innovative individualized approaches to autism are presented in the following 14 papers, which encompass studies from diverse research groups all around the world.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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The Relative Utility of Concurrent Sources of Information for Diagnosis of Autism Spectrum Disorder in Early Childhood

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The development of effective screening methods for Autism Spectrum Disorder (ASD) in early childhood remains a public health priority for communities around the world. Little is known regarding the concurrence between parent concerns about ASD and formal ASD diagnostic methods. This study aimed to examine the relationships among *a priori* parental ASD concern, ADOS classification, and a physician specialist's diagnosis. One hundred and thirty-four toddlers (74% male; mean age = 31.8 months, SD 4.4) received an evaluation at a university center specializing in ASD and neurodevelopmental disorders. Correspondence between *a priori* parental ASD suspicion and physician diagnosis of ASD was 61% ($p = 0.028$). Correspondence between *a priori* parental suspicion of ASD and ADOS ASD classification was 57% ($p = 0.483$). Correspondence between ADOS classification and physician diagnosis of ASD was 88% ($p = 0.001$). Our results have implications for evaluations in low resource regions of the world where access to physician specialists may be limited; the high correspondence between ADOS classification and a physician specialist's diagnosis supports the use of trained ADOS evaluators, such as field health workers or early childhood educators, in a tiered screening process designed to identify those most in need of a specialist's evaluation. Our results also have implications for public health efforts to provide parent education to enable parents to monitor their child's development and share concerns with their providers. Parent awareness and expression of concern coupled with timely responses from providers may lead toward earlier identification of ASD, and other neurodevelopmental disorders, and hence, generate opportunities for earlier and more personalized intervention approaches, which in turn may help improve long-term outcomes. Empowering parents and community members to screen for ASD may be especially important in regions of the world where access to formal diagnosis is limited.

Keywords: neurodevelopmental disorders, developmental disabilities, Autism, diagnosis, ADOS, parent concern, screening, evaluation

INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by persistent deficits in social communication and interaction and restricted, repetitive patterns of behavior, interests, or activities that are present during a child's development and cause clinically significant impairments in their functioning (1). The prevalence of ASD in 8 year-old children in the United States is estimated to be 1.68% (2).

Early intervention in children with ASD may reduce severity of symptoms so greatly that up to 25% of children identified as early as 24 months and as late as 60 months may reach an average range of cognitive, adaptive, and social skills, thereby reaching "optimal outcome" [e.g., (3–5)]. Furthermore, a randomized clinical trial examining the effects of an early intensive behavioral intervention on children with ASD aged 30 months or less determined that there were no significant differences between intervention and control groups immediately following the intervention, although the intervention group demonstrated significant improvements in core symptoms of ASD and adaptive behaviors compared to the control group at a 2-year follow-up (6). Recent efforts to promote early intervention have improved early identification, and although formal assessment for ASD may now take place as early as 12 months of age, the average age of attaining a diagnosis in the general community in the last decade has stalled at 64.5 months in the United States (7, 8). However, current practice parameters aim to support the effort to identify ASD at a young age so that intervention may begin early, as intervention for ASD may lead to better outcomes when it begins at younger ages [e.g., (9–12)]. In contrast to general pediatric settings, centers focusing on ASD often make the diagnosis at 18 to 24 months of age, but these settings are typically staffed with physicians with specialty training in diagnosing early childhood neurodevelopmental disorders, such as developmental and behavioral pediatricians and pediatric neurologists (13, 14). Despite recent improvements, the barriers to early identification and diagnosis of ASD remain significant and the missed opportunities for early and optimal outcomes are profound.

Zuckerman et al. (15) noted that parents of children diagnosed with ASD reported concerns about their child's development as early as 24 months of age. Similarly, Chawarska et al. (16) noted that 50% of parents of children who were diagnosed with ASD at 4 years of age had concerns when their child was between 18 months and 4 years of age. In a study of siblings of children with ASD, parent concerns about the sibling's development at 14 months of age have been identified as an indicator of later diagnosis of ASD (17), suggesting possible earlier awareness among parents who already have a child diagnosed with ASD. Parental concern about ASD most frequently begins with recognition of atypical development of communication skills (16); however, parental observations of impaired social interactions (e.g., lack of eye contact, poor response to hearing one's name, seeming socially withdrawn) followed by delays in language development (e.g., delayed speech or absence of speech) have been identified as the most significant warning signs for parents (18). Research indicates that parents of children who were later diagnosed with ASD were more likely to receive a passive and less proactive response (e.g., reassurance that behavior was normal, too early to tell) from their providers compared to parents of children who were later diagnosed with intellectual disability/developmental disability who received proactive provider responses [i.e., further developmental testing, specialist referrals; (15)].

Field health workers and school professionals may play a pivotal role in the early identification of children at-risk for ASD and can help ensure referral into early intervention (19). Educators are posed to be particularly familiar with typical early childhood development and have the opportunity to encourage parents to seek further neurodevelopmental evaluation when there are concerns (20). Promoting the early identification of developmental difficulties across educational and healthcare systems may increase the likelihood that children in need of intervention will receive it at a younger age. As such, educational and public health systems are positioned as additional safety nets to ensure early identification and intervention, which are particularly important for children whose providers may take a wait-and-see approach or for whom access to trained medical providers is limited.

Given the importance of early diagnosis and the known barriers that impede or delay interventions, there has been an effort to have greater involvement of professionals in the general community aid in more readily identifying children who may have developmental disorders. Branson et al. (20) suggested creating a universal developmental screening in community childcare programs with a specific component for identifying children at risk of ASD. Further, childhood educators may play a vital role in providing early classroom intervention. Brodzeller et al. (21) recommended a balance of research-based interventions and adaptations in early education to encourage children with ASD to participate and learn in settings with peers who do not have disabilities. Before implementing these interventions, however, identification of ASD is needed.

A "gold standard" assessment tool for classifying ASD is the Autism Diagnostic Observation Schedule (ADOS), which evaluates communication, social interactions, play, and restricted and repetitive behaviors observed during semi-structured tasks and is now in its second edition [ADOS-2; (8, 22, 23)]. The ADOS has demonstrated 77% agreement with a multi-disciplinary team diagnosis, not including a physician (24). However, multidisciplinary centers for ASD are scarce, waiting times are often long, and individuals who are trained in the administration of the ADOS and who have been found to reach inter-rater reliability by research or clinical standards are few (25). Little is known about the relative utility of various sources of information in early childhood, including parental concerns about a potential diagnosis of ASD, physicians' clinical diagnoses, and formal standardized evaluations, such as ADOS evaluations. There is a need for research demonstrating the concurrence between these sources of information and subsequent ASD diagnosis, especially as it may help providers and educators better respond to and understand the importance of parental concerns about early development.

The objectives of this study were to examine the relationships among *a priori* parental ASD concern, ADOS classification, and a physician specialist's diagnosis [at an autism center with specialists using the American Psychiatric Association Diagnostic and Statistical Manual of Mental Disorders 5th Edition DSM-5; (1)]. Specifically, we predicted that *a priori* parental concern for ASD would be significantly correlated with ADOS classification and physician diagnosis, and we hypothesized that there would be strong, statistically significant agreement between ADOS classification and a physician's diagnosis.

METHODS

Participants

This study was approved by the local Institutional Review Board, and informed consent for participation was obtained from all parents of toddlers in this study. Participants were 134 toddlers (enrolled at age 23–39 months) with various developmental concerns whose parents were seeking a neurodevelopmental evaluation at a university-affiliated clinic with expertise in autism and neurodevelopmental disorders between May 2013 and June 2014. Participants were included if they were between 24 and 39 months at the time of their scheduled evaluation, were scheduled for or recently had a clinical evaluation for ASD at this clinic, and were English speaking.

Measures

Parent report at the initial telephone intake provided the information used to assign children to one of two study groups. If parents reported a specific suspicion of ASD, participants were grouped in the "*a priori* ASD suspicion" group. If parents reported any other developmental concern without specific concerns for ASD (e.g., speech delay concern, general behavioral concern, general developmental concern, or motor development concern) participants were assigned to the "no *a priori* ASD suspicion" group. During the physician visit, parents reported on the child's medical history, developmental and behavioral patterns, as well as social and family history.

The Autism Diagnostic Observation Schedule (ADOS) "is a semi-structured, standardized assessment…for individuals who have been referred because of possible autism…" (26). The ADOS provides a classification of *autism, autism spectrum,* or *non-spectrum* for individuals based on ratings of behaviors observed in the domains of [1] communication and [2] reciprocal social interaction during the assessment time sample. For the current study, ADOS modules 1 and 2 were used; scores that met the threshold of either *autism* or *autism spectrum* were coded as meeting ASD ADOS classification; *non-spectrum* scores were coded as meeting non-ASD ADOS classification. At the time of this study, the first version of the ADOS was used as the second version of the ADOS (ADOS-2) was not yet available.

A board-certified developmental-behavioral pediatrician or a board-certified child neurologist evaluated the child (independent of the ADOS evaluation) and provided a diagnostic impression of ASD using DSM-5 criteria (1). Physicians conducted a 90-min evaluation with each child and parent that included reviewing the child's medical history, developmental milestones and behavioral patterns, family and social history, and observing and examining the child.

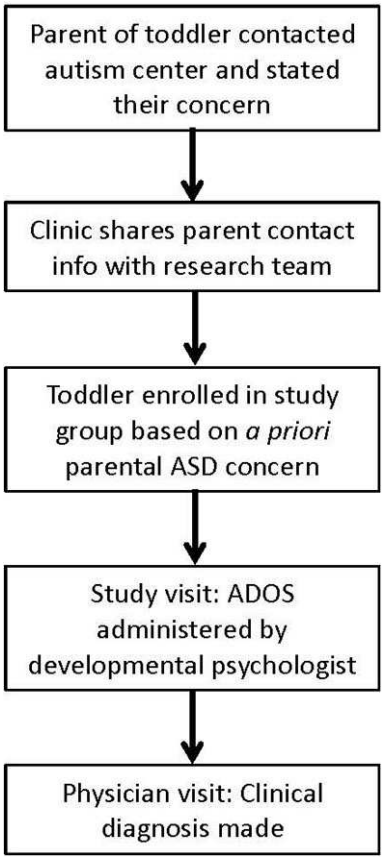

FIGURE 1 | Timeline of procedure from first contact with the autism center to physician's clinical diagnosis.

Procedures

Participants were assigned to study groups according to parental report specifying concerns about ASD or another developmental concern during a standard scripted initial telephone intake, which was conducted by the clinic staff with all parents who called. If parents consented to be contacted for research, clinic staff shared patient contact information with the study team. Upon being contacted by the study team, if parents consented, their toddler was enrolled in the study.

The ADOS was conducted during a study visit by a developmental psychologist who had expertise in assessing children with ASD and had completed ADOS training specific to attaining reliability to a standard acceptable for research and clinical purposes. A diagnosis was made at a separate clinic visit by a developmental-behavioral pediatrician or a pediatric neurologist who was blind to ADOS classification results (**Figure 1**). After recording their clinical diagnosis, physicians were provided ADOS results to assist in their clinical care of these patients.

TABLE 1 | Demographic characteristics of 134 child participants: *a priori* ASD suspicion group ($n = 76$) and no *a priori* ASD suspicion group ($n = 58$).

Child characteristic	Full sample ($n = 134$)	*a priori* ASD suspicion ($n = 76$)	No *a priori* ASD suspicion ($n = 58$)
Mean age (months)	31.8 (SD 4.4)	31.65 (SD 4.4)	31.93 (SD 4.5)
Gender			
Female	25.6%	25.3%	25.9%
Male	74.4%	74.7%	74.1%
Ethnicity			
Hispanic or Latino	36.6%	36.8%	36.2%
Not Hispanic or Latino	61.9%	63.2%	60.3%
Decline to state	2.2%	0.0%	3.4%
Race			
White	43.6%	41.3%	46.6%
African American	1.5%	2.5%	0.0%
Native American	0.7%	1.3%	0.0%
Asian American	24.2%	28.1%	18.9%
Native Hawaiian	0.0%	0.0%	0.0%
Other Pacific Islander	1.6%	0.0%	3.4%
None of the above	2.3%	2.7%	1.7%

Some categories may not add to exactly 100% due to rounding. The categories for race do not add up to 100% because parents of only two Hispanic/Latino children selected a race (both selected White). No race was reported for the other Hispanic children in the sample.

Analyses

Contingency tables summarized the frequency distributions of participants across ADOS classification, parental *a priori* ASD suspicion, and physician diagnosis. Fisher's Exact Tests and chi-square tests assessed for significant correspondence between groups.

RESULTS

Participant and Parent Characteristics

Participant age ranged from 23 to 39 months (mean age 31.8 months, SD 4.4) at the time of consent and enrollment. Thirty-seven percent of toddlers were Hispanic. Within the Hispanic category, 73.1% reported they were Mexican, 4.9% were Puerto Rican, 19.5% reported other Hispanic, and 2.4% declined to state. Sixty-two percent of toddlers were non-Hispanic. Parents reported on child race, and data indicated that children were 43.6% White, 24.2% Asian American, 1.5% African American, and 1.6% Pacific Islander. Seventy-four percent of participants were male (**Table 1**). Parents of 76 (57%) children suspected ASD, and parents of 58 (43%) children had other developmental concerns.

Parents of participants were on average 35.4 years old (SD 7.7). Toddlers were brought in by their biological mother 78.2% of the time and by their biological father 15.8% of the time; 2.3% were brought by an adoptive mother, and 3.8% were brought by a foster mother or other legal guardian. Nearly 77 percent of the toddlers' parents were married. Approximately fifty-three percent of parents had obtained a Bachelor of Arts degree or higher (**Table 2**).

TABLE 2 | Demographic characteristics of 134 participating parents: *a priori* ASD suspicion group ($n = 76$) and no *a priori* ASD suspicion group ($n = 58$).

Parent characteristic	Full sample ($n = 134$)	*a priori* ASD suspicion ($n = 76$)	No *a priori* ASD suspicion ($n = 58$)
Mean age	35.4 (SD 7.7)	35.9 (SD 7.2)	34.77 (SD 8.4)
Relationship to the child			
Biological mother	78.2%	74.7%	82.8%
Adoptive mother	2.3%	4.0%	0.0%
Foster mother	1.5%	2.7%	0.0%
Biological father	15.8%	16.0%	15.5%
Other (e.g., grandmother)	2.3%	2.7%	1.7%
Marital status			
Married	76.7%	77.3%	75.9%
Divorced	2.3%	2.7%	1.7%
Widowed	0.8%	1.3%	0.0%
Separated	3.8%	2.7%	5.2%
Never married	6.8%	6.7%	6.9%
Living with a partner	9.8%	9.3%	10.3%
Household income			
$200,000 or more	8.2%	9.2%	6.9%
$100,000–$199,000	20.1%	14.5%	27.6%
$75,000–$99,000	17.2%	21.1%	12.1%
$50,000–$74,999	17.9%	22.4%	12.1%
$30,000–$49,999	6.7%	5.3%	8.6%
$20,000–$29,999	9.7%	10.5%	8.6%
$10,000–$19,999	6.0%	6.6%	5.2%
Below $10,000	6.7%	2.6%	12.1%
Decline to state	6.7%	6.6%	6.9%
Education level			
Professional or doctoral degree	9.8%	9.2%	10.3%
Master of Arts/Sciences degree	16.5%	15.8%	17.2%
Bachelor of Arts degree	27.1%	30.3%	22.4%
Associates degree or vocational program	9.1%	8.0%	10.3%
Some college	20.3%	21%	18.9%
High school diploma	15.8%	14.5%	17.2%
Some high school	3.6%	1.3%	6.8%

Some categories may not add to exactly 100% due to rounding.

The appropriate ADOS module was used for each child, based on guidelines in the ADOS manual. ADOS module 1 was administered to 93.9% of participants, and ADOS module 2 was administered to 6.1% of participants.

Correspondence Between Parental *a priori* ASD Suspicion and Physician Clinical Diagnosis

Correspondence between *a priori* ASD suspicion and diagnosis of ASD by a physician was 61%, χ^2 (1, $N = 132$) =

TABLE 3 | Correspondence of ASD diagnoses among ADOS classification, physician clinical diagnosis, and *a priori* parental suspicion.

	Correspondence (%)	*p*-value
ADOS classification and physician clinical diagnosis ($n = 132$)	87.9	0.001
ADOS + and physician diagnosis ASD +	84.1	
ADOS– and physician diagnosis –	3.8	
ADOS – and physician diagnosis +	2.3	
ADOS + and physician diagnosis –	9.8	
A Priori Parental Suspicion and ADOS Classification ($n = 134$)	56.7	0.483
Parental suspicion + and ADOS +	53.7	
Parental suspicion – and ADOS -	3.0	
Parental suspicion – and ADOS +	40.3	
Parental suspicion + and ADOS -	3.0	
A Priori Parental Suspicion and Physician Clinical Diagnosis ($n = 132$)	61.4	0.028
Parental suspicion + and physician diagnosis +	52.3	
Parental suspicion – and physician diagnosis -	9.1	
Parental suspicion – and physician diagnosis +	34.1	
Parental suspicion + and physician diagnosis -	4.5	

N = 134.

4.67, $p = 0.03$. Fifty-three percent of toddlers with an *a priori* ASD concern received a physician diagnosis of ASD; nine percent of children had no *a priori* ASD concern and received a non-ASD physician diagnosis. Thirty-four percent of toddlers who did not have an *a priori* ASD concern received a physician diagnosis of ASD; five percent of children who had an *a priori* ASD concern received a non-ASD diagnosis from a physician (**Table 3**).

Correspondence Between Parental *a priori* ASD Suspicion and ADOS Classification

Correspondence between parental ASD suspicion and the ADOS classification was non-significant at 57% ($p = 0.48$, Fisher's Exact Test). Fifty-four percent of toddlers had an *a priori* ASD concern and an ASD ADOS classification. Forty percent of toddlers did not have an *a priori* ASD concern and an ASD ADOS classification. Four percent of toddlers with an *a priori* ASD concern and 4% of toddlers without an *a priori* ASD concern met non-ASD ADOS classification criteria.

Correspondence Between ADOS Classification and Physician Clinical Diagnosis

Correspondence between the independently obtained ADOS classification and physician diagnosis of ASD by a physician was 88% ($p = 0.001$, Fisher's Exact Test). Eighty-four percent of toddlers received an ADOS ASD classification as well as a DSM-5 medical diagnosis of ASD by a physician, and 4% received a non-ASD ADOS classification and a non-ASD diagnosis by a physician. Two percent of toddlers received a non-ASD ADOS classification and an ASD diagnosis by a physician, and 10% received an ASD ADOS classification and a non-ASD diagnosis by a physician.

DISCUSSION

The moderate and significant correspondence (61%) between parental concern about development and physician diagnosis of ASD suggests that parent concerns regarding development and possible ASD warrant further clinical evaluation. Similar results were observed in a study that indicated parents with very early developmental concerns not specific to ASD, were more likely to receive a later diagnosis of ASD, even when they often voiced concerns earlier than parents with specific concerns about ASD (18). There was moderate, yet non-significant correspondence (57%) between *a priori* parental ASD concern and ADOS ASD classification.

Community professionals, such as early childhood educators or public health community workers may help bridge the gap between early and late identification by raising awareness of developmental concerns earlier [e.g., (20)]. In a review on different ages of diagnosis by physician, Daniels and Mandell (27) noted greater parental concern about initial symptoms as a factor associated with earlier ASD diagnosis. This finding highlights the importance of attending to parent concerns in an effort toward aiding early identification and diagnosis. It also highlights the importance of community health education to increase parent awareness of early symptoms of ASD in order to improve parent knowledge and ability to recognize early symptoms of ASD, as it is likely that parent knowledge about development and ASD differs widely across communities and countries. Although it is critical to respond immediately to parental concerns, these concerns may be better understood if used as part of a comprehensive evaluation that includes data from other sources (e.g., early childhood educators, a specialist's evaluation and standardized evaluation tools).

Our study identified a high correspondence (88%) between ADOS classification and physician diagnosis, indicating that ADOS classification is concordant with diagnostic impressions of board-certified physicians specializing in ASD and neurodevelopment. Our study demonstrates that experienced physician diagnoses of ASD are highly consistent with the ADOS, a "gold standard" research tool for the identification of ASD. In addition, it demonstrates the utility of the ADOS as a tool for identifying ASD that can be used as part of a diagnostic evaluation, suggesting that in practice areas where access to physicians with expertise in neurodevelopmental disorders is limited, having trained ADOS evaluators may assist primary care physicians, educators, and public health professionals in making an ASD diagnosis more easily, thus leading to earlier intervention.

Although our data demonstrate that there is a low degree of disagreement between the classification of ASD by the ADOS and by experienced physicians, they also call attention to the importance of a comprehensive evaluation, given the potential for false positives and false negatives if the ADOS or a similar standardized assessment tool is used alone. In 10% of cases, there was an ADOS-based ASD classification but no ASD physician diagnosis, whereas in 2% of cases there was a non-ASD ADOS classification and an ASD physician diagnosis. *Post-hoc* analyses revealed that among patients in our study whose parents expressed concerns about ASD but whose children were not diagnosed with ASD by the physician, a number of other diagnoses were recorded, including the following: global developmental delay, language disorders, cerebral dysfunction, neurological abnormalities, intellectual disability, behavior disorders, and hypotonia. In summary, although the ADOS classification is a strong indicator of whether a child will go on to receive a diagnosis of ASD from an experienced physician, it remains important to take into account a child's comprehensive developmental history and medical evaluation.

Several limitations of the current study should be noted. First, our inclusion criteria were focused on a restricted age range (24-39 months old at the time of evaluation, with some children enrolled at 23 months and scheduled for evaluation at 24 months or later), and results may vary in younger or older age groups. We expect that agreement between sources may remain strong or become even stronger in older children, as deviations from typical development may become clearer; whether or not this level of agreement is observed in the first 2 years of life should be investigated in future research. Because it is likely that parent understanding of infant and toddler development may impact their assessment of and potential concerns about their child's development, future research should also assess parental knowledge of early developmental milestones and symptoms of ASD.

A second limitation is that the ADOS may be difficult to administer in low resource settings, where community health workers are already over-burdened and the costs of administering the ADOS may be prohibitive. In such settings, the development and testing of low-cost screening tools, including brief questionnaires and mHealth screening tools, could be used to identify children in need of further evaluation. Digital screening tools could be used by community health workers, community advocates, or by parents themselves. One advantage of digital tools is that they provide the possibility of programming the application so that when scores cross a threshold, the application can generate recommendations for follow up with a community health care worker or clinician, supporting a tiered approach to screening and evaluation that is sensitive to local conditions. Digital tools also could embed educational information related to developmental milestones and symptoms of ASD, providing both education and screening. Such tools could be used in communities where ADOS evaluators and physician specialists are scarce. For example, in Africa, researchers (28) noted the importance of raising public awareness of ASD and addressing screening in different settings, including community settings, health care services, and schools. The authors noted that, "the public, parents, and professionals needed basic knowledge about child development and autism spectrum disorder to help identify children with autism spectrum disorder" (p. 6). They described the importance of using screening and assessment tools that consider local conditions and discussed further limitations of the ADOS, such as the inclusion of tasks involving items that may not be familiar to some African children, which would adversely affect the validity of ADOS scores. This points to the necessity of considering whether or not screening tools are culturally appropriate for the communities in which they are applied. Clearly much more research is needed to develop and test culturally appropriate, feasible, scientifically rigorous, and meaningful methods of screening children in diverse communities.

CONCLUSIONS

Our results illustrate the importance of educating parents about typical development and specific symptoms that indicate risk for ASD or other neurodevelopmental disorders. For primary care providers, community health workers, and early childhood educators, our results highlight the importance of responding immediately to parent concerns about their toddler's development to provide timely referral for further evaluation. This research supports a call for increased efforts by national organizations, primary health providers, early childhood educators, and community health organizations to educate parents about child development and encourage parents to express their concerns to their primary care providers as early as possible. This emphasis on parent education –along with timely responses from providers—may further existing trends toward earlier identification of ASD, and other neurodevelopmental disorders, and hence, generate opportunities for earlier and more personalized intervention approaches, which may help improve long-term outcomes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of California, Irvine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SA, MA, KL, and WG: study concept and design. MA, SA, and KL: analysis and interpretation of data. SA, MA, KL, and SS: drafting of manuscript. SA, MA, KL, WG, YG, JD, and SS: critical revision of manuscript for important intellectual content. KL:

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MINI REVIEW
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Review of Autism Profiles and Response to Sertraline in Fragile X Syndrome-Associated Autism vs. Non-syndromic Autism; Next Steps for Targeted Treatment

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Given significant genetic, molecular, and phenotypic overlaps, researchers have begun to investigate whether targeted treatments for Fragile X Syndrome (FXS) could also be beneficial for patients with Autism Spectrum Disorder (ASD). For example, low-dose sertraline, an SSRI, was used in two recent controlled trials in children with FXS and ASD. The first trial recruited 52 children with FXS, 32 of which were also diagnosed with ASD; the second trial recruited 58 children with non-syndromic ASD. One focus of the present study is to compare the response to sertraline between the FXS-associated ASD and non-syndromic ASD groups. Another focus is to compare baseline ASD-related characteristics between the groups and review these differences within the context of recent literature comparing these populations. Our comparison showed more severe ASD profiles in children with non-syndromic ASD vs. FXS-associated ASD. Regarding response to sertraline, the FXS-ASD group displayed significant improvements in language development, while the non-syndromic group did not show any significant improvements. One possible explanation for this differential response is the distinct anxiety profiles that are seen in these two groups. The heightened anxiety phenotype seen in those with FXS-ASD may have led to a greater relief of anxiety symptoms with sertraline compared to those with non-syndromic ASD; this, in turn, could have led to measurably greater developmental gains. Further research is required to solidify this connection between anxiety relief and developmental gains in these populations.

Keywords: Fragile X Syndrome, Autism spectrum disorder (ASD), targeted treatment, sertraline, anxiety

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INTRODUCTION

Autism spectrum disorder (ASD) is a behaviorally defined neurodevelopmental disorder that is characterized by impaired social and language development, repetitive behaviors, and restricted interests, as well as hyper or hypo-reactivity to sensory inputs. The etiology of ASD is complex and not well-defined, with over 500 different genetic mutations, as well as epigenetic interactions

associated with the development of the disorder (1–3). Despite the often-multifactorial nature of the disorder, ASD can also be caused by single gene mutations. The most common single-gene cause of ASD is Fragile X Syndrome (FXS). Approximately 60% of males and 20% of females with FXS meet the criteria for ASD. The clinical presentation of FXS and idiopathic ASD overlap significantly, with a number of neurologic and behavioral characteristics seen in both conditions. These include language deficits, poor eye contact, repetitive behaviors, perseverative speech, hypersensitivity to environmental stimuli, ADHD, anxiety, and social deficits (4–6).

Unlike ASD, the etiology of FXS—the most common inherited cause of intellectual disability—is well-defined. It arises from a full mutation repeat expansion (>200 CGG repeats in the 5′ untranslated region) in the *FMR1* gene on the X chromosome. This expansion results in the methylation and subsequent silencing of the *FMR1* gene, leading to drastically reduced levels of FMRP, the protein product of *FMR1*. The amount of FMRP produced in an individual directly correlates with his or her degree of cognitive impairment, with higher levels found in affected individuals with IQ scores above 70 (7, 8). FMRP is an mRNA binding protein involved in the transport and translational regulation of a number of dendritic mRNAs (9). Acting at the ribosomal level, it regulates the translation of the proteins involved in synaptic maturation and integrity. Compromised FMRP levels lead to abnormal dendritic spine density, abnormal synaptic plasticity, and immature, elongated dendritic spine morphology (10, 11). The cognitive and behavioral deficits seen in FXS are associated with the synaptic dysfunction that stems from the loss of FMRP.

The molecular abnormalities seen in individuals with FXS have many similarities with those seen in individuals with ASD. Investigation of ASD-related genes, mostly through analysis of copy number variants (CNVs) and point mutations associated with ASD, has implicated three broad domains impacted in ASD pathophysiology: synaptic function, neuronal signaling and development, and chromatin regulation (12–16). Importantly, two of these three domains—synaptic function and neuronal signaling and development—are compromised in FXS as well. Recent studies have shown that FMRP binds to up to 50% of all genes associated with ASD (1), and the CNVs in genes responsible for postsynaptic regulation of FMRP are also associated with ASD (17). These genetic overlaps provide a possible explanation for why synaptic dysfunction and altered neuronal signaling are present in both ASD and FXS, as well as why the two conditions share such overlapping phenotypes.

Given these commonalities, researchers have started investigating whether treatments targeting the neurobiological dysfunction in FXS may also be effective in ASD. For example, two controlled trials of the selective serotonin reuptake inhibitor (SSRI) sertraline were recently completed, first in children with FXS and then in children with idiopathic (non-syndromic) ASD (18, 19). In young children with ASD, serotonin production and levels are abnormally low, especially in the first 5 years of life (20). This early developmental window is when synaptic formation occurs most rapidly. Thus, an SSRI could exert its greatest beneficial effect in these early years (18). In addition to improving low serotonin levels, there is evidence that SSRIs stimulate brain-derived neurotrophic factor (BDNF) in certain mouse models (21). BDNF is active at synapses and is involved in synaptic maturation, neurogenesis, and plasticity (21–23). Given the molecular deficits common to FXS and ASD, it is apparent why SSRIs have emerged as an intriguing option as a targeted treatment for both disorders.

The initial rationale for the two aforementioned trials came when a retrospective chart review showed that low-dose sertraline in 45 children with FXS, improved both expressive and receptive language trajectory (24). The first of the two controlled trials under comparison in this study enrolled 52 children with FXS from 2012 to 2015, 32 of these subjects were also diagnosed with ASD. The second controlled trial, which ran from 2015 to 2018, enrolled 58 children with non-syndromic ASD. One of the primary goals of this review is to compare the response to sertraline in the 32 children with fragile X syndrome-associated autism (FXS-ASD), as reported by Greiss Hess et al. (18), with the 58 children with non-syndromic ASD, as reported by Potter et al. (19). This review will also compare baseline data from the FXS-ASD and non-syndromic ASD groups concerning language development, autism severity, and overall cognition. These baseline comparisons will serve to strengthen the evidence cited in recent literature, which outlines that there are qualitatively different autism profiles in these two groups (6, 25). Understanding these differences, moreover, can have important implications for how we utilize targeted treatments in the future.

MATERIALS AND METHODS

Participants and Design

Both trials under analysis were double-blind, placebo-controlled studies in children between the ages of 2 and 6 years. The two protocols were designed to be as similar as possible to maximize our ability to directly compare and contrast the effects on the two different populations. Both trials followed the same structure with a total of three study visits: an initial visit that included a detailed physical exam, diagnostic testing, and developmental testing; a 3-months visit that involved side effects and safety monitoring; and a 6-months visit during which testing was repeated to assess for developmental gains.

For the first trial, the inclusion criteria were molecular documentation of FXS, having a primary caregiver who was English speaking, and willingness to travel and participate in the trial. The exclusion criteria included whether they had a central nervous system (CNS) disease other than FXS or any other serious comorbid medical disorder. For the second trial, inclusion criteria were documentation of ASD as verified using both the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) and Autism Diagnostic Observation Schedule, Second Edition (ADOS-2), concurrent enrollment in at least one community or school intervention for ASD, having a primary caregiver who was English speaking, and willingness to travel and participate in the trial. The exclusion criteria included a previous diagnosis of the FXS full mutation or identification of the full mutation on initial visit blood

testing, current or past SSRI treatment, and any other serious comorbid medical disorders. Though individuals with FXS were excluded from this latter trial, there was one patient from this study whose autism was associated with another genetic syndrome—Beckwith-Wiedemann Syndrome. However, the other 57 children from this trial had purely idiopathic ASD not associated with a genetic syndrome.

The study drug was administered in liquid form (20 mg per mL). Subjects ages 2–3 years received sertraline liquid or placebo liquid in a dose of 2.5 mg per day (0.125 mL). Subjects ages 4–6 years received a dose of 5.0 mg per day (0.25 mL). The doses were based on those used in the retrospective study that originally suggested sertraline may help improve the trajectory of language development (24).

Assessments

Developmental assessments for both studies were conducted in the clinic at the initial study visit and the final 6-months visit. Both trials included the following study assessments: Mullen Scales of Early Learning (MSEL), Clinical Global Impression—Improvement Scale (CGI-I), Preschool Language Scale—Fifth Edition (PLS-5), Sensory Processing Measure—Preschool Edition (SPM-P), and Vineland Adaptive Behavior Scale-II (VABS-II). Both trials conducted the ADOS-2 for diagnostic purposes at the initial study visit only. Despite the majority of assessments overlapping between both studies, some assessments were only used in one study. For example, the McArthur-Bates Communicative Development Inventories (CDI) and Parenting Stress Index—Fourth Edition (PSI-4) were only used in the FXS trial, while the Aberrant Behavior Checklist (ABC) was only used in the non-syndromic ASD trial. Only assessments used in both trials were analyzed in this comparison.

For both trials, language development as measured by the MSEL Expressive Language scores were the primary outcome measure. Accordingly, language development was a focus of our baseline comparison, which analyzes the baseline MSEL Expressive Language (EL) and Receptive Language (RL) raw scores, as well as PLS-5 Expressive Communication (EC) and Auditory Comprehension (AC) raw scores. The MSEL Early Learning Composite (ELC) standard score, thought to be representative of an IQ equivalent in children with ASD, will also be analyzed. Lastly, this comparison will use ADOS-2 Social Affect (SA) and Restrictive and Repetitive Behavior (RRB) scores to better characterize the distinct autism profiles seen in the two populations.

Statistical Analysis

The baseline ADOS-2 scores, MSEL scores, and PLS-5 scores between the FXS-ASD and non-syndromic ASD groups were analyzed using non-parametric, two-sample Mann-Whitney *U*-tests carried out with the statistical package SPSS, version 26. Analysis of the response to sertraline with respect to MSEL EL and RL raw scores was carried out and described by Greiss Hess et al. and Potter et al., respectively (18, 19).

RESULTS

Baseline Data

Our results showed that ADOS-2 SA scores (14.051 vs. 11.125; $p < 0.01$) as well as RRB scores (5.258 vs. 3.875; $p < 0.005$) were both significantly increased in the non-syndromic ASD group relative to the FXS-ASD group, indicating more severe autistic behaviors in the non-syndromic ASD group (**Table 1**). Effect sizes for SA and RRB scores showed small to medium strengths of association ($r = 0.28$ and $r = 0.32$, respectively). No differences in baseline MSEL EL (18.862 vs. 17.645; $p = 0.769$) or RL (22.344 vs. 21.806; $p = 0.880$) scores were found between the two groups, nor was there any difference in EC scores on PLS-5 (22.95 vs. 23.44; $p = 0.61$). However, the lower AC scores on PLS-5 in the non-syndromic ASD group relative to the FXS-ASD group approached statistical significance (22.70 vs. 27.19; $p = 0.075$) and had a small strength of association ($r = 0.19$), suggesting marginally more advanced auditory comprehension in those with FXS-ASD. Notably, the higher Early Learning Composite (ELC) standard score in the non-syndromic ASD group approached statistical significance as well (57.67 vs. 51.06; $p = 0.077$; $r = 0.19$).

Response to Sertraline

According to the MSEL EL raw scores, those with FXS-ASD experienced a significant improvement with sertraline vs. placebo (23.5 vs. 17.6; $p < 0.005$) (18), while those with non-syndromic ASD experienced no difference between sertraline treatment and placebo (20.3 vs. 21.8; $p = 0.547$) (19). Regarding the MSEL RL scores, there was no difference between sertraline and placebo in either the FXS-ASD group or the non-syndromic ASD group (18, 19).

DISCUSSION

One of the main goals of this study was to further contribute to the body of knowledge regarding phenotypic similarities and the differences between non-syndromic ASD and fragile X-associated ASD, and our results support the findings of recent studies showing qualitatively different autism profiles between these two populations. Wolff et al. (25), comparing age-matched boys with FXS-ASD and non-syndromic ASD, analyzed five ADOS-2 measures and found that boys with FXS-ASD were less impaired in regards to social smiling, quality of social overtures, and facial expressions relative to boys with non-syndromic ASD. Moreover, McDuffie et al. (6) found that boys with FXS-ASD demonstrated more social smiling, more motivation to engage in triadic interactions, and more non-verbal gestures relative to age-matched boys with non-syndromic ASD. Furthermore, among verbal participants, those with FXS-ASD were more likely to engage in social conversation. These reports of less social impairment in individuals with comorbid FXS and ASD are concordant with the results of the present study, which found significantly lower social affect domain scores in children with FXS-ASD relative to non-syndromic ASD.

These findings also demonstrate fewer restrictive and repetitive behaviors in children with FXS-ASD, which also

TABLE 1 | Comparison of baseline ADOS-2, MSEL, and PLS-5 baseline scores.

	Non-syndromic ASD			Fragile X-associated ASD		
	N	**Mean**	**Standard deviation**	***N***	**Mean**	**Standard deviation**
ADOS-2 SA score	58	13.98	4.54	32	11.13	4.44
ADOS-2 RRB score	58	5.26	2.08	32	3.88	1.58
MSEL EL raw score	58	18.96	10.92	31	17.65	8.65
MSEL RL raw score	58	22.34	9.78	31	22.10	7.63
MSEL ELC standard score	58	57.67	12.77	32	51.06	3.92
PLS-5 AC raw score	56	22.70	12.83	31	27.19	10.19
PLS-5 EC raw score	56	22.95	11.82	31	23.44	8.85
			Mean difference			**Effect size (*r*)***
ADOS-2 SA score			2.85 ($p = 0.008$)			0.28
ADOS-2 RRB score			1.38 ($p = 0.002$)			0.32
MSEL EL raw score			1.31 ($p = 0.77$)			0.03
MSEL RL raw score			0.24 ($p = 0.88$)			0.02
MSEL ELC standard score			5.61 ($p = 0.077$)			0.19
PLS-5 AC raw score			4.49 ($p = 0.075$)			0.19
PLS-5 EC raw score			0.49 ($p = 0.61$)			0.05

**Interpretation of Pearson r coefficient according to Cohen (1988): 0.1–0.3—small strength of association; 0.3–0.5—medium strength of association; 0.5–1.0—large strength of association (26). **SA, Social Affect; RRB, Restrictive and Repetitive Behaviors; EL, Expressive Language; RL, Receptive Language; ELC, Early Learning Composite; AC, Auditory Comprehension; EC, Expressive Communication.*

supports McDuffie et al.' report that boys with FXS-ASD exhibit less insistence on sameness relative to boys with non-syndromic ASD (6). Regarding baseline language development, the FXS-ASD group showed modestly advanced auditory comprehension relative to the non-syndromic ASD group but no difference in expressive language or communication. Notably, MSEL ELC scores—thought to be an IQ equivalent for individuals with ASD (27)—were slightly higher in the non-syndromic ASD group. These results are again consistent with previous evidence showing higher levels of non-verbal IQ in non-syndromic ASD relative to FXS-ASD (6).

Understanding the differences and similarities between FXS-ASD and non-syndromic ASD profiles is important clinically, specifically in answering the question of whether treatments targeting the core symptoms of FXS can also be useful for individuals with non-syndromic ASD. Some speculate that the potential for common treatments for these two conditions negatively correlates with an increasing number of differences identified between the two conditions (6). This is a valid concern, and it may provide a rationale for why, contrary to what the authors hypothesized, sertraline did not stimulate language gains in children with non-syndromic ASD like it did in children with FXS-ASD.

We believe that the underlying basis for this incongruent response to sertraline can in part be explained by the differing anxiety profiles between the two groups. Individuals with FXS have more significant GABA deficits, leading to impaired habituation to sensory stimuli, greater sympathetic responses, and a heightened anxiety phenotype relative to individuals with non-syndromic ASD (19, 28). Recent studies have shown that certain components of ASD symptomatology seen in those with FXS-ASD, including repetition of words or phrases as well as gaze avoidance, may be due to heightened anxiety, secondary to FXS rather than solely due to the social impairment seen in those with ASD without FXS (29–31). This suggests different mechanistic underpinnings behind overlapping phenotypes between FXS-associated and non-syndromic ASD. Given this, it is possible that the FXS-ASD population, with a greater degree of anxiety than those with non-syndromic ASD, experienced more appreciable anxiety relief while taking sertraline. This, in turn, could have facilitated the behavioral or attentional improvements that led to measurably greater developmental and language gains by the end of the study (19, 30). Moreover, this anxiety link explains why, in our baseline analysis, the FXS-ASD group had better auditory comprehension than the non-syndromic ASD group despite equivalent levels of expressive communication. This supports the notion that children with FXS-ASD have a better understanding of language and communication than children with non-syndromic ASD at baseline, but their underlying anxiety manifests as an apparent deficit in expressive language.

These hypotheses must be viewed with caution, however, and have two main limitations. First, baseline levels of anxiety were not included in the study protocol described by Greiss Hess et al., so analysis of anxiety levels between the two study populations was not possible. Second, there is limited evidence that this low dose of sertraline is effective in treating anxiety, as the usual lowest starting dose for sertraline when treating pediatric anxiety and depression is 12.5–25 mg (32). However, these recommendations are typically for older children, as SSRIs are not frequently started in children under 6 years old and

thus have not been well-studied in this age group. Furthermore, we have seen anecdotal evidence in our clinical practice that 2.5–5.0 mg of sertraline can improve anxiety in this very young patient population.

The next steps in regards to sertraline as a targeted treatment should involve narrowing focus on the study population in whom we have already seen demonstrable benefits, young children with FXS-ASD. Future research on the topic should involve clear pre and post-treatment anxiety levels to answer the question of whether this dosage of sertraline can improve anxiety in affected children of this age. It is important to note, though, that the complex and varying clinical picture seen in ASD can complicate the evaluation of anxiety in these patients. Thus, future studies that analyze this must be sure to evaluate the different possible manifestations of anxiety in these patients in addition to social withdrawal. This includes irritability—which can manifest itself in a number of ways such as tantrums, aggression, and self-injurious behavior—as well as hyperactivity behaviors such as excessive movement or inability to sit still. Thus, a detailed evaluation of anxiety in this patient population is required.

If this data is gathered and analyzed within the context of pre and post-treatment language development markers as well as ADOS-2 scores, it could elucidate whether improving anxiety will drive improvements in language development and autism severity, as the authors of this study hypothesize. Such data would accomplish three main goals: it would provide novel evidence that low-dose sertraline is effective in treating anxiety in this specific patient population. It would further solidify the argument that increased anxiety in FXS-ASD is the primary driver of social withdrawal, in contrast to the intrinsic social deficits seen in non-syndromic ASD. Finally, it would help optimize our use of targeted treatments within these populations in the future.

AUTHOR CONTRIBUTIONS

AR, LP, and RH contributed to the conception and design of the study. AR and HB performed the statistical analyses. AR and IP wrote the first draft of the manuscript. AS administered developmental and language assessments. All authors contributed to manuscript writing, revision, read, and approved the submitted version.

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Phenotypic Subtyping and Re-analyses of Existing Transcriptomic Data from Autistic Probands in Simplex Families Reveal Differentially Expressed and ASD Trait-Associated Genes

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Autism spectrum disorder (ASD) describes a collection of neurodevelopmental disorders characterized by core symptoms that include social communication deficits and repetitive, stereotyped behaviors often coupled with restricted interests. Primary challenges to understanding and treating ASD are the genetic and phenotypic heterogeneity of cases that complicates all omics analyses as well as a lack of information on relationships among genes, pathways, and autistic traits. In this study, we re-analyze existing transcriptomic data from simplex families by subtyping individuals with ASD according to multivariate cluster analyses of clinical ADI-R scores that encompass a broad range of behavioral symptoms. We also correlate multiple ASD traits, such as deficits in verbal and non-verbal communication, play and social skills, ritualistic behaviors, and savant skills, with expression profiles using Weighted Gene Correlation Network Analyses (WGCNA). Our results show that subtyping greatly enhances the ability to identify differentially expressed genes involved in specific canonical pathways and biological functions associated with ASD within each phenotypic subgroup. Moreover, using WGCNA, we identify gene modules that correlate significantly with specific ASD traits. Network prediction analyses of the genes in these modules reveal canonical pathways as well as neurological functions and disorders relevant to the pathobiology of ASD. Finally, we compare the WGCNA-derived data on autistic traits in simplex families with analogous data from multiplex families using transcriptomic data from our previous studies. The comparison reveals overlapping trait-associated pathways as well as upstream regulators of the module-associated genes that may serve as useful targets for a precision medicine approach to ASD.

Keywords: ASD subgroups, transcriptomic analysis, simplex families, ASD trait-associated genes, comparison with multiplex population

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder in which symptoms typically appear within the first 3 years of life. According to the Diagnostic and Statistical Manual of Mental Disorders-5th Edition (DSM-5), a diagnostic guide created by the American Psychiatric Association for mental disorders (1), individuals with ASD are affected in two core domains: social communication and repetitive, stereotyped behaviors often with restricted interests. DSM-5 differs from the previous DSM-4 diagnostic guide in that a third core domain (i.e., language development and pragmatics) is now integrated into social communication. Moreover, individuals who were previously classified into one of several related conditions, including Autistic Disorder, Asperger's Syndrome, and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS) are now combined under "Autism Spectrum Disorder." Nonetheless, individuals with ASD manifest a wide variety of symptoms within the core domains, with highly variable severity. Thus, even though the clinical definition for ASD has been condensed, the genetic and phenotypic heterogeneity of affected individuals still complicates omics studies of ASD. In addition, there are no clear relationships between gene expression profiles and autistic traits in ASD individuals.

Recently, researchers have explored reducing clinical heterogeneity by selecting individuals with specific ASD traits based on severity scores from the Autism Diagnosis Interview-Revised (ADI-R) diagnostic instrument (2–5). For research purposes, the ADI-R diagnostic questionnaire is considered the gold standard behavioral test for ASD (6), whereas the DSM-5 is used for screening in clinical practice. The ADI-R, based on the DSM-4 guidelines, focuses on behavioral assessment in three main areas: reciprocal social interaction, communication and language, and repetitive stereotyped behaviors, including restricted interests. Previous studies in our laboratory identified four phenotypic groups in multiplex families (having more than one affected child) by multivariate cluster analyses of 123 severity scores on 63 items from the ADI-R (4). In addition, we validated biological differences among these subgroups by transcriptomic analyses of three of the subgroups in comparison to a control group (7). Moreover, class prediction analyses of differentially expressed genes (DEGs) from these analyses identified a limited number of DEGs that could differentiate cases from controls, thus exhibiting potential for use as biomarkers (8).

For genetic and quantitative trait association analyses, we further divided the three core domains probed by the ADI-R (social, language, and repetitive behaviors) into subcategories (including spoken language, non-verbal communication, play skills, social interactions, and insistence on sameness) which led to the identification of both subtype- and trait-associated genetic variants (9, 10). Savant skills, which are present in roughly 10% of individuals with ASD, is also a trait of interest and were present at higher frequency in one of the subgroups used for gene expression profiling (7).

Despite the demonstrated association of genetic variants and autistic traits, there is still relatively little understanding of the relationship between gene expression and ASD traits. In this regard, Weighted Gene Co-expression Network Analysis (WGCNA) is a software tool designed to explore the relationships between gene expression profiles and external information, such as case-control status or a specific condition (11). It has been applied recently in research on Alzheimer's disease (12), bipolar disorder (13), pancreatic cancer (14), and renal cell carcinoma (15) to identify gene networks dysregulated within the respective diseases. Co-expression analysis has also been implemented in previous research studies on ASD (16–21), but there has yet to be WGCNA analyses focused on autistic traits.

The primary goals of this study are to: (1) determine whether subtyping of the ASD probands from simplex families (having only one affected child) by cluster analyses of scores from the ADI-R improves the ability to determine gene expression (i.e., biological) differences between subtyped cases and controls over unsegregated cases and controls, (2) use WGCNA to investigate the association of gene networks with various traits of ASD, and (3) compare the gene expression modules and pathways associated with selected autistic traits in simplex families vs. those in multiplex families to identify both differences and similarities.

MATERIALS AND METHODS

The main goal of this study was to re-analyze existing transcriptomic data on a group of autistic probands and their respective siblings from the Simons Simplex Collection (SSC) in order to determine whether subtyping of ASD probands by multivariate cluster analysis of ADI-R item severity scores can facilitate detection of gene expression differences between cases from phenotypic subgroups and their non-autistic siblings. In addition, we sought to interrogate and compare relationships between gene networks and autistic traits in cases from both simplex and multiplex families. **Figure 1** shows the overall analytical workflow for this study.

Phenotypic Subtyping for Simplex Families

Raw ADI-R scoresheets for 1,900 individuals with ASD (i.e., probands) were obtained from the SSC (New York, NY). As described previously for multiplex families (4), 123 scores on 63 ADI-R items for each proband were subjected to K-means cluster (KMC) analysis. This analysis showed an optimum separation of cases with $K = 3$, indicating three phenotypic subgroups. This was in contrast to the four phenotypic subgroups that optimally distinguished ASD cases in multiplex families (4). Unsupervised principal component analysis (PCA), a dimensionality reduction tool, was then used to more clearly visualize the distribution of cases from these three subgroups based on their respective ADI-R severity scores (**Figure 2**).

Acquisition of Transcriptomic Data for Individuals With ASD and Their Siblings

Normalized gene expression data from a study by Luo et al. (22) on lymphoblastoid cell lines (LCL) from 412 individuals (combined cases and controls) included in the SSC were downloaded from the Gene Expression Omnibus (GEO) data repository using the GEOquery R package (accession number

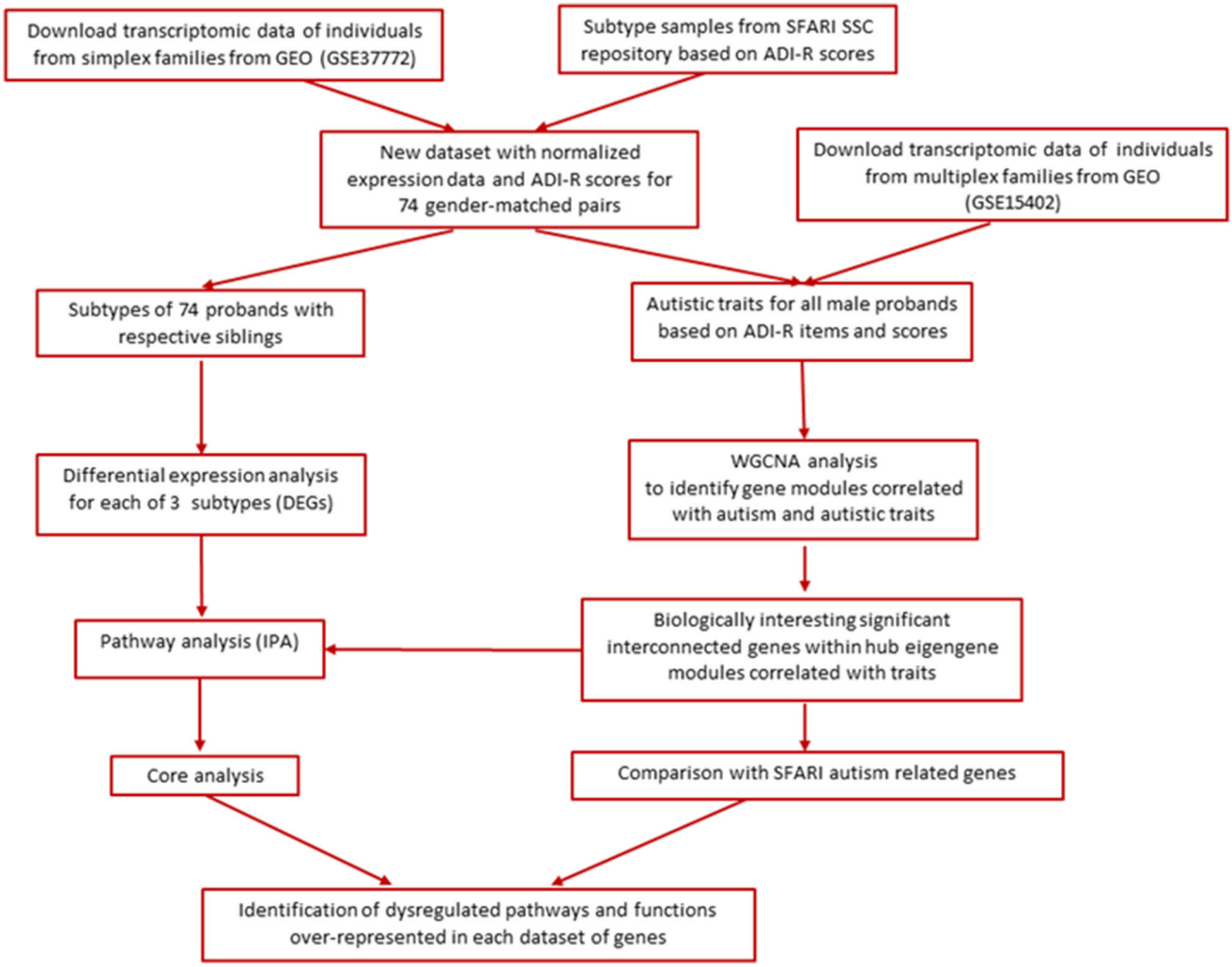

FIGURE 1 | Overall analytical workflow.

GSE37772). The expression data had been obtained using the Illumina Whole Human Genome Array Human REF-8 version 3.0 following the manufacturer's standard protocol (22). Among the set of individuals included in the study were 168 pairs of cases and controls (each pair from the same family), including 98 sibling pairs matched for sex. The sample identification numbers (IDs) of the probands in this group were cross-referenced against those for whom complete ADI-R scoresheet data were available for the subtyping analyses described above. This resulted in the identification of 74 pairs of sex-matched cases and sibling controls with both gene expression and ADI-R data (for cases only). The demographic information on these individuals is shown in **Supplementary Table 1**. Based on the cluster analyses of ADI-R scores, the probands (together with their respective unaffected siblings) were distributed into three phenotypic subgroups for transcriptomic analyses. These subgroups were identified as: Language (7 pairs), Intermediate (26 pairs), and Mild (41 pairs).

For WGCNA analyses of autistic traits in individuals with ASD from multiplex families, normalized gene expression data from a study by Hu et al. (7) were downloaded from GEO (accession number GSE15402). These data were obtained using custom-printed TIGR 40K human arrays containing 39,936 human cDNA probes, as previously described. ADI-R data for the individuals in this study were obtained from the Autism Genetics Research Exchange, with subtyping performed on adjusted ADI-R scores as previously described (4).

Identification of Subgroup-Associated Differentially Expressed Genes (DEGs)

Differential gene expression analysis was performed using Multi-experiment Viewer (MeV) software for microarray analyses (23) which employed an 80% data filter, meaning that each gene included in the study had an expression value in at least 80% of the samples. *T*-tests with bootstrapping permutation, which randomly regrouped samples 1,000 times, were conducted on the normalized data from cases within each subgroup and their respective sibling controls to identify significant DEGs with a critical p-value set at ≤ 0.05. Principal component analysis (PCA) was used to visualize the separation of cases and controls based

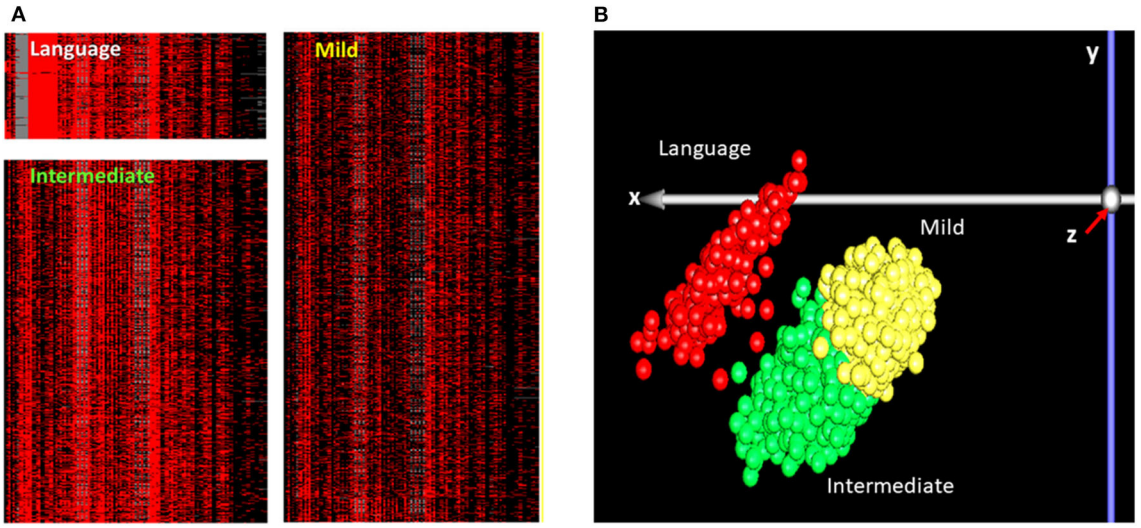

FIGURE 2 | Phenotypic subgroups of individuals with ASD which were identified by: **(A)** K-means cluster (KMC) analysis of 123 scores on 63 ADI-R items for 1,900 individuals with ASD, with K = 3; **(B)** Graphical representation of a principal component analysis (PCA) of the ADI-R scores represented in panel **(A)** for 1,900 ASD cases from the SSC. For the KMC analysis **(A)**, each row represents an individual with ASD and each column represents the respective individual's score on a specific ADI-R item. Severity of ADI-R item scores are indicated by the intensity of red in the heatmaps, with bright red indicating a score of 3, lighter shades of red indicating scores of 2 and 1, and black representing a score of 0, which is equivalent to "normal." The bright red block of columns in the "Language" subgroup corresponds to items related to deficits in spoken language on the ADI-R diagnostic instrument. Based on the KMC analysis, the subgroups were labeled as "Language" for the severely language-impaired subgroup, "Mild" for the subgroup with the lowest severity profile across the 123 ADI-R scores, and "Intermediate" for the subgroup exhibiting an intermediate severity profile between the Language-impaired and Mild subgroups. PCA was used to reduce the dimensionality of the multivariate data (i.e., 123 scores per individual) in order to better visualize the clusters of individuals with similar severity profiles across all ADI-R items. The *x*, *y*, and *z* axes represent the first, second, and third principal components from the PCA analysis **(B)**. Each point on the graph represents an individual, with red representing individuals in the Language subgroup, green representing individuals in the Intermediate subgroup, and yellow representing individuals in the Mild subgroup.

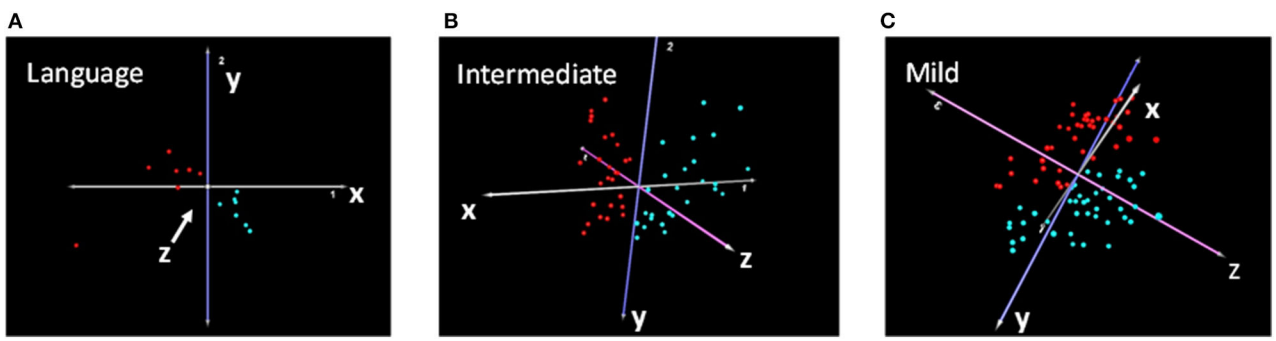

FIGURE 3 | Principal component analyses showing separation of cases (red) and controls (turquoise) based on DEGs from transcriptomic analyses of the **(A)** Language, **(B)** Intermediate, and **(C)** Mild cases and their respective sibling controls.

on the subgroup-associated DEGs. Based on the PCA results, we used a more stringent p-value of ≤ 0.03 to optimize the separation of Mild cases from their respective sibling controls.

Classification of Clinical Autistic Traits

Another goal of this study was to investigate the correlation between gene expression profiles and autistic traits. The three core domains considered in the ADI-R diagnostic tool (communication and language, social interaction, and repetitive stereotyped behaviors were further parsed into six primary traits as shown in **Supplementary Table 2**, along with the ADI-R items corresponding to each trait. The cumulative severity score across the items comprising each trait was used as the specific trait score for each individual. The six traits included impairment of verbal communication (Verbal), non-verbal communication (Non-verbal), social interaction (Social), play skills (Play), insistence on sameness and rituals (Sameness), and presence of savant skills (Savant).

Weighted Gene Co-expression Network Analysis (WGCNA)

WGCNA is an R-package primarily designed for co-expression analysis of transcriptomic data (11). In this study, WGCNA analyses were performed on normalized expression data from

all detectable genes to identify correlated gene networks associated with each of the autistic traits in both simplex and multiplex families. The threshold of signed R^2 was set to 0.85 prior to network construction, as this value is used to calculate the unsigned co-expression topology overlap necessary for constructing the network. Gene clusters, called modules, were then merged if the correlation values for the eigengenes were above 0.8. Hub modules were selected based on significant correlation (Pearson's correlation coefficient, $p \leq 0.05$) with external information (i.e., autistic trait severity scores). For WGCNA analyses of autistic traits in simplex families, normalized gene expression data for 24,526 probes (corresponding to 18,415 genes) from 63 male probands were used to identify gene networks that associated with the six autistic traits. Similarly, WGCNA analysis of autistic traits in multiplex families utilized normalized gene expression data for 28,592 probes (corresponding to 11,129 genes) from 81 male individuals with ASD. This transcriptomic data was derived from the study of 7).

Pathway and Functional Analyses of DEGs and Module Genes From WGCNA

Ingenuity Pathway Analysis (IPA) software (Qiagen, Germantown, MD) was used for network prediction analyses to identify canonical pathways, biological functions, and diseases enriched among DEGs from our subtype-dependent transcriptomic analyses as well as those over-represented among genes in significant trait-associated gene modules from WGCNA analyses. The Fisher Exact Test, as implemented by IPA software, was used to determine the significance of enrichment with respect to a given pathway, function, or disease using IPA Knowledgebase as a reference gene set. IPA was also used for comparison analysis of pathways and upstream regulators associated with DEGs from simplex and multiplex samples.

Hypergeometric Distribution Analyses for ASD Gene Enrichment Among Module Genes

Trait-associated module genes were compared to a list of 910 known autism risk genes in the SFARI Gene database (24). Venny 2.1.0, an online software package for creating Venn diagrams (25), was used to identify overlapping genes between the SFARI gene set and the trait genes https://bioinfogp.cnb.csic.es/tools/venny/. The CASIO Keisan Online Calculator http://keisan.casio.com/exec/system/1180573201 was then used to determine hypergeometric distribution probabilities for over-representation of module genes within the SFARI dataset with significance determined by an upper cumulative $q \leq 0.05$.

RESULTS

Differential Gene Expression Analysis of Each Subtype in Simplex Families

Differential gene expression analysis of each subtype shown in **Figure 2** resulted in a total of 774, 384, and 274 differentially expressed transcripts corresponding to 765, 377, and 270 DEGs for the Language, Intermediate, and Mild subgroups, respectively (**Supplementary Tables 3–5**). PCA analyses based on these subtype-associated DEGs showed almost complete separation of cases from controls within each phenotypic subgroup, as illustrated in **Figure 3**. In addition, a separate gene expression analysis of all cases (combined subgroups) and controls was performed to assess the result of heterogeneity reduction in identifying DEGs and associated biological processes (**Supplementary Table 6**).

Pathway Analysis of DEGs From Each Subtype in Simplex Families

IPA was used to identify over-represented canonical pathways, diseases, and neurological functions among the DEGs from each of the three ASD subgroups. **Table 1** shows a comparison of autism-relevant canonical pathways significantly enriched among DEGs from the three subgroups. As shown, there are a much greater number of significantly over-represented pathways relevant to ASD in the Language subgroup in comparison to those over-represented in the Intermediate and Mild subgroups. Notably, all of the pathways shown are also enriched among genes from the SFARI Gene database (data not shown). Interestingly, there were no significant autism-associated pathways enriched among DEGs obtained when all 74 cases were combined for expression analysis as shown in **Supplementary Table 7**, which

TABLE 1 | Comparison of significantly over-represented ASD-relevant canonical pathways in three phenotypic subgroups of ASD (Language, Intermediate, and Mild).

Ingenuity canonical pathways	Language	Intermediate	Mild
(implicated in ASD)		–log(*p*-value)*	
Axonal Guidance Signaling	3.61		1.44
Protein Kinase A Signaling	3.44		
Actin Cytoskeleton Signaling	3.33		
CDK5 Signaling	3.22		
cAMP-mediated signaling	2.69	1.67	
Androgen Signaling	2.46		
Synaptogenesis Signaling Pathway	2.03		2.14
Melatonin Signaling	2.02		
VDR/RXR Activation	2.00		
Ephrin Receptor Signaling	1.99		
Neuregulin Signaling	1.93	1.78	2.36
GABA Receptor Signaling	1.93		
Gap Junction Signaling	1.90		
CREB Signaling in Neurons	1.84		
Synaptic Long Term Depression	1.74		
PI3K/AKT Signaling	1.66		
Synaptic Long Term Potentiation	1.62		
Estrogen Receptor Signaling	1.58		
Neurotrophin/TRK Signaling	1.44		
ERK/MAPK Signaling	1.41		
Netrin Signaling		1.71	
PTEN Signaling		1.39	

**Negative logarithm of the Fisher Exact p-value indicating the probability that the indicated pathway is not over-represented among the genes in the respective datasets. A –log(p-value) of 1.3 is equivalent to a p-value of 0.05. Only significant values are shown.*

provides a complete list of significant pathways for both subgroup and combined group analyses.

Table 2 shows an IPA-generated comparison of over-represented neurological diseases and functions that are both shared and unique among the ASD subtypes and the combined case group. Not surprisingly, the Language subgroup is associated with more neurological functions than either of the other two subgroups or the combined group. This finding is consistent with the phenotypic data which suggests that the Language subgroup is the most severely affected according to ADI-R severity scores (see **Figure 2**). Cognitive impairment also occurs exclusively within the confines of the Language subtype. Interestingly,

TABLE 2 | Comparison of significantly over-represented neurological functions and diseases in three phenotypic subgroups of ASD as well as in the combined case group.

Neurological diseases and functions	Language	Intermediate	Mild	Combined
		–log(*p*-value)*		
Morphology of nervous system	6.49	4.52		3.09
Organization of cytoskeleton	6.03			
Motor dysfunction or movement disorder	5.82			
Neurological signs	5.60			
Neurotransmission	5.59	3.68		2.37
Movement Disorders	5.58			
Schizophrenia spectrum disorder	5.35		3.16	
Severe psychological disorder	5.11			
Abnormal morphology of nervous system	4.90	4.11		2.32
Cognition	4.89	3.87		2.99
Synaptic transmission	4.73	3.22		
Memory	4.36	2.80		
Potentiation of synapse	4.12			
Morphology of brain	3.93	3.27		
Learning	3.90	2.92		2.78
Neuronal cell death	3.87			
Cognitive impairment	3.86			
Long-term potentiation	3.83			
Development of neurons	3.77		2.54	
Organization of actin cytoskeleton	3.71			
Growth of neurites	3.64			
Neuritogenesis	3.38			
Outgrowth of neurites	3.24			
Secretion of neurotransmitter	3.17		3.40	
Morphology of dendritic spines	3.09			
Polarization of neurites	3.04			
Complete agenesis of corpus callosum		3.67		
Extension of dendrites		3.33		
Sensory disorders		3.25		
Epilepsy or neurodevelopmental disorder			4.58	2.90
Seizure disorder			3.41	
Epilepsy			3.35	
Polarization of neuroglia			3.18	
Complexity of dendritic trees				4.10
Migration of neurons				3.46
Size of neurons				3.40
Size of dendritic trees				3.38
Guidance of axons				3.19
Sensory system development				3.06
Emotional behavior				3.04

**Negative logarithm of the Fisher Exact p-value which indicates the probability that the indicated disease or function is not over-represented in the respective dataset of genes. A –log(p-value) cutoff of 3 which is equivalent to a p-value of 0.001 was used to select diseases and functions from each group shown here.*

sensory disorders and epilepsy, which are cormorbidities frequently associated with ASD (26, 27), are enriched within DEGs in the Intermediate and Mild subgroups, respectively. None of these subgroup-associated comorbid disorders were identified when all cases were combined.

WGCNA Analyses of Autistic Traits in Simplex Families

WGCNA was used to identify gene clusters (modules) correlated with clinical autistic traits in 63 male probands using their respective cumulative ADI-R trait scores and normalized expression data for all detectable genes. Inasmuch as ASD is a neurodevelopmental disorder predominantly affecting males (28), we decided to focus on male probands to avoid confounding effects related to sex. Module-trait relationships determined by WGCNA revealed that only the verbal, non-verbal, and social interaction traits were significantly correlated with specific gene modules (**Figure 4**). Pathway analyses were then conducted on all genes within the significant modules for each of these traits as described below. These included the greenyellow, purple, and red modules for the verbal trait, brown, green, red, tan, and turquoise modules for the non-verbal trait, and the red and tan modules for the social trait.

Network Prediction Analyses of Genes Associated With Autistic Traits in the Simplex Population

IPA was used to identify canonical pathways that were enriched among the module genes collectively contributing to verbal, non-verbal, and social deficits in individuals with ASD from the simplex population. The complete sets of significantly over-represented pathways for each trait are presented in **Supplementary Table 8** together with the pathways associated with the combined ASD traits, using genes from all seven significant modules in an IPA core analysis. **Table 3** summarizes the ASD-relevant canonical pathways among the top 25 over-represented pathways associated with verbal, non-verbal and social traits, while **Figure 5** shows the overlap among all significant pathways associated with each trait. As shown, there are both unique and overlapping trait-associated pathways, with the non-verbal trait exhibiting the largest number of pathways previously implicated in ASD. Aside from the pathways shared among the three traits, the non-verbal trait is also uniquely associated with several neurological functions including reelin, NGF, neurotrophin, and synaptogenesis signaling in addition to estrogen receptor and NFkB signaling. Interestingly, mitochondrial dysfunction, which affects some individuals with ASD (29–32), was uniquely associated with deficits in social interaction. With respect to neurological diseases, only modules correlated with the non-verbal trait are enriched with genes that are significantly associated with mental retardation (p = 9.25E-13; 189 genes) and cognitive impairment (p = 1.50E-09; 265 genes), which are comorbidities frequently seen in ASD (27, 33). In addition, non-verbal communication is the only trait correlated with module genes that are explicitly associated with autism spectrum disorder or intellectual disability (p = 1.18E-13; 237 genes), suggesting that deficits in non-verbal communication are major contributors to the overall phenotype of ASD in the simplex population.

WGCNA Analysis of Autistic Traits in Multiplex Families

WGCNA was also used to identify gene modules that correlated with selected autistic traits in males from multiplex families that were included in our previous gene expression study of

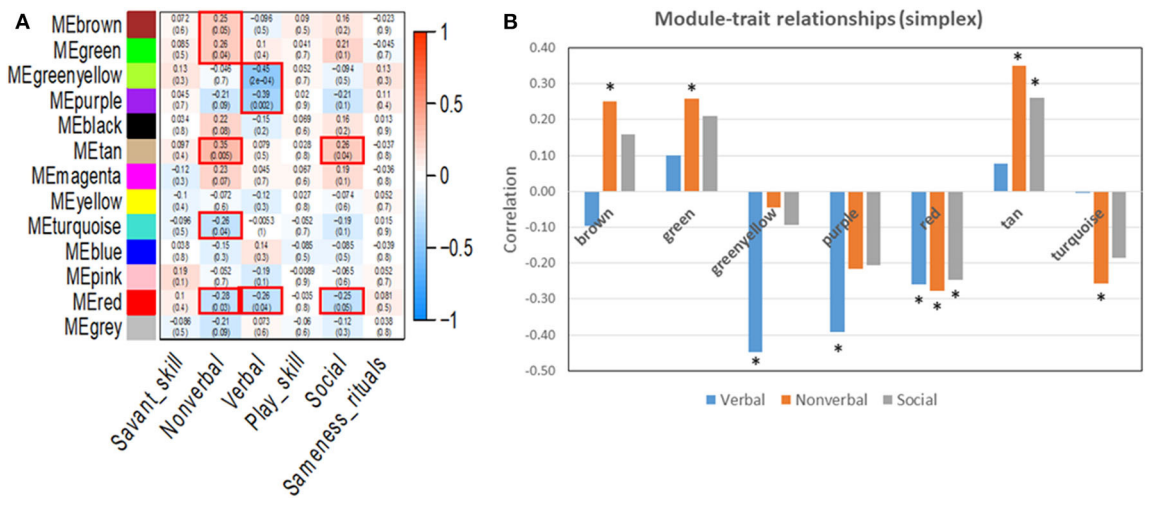

FIGURE 4 | (A) Module-trait relationships resulting from WGCNA analyses of autistic traits in the simplex population. Significant modules with $p \leq 0.05$ are outlined in red. **(B)** Correlation values for the trait-associated gene modules that are significantly correlated with at least one autistic trait. *$p \leq 0.05$. Each colored module represents a network of genes with correlated expression among its members. Six autistic traits were evaluated for correlated gene expression networks. Three traits (verbal, non-verbal, and social) showed significant association with at least one module.

TABLE 3 | Significantly over-represented ASD-relevant pathways among the top 25 associated with the verbal, non-verbal, and social traits in the simplex population.

Ingenuity canonical pathways	–log(*p*-value)*
Verbal communication	
Regulation of eIF4 and p70S6K Signaling	5.07
ERK/MAPK Signaling	4.86
EIF2 Signaling	4.86
mTOR Signaling	3.82
Non-verbal communication	
Regulation of eIF4 and p70S6K Signaling	8.35
Protein Ubiquitination Pathway	7.5
mTOR Signaling	7.48
Ephrin Receptor Signaling	7.21
Estrogen Receptor Signaling	6.8
EIF2 Signaling	6.51
Reelin Signaling in Neurons	6.25
p70S6K Signaling	6.12
NGF Signaling	6.09
Synaptogenesis Signaling Pathway	5.94
ERK/MAPK Signaling	5.92
Social interaction	
ERK/MAPK Signaling	3.34
ATM Signaling	3.06
Androgen Signaling	2.95

**Negative logarithm of the Fisher Exact p-value which represents the probability that the pathway is not over-represented among the respective dataset of genes, where a value of 1.3 is equivalent to a p-value of 0.05.*

ASD subtypes (7) for the purpose of comparison with the data from simplex families in this study. **Figure 6A** shows that, in contrast to the results for probands from simplex families, more modules (18 in all) are highly correlated with five autistic traits. The significant modules and their correlation with each of the five traits (verbal, non-verbal, play, insistence on sameness and rituals, and savant skills) are highlighted in the barplot (**Figure 6B**). Interestingly, modules correlating with savant skills show inverse correlation with respect to other autistic traits sharing the same modules. By contrast, no modules were correlated with savant skills in the simplex population.

Network Prediction Analyses of Genes Associated With Autistic Traits in the Multiplex Population

IPA was used to identify pathways and functions over-represented among module genes significantly associated with five ASD traits in the multiplex population. The gray module was not included in the pathway analysis because it represents the default module comprised of genes not correlated in any other module. The complete lists of significant trait-associated pathways are provided in **Supplementary Table 9**. **Table 4** summarizes the ASD-relevant pathways among the top 25 over-represented pathways associated with four autistic traits (verbal, non-verbal, insistence on sameness, and savant skills), while **Figure 7** shows the overlap among all significant pathways associated with the four traits. Although several metabolic pathways were significantly enriched for play skills, there were no neurological signaling pathways that are relevant to ASD. In contrast to the results obtained with the simplex population, there are more ASD-related pathways associated with the verbal than with the non-verbal traits. Estrogen receptor signaling and the protein ubiquitination pathway are among the most significantly over-represented pathways associated with three of the five traits (verbal, non-verbal, and savant), with –log(*p*-values) for pathway enrichment ranging from 7.9 to 12.5 and from 8.6 to 11.3, respectively. Mitochondrial dysfunction and oxidative phosphorylation are also significantly over-represented among genes associated with the non-verbal and savant traits. As for the simplex samples, there are both unique and overlapping ASD-associated pathways among the four traits represented in **Figure 7**. There is also overlap with the shared trait-associated pathways from the simplex analyses (**Figure 5**), but with additional pathways, including estrogen receptor signaling, axon guidance, PTEN, PI3K/AKT, and neuregulin signaling, shared among all four traits in the multiplex population.

With respect to neurological diseases, motor dysfunction and movement disorders are significantly associated with verbal (p = 5.7E-22), non-verbal (p = 3.69E-09), and savant (p = 8.36E-10) traits, while a related disorder, ataxic gait, is associated with play skills (p = 4.09E-04) and insistence on sameness or ritualistic behaviors (p = 5.79E-06). Genes for cognitive impairment are enriched for both verbal (p = 1.9E-15) and savant (p = 1.26E-08) traits, although the shared modules that are significant for these two traits show inverse correlation with respect to gene expression and cumulative trait score.

To better distinguish pathways differentiating verbal and savant skills, IPA was used to analyze genes in modules unique to each trait (i.e., not shared with any other trait). For the verbal trait, these 8 modules included the black, blue, brown, cyan, darkgreen, greenyellow, plum1, and skyblue. For the savant trait, these modules included grey60, lightyellow, royalblue, and tan. **Table 5** shows the most significant ASD-relevant pathways among the top 50 associated with each set of trait genes. Interestingly, the pathways associated with the verbal trait are enriched in genes involved in neuronal processes (e.g., axon guidance, synaptogenesis, reelin, ephrin receptor, and neuregulin signaling), whereas the top pathways associated with the savant trait are related to NFkB, PI3K/AKT, ERK/MAPK, and downstream signaling pathways involved in inflammation, protein synthesis and cell growth. While both traits are associated with genes involved in motor dysfunction and movement disorders (p = 2.63E-11 for verbal; p = 7.30E-03 for savant), the savant skills are also associated with genes linked to severe mental retardation (p = 2.00E-03) and cognitive impairment (p = 4.89E-03), thus highlighting their relevance to intellectual ability and cognition.

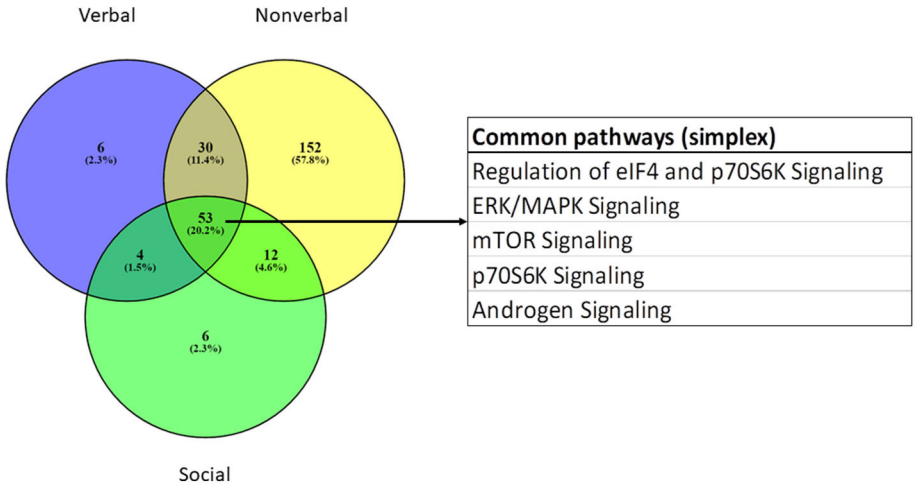

FIGURE 5 | Venn diagram showing overlap of all over-represented canonical pathways associated with genes correlated with verbal, non-verbal, and social traits in the simplex population.

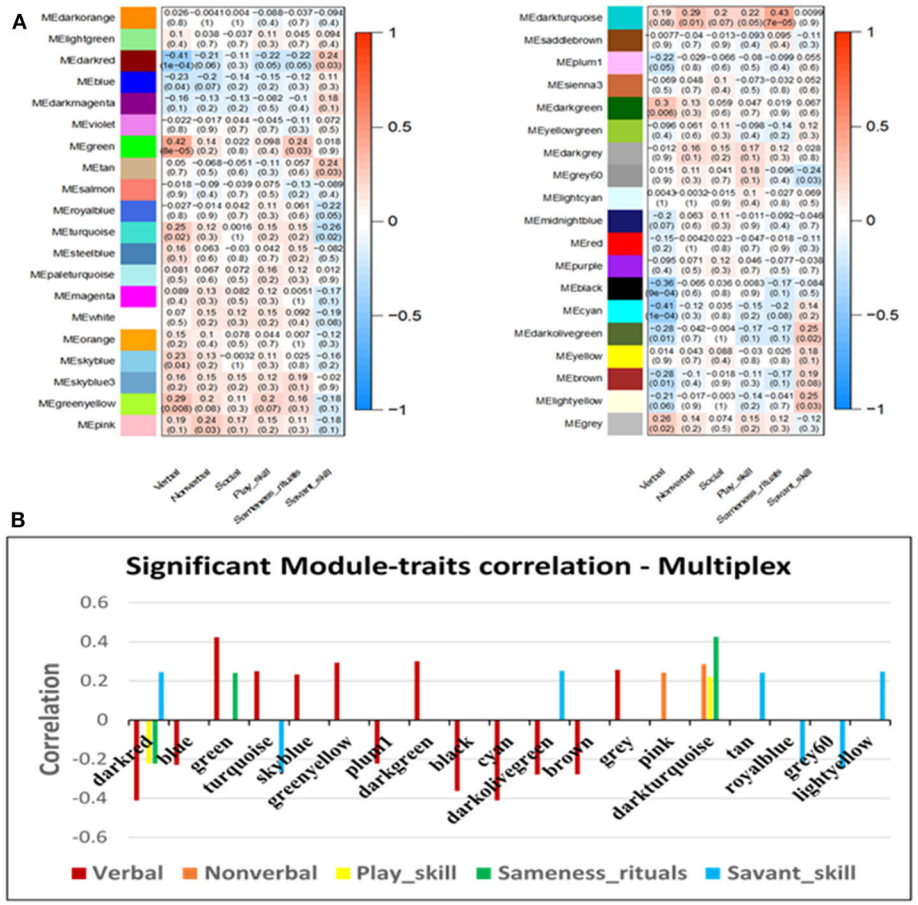

FIGURE 6 | (A) Module-trait relationships resulting from WGCNA analyses of autistic traits in the multiplex population. **(B)** Correlation values for the trait-associated gene modules that are significantly correlated with at least one autistic trait, with $p \leq 0.05$. Each colored module represents a network of genes with correlated expression among its members. Six autistic traits were evaluated for correlated gene expression networks. Five autistic traits (verbal, non-verbal, play, insistence on sameness or rituals, and savant) showed significant association with at least one module.

Comparison of Genes, Biological Pathways, and Functions Associated With Autistic Traits in Simplex and Multiplex Populations and Their Relevance to ASD

While the WGCNA analyses of autistic traits clearly show differences in the gene networks associated with ASD in simplex and multiplex families, we also sought to find similarities as well as differences among all the trait-associated genes and biological networks. A direct comparison of all trait-associated genes from the simplex (4,649 genes) and multiplex (6,081 genes) individuals with ASD shows an overlap of 1,754 genes. Hypergeometric distribution analyses were then conducted to determine the over-representation of 910 autism risk genes (downloaded in May, 2020) from the SFARI Gene database within each set of trait-associated genes as well as the overlapping set of genes. These analyses show that trait genes from both simplex and multiplex populations as well as the overlapping set are significantly enriched in SFARI genes, with hypergeometric distribution upper cumulative q-values of 0.012 (simplex), 7.62E-06 (multiplex), and 0.044 (overlapping), thus confirming relevance of these genes to ASD.

IPA was then used to identify canonical pathways over-represented among the overlapping genes. **Table 6** shows that the 1,754 shared trait genes are over-represented in pathways that reflect many of the top pathways enriched among module genes correlated with all ASD traits in simplex and multiplex populations (**Table 7**). Despite the fact that there are hundreds to thousands of genes in each of the gene modules correlated with autistic traits, a comparison analysis of the upstream regulators of these genes reveals that relatively few genes can potentially regulate hundreds of trait-associated genes in both simplex and multiplex populations (**Table 7**). HNF4A, TP53, and ESR1 are also the top three upstream regulators of the overlapping genes in the simplex and multiplex samples (p-values of overlap: 2.48E-25, 7.8E-23, and 1.06E-20, respectively).

TABLE 4 | Significantly over-represented ASD-relevant pathways among the top 25 associated with four autistic traits in the multiplex population.

Ingenuity canonical pathways	–log(p-value)*
Verbal communication	
Estrogen Receptor Signaling	12.5
Axonal Guidance Signaling	11.1
Protein Kinase A Signaling	10.8
Synaptogenesis Signaling Pathway	9.92
p70S6K Signaling	9.81
Regulation of eIF4 and p70S6K Signaling	9.65
Androgen Signaling	9.17
Protein Ubiquitination Pathway	8.64
Non-verbal communication	
Protein Ubiquitination Pathway	10.7
Estrogen Receptor Signaling	10.4
Mitochondrial Dysfunction	8.06
Oxidative Phosphorylation	7.91
Play skills	
None specifically associated with ASD	
Insistence on sameness (ritualistic behavior)	
NF-κB Signaling	4.06
PTEN Signaling	2.31
Androgen Signaling	2.1
Savant skills	
Protein Ubiquitination Pathway	11.3
Mitochondrial Dysfunction	8.44
Oxidative Phosphorylation	8.39
Estrogen Receptor Signaling	7.9
Regulation of eIF4 and p70S6K Signaling	6.05
Androgen Signaling	5.97
ERK/MAPK Signaling	5.25
Protein Kinase A Signaling	5.16

**Negative logarithm of the Fisher Exact p-value which represents the probability that the pathway is not over-represented among the respective dataset of genes, where a value of 1.3 is equivalent to a p-value of 0.05.*

DISCUSSION

The Advantage of Phenotypic Subtyping in ASD

A primary goal of this study was to test the hypothesis that reducing the heterogeneity of ASD by subtyping cases according to clinical severity across a broad spectrum of autistic behaviors and traits will improve upon the ability to identify DEGs together with their associated pathways and biological functions in each of the subgroups relative to the combined set of cases. In this study, we apply multivariate cluster analyses of ADI-R scores to divide individuals with ASD from simplex families included in the SSC into three clinically distinct subgroups and perform gene expression profiling of each subgroup using existing transcriptomic data from a published study. In contrast to our study, the Luo et al. study (22) analyzed the expression outliers in individual pairs of case-control siblings and correlated them with CNVs from the same individuals. Here, we are interested in using the transcriptomic data to identify DEGs between probands and their respective unaffected siblings in each of the phenotypic subgroups in order to detect biological differences between cases and controls that associate with the differences in the severity of specific clinical symptoms. While we have previously demonstrated that this subtyping strategy improved upon the biological information obtained on ASD subgroups derived from multiplex families (7), this approach has never been used to analyze transcriptomic data from simplex families. Our results show that: (1) significant DEGs can successfully separate cases from controls (**Figure 3**) in each of the three subtypes derived from the ADI-R cluster analyses, and (2) the DEGs in each subgroup are differentially enriched in autism-related canonical pathways, diseases and biological functions (**Tables 1**, **2**). Notably, despite the fact that the Language subgroup is comprised of the fewest number of samples (seven pairs), transcriptomic and pathway prediction analyses show that DEGs from this group are the most enriched in autism genes (as reflected by a hypergeometric distribution q-value of 0.001 for enrichment in SFARI genes), pathways, and functions relevant to ASD. Among

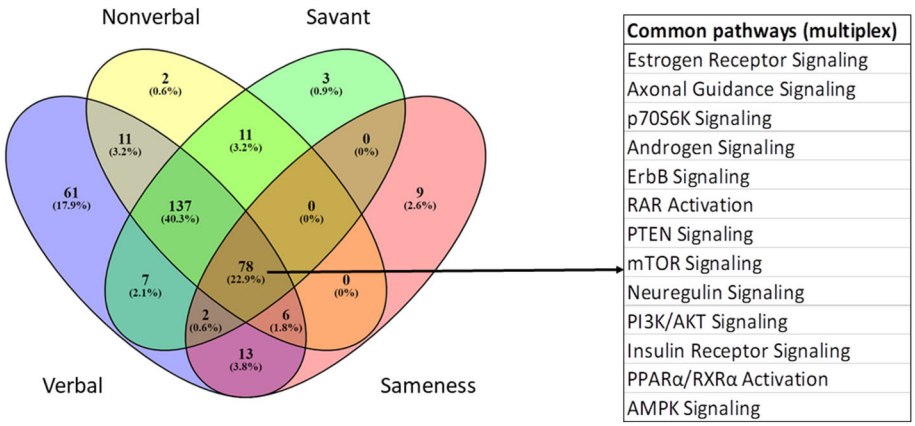

FIGURE 7 | Venn diagram showing overlap of all over-represented canonical pathways associated with genes correlated with verbal, non-verbal, savant, and insistence on sameness traits in the multiplex population.

TABLE 5 | Significantly over-represented canonical pathways among genes in modules unique to verbal and savant traits.

Ingenuity canonical pathways (modules unique to verbal)	–log(*p*-value)*	Ingenuity canonical pathways (modules unique to savant)	–log(*p*-value)
Axonal Guidance Signaling	11.1	NF-κB Signaling	4.49
Synaptogenesis Signaling Pathway	7.54	PI3K/AKT Signaling	2.68
Protein Kinase A Signaling	6.44	ERK/MAPK Signaling	2.40
Wnt/β-catenin Signaling	5.49	CREB Signaling in Neurons	2.14
p70S6K Signaling	5.42	PTEN Signaling	2.00
Reelin Signaling in Neurons	4.94	Protein Kinase A Signaling	1.98
Ephrin Receptor Signaling	4.91	Regulation of eIF4 and p70S6K Signaling	1.97
PTEN Signaling	4.67	p70S6K Signaling	1.88
EIF2 Signaling	4.19	Androgen Signaling	1.81
Neuregulin Signaling	4.14	mTOR Signaling	1.69

**Negative logarithm of the Fisher Exact p-value which represents the probability that the pathway is not over-represented among the respective dataset of genes, where a value of 1.3 is equivalent to a p-value of 0.05.*

the significantly enriched ASD-relevant pathways associated with the Language phenotype are those involved in axonal guidance, actin cytoskeleton signaling, synaptogenesis, GABA receptor, and neuregulin signaling (**Table 1**). By contrast, DEGs from the Intermediate and Mild subgroups are enriched in fewer pathways related to ASD. Interestingly, when all 73 cases were combined for transcriptomic and functional analyses, none of the canonical pathways over-represented within the resulting dataset of DEGs were specifically associated with ASD (**Supplementary Table 7**). Similarly, with respect to neurological diseases, the Language subtype exhibits the largest number of functions and comorbid disorders associated with ASD, including movement disorders and cognitive impairment. By contrast, the Intermediate and Mild subtypes are associated with sensory disorders and epilepsy, respectively (**Table 2**). These findings thus demonstrate that the reduction in heterogeneity of ASD cases within the simplex population helps to reveal significant but different biological processes and disorders associated with each subtype of autism based on gene expression profiles. We have recently demonstrated that application of this subtyping method to DNA methylation data from probands in simplex families similarly enhances the ability to identify subtype-associated genes in differentially methylated regions (DMRs) in the same three phenotypic subgroups represented in this study (34). Moreover, 1.6 times more DMR-associated genes are detected among the three subgroups in comparison to those detected when all cases are combined into a single case group, again demonstrating the value of heterogeneity reduction and phenotype definition for genome-wide omics analyses. Together, these gene expression and methylation studies reveal dysregulated ASD subtype-dependent genes, pathways, biological functions, and gene regulatory mechanisms that may aid in the design of therapeutic strategies for each phenotypic subgroup.

Biological Associations With Autistic Traits in Simplex Families

While WGCNA has been primarily used to identify gene modules that correlate with a specific disease state (e.g., autism, Alzheimer's disease, bipolar disorder, renal and pancreatic

TABLE 6 | Top 25 canonical pathways significantly over-represented among overlapping genes associated with all traits in simplex and multiplex populations.

Ingenuity canonical pathways	–log(*p*-value)*
Molecular Mechanisms of Cancer	8.31
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	6.85
ATM Signaling	6.64
Estrogen Receptor Signaling	6.37
Senescence Pathway	6.23
Hypoxia Signaling in the Cardiovascular System	6.16
Cyclins and Cell Cycle Regulation	6.16
PI3K Signaling in B Lymphocytes	6.08
p70S6K Signaling	5.55
Cell Cycle Control of Chromosomal Replication	5.24
Protein Ubiquitination Pathway	5.19
Synaptogenesis Signaling Pathway	5.03
Regulation of eIF4 and p70S6K Signaling	5.03
Reelin Signaling in Neurons	4.85
Pyridoxal 5′-phosphate Salvage Pathway	4.8
ErbB4 Signaling	4.68
B Cell Receptor Signaling	4.5
NGF Signaling	4.45
Protein Kinase A Signaling	4.15
Opioid Signaling Pathway	4.12
Sumoylation Pathway	4.11
Systemic Lupus Erythematosus In B Cell Signaling Pathway	4.02
Natural Killer Cell Signaling	4
Ephrin Receptor Signaling	4
GM-CSF Signaling	3.93

**Negative logarithm of the Fisher Exact p-value which indicates the probability that the indicated pathway is not over-represented in the respective dataset of genes; a value of 1.3 is equivalent to a p-value of 0.05.*
Boldface font indicates association with ASD.

TABLE 7 | Comparison of the most significant over-represented canonical pathways and upstream regulators associated with gene modules correlated with all traits in simplex and multiplex ASD populations.

Canonical pathways	Simplex	Multiplex
(All traits combined)	**–log (*p*-value)***	
Molecular Mechanisms of Cancer	11.32	16.83
Regulation of eIF4 and p70S6K Signaling	11.02	11.41
Estrogen Receptor Signaling	6.63	13.43
ATM Signaling	13.32	6.67
Senescence Pathway	10.76	8.97
Protein Ubiquitination Pathway	8.80	9.29
EIF2 Signaling	9.55	8.48
p70S6K Signaling	6.92	11.01
B Cell Receptor Signaling	5.64	11.40
Hepatic Fibrosis Signaling Pathway	0.97	15.96
mTOR Signaling	9.11	7.56
Protein Kinase A Signaling	4.03	12.29
Huntington's Disease Signaling	6.58	9.18
Synaptogenesis Signaling Pathway	5.81	9.86
PI3K Signaling in B Lymphocytes	6.02	9.59
Thrombin Signaling	3.11	11.79
Hereditary Breast Cancer Signaling	6.54	8.35
Axonal Guidance Signaling	2.31	12.48
Ephrin Receptor Signaling	7.40	7.35
Hypoxia Signaling in the Cardiovascular System	9.70	4.74
Upstream Regulators (All traits combined)	**Simplex *p*-value of overlap***	**Multiplex (# target genes)**
HNF4A	1.86E-59 (728)	3.19E-46 (821)
TP53	7.66E-25 (559)	3.85E-49 (761)
ESR1	9.81E-16 (396)	5.86E-54 (606)
MYC	1.57E-14 (353)	9.34E-30 (485)
beta-estradiol	7.42E-08 (525)	2.30E-35 (791)

**Negative logarithm of the Fisher Exact p-value which represents the probability that the pathway is not over-represented among the respective dataset of genes, where a value of 1.3 is equivalent to a p-value of 0.05.*
Bold font indicates association with ASD.
**Indicates the probability that the trait-associated genes are not among those known to be regulated by the indicated upstream regulator.*

cancer), in this study, we use WGCNA to identify gene modules that may relate to specific traits of ASD. These traits included deficits in verbal ability (spoken language), non-verbal communication, play skills, social interaction, insistence on sameness/rituals, and manifestation of savant skills. As shown in **Figure 4**, seven modules were significantly correlated with at least one of three traits (verbal, non-verbal, and social), with the non-verbal trait associated with the largest number of ASD-related pathways. Interestingly, all seven modules were also identified in autism brain co-expression networks (16), suggesting relevance of our results to neurological processes impacted in the autistic brain. Separate network prediction analyses of genes in all of the modules correlated with each trait revealed both overlapping and unique canonical pathways associated with each trait, as summarized in **Figure 5**. Among the shared pathways are those involving regulation of eIF4 and p70S6K signaling as well as ERK/MAPK, mTOR, and androgen signaling, all of which have been implicated in ASD (7, 35–40).

Biological Associations With Autistic Traits in Individuals From Multiplex Families

Although we have previously reported on differential gene expression in three subtypes of ASD from multiplex families (7), we were interested in applying WGCNA to study the correlation of autistic traits in this population with gene networks in order to compare the results with those from the simplex population, which was the main focus of this study. **Figure 6** shows that

many more gene modules can be correlated with autistic traits in individuals from multiplex families than from simplex families. In addition, each of five autistic traits (verbal, non-verbal, play, insistence on sameness, and savant skills) could be correlated with at least one gene module. In particular, the verbal trait is associated with 13 modules, two each for non-verbal and play skills, three for insistence on sameness, and seven for savant skills. The majority of these modules, with the exception of plum1 and darkolivegreen, were also found among co-expression networks in the autistic brain (16). In contrast to the results from the simplex population, the verbal trait is associated with the largest number of pathways implicated in ASD while the non-verbal trait is associated with relatively few ASD-relevant pathways (**Table 4**). Interestingly, the Language subgroup in the multiplex population accounts for 34.1% of the 1,954 individuals for whom ADI-R scores were available. In the simplex population, the Language subgroup makes up only 11.1% of the 1,900 probands for whom ADI-R scoresheets were obtained. It is also notable that savant skills show inverse correlation with other autistic traits sharing the same modules, suggesting that the presence of savant skills may counteract at least in part the severity of some autistic traits. In other words, the direction of changes in gene expression associated with the presence of savant skills may be opposite to that which is associated with other autistic traits. By contrast, we did not find any modules that correlated with savant skills in probands from the simplex population. It is noted that one subgroup of individuals with ASD (called the "Savant" subgroup) in the multiplex population that was included in our previous gene expression profiling analyses exhibited a higher frequency of savant skills than individuals in the other three subgroups (4, 7). The Savant subgroup was not distinguished within the simplex population, which instead had a higher percentage of individuals in the Mild subgroup (52.5%). Thus, the larger number of trait scores related to savant skills in the multiplex population may account in part for the ability to identify gene modules correlated with this trait.

Similarities and Differences Associated With ASD Traits in Simplex and Multiplex Populations

The trait-associated genes in both simplex and multiplex populations are enriched in autism-risk genes from the SFARI Gene database, but the significance of the enrichment is greater for the multiplex population by ∼4 orders of magnitude. Likewise, the number of trait-associated modules (excluding gray) are considerably greater for the multiplex population (18 vs. 7 modules). These results suggest that there may be a greater burden with respect to gene dysregulation and network disruption in individuals with ASD from multiplex families. Despite these differences, there are shared canonical pathways that appear to underlie ASD traits in both populations albeit to different extents, as shown in **Table 7**. Interestingly, many of the trait-associated genes in both populations can be regulated by a handful of potent upstream regulators, in particular, HNF4A, TP53, ESR1, MYC, and estradiol. The tumor suppressor gene, *TP53*, and the proto-oncogene *MYC* are both regulators of cell cycle progression, growth, and apoptosis, processes that are notably deregulated in ASD (36, 41). The estrogen receptor 1, ESR1, as well as its ligand estradiol, are critically important to brain development and sexual differentiation (42, 43), and have been implicated in a number of studies on ASD (44, 45). Hepatocyte nuclear factor 4, HNF4A, is a transcription factor known to play a major role in liver and gastrointestinal diseases (46). While HNF4A has never before been associated specifically with ASD, it has been reported to be involved in Parkinson's disease (47) and major depressive disorder (48) as well as in regulation of circadian rhythm (49). Notably, circadian rhythm and sleep disorders have previously been associated with ASD by clinical, genetics, and transcriptomic analyses (7, 26, 50–52). This study shows that HNF4A may also disrupt a large number of genes and pathways associated with autistic traits and, in addition, serve as a link to gastrointestinal disorders, such as diarrhea, constipation, inflammatory bowel disease and colitis, which are comorbidities in some individuals with ASD (26, 53–55). While none of these upstream regulators are considered autism risk genes themselves, our findings suggest that therapeutic agents targeted toward these master regulators may have a significant impact on many downstream pathways associated with the autistic phenotype.

Limitations and Future Considerations

An obvious limitation of this study is that our results are based on transcriptomic analyses of lymphoblastoid cell lines. It has been argued that peripheral tissues are not the ideal experimental model to understand brain development despite the fact that many neurological functions and signaling pathways relevant to autism have been identified in LCL and other peripheral tissues, such as whole blood, lymphocytes, and natural killer cells (7, 56–62). In addition, the extensive overlap of trait-associated gene modules (18 out of 20) from this study with those resulting from transcriptomic analysis of the autistic brain (16) suggests that LCL are a useful surrogate model for investigations of the pathobiology underlying ASD. Another limitation is that sample sizes are small, especially for the Language subtype, even though the largest number of DEGs and more ASD associated pathways and biological functions were identified for this subgroup compared to the other two subgroups that have 3.7–5.8 times more cases. Moreover, only male probands were used for the WGCNA analyses of autistic traits. Future studies should include additional samples in each of the phenotypic subgroups to confirm the DEGs and pathways reported here as well as more females to allow a separate WGCNA analysis of autistic traits in females.

CONCLUSIONS

This study shows that heterogeneity reduction by phenotypic subtyping of individuals with ASD enhances the ability to identify more differences in autism-relevant gene expression, pathways, and functions between probands and siblings from simplex families in comparison to those obtained with a combined case group. These findings thus replicate those of our earlier study that applied this subtyping approach to transcriptomic analyses

of the multiplex ASD population. While we used multivariate cluster analyses of ADI-R severity scores for the subtyping, we anticipate that other approaches to reduce heterogeneity among individuals with ASD (e.g., by comorbidities, immune status, physical abnormalities (like head size), or functional MRI profiles) may also improve the ability to detect biological differences at the genome-wide level.

To our knowledge, this is the first study to use WGCNA to analyze gene networks in the context of a continuous severity spectrum of autistic traits rather than with a dichotomous diagnosis of ASD. Perhaps the most important findings from this study are the associations of specific ASD traits with expressed gene networks and embedded pathways that may serve as useful targets for precision medicine in autism spectrum disorders.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Gene Expression Omnibus (GEO); Accession numbers GSE37772 and GSE15402.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by GWU Institutional Review Board, Office for Human Research. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

VH conceived of and designed the study, performed subgroup phenotyping, pathway, functional and hypergeometric analyses, and wrote the manuscript. CB was responsible for gene expression and WGCNA analyses as part of her Master's thesis research and contributed to manuscript preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.578972/full#supplementary-material

Supplementary Table 1 | Demographic information on the individuals from the SSC population included in this study.

Supplementary Table 2 | Clinical autistic traits classification and included trait-associated ADI-R items.

Supplementary Table 3 | Differentially expressed genes in the Language subtype of ASD.

Supplementary Table 4 | Differentially expressed genes in the Intermediate subtype of ASD.

Supplementary Table 5 | Differentially expressed genes in the Mild subtype of ASD.

Supplementary Table 6 | Differentially expressed genes in the combined case group.

Supplementary Table 7 | All significant over-represented canonical pathways associated with DEGs in the subtypes of ASD and the combined case group.

Supplementary Table 8 | All significant over-represented canonical pathways associated with module genes corresponding to verbal, non-verbal, social, and all (combined) traits in the simplex population.

Supplementary Table 9 | All significant over-represented canonical pathways associated with module genes corresponding to verbal, non-verbal, play, insistence on sameness, savant, and all (combined) traits in the multiplex population.

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A Pilot Investigation of the Social Attention and Communication Surveillance (SACS) Tool for the Early Identification of Autism in Tianjin, China (SACS-C)

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Introduction: Autism spectrum disorder (ASD) comprises difficulties in social communication and restrictive and repetitive behaviors. Despite an increased global prevalence, little remains known about early detection and diagnosis of autism in Mainland China. Our aim was to conduct a pilot investigation of the implementation of an Australian tool, Social Attention and Communication Surveillance (SACS), in Tianjin, China (SACS-C) by trained professionals to identify autism early compared to the Checklist for Autism in Toddlers-23 (CHAT-23) completed by parents and professionals.

Materials and Methods: A total of 10,514 children were monitored across 61 Community Health Service Centres in six Tianjin districts on the SACS-C at 12, 18, and 24 months of age following a half-day training of 225 child health practitioners. Children deemed at "high likelihood" for autism on either the SACS, CHAT-23, or both, were referred for developmental assessments at the Tianjin Women and Children's Health Centre (TWCHC).

Results: A total of 87 children (0.8%) were identified at "high likelihood" on the SACS-C, of whom 57 (66%) were assessed for autism; 24 children were subsequently diagnosed with autism (42.1%), and the remaining 33 (57.9%) were diagnosed with developmental and/or language delays. The SACS-C had a positive predictive value (PPV) of 42.1%, a negative predictive value (NPV) of 99.8%, and sensitivity and specificity of 53.3 and 99.7%, respectively. Only 21 children were identified at "high risk" for autism on the CHAT-23 (0.2%), over four times fewer children than the SACS-C, with 14 children assessed for autism (66%); nine were diagnosed with autism (64.3%) and the remaining five children were diagnosed with developmental and/or language delays. The CHAT-23 had an overall PPV of 64.3%, NPV of 99.6%, sensitivity of 27.3%, and specificity of 99.9%.

Conclusion: This was the first large-scale study identifying autism in 12–24-month-old children in China. We ascertained the feasibility of training community health practitioners

to monitor infants and toddlers for the early signs of autism, and determined the effectiveness of their use of SACS-C which had a better balance between accuracy and sensitivity in detecting autism in contrast to the CHAT-23 which missed the majority of children with autism (72.7%) vs. the SACS-C (46.7%). Given the emphasis on identifying as many children with autism as possible in Mainland China, SACS-C was identified as the tool of choice by the TWCHC. However, more work is needed to improve the psychometric properties in using the SACS-C in Mainland China so that it is comparable to its use in Australia.

Keywords: early detection, screening, autism spectrum disorder, developmental surveillance tool, China

INTRODUCTION

Autism spectrum disorder (ASD) comprises significant difficulties in social attention, communication and the presence of sensory and restrictive and repetitive behaviors (1). Early developmental surveillance and screening plays a vital role in the early identification, detection and diagnosis of autism, which allows access to early intervention, leading to better child outcomes and improved quality of life (2–5). In Mainland China, early clinical manifestations and symptoms of autism are not widely recognized, often being described as the "lonely disease" (6). In 1982, Dr. Tao Guotai from Nanjing Brain Hospital reported the first four cases of autism in Mainland China (6). Increasing numbers of children are now diagnosed with autism in China, particularly following the improved knowledge and awareness about this condition (7).

The prevalence of autism in the US was recently reported to be 1 in 54 children aged 8 years (8), whilst in the UK it is 1 in 64 (9), Australia 1 in 70 (10), and 1 in 38 in South Korea (11). There remains limited knowledge about the prevalence of autism in Mainland China. A meta-analysis of 18 studies found a wide range in prevalence rates from 2.8 to 30.4 per 10,000, with the pooled prevalence of autism being 12.8 per 10,000 (95% CI: 9.4–17.5) (12), much less than that reported above. More recently a meta-analysis in 2018 found a pooled ASD prevalence of 39.2 per 10,000 (95% CIL 28.4–50.0) and specific prevalence of autism as 10.2 per 10,000 (95% CI: 8.5–11.9) (13). Furthermore, a 2019 study used the Childhood Autism Spectrum Test (CAST) screening tool to ascertain autism prevalence in the Chinese cities of Jilian City, Shenzhen City, and Jiamusi City, finding that autism prevalence estimates were similar to Western prevalence rates in Jilian City (1.08%; 108 per 10,000) but lower in Shenzhen City and Jiamusi City with rates of 0.42% (42 per 10,000) and 0.19% (19 per 10,000), respectively (14).

Sun et al. (15) found the strongest determinant of the variability in prevalence estimates was the screening tool used, and found that studies using the Autism Behavior Checklist (ABC) (16), and the Clancy Autism Behavior Scale (CABS) (17), produced lower prevalence estimates, whilst studies that used the Checklist for Autism in Toddlers (CHAT) (18) reported higher prevalence estimates for autism (15). The authors also noted age at screening as another strong determinant in the prevalence estimates. Fifteen of the 18 studies focused on children screened between the ages of 2–6 years and a further seven focused on children aged 6–14 years and older. Whilst most of the studies included in the systematic review were young children, mean age at diagnosis for children in Mainland China was not reported (15). However, a recent study did report the mean age at diagnosis in Mainland China, with an average age at diagnosis being 3.3 years for Chinese children aged 6–14 years of age (19).

The CHAT (18) and its modified versions (M-CHAT, CHAT-23) are frequently used screening tools in Chinese populations in Mainland China (15, 20). The CHAT is more rigid with a specific applicable age of 18-months; it is a nine-item questionnaire for parents and contains five child observations by professional (18). It has since been further validated, evaluated and modified into the M-CHAT (21), and CHAT-23 versions, with the latter designed for Chinese children (22). CHAT-23 has a validity and reliability scores of 94 and 88%, respectively (23), as well as a sensitivity and specificity of 93 and 85% (22). Despite these seemingly high sensitivities and specificities reported, the age groups screened were wide (or unclear), and none of these studies were exclusively conducted in low-risk, community-based populations. There is, therefore, a gap in the literature on developmental surveillance and community-level screening procedures for infants and toddlers in the general population in China. There is also a lack of professional education available to Chinese primary-care professionals on the early signs of autism (24).

A recent systematic review jointly undertaken by Australian and Chinese scholars (24) reported that whilst screening tools currently used in China have reasonable psychometric properties for identifying autism in clinical populations, there appear to be no studies undertaken with community-based samples. They stressed the need to align the screening and diagnostic systems in Mainland China with best practice in autism screening and diagnosis (25–29). Prioritizing the need for community-based screening in the general population with psychometrically and culturally validated tools is needed together with follow-up of children deemed at high-likelihood of autism at the community level so that they are assessed and diagnosed by a specialized multidisciplinary clinical team (24).

A developmental surveillance tool designed for use in low-risk populations within community-based settings is the Social Attention and Communication Surveillance (SACS) tool. Designed and implemented in Australia, this tool has an excellent Positive Predictive Value (PPV; 81–83%), Negative Predictive Value (99%), Sensitivity (82–84%), and Specificity (99–99.5%) for identifying children with autism between 11 and 30 months of age (26, 30). Moreover, following diagnosis at age 24 months

using gold standard tools, diagnostic stability is high at 88.3% between 24 and 48 months of age (25). On the strength of these findings, the SACS tool has been implemented state-wide throughout the universal Victorian and Tasmanian Maternal and Child Health (MCH) Services in Australia, where children are routinely monitored 10 times from birth to 3.5 years. The SACS is administered at 12, 18, and 24 months of age by trained MCH Nurses to identify the early signs of autism during children's routine check-up (30). Importantly, the availability of universal developmental surveillance of babies by medical professionals in China, undertaken within Women and Child Health Centres, provides an ideal platform for monitoring the early signs of autism to promote early identification and diagnosis of autism.

Tianjin is the fourth largest city in China, consisting of 16 county-level administrative areas. Over 100,000 babies are born in Tianjin every year, which, based on the estimated prevalence of autism was 27.5 per 10,000 (31), equates to 1,000–2,000 babies potentially born with autism each year. A thoroughly developed women and child health care system in this city, the Tianjin Women and Children's Health Centre (TWCHC), has made it an ideal test site for the implementation of screening for various child conditions such as congenital heart disease (32), developmental dysplasia of the hip (33), and congenital cataract (34). However, developmental surveillance and screening for autism had not yet been implemented.

Our study objective was to conduct a pilot investigation of a Mandarin translation of the SACS, –SACS-China (SACS-C) – in Tianjin, by comparing the outcomes of implementing the SACS-C with the CHAT-23, as described above, and which has been widely used with Chinese children (22, 35, 36). The study comprised two aims: firstly, to ascertain the feasibility of training early child health experts to monitor babies and toddlers for early signs of autism in Tianjin; and, secondly, to determine the performance of two tools (SACS-C and CHAT-23) to enable selection for use in early identification for autism in the TWCHC.

METHOD

Study Setting

Tianjin has a three-level maternal and child health care system, consisting of a city level administrative centre (the TWCHC), Women and Children's Health Centres at a district level, and the community level health centres (CHC). In Tianjin, children's health status and development are monitored in the community health centers by qualified medical health practitioners. The CHC services are offered to all families with children younger than 7 years, with an emphasis on child health surveillance and screening (37). As part of this service, routine health checks for children in the community are scheduled from birth to 7 years of age. It is expected that children under 12 months are examined every 3 months, children between 12 and 36 months are examined every 6 months, and children over 36 months are examined once a year (37). Every year, over 90% of babies in Tianjin access the CHC service soon after birth, with attendance remaining relatively high within the first 2 years; this service has enormous potential to identify infants at high-likelihood of autism.

Participants

From May 2013 to July 2014, a total of 10,514 children were monitored through 61 CHCs in six selected districts in the urban areas in Tianjin (see star in **Figure 1**). In 2010, 4.3 million out of 13 million residents lived in the six central urban districts. The districts in this study were chosen based on proximity to facilitate ease of referral to the diagnostic center at the TWCHC, which is in the city center.

While all 10,514 children were monitored with the SACS-C, only a subset ($n = 6,744$; 64%) were also screened with the CHAT-23. Many children in the original SACS studies (26, 30) were seen at each of the 12, 18, and 24 months checks. However, in this pilot study, children were only monitored twice on the SACS-C (at 12, 18 and/or 24 months) due to funding restrictions. Initially children were monitored on the SACS-C at 12-months ($n = 3,178$), 18-months ($n = 3,757$), and 24-months ($n = 3,579$) of age. As the SACS-C is a developmental surveillance tool administered at different time points, the majority of the cohort (66%) initially monitored at 12, 18, or 24 months were also monitored again by the health practitioners 6 months later; 79% of 12-month-olds ($n = 2,497$), 78% of 18-month-olds ($n = 2,911$), and 42% of 24-month-olds ($n = 1,494$). Children within the age limits of the CHAT-23 at 18-months ($n = 3,683$) and 24-months ($n = 3,061$) were only monitored once, given its use as a "once-off" screening tool. The average age of all children monitored in the study was 18.70 months (SD 4.99), with the sample comprising 52% boys and 48% girls. Detailed age, gender and assessment characteristics are shown in **Table 1**.

Measures

Translation of SACS Checklists

For effective implementation in Tianjin, the SACS was translated from English into Chinese by one of the authors (CW) and further validated by a practitioner from the TWCHC. The SACS-C was then back translated to English, this English version checked by the first author (JB) and this process was repeated twice between CW, Chinese colleagues and JB, with modifications made to be in line with the "meaning" of the original instrument. Authors CW and JB then evaluated both the English and Chinese versions to ensure these were comparable in meaning. A summary of the behaviors monitored with the SACS-C, highlighting the "key items," are presented in **Supplementary Table 1**.

Training of Community Practitioners on SACS-C

In March 2013, 225 child health practitioners from 61 communities within the six districts in Tianjin received a 3-hour-training workshop by the authors of the SACS (JB & CD). The workshops focused on typical and atypical social-communicative development, the early signs of autism, and the administration of the SACS items. Simultaneous translation from English to Mandarin was undertaken during the workshops (by CW), with all written content also translated and then back translated by the CW and JB.

The SACS authors (JB and CD) also observed administration of the SACS-C with two children at each of the ages of 12, 18, and 24-months, undertaken by a number of the trained health practitioners at TWCHC, and provided in-person

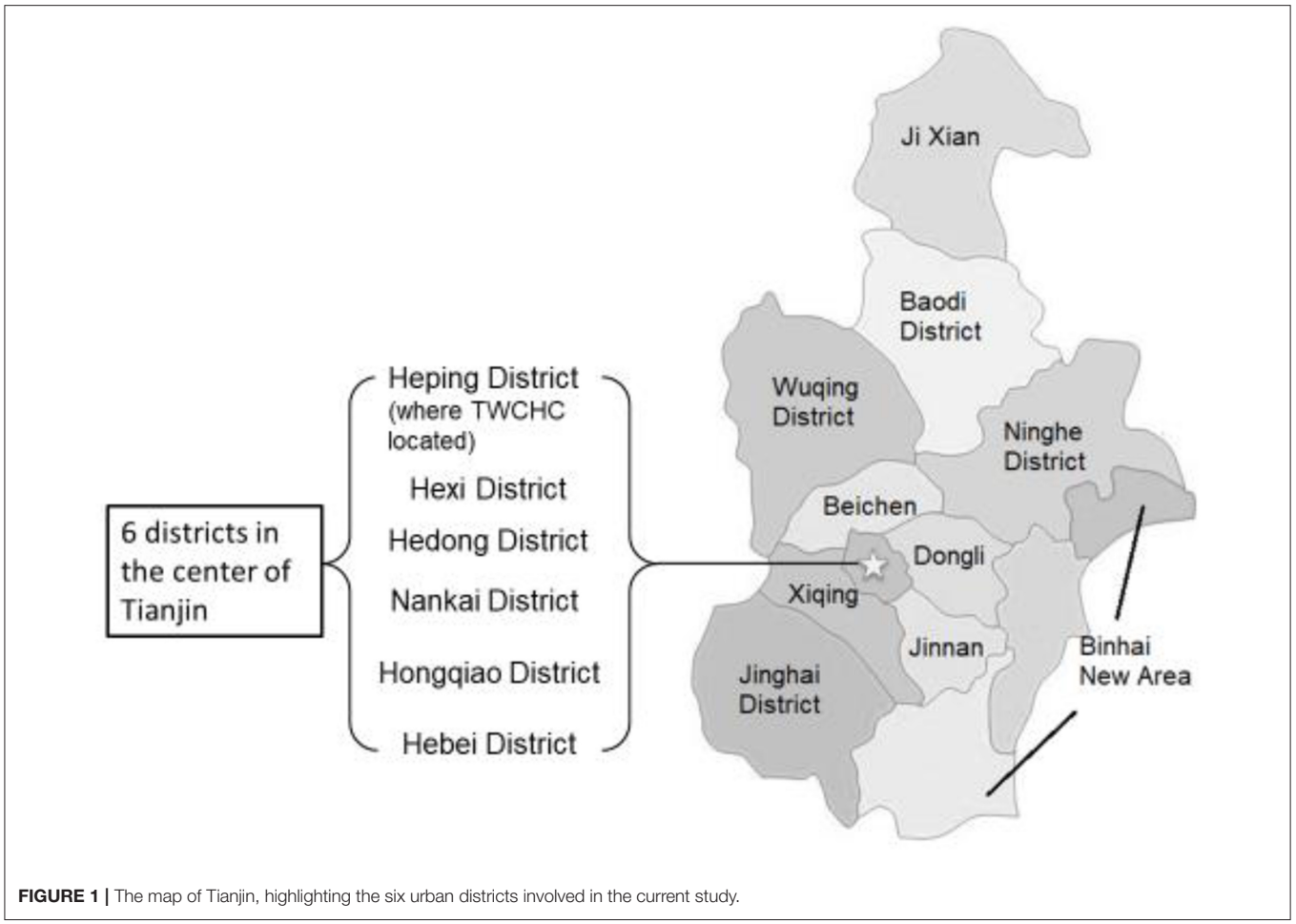

FIGURE 1 | The map of Tianjin, highlighting the six urban districts involved in the current study.

TABLE 1 | Demographics characteristics of children administered the SACS-C and CHAT 23.

	SACS-C				CHAT-23		
Age (months)	**12**	**18**	**24**	**Overall**	**18**	**24**	**Overall**
n	3,178	3,757	3,579	10,514	3,683	3,061	6,744
Age M (SD)	12.36 (0.60)	18.44 (0.70)	24.6 (1.24)	18.70 (4.99)	18.45 (0.69)	24.23 (0.33)	21.08 (2.93)
Gender							
Male (%)	1,653 (30.2)	1,954 (35.7)	1,861 (34.1)	5,468 (100) (52.0)	1,915 (54.6)	1,592 (45.4)	3,507 (100) (52.0)
Female (%)	1,525 (30.2)	1,803 (35.7)	1,718 (34.1)	5,046 (100) (48.0)	1,768 (54.6)	1,469 (45.4)	3,237 (100) (48.0)
Total (%)	3,178 (30.2)	3,757 (35.7)	3, 579 (34.1)	10,514 (100)	3,683 (54.6)	3,061 (45.4)	6,744 (100) (100)

CHAT-23, Checklist for Autism in Toddlers-23; SACS-C, Social Attention and Communication Surveillance-China Tool; n, number of participants; M (SD), mean (standard deviation).

feedback on these administrations. These health practitioners then assisted CHC practitioners in any queries relating to SACS-C administration and scoring.

SACS-C Implementation

Following training, the SACS-C was implemented as part of the routine health checks in the CHCs. Community health practitioners initially undertook a physical examination of the child, and the child was monitored on the SACS-C in the presence of a parent/caregiver. The practitioners, who had been trained on how each item was to be administrated at each developmental age, recorded whether the child's behaviors were typical or atypical (as opposed to present or absent) on a form provided for each child. Children were considered at "high-likelihood" for autism if they did not engage in three of the five "key" items at 12, 18, and/or 24-months-of-age. Practitioners were instructed to

administer items up to a maximum of three times (e.g., calling a child's name). In the minority of cases where practitioners were unable to elicit a behavior because of the child being ill, distressed, or asleep, detailed parental/caregiver report was used to mark the item as typical or atypical. Children who were identified as "high-likelihood" for autism were referred to the TWCHC for a follow-up developmental and diagnostic assessment for autism by two autism specialist pediatricians.

CHAT-23 Training and Implementation

The health practitioners were also trained on the use of the CHAT-23 by one of the authors (CW), who is a native Chinese speaker. CHAT-23 is popular in Chinese-speaking areas, and the Chinese version of the test is available. The training focused on how to identify the passing or failing in each item, and the referral standards. The CHAT-23 comprises two parts: Part A is a parent questionnaire with 23 questions regarding children's behaviors, and Part B comprises seven-key item scored based on observations of the child, conducted by the health practitioner. If a child fails six or more items of the total of 23 parent completed items in Part A, they are administered Part B. If the child had two or more failed items in Part B, the child was identified as "high-likelihood" for autism on the CHAT-23 and referred to the TWCHC for a further assessment. Notably, unlike the SACS-C, which is a developmental surveillance tool administered across the second year of life, the CHAT 23 is administered only once between 18 and 24 months of age and is appropriate after 18 months of age, as it is a screening tool designed to be administered at one point in development.

Procedure

Study recruitment was conducted through advertisements on clinic noticeboards, as well as brochures on the early signs of autism displayed and issued to parents in the visiting room. The SACS-C and CHAT-23 were introduced during children's routine health checks. Firstly, parents filled out Part A of the CHAT-23 in the waiting room (if the child was aged between 18- and 24-months). Children then underwent their routine health checks with the health practitioner, including measurement of height and weight. The health practitioners reviewed parent responses on Part A of the CHAT 23 and followed this with Part B of CHAT-23 (if the child had failed Part A); the SACS-C was then administered by the practitioner for all children. If a child was deemed "high-likelihood" for an autism on either the SACS-C, CHAT-23, or both, the health practitioners advised parents about their concerns regarding the child's development in social attention and communication. Parents were told that the monitored behaviors were important developmental milestones that need to be assessed further, and they were then referred to the TWCHC for a further developmental and diagnostic assessment for autism.

This study was approved by the Tianjin Women and Children's Health Centre (TWCHC) Human Ethics Committee.

Data Collection and Quality Control

Quality Control was undertaken during the entire data collection process. During the early stages of data collection, nominated staff from TWCHC and students from Nankai University (NU) were sent to the six districts, with one person allocated per district. They assisted the community health practitioners to correctly administer, score, and use of the SACS-C and CHAT-23 with the children. Furthermore, one staff member from TWCHC (JW) and Nankai University (CW) visited approximately 35% of the communities, thus ensuring correct administration of the two tools in all six districts, and accurate completion of the checklists. They also provided feedback to the health practitioners on the use of the tools and the referral procedure. Additionally, mid-way through data collection, a TWCHC staff member (JW) and one student from Nankai University re-visited ∼30% of the communities to check project implementation.

The SACS-C and CHAT-23 data sheets were initially stored in secure cabinets in the local CHCs and transferred to TWCHC at the end of the data collection phase, where they were stored in a secure cabinet. Each child was assigned a unique identification number, used to link child and assessment details. Health practitioners also entered the data from the record forms into a database at each CHC. Both the hard copy form and the database from each different district was then collected, and data entered for a second time at the TWCHC. Students from NU were involved in the second data entry process. Epidata 3.1 was used for the double data entry, and all the statistical analysis was undertaken using SPSS 21.0; the final database was stored in an encrypted computer at TWCHC and Nankai University.

Assessment Protocols for Children at "High-Likelihood" for Autism

The diagnostic procedure for autism in China involves a four-step process: (1) Collecting the medical history, including the clinical symptoms related to autism, the child's growth and developmental information, and family history; (2) Conducting cognitive assessments, including observing the child's behavioral symptoms and communication. Based on their clinical experience, each physician sets up an environment and activities to observe the child's behaviors (no one specific standard applied); (3) physical and neurological examination, including laboratory tests and administration of psychological assessments to assist the diagnosis if needed; (4) Before diagnosing as autism, other conditions such as language developmental disorders, intellectual disability, childhood schizophrenia and mental illnesses and other developmental disorders are excluded (differential diagnosis).

Two pediatricians from the TWCHC [Dr. Liang, Associate Chief Physician has 14-years of experience in diagnosing children's psychological and behavior disorders, and was trained on the Autism Diagnostic Observation Scale (ADOS); Dr. Yao, Chief Physician, has more than 10 years of experience in diagnosing children's psychological and behavior disorders] undertook the assessments and diagnosis of the referred children. The assessment tools commonly used with the referred children included the ASD Behavior Checklist (ABC) (17), Gesell Development Scale (GDS) (38), and Infants-Junior Middle School Students Social-Life Abilities Scale (S-M scale) (39). These tests were not used on all children but selected at the discretion of the clinicians based on signs the children were displaying. A final diagnosis of autism or Non-autism was then made on the basis of the above tests and clinical judgment. The above assessment

scales conducted for the children identified "high-likelihood" on SACS-C and CHAT-23 are listed in **Table 2**.

Follow-Up

Approximately 80% of the children monitored by the health practitioners in this study were followed-up in kindergartens from the six districts in Tianjin when they were aged between 3 and 4 years of age, to identify any "false negatives" from the surveillance and screening procedure undertaken between 12- and 24-months of age. JW from TWCHC and at least two trained practitioners from every district-level Women and Children's Health Centers visited the municipal and district kindergartens, respectively. Firstly, observation sheets were issued to the teachers in advance, which listed eight atypical behaviors and/or developmental concerns (see **Supplementary Table 2**), and they were asked to nominate children demonstrating those behaviors. Secondly, interviews were conducted with teachers regarding children who were identified as showing atypical behaviors to obtain more information about their behavior and development. The interviewers then observed the nominated children in the class, focusing on their social-communication skills and overall development. If the children were indeed showing atypical behaviors/development, they were referred to TWCHC for a further assessment and diagnosis by the two pediatricians.

Early Intervention

Children diagnosed with autism were referred to an autism intervention organization. Children who were diagnosed with other delays and disorders were referred to one of the child development intervention institutes, and their parents were taught some simple interventions by the clinicians, such as increasing social activities with other peers, encouraging more eye contact, and applying effective reinforcers to decrease behavioral problems.

RESULTS

Children Tested on Both the SACS-C and CHAT-23

Of the children assessed on both the SACS-C and CHAT-23 (n = 6,744), 21 were flagged as "high-likelihood" on the CHAT-23, and 52 were flagged as "high-likelihood" on SACS-C, with 17 children identified as being at "high-likelihood" on both tools (see **Table 4**). The Positive Predictive Value (PPV) of children with "high-likelihood" on both SACS-C and CHAT-23 was 81.0%, whilst the Negative Predictive Value (NPV) was 99.2%.

Psychometric Properties of SACS-C

Of the 10,514 children monitored with the SACS-C, 87 children were identified as "high-likelihood" (0.83% of the sample). Of these children at high-likelihood, 27.6% were identified at 12-months of age, 34.5% at 18-months of age, and 37.9% at 24-months of age. Only 57 (65.5%) of the 87 high-likelihood children were assessed for autism, as 30 families declined the invitation for a developmental assessment (**Table 3**). Of the 57 children assessed, 24 were diagnosed with autism (42.1%), and 25 (43.9%) children were diagnosed with developmental and/or language delays/disorders (DD/LD); a further 8 (14.0%) children were determined to be typically developing (TD).

The positive predictive value (PPV) for the SACS-C was 42.1% for autism and 86.0% for all developmental delays/disorders when used between 12 and 24-months of age. At the 2-year post-assessment follow-up, an additional 21 children were identified and diagnosed with autism; these children had previously been identified as "not high-likelihood" on the SACS-C when seen between 12- and 24 months, resulting in a Negative Predictive Value (NPV) of 99.8% for autism. Sensitivity and specificity for autism on the SACS-C was 53.3 and 99.7%, respectively. The estimated prevalence of autism among the study population monitored by the SACS-C (including follow-up) was 0.55%.

Psychometric Properties of CHAT-23

Of the 6,744 children also monitored with the CHAT-23, 21 children were identified as "high-likelihood" (0.31% of the sample), with 57% identified at 18-months and 43% at 24-months. However, as seven families declined an offer for a developmental assessment, only 14 children at "high-likelihood" for autism was assessed at the TWCHC. Of these, nine were diagnosed with autism (64.3%), four were diagnosed with developmental and/or language delays/disorders (DD/LD), with one child identified as typically developing (TD) (see **Table 3**). The CHAT-23 had an overall PPV of 64.3% for autism and 92.9% for all developmental delays/disorders. At the 2-year post-assessment follow-up, similar to SACS-C, an additional 24 children were identified and diagnosed with autism among children originally defined as "low-likelihood" on the CHAT-23, thus resulting in an NPV of 99.6%. Sensitivity and specificity for autism on the CHAT-23 was 27.3 and 99.9%, respectively. The estimated prevalence of autism among the study population using the CHAT-23 (including follow-up) was 0.56%.

DISCUSSION

This is the first large-scale study on developmental surveillance for autism in infants and toddlers among children in China. The findings demonstrated the feasibility of implementing developmental surveillance for autism within the Tianjin, Mainland China. They also indicated that the SACS-C tool was effective in identifying autism in a community-based sample at an early age. The SACS-C was found to have higher sensitivity compared with CHAT-23 (53.33 vs. 27.27%, respectively), but a lower PPV (42.11 vs. 64.29%). For both measures, the PPV increased with increasing age of screening, from 12 to 24 months of age, and at the age of 24 months, the PPV of SACS-C and CHAT-23 were the same (both PPV = 66.7%). A possible explanation is that for older children, the atypical behaviors are more prevalent and detectable by both parents and health practitioners. The specificity and NPV of the two tools were also very similar (SACS 99.7, 99.8%; CHAT-23 99.9, 99.6%, respectively). However, the results showed that the SACS-C identified many more children with autism than the CHAT-23 (0.83 vs. 0.31%), with the latter missing more children during these early years.

TABLE 2 | Sample characteristics of assessed children grouped according to age and diagnosis at each health check 12, 18, and 24 months.

	Group					
	Autism		**DD/LD**		**TD**	
SACS-C at 12-month-check						
SACS-C ($n = 16$)	3		9		4	
Mean age of identification (SD)	12.09 (0.11)		12.27 (0.24)		12.11 (0.19)	
Mean age of assessment (SD)	15.05 (2.86)		13.07 (1.09)		15.70 (4.53)	
Gender (male: female)	3:0		6:3		2:2	
Tests						
ABC	$n = 1$	78.00	$n = 3$	19.33 (8.51)	$n = 1$	18.00
Development scale	$n = 2$	68.65 (10.96)	$n = 8$	78.13 (8.56)	$n = 4$	89.63 (7.36)
S-M	n = 1	9.0	$n = 4$	9.75 (0.50)	n =1	10.00
SACS-C at 18-month-check						
SACS-C ($n = 20$)	7 (5 "high-likelihood" on CHAT)		10 (2 "high-likelihood" on CHAT)		3 (1 "high-likelihood" on CHAT)	
Mean age of identification (SD)	18.50 (0.56)		18.06 (0.43)		18.23 (0.26)	
Mean age of assessment (SD)	21.38 (7.097)		22.03 (10.54)		22.18 (1.18)	
Gender (male: female)	7:0		8:2		1:2	
Tests						
ABC	$n = 2$	47.50 (7.78)	$n = 6$	31.83 (18.23)	$n = 2$	18.50 (7.78)
Development scale	–	–	$n = 6$	78.10 (8.68)	$n = 2$	88.60 (3.68)
S-M	$n = 5$	9.20 (0.48)	$n = 8$	8.75 (3.66)	$n = 2$	10.00 (0.00)
CHAT-23 at 18-month-check						
CHAT-23 ($n = 8$)	5 (5 "high-likelihood" on SACS-C)		2 (2 "high-likelihood" on SACS-C)		1 (1 "high-likelihood" on SACS-C)	
Mean age of identification (SD)	18.48 (0.68)		18.12 (0.12)		18.53	
Mean age of assessment (SD)	22.39 (8.43)		34.89 (23.60)		23.03	
Gender (male: female)	5:0		2:0		1:0	
Tests						
ABC	$n = 2$	47.50 (7.78)	$n = 1$	58.00	$n = 1$	24.00
Development scale	–	–	$n = 1$	77.90	$n = 1$	91.20
S-M	$n = 3$	9.33 (0.58)	$n = 1$	12.00	$n = 1$	10.00
SACS-C at 24-month-check						
SACS-C ($n = 21$)	14 (4 "high-likelihood" on CHAT)		6 (0 "high-likelihood" on CHAT)		1 (0 "high-likelihood" on CHAT)	
Mean age of identification (SD)	24.98 (1.92)		27.09 (2.78)		26.74	
Mean age of assessment (SD)	25.82 (2.13)		27.92 (2.16)		27.04	
Gender (male: female)	13:1		3:3		1:0	
Tests						
ABC	$n = 12$	44.08 (17.93)	$n = 1$	54.00		
Development scale			$n = 4$	65.65 (18.03)	$n = 1$	91.10
S-M	$n = 9$	7.22 (0.44)	$n = 2$	8.00 (1.41)		
CHAT-23 at 24-month-check						
CHAT-23 ($n = 6$)	4 (4 "high-likelihood" on SACS-C)		2 (0 "high-likelihood" on SACS-C)		0	
Mean age of identification (SD)	24.22 (0.11)	24.38 (0.33)	–			
Mean age of assessment (SD)	25.06 (1.65)	34.27 ± 14.22	–			
Gender (male: female)	3:1	0:2	–			
Tests						
ABC	$n = 4$	56.25 (11.76)	$n = 1$	51.00	–	
S-M	$n = 1$	7.00	–	–	–	

*ABC, ASD Behavior Checklist; CHAT-23, Checklist for Autism in Toddlers-23; Development scale, Gesell Development Scale; S-M, Infants-Junior Middle School Students Social-Life Abilities Scale; SACS-C, Social Attention and Communication Surveillance-China tool; n, number of participants; SD, standard deviation. **Tests, These tests were not used on all children but selected at the discretion of the clinicians based on signs the children were displaying. –, not applicable/administered.*

Previous studies and meta-analyses have reported considerable variability in prevalence estimates, ranging from 1.8 to 426.4 per 10,000 (12, 15, 40). These studies indicated that compared with estimates of around 1% in developed countries, the reported prevalence of autism in Mainland China is much lower (12, 15, 40). Sun et al. reported an estimated prevalence of

TABLE 3 | Assessment characteristics of children administered the SACS-C and CHAT 23.

	SACS-C				CHAT-23		
Age (months)	**12**	**18**	**24**	**Overall**	**18**	**24**	**Overall**
n	3,178	3,757	3,579	10,514	3,683	3,061	6,744
Assessed (%)	16 (28.1)	20 (35.1)	21 (36.8)	57 (100)	8 (57.1)	6 (42.9)	14 (100)
Autism (%)	3 (12.5)	7 (29.2)	14 (58.3)	24 (100)	5 (55.6)	4 (44.4)	9 (100)
DD/LD (%)	9 (36.0)	10 (40.0)	6 (24.0)	25 (100)	2 (50.0)	2 (50.0)	4 (100)
TD (%)	4 (50.0)	3 (37.5)	1 (12.5)	8 (100)	1 (100)	0	1 (100)
Declined assessment (%)	8 (26.7)	10 (33.3)	12 (40.0)	30 (100)	4 (57.1)	3 (42.9)	7 (100)
Total "high-likelihood" (%)	24 (27.6)	30 (34.5)	33 (37.9)	87 (100)	12 (57.1)	9 (42.9)	21 (100)
PPV Autism %	18.75	35.0	66.7	42.1	62.5	66.7	64.3
PPV all disorders %	75.0	85.0	95.2	86.0	87.5	100.0	92.9

*DD/LD, Developmental Delay or Language Delay; TD, Typically Developing (TD); CHAT-23, Checklist for Autism in Toddlers-23; SACS-C, Social Attention and Communication Surveillance-China tool; PPV, Positive Predictive Value; n, number of participants. **Total "high-likelihood" equals to total children deemed high-likelihood for autism.*

TABLE 4 | The number of children deemed at "high" (positive) and "low" (negative) likelihood for autism following screening on SACS-C and CHAT-23.

	CHAT positive	**CHAT negative**	**Total**
SACS-C positive	17	35	52
SACS-C negative	4	6,688	6,692
Total	21	6,723	6,744

CHAT-23, Checklist for Autism in Toddlers-23; SACS-C, Social Attention and Communication Surveillance-China tool.

119 per 10,000 among 737 school-age (6–10 years) children (7). In our study population based in Tianjin City, the rate of autism was estimated to be 0.43% (1 in 233) based on the SACS-C and 0.49% on the CHAT-23 (1 in 204). This estimate is similar to the prevalence in Shenzhen City, with an estimate of 0.42% (42 per 10,000 95% CI 20–89) (14). Our lower estimated prevalence rates could possibly be explained by the lack of knowledge of and experience with the early signs of autism, leading to a lower detection rate (24).

When the two screening tools were compared in this study, the SACS-C demonstrated a better balance between accuracy (PPV) and sensitivity in identifying autism in infants and toddlers compared to the CHAT-23. There are also a number of advantages of using SACS-C; firstly, the SACS-C is potentially more objective because the community health practitioners directly observed and rated the SACS-C items, whereby their administration of the CHAT-23 is based on parents responses in the first instance, who are likely to be less knowledgeable about autism. (15) Secondly, the SACS-C had a higher sensitivity, detecting more autism cases in the community-based population, which is essential as it is the ultimate aim of screening (26). Although SACS-C had a lower PPV than the CHAT-23, the higher PPV of the CHAT-23 came at the cost of fewer referrals, and lower sensitivity. Also, when looking at the 24-month data, the SACS-C and CHAT-23 had identical PPVs. Finally, the SACS-C is a developmental surveillance tool, so that repeated monitoring is conducted across the second year of life, ensuring the tool is able to identify children with autism at subsequent checks if they are not initially identified, rather than being a single screen at a given period.

A significant strength of this study was the successful training of community health professionals that enabled the community-based surveillance of infants and toddlers in Tianjin, Mainland China for autism and related conditions. However, there are a few study limitations that should be noted. The lower sensitivity and PPV of SACS-C, compared to the original SACS (30), could be due to a few factors, such as possible cultural differences in administration of the SACS-C, limited knowledge and experience of community health professionals in early autism symptoms presentation and detection prior to this pilot study, differences between the two community health systems, and differences in the diagnostic procedures.

The diagnostic procedures for autism in China differed to those undertaken in Australia and varied according to the pediatricians preference. For example, the diagnostic assessments were not conducted using gold standard diagnostic tools such as ADOS (41), and Autism Diagnostic Interview-Revised (ADI-R) (42). Given that the percentage of children identified as "high-likelihood" on SACS-C (0.83%) was similar to the rate of children at "high-likelihood" for autism in the original SACS (1.04%) (30), it is possible that the diagnostic assessments conducted in Tianjin were not identifying as many children with autism that did indeed have autism, and instead diagnosed children with other conditions instead.

This pilot study implemented the SACS-C in Tianjin, China, and effectively compared its performance with that of CHAT-23 in a large community-based sample. In so doing, the feasibility of successfully training community health practitioners to monitor infants and toddlers for the early signs of autism using SACS-C was established. The SACS-C was found to be efficacious and culturally valid for use with Tianjin infants and toddlers aged 12- to−24-months. The SACS-C revealed a good balance between accuracy and sensitivity in detecting autism compared to the CHAT-23, which missed the majority of children on the spectrum (72.7 vs. 46.7%). Given these findings, it was found that newly-trained community health practitioners can identify

and refer more infants and toddlers with the early signs of autism on SACS-C than CHAT-23, indicating that the SACS-C is a viable alternative to be implemented in the CHC system in Tianjin. Based on the findings from this study, the team at the TWCHC selected SACS-C as the preferred autism developmental surveillance tool, such that it was incorporated into the 7-year Tianjin Women and Child Health Plan (2013–2020). Infants and toddlers in Tianjin have since been monitored for autism using the SACS-C following the training of all early child health professionals in Tianjin. However, future research is needed to improve the psychometric properties of the SACS-C in Mainland China so that it is comparable to its use in Australia.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Tianjin Women and Children's Health Centre (TWCHC) Human Ethics Committees. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JiW conducted the analyses, contributed to the interpretation of the results, and drafted the initial manuscript. JB, CW, GL, and CD developed the study design, contributed to data analysis and interpretation, and reviewed drafts, with JB coordinating these tasks. YL conducted the developmental assessment for children referred for assessment. JinW and IA contributed to the literature review and review of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.597790/full#supplementary-material

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The ADNP Syndrome and CP201 (NAP) Potential and Hope

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Activity-dependent neuroprotective protein (*ADNP*) syndrome, also known as Helsmoortel-Van Der Aa syndrome, is a rare condition, which is diagnosed in children exhibiting signs of autism. Specifically, the disease is suspected when a child is suffering from developmental delay and/or intellectual disability. The syndrome occurs when one of the two copies of the *ADNP* gene carries a pathogenic sequence variant, mostly a *de novo* mutation resulting in loss of normal functions. Original data showed that *Adnp*$^{+/-}$ mice suffer from learning and memory deficiencies, muscle weakness, and communication problems. Further studies showed that the ADNP microtubule-interacting fragment NAP (called here CP201) resolves, in part, *Adnp* deficiencies and protects against *ADNP* pathogenic sequence variant abnormalities. With a clean toxicology and positive human adult experience, CP201 is planned for future clinical trials in the *ADNP* syndrome.

Keywords: ADNP, ADNP syndrome, CP201 (NAP, davunetide), microtubules (MT), *Adnp*$^{+/-}$ mice, tau

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BACKGROUND

The *ADNP* syndrome (https://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=EN&Expert=404448; https://rarediseases.info.nih.gov/diseases/12931/adnp-syndrome) traits include limitations of social interactions and communication along with stereotypic, repetitive behavior, and restricted interest (1). *ADNP de novo* mutations (pathogenic sequence variants) causing syndromic autism were first described by O'Roak et al. and later extended by Helsmoortel et al. as reviewed in the laboratory of Illana Gozes, the discoverer of the *ADNP* gene (2–8).

The human *ADNP* gene is ∼40 kilobases long and contains five exons and four introns (5). The gene is located on the q13.13 band of chromosome 20 (8, 9). The protein comprises 1,102 amino acids including asparagine–alanine–proline–valine–serine–isoleucine–proline–glutamine (NAPVSIPQ), which is an 8-amino-acid neuroprotective peptide called NAP (also discovered by the Gozes Laboratory). NAP is referred here to as CP201 (1, 4, 7, 8).

The *ADNP* gene is one of the most prevalent single mutated genes within the autism spectrum disorders (ASDs) (5, 6, 10, 11). According to the original description, the *ADNP* syndrome is estimated to account for 0.17% of all cases of ASD (4, 12).

More than 400 genes are regulated by ADNP, which are critical for brain formation, organ development, cognition, and motor function (6, 13–16). In the nucleus, ADNP is a member of a chromatin remodeling complex that is responsible for RNA transcription and splicing (13, 17–19). In the cytoplasm, ADNP has been shown to correlate with the microtubule (MT)–associated protein Tau, leading to dynamic Tau expression and protection against Tau pathology (hyperphosphorylation) (20). Tau hyperphosphorylation has been associated

with neurodegeneration along with cognitive decline (6, 20, 21). Importantly, ADNP interacts directly with the MT end-binding proteins (EB1 and EB3). When there is a mutation and one of the *ADNP* alleles is lost (or dysfunctional), there is a disruption in the MT–EB protein interaction (6). This causes a negative impact on brain formation leading to decreased learning skills and memory (5).

The syndrome occurs when one of the two copies of the *ADNP* gene is mutated and loses its normal function (4). The mutation is most often a *de novo* (4). In this respect, $Adnp^{+/-}$ mice suffer from learning and memory deficiencies, muscle weakness, and communication problems. Data have shown the resolution of these symptoms with the administration of CP201, which also reduces neurodegeneration (20, 22). Mice with both *Adnp* genes deleted ($Adnp^{-/-}$) do not survive, as Adnp is critical for neural tube closure and further brain formation (4, 23). Most recent data showed direct protection of CP201 against deleterious effects of ADNP pathogenic sequence variants spanning the ADNP protein (24, 25) as detailed below.

Symptoms

Children are delivered on time (normal length and weight) (1, 26). Dysmorphic facial features are common, including a prominent forehead, high hairline, widely spaced and down-sloping eyes, posteriorly rotated ears, large head, long flat philtrum, thin upper lip, and a flat/broad nasal bridge ((4); https://www.adnpfoundation.org/). Other symptoms include seizures, hypotonia, feeding difficulties, gastroesophageal reflux disease, constipation, vomiting, heart defects (atrial septal defects and mitral valve prolapse), brain abnormalities (anxiety, aggressiveness, obsessive compulsive disorder), delayed milestones, severe cognitive delays, language disorder, motor skill disorder, undescended testicles, bilateral cryptorchidism, congenital hernia, and visual disturbances (hypermetropia, strabismus, and ptosis) (1, 5, 26). The main, similar features include gross and fine motor delay, along with intellectual disability (ID) and speech delay (10, 26–28).

Musculoskeletal defects have also been noted. These include joint hyperlaxity and multiple hand abnormalities, including, but not limited, to clinodactyly and abnormal phalanges (1). These children are also plagued with recurrent infections of the upper respiratory and urinary tracts (1). Abnormalities seen on brain magnetic resonance imaging (MRI) include wide ventricles, white matter lesions, and choroid cysts (1).

Diagnosis

Diagnosis is usually made by identifying a heterozygous *ADNP* mutation through molecular genetic testing using whole-exome sequencing (4, 5, 10). Other molecular testing approaches are acceptable including single-gene testing and multigene panels (4). Commonly, the mutation is a *de novo* mutation, meaning it is a spontaneous pathogenic sequence variant within the DNA (5, 10).

Early tooth eruption is a common trait found in these children (6). Usually, by the end of their first birthday, the children have a full mouth of teeth including their molars. This premature teething can be an early diagnostic marker for *ADNP* mutations, which can pave the way to early intervention and personalized treatment (6).

STANDARD OF CARE

Currently, there is no cure for this disease, and the prognosis for this syndrome is unknown (26). Although there is no standard of care for these children, they are symptomatically treated with walkers and surgically treated for atrial septal defects, ventricular septal defects, cardiovascular valve prolapse, imperforate anus, and astigmatism, along with other anatomical defects (10). Occasional treatments with risperidone have also been reported. Specifically, one case study on a 2½-year-old patient described that application of antipsychotic medication resulted in a significant resolution of behavioral outbursts, leading to progress in language acquisition (29).

Additional current treatments include physical therapy, occupational therapy, behavioral therapy, sensory processing therapy, and music and water therapy. Improvement with therapeutic intervention would prove beneficial to these patients and to caregivers (5, 10).

Rationale for Drug Development

ADNP syndrome is a chronically debilitating disease to which there is no approved treatment. Although current pharmacological treatments can be effective at treating children symptomatically, a treatment to help with ID could potentially be life changing. Usually, treatment of these children involves multiple specialists that include neurologists, cardiologists, and surgeons (https://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=EN&Expert=404448; https://rarediseases.info.nih.gov/diseases/12931/adnp-syndrome) (26).

This disease affects not only the child, but also the parents and the health care system. In the first few years of life, a child may go through multiple surgeries, including open heart surgery with ADNP involved in heart development (13) and affecting congenital heart diseases (5, 30). The cognitive impairments (ID) may be very severe; a 7-year-old may behave like a 16-month-old, and the language of a 3-year-old may be equivalent to a 12-month-old, as words are unrecognizable (5, 10, 31).

Looking at more than anecdotal case highlights, an extensive worldwide cohort of 78 individuals with *ADNP* syndrome was collected (2014–2016). The comprehensive results are published including clinician and parental interviews (26). In summary, clinical features include ID, autistic traits, severe motor and language development delays, and common facial appearances (outlined above). Behavioral problems, sleep irregularities, epilepsy, visual problems, hypotonia, congenital heart defects, gastrointestinal irregularities, short stature, endocrine (hormonal) deficiencies, and brain abnormalities (MRI) were described as common comorbidities. All these emphasize a need for further drug development.

Although rare, there have been at least two cases of childhood deaths (personal Facebook, https://www.facebook.com/TeamKnoxJoseph/; https://www.adnpkids.com/adnp-angels.html) with one recently published (25).

Postmortem analysis was conduct on a 7-year-old boy, heterozygous for *ADNP de novo* pathogenic sequence variant c.2244Adup/p.His559Glnfs*3. The child had autism, motor delays, severe ID, and seizures. He died following liver transplantation and multiple organ failure. A comparison to young adult with no tauopathy emphasizes the disease severity (25). Thus, a widespread child brain tauopathy paralleled by extensive transcriptomic alternations was discovered. Tauopathy was explained by direct ADNP mutation inhibition of Tau–MT binding (25). As tauopathy is a progressive condition, treatments halting tauopathy progression are required (24).

Therefore, the *ADNP* syndrome, in some cases, may be a devastating disease that does not allow children suffering from this disease to integrate into society due to multiple serious medical problems such as feeding difficulties, developmental delays (memory loss, limited speech), anatomical defects, and limited mobility.

To this end, CP201 is being developed for the treatment of the *ADNP* syndrome. This is based on reports of CP201 administration in heterozygous (haploinsufficient) mouse models of ADNP that has shown amelioration of some cognitive abnormalities along with restoration of learning and memory, skeletal strength, and vocalization with a reduction in neurodegeneration (22, 32–36). This is further based on CP201 mechanism of action as illustrated below.

DRUG CANDIDATE: CP201 (NAP) MECHANISM OF ACTION

ADNP is critical for the brain, influencing brain development, brain injury protection, and aging. ADNP has been associated with EB1/EB3 (end-binding proteins) through the CP201 active motif mediating MT neuroplasticity (37). ADNP deficiency in mice impedes axonal transport (6, 38). Neuronal communication depends on MT integrity, and disruption results in delayed cognition. CP201 is brain bioavailable, benefiting synaptic development by promoting neuronal cell survival, synaptic maturation, neuroplasticity, and axonal transport. Alternative names include AL-108, NAP, NAPVSIPQ (molecular weight, 824.9 Da), and davunetide. CP201 is an intranasal (IN) investigational drug product constituting a multidispensing, metered nasal spray pump device including an aqueous solution of davunetide. It is packaged in a mechanical multi-dose device designed for the IN application of solutions.

Specifically, CP201 exhibits brain bioavailability (39, 40) and cellular bioavailability (41). The mechanism of action of CP201 is through its interaction with the MT EB-interacting motif (SxIP) in ADNP (binding to the neuroactive proteins EB1 and EB3) (37). CP201 is shown to enhance ADNP-EB1/EB3/Tau interaction (6, 42, 43) even in the face of ADNP mutations (24, 25).

By binding to EB1/EB3 and promoting other SxIP-containing proteins including ADNP to associate with EBs, CP201 also enhances MT impact on neuroplasticity and neuroprotection (37). Furthermore, by binding CP201 and EB1/EB3, the Tau–MT interaction is dramatically increased leading to neuron/brain protection (6). As such, CP201 promotes formation of mature dendritic spines (post synapse) (17, 22), enhances MT invasion to the tip of the growth cone (pre-synapse) (33, 44) and protects MT-dependent axonal transport (6, 38). This explains the breadth and efficiency of CP201's neuroprotective capability along with its neurotrophic capacities (6, 37). Heterozygous mutations of *Adnp* ($Adnp^{+/-}$) result in Tau (MT associated protein) hyperphosphorylation paralleled by cognitive deficits. CP201 enhances Tau–MT binding and inhibits Tau hyperphosphorylation and aggregation, therefore, reversing ADNP deficiency (21).

Specifically, cellular expression of heterozygous ADNP truncating mutations (representing the majority of the *ADNP* syndrome cases, e.g., ADNP p.Ser404* or p.Tyr719*, or p.Arg730*) reduced Tau–MT interactions (25) and impaired MT dynamics, in cell culture models (24). We have previously shown that CP201 enhanced the interaction of the intact ADNP with MT-Tau (6, 37). Thus, treating the ADNP-mutated cells with CP201 protected against ADNP mutation-induced MT dysfunction (24, 25). These results suggest that CP201-induces increased interaction of the intact ADNP with MT-Tau (6) and provides cellular protection (37), in the face of ADNP pathogenic sequence variants (25).

Furthermore, autophagy, a major cellular regulatory mechanism, is dependent on MTs, and ADNP binding to the MT associated protein 1 light chain 3 (LC3) is enhanced by CP201, protecting autophagy (45).

We propose CP201 as a first-in-class drug candidate, leading to the discovery of new routes to combat devastating brain diseases associated with the loss of essential cellular functions that culminate in loss of crucial daily functions (37).

Preclinical Studies

Toxicology and pharmacology studies in animals were conducted with davunetide, the Drug Substance (DS). The DS used was of similar purity and quality as the batch used to produce the clinical supplies described here. For intravenous (IV) administration in the non-clinical acute dog toxicity study and the safety pharmacology studies, davunetide was dissolved in sodium chloride for injection. For IN administration in the non-clinical toxicity studies, davunetide was dissolved either in sodium chloride for injection or in a solution containing 7.5 mg/mL sodium chloride, 1.7 mg/mL citric acid monohydrate, 3 mg/mL disodium phosphate dihydrate, and 0.01% benzalkonium chloride. The IN toxicology studies used the same formulation composition as for the davunetide clinical supplies, except that the concentration of benzalkonium chloride used in the toxicology studies was twice the concentration in the clinical formulation (0.01% for animal studies vs. 0.005% for clinical supplies). Benzalkonium chloride is used as a preservative or bacterial-static agent commonly found in IN drugs. Proof-of-concept studies are described below.

The safety of davunetide was studied in various modes of administration (IN or IV) in a broad spectrum of doses (up to 300 mg/kg per day) and in several animal model (rats, dogs, and mice), as well as studies in juvenile animal performed in 6-week-old rats and 4–5-month old beagle dogs. The studies included safety pharmacology, acute dose toxicity, repeat dose toxicity

TABLE 1 | *In vitro* preclinical proof-of-concept studies.

Study title	Purpose	Assay and concentrations	Results	Conclusion
The CP201 motif of ADNP regulates dendritic spines through microtubule end binding proteins (37)	To evaluate the requirement of the SxIP motif (microtubule interacting motif) in CP201 and ADNP in the modulation of synaptic plasticity and cell protection	Primary neurons, COS7 cells, PC12 cells (CP201, 10^{-15}-10^{-9} M) Measurements of protein characteristics for dendritic spines. Immunochemistry, immunopercipitation EBs RNA silencing Affinity chromatography and cell survival assays	CP201 increased dendritic spine plasticity and protected neurons through the SxIP motif, while enhancing endogenous ADNP interaction with microtubules	The identified SxIP shared by CP201/ADNP is directly implicated in synaptic plasticity, explaining the wide scope and potency of neurotrophic/neuroprotective capacities
ADNP/CP201 dramatically increase microtubule end-binding protein-Tau interaction: a novel avenue for protection against tauopathy (6) even in the face of multiple ADNP mutations (24, 25)	To evaluate the effect of CP201 on Tau–microtubule interaction through the SxIP motif	N1E-115 neuroblastoma neuronal cell model. Immunochemistry, cell transfection with fluorescent proteins, and live cell imaging. Mutations tested include ADNP-p.Ser404*, p.Tyr719*, and p.Arg730*. NIH3T3 fibroblasts, cell transfection with Tau and live cell imaging; immunopercipitation (CP201, 10^{-12} M)	CP201 augmented microtubule dynamics in N1E-115 neuroblastoma neuronal model. CP201 dramatically increased Tau–microtubule interaction through its SxIP motif and protected NIH3T3 cells against zinc intoxication, only if these cells were transfected with Tau	Microtubule–Tau binding is identified as a new site for endogenous ADNP neuroprotection, and a target for drug development, with CP201 as a lead compound
Premature primary tooth eruption in cognitive/motor-delayed ADNP-mutated children and activity-dependent neuroprotective protein deficiency models synaptic and developmental phenotypes of autism-like syndrome (6, 22, 25, 47)	To compare gene expression patterns in ADNP patient-derived lymphoblastoid cells (LCLs) to *Adnp*$^{+/-}$ mouse hippocampal, cortical, and splenic expression levels and evaluate protection by CP201	Cellular mutations tested: ADNP-p.Arg216*, ADNP-p.Lys408Valfs*31, and ADNP-p.Tyr719* CP201 was administered *in vivo* (intranasal) at 0.5 μg/mouse, 1 month of daily intranasal administrations (starting at 2 months of age)	1,442 common genes were differentially expressed in all three different ADNP-mutated cell lines compared to the control cell line. RNA transcripts changed by *ADNP* deficiency and reversed by CP201 treatment in the mouse spleen were also found to be changed by various human ADNP mutations in the ADNP-mutated cells	Tested *ADNP* syndrome pathogenic mutations cause loss of function (ADNP haploinsufficiency). Gene expression patterns affected by ADNP loss of function are partially ameliorated by CP201 treatment
Cellular and animal models of skin alterations in the autism-related *ADNP* syndrome (32).	Test the involvement of ADNP in skin function and CP201 ameliorative effects on dermal thickness and wound healing	ADNP Tyr719* patient-derived skin cells, 100 or 600 nM CP201 or mouse *Adnp*$^{+/-}$ fibroblasts, 180 nM CP201	Ameliorative effects of drug treatment on skin abnormalities, specifically wound healing, which seems to be impaired (see also *in vivo* results, **Table 2**)	A new activity of ADNP was discovered in the skin that may serve to characterize the clinical phenotype of patients with *ADNP* syndrome. The study further provides a therapeutic option for skin deficits in these patients

in various lengths and designs, genotoxicity, pharmacokinetic analysis, and drug–drug interaction where the inhibition of CYPs was studied. The product was well tolerated in the non-clinical studies and did not demonstrate any test article related adverse events (39, 40). Davunetide demonstrated a maximal NOAEL of 20 mg/kg per day in IN administration in dogs, which is equivalent to 11.11 mg/kg per day in humans.

Proof of Concept Studies

In vitro

CP201 (NAP) was extensively studied in multiple *in vitro* studies. A previous review summarized the pharmacology up to 2017 (46). Here, *in vitro* studies related to the mechanism of ADNP are summarized in **Table 1**. These studies demonstrate that CP201 directly affects ADNP mechanisms. In neuronal cell cultures, CP201 increased dendritic spine plasticity and protected neurons through the SxIP motif, while enhancing endogenous ADNP interaction with MTs and Tau.

In vivo

The Adnp$^{+/-}$ *Mouse Model*

The *Adnp*$^{+/-}$ mouse model predicted the *ADNP* syndrome (20). This mouse line exhibits developmental delays and synaptic dysfunctions mimicking children with *ADNP* syndrome. A survey of 78 children carrying pathogenic *ADNP* sequence variants spanning the entire protein suggested partial loss of similar functions, with potentially some increased severity in ADNP p.Tyr719* children (the most prevalent group) (26). Importantly, the mutated (mostly truncated) and the intact *ADNP* alleles are both expressed in *ADNP* syndrome human

TABLE 2 | *In vivo* preclinical proof-of-concept studies.

Study title	Purpose	Animal model	Study description	Results	Conclusion
The ADNP snippet NAP reduces Tau hyperphosphorylation and enhances learning in a novel transgenic mouse model (20)	To generate a transgenic mouse devoid of one *Adnp* allele and to assess CP201 activity *in vivo*	*Adnp*/$^{+/-}$ mice (*Adnp*$^{-/-}$ was embryonic lethal) (23)	Newborn mice administered daily with SC CP201 (25–500 μg/kg) and subjected to behavioral testing. In a separate experiment, CP201 was administered IN daily over a 2-week period to 2- and 9-month-old male mice (0.5 μg/5 μL/mouse per day.	*Adnp*$^{+/-}$ mice exhibited cognitive deficits, significant increases in pathological phosphorylated Tau compared with *Adnp*$^{+/+}$ mice. CP201 treatment partially ameliorated cognitive deficits and reduced Tau hyperphosphorylation in the *Adnp*$^{+/-}$ mice	These results imply that ADNP is critical for brain activity, participating in normal cognitive function. *Adnp*-deficient mice were shown to be a model for evaluation of cognitive enhancers, such as CP201, which ameliorated cognitive deficits associated with ADNP deficiency
ADNP is an alcohol-responsive gene and negative regulator of alcohol consumption in female mice (35)	To assess the ADNP/CP201 role in the regulation of alcohol consumption	*Adnp*$^{+/+}$ and littermates, *Adnp*$^{+/-}$ mice (outbred with the ICR strain for 30 generations) (21)	*Adnp* $^{+/-}$ or *Adnp*$^{+/+}$ mice (25–30 g, n = 14–15/experimental group) were given continuous access to two bottles: water and 10% alcohol, for 4 weeks. After 1 week of drinking without treatment, all mice received vehicle treatment for 1 week, followed by intranasal CP201 (0.5 μg/5 μL) treatment for 2 weeks, 5 days per week	The *Adnp*$^{+/-}$ female mice showed higher alcohol consumption and preference, compared to *Adnp*$^{+/-}$ females. Daily intranasal administration of CP201 normalized alcohol consumption in the *Adnp*$^{+/-}$ females	ADNP is a potential new biomarker and regulator of alcohol-drinking behaviors. CP201 corrected the phenotype, suggestive of corrected obsessive/addictive phenotype
Activity-dependent neuroprotective protein deficiency models synaptic and developmental phenotypes of autism-like syndrome (22)	The study was conducted to correlate one-to-one the children phenotype to the *Adnp*$^{+/-}$ mouse phenotype, and to assess CP201 protection and target engagement	A unique neuronal membrane tagged GFP-expressing *Adnp*$^{+/-}$ mouse line allowing *in vivo* synaptic pathology quantification	For dendritic spine determinations, 3-month-old *Adnp*-GFP mice were treated for 9 consecutive days with either intraperitoneal CP201 injection (0.4 μg) diluted in 0.1 mL saline or with 0.1 mL saline as vehicle. On day 9, mice were perfused, and brains were subjected to immunohistochemistry	ADNP deficiency reduced dendritic spine density and altered synaptic gene expression, both of which were partly ameliorated by CP201 treatment. *Adnp*$^{+/-}$ mice further exhibited global developmental delays, vocalization impediments, gait/motor dysfunctions, and social/object memory impairment, all partially reversed by daily CP201 administration (systemic/intranasal)	This study associated ADNP-related synaptic pathology to developmental/behavioral functions, establishing CP201 *in vivo* target engagement. The study further identified potential future biomarkers. The results of the study provide incentive to clinical development of CP201 in the *ADNP* syndrome
Hanging wire test: *Adnp*$^{+/-}$ mice display decreased latency to fall in an age- and sex-dependent manner—NAP protects (22, 47)	To assess the effect of CP201 on reduced grip strength	*Adnp*$^{+/+}$ and *Adnp*$^{+/-}$ mice	2-month-old mice (n = 3–4 males or 6–8 females per experimental group) were treated daily, five times a week for 5 weeks with 0.5 μg NAP/mouse per day by intranasal administration. Grip strength was measured by hanging wire tests	The time it took *Adnp*$^{+/-}$ CP201-treated mice to fall off the inverted cage lid was 90 s, similar to the time it took the *Adnp*$^{+/+}$ mice to fall off, as opposed to ∼15 s for the *Adnp*$^{+/-}$ mice. Sexual dichotomy was also observed in *Adnp*$^{+/-}$ mice ($p < 0.05$)	Male *Adnp*$^{+/-}$ mice exhibited decreased latency to fall, as compared to *Adnp*$^{+/+}$ mice, which was improved by CP201 administration
Grip strength test: *Adnp* mice exhibit significant decreased grip force—NAP protects (22, 47)	To assess the effect of CP201 on reduced grip strength	*Adnp*$^{+/+}$ and *Adnp*$^{+/-}$ mice	Two-month-old mice were treated by daily intranasal administration of 0.5 μg CP201/mouse, five times a week for 5 weeks. Grip strength was measured by using the Ugo Basile 47200-Grip-Strength Meter	*Adnp*$^{+/-}$ mice demonstrated decreased strength for males and females as opposed to the strength displayed by the male and female *Adnp*$^{+/+}$. The treatment of CP201 restored the grip strength of the *Adnp*$^{+/-}$ mice for males and females, respectively. Sexual dichotomy was also observed in *Adnp*$^{+/-}$ mice ($p < 0.05$)	*Adnp*$^{+/-}$ male mice exhibited reduced muscle strength vs. *Adnp*$^{+/+}$ mice, with CP201 significantly improving it.

(Continued)

TABLE 2 | Continued

Study title	Purpose	Animal model	Study description	Results	Conclusion
NAP treatment protected against vocalization deficiency in *Adnp*$^{+/-}$ mice (22, 47)	To assess the effect of CP201 on speech deficits	*Adnp*$^{+/+}$ and *Adnp*$^{+/-}$ mice	Ultrasonic vocalizations (USVs) were recorded in 8-day-old pups, subjected to daily subcutaneous injections of NAP (25 μg/mL saline) or saline (20 and 40 μL on postnatal days 1–4 and 5–7).	The *Adnp*$^{+/-}$ mice had a decrease in the number of vocalizations per minute at approximately ½ the number seen in the *Adnp*$^{+/+}$ mice. When the *Adnp*$^{+/-}$ mice were treated with CP201, the number of vocalizations increased to over 18 vocalizations per minute	CP201 administration increased vocalization in the *Adnp*$^{+/-}$ mice, suggesting that CP201 has the potential to treat vocal communication deficits
Cellular and animal models of skin alterations in the autism-related *ADNP* syndrome (32)	Test the participation of ADNP in skin function and CP201 ameliorative effects on dermal thickness and wound healing	*Adnp*$^{+/+}$ and *Adnp*$^{+/-}$ mice. ADNP p.Tyr719* patient derived skin.	Sonography in the patient revealed thin skin. Dermal thickness measurements in the mice in the presence and absence of CP201 treatment. Nasal CP201 application (0.5 μg CP201 in 5 μL vehicle solution) was performed daily, once a day, for 6 weeks (5 days a week). Vehicle-treated mice were maintained until the age of 4.5 months	The human and the *Adnp*$^{+/-}$ mice had thinner skin, which was normalized by CP201 treatment	The study discovered a new activity of the autism-linked ADNP in the skin. This activity may serve to define the clinical phenotype of patients with *ADNP* syndrome. Furthermore, the results suggest CP201 as an attractive medication for skin problems in ADNP patients
Microbiota changes associated with ADNP deficiencies: rapid indicators for NAP (CP201) treatment of the *ADNP* syndrome and beyond (36)	As the microbiome interacts with brain function, we investigated the effects of the *Adnp*$^{+/-}$ genotype on microbiota composition in our *Adnp*$^{+/-}$ mouse model	*Adnp*$^{+/+}$ and *Adnp*$^{+/-}$ mice (1-month-old on the first treatment day)	DNA obtained from fecal bacterial loads was subjected to PCR to identify different microbiota with and without CP201 treatment (nasal application 0.5 μg/5 μL/mouse per dose, daily for 45 days)	A highly significant sexually dichotomized *Adnp* genotype effect and amelioration by CP201 was observed as described below. Most of the commensal bacterial microbiota tested were affected by the *Adnp* genotype and corrected by CP201 treatment in a male sex-dependent manner. A female *Adnp*$^{+/-}$ genotype linked decrease (contrasting with a male increase) was observed in the *Lactobacillus* group. Significant correlations were found between specific bacterial group loads and behavior in the open-field and the three-chamber apparatus measuring social behavior.	ADNP deficiency–associated changes in commensal gut microbiota compositions and a sex-dependent biomarker for the *ADNP* syndrome were discovered. Strikingly, a rapidly detected CP201 treatment-dependent biomarkers within the gut microbiota was also discovered. Because gut microbiota are closely associated with immune responses, and CP201 modulates the immune response toward an anti-inflammatory response (48, 49), microbiota and immune markers are now under patent protection (Ramot at Tel Aviv University)
Age- and sex-dependent ADNP regulation of muscle gene expression is correlated with motor behavior: possible feedback mechanism with PACAP (16)	Understand the involvement of ADNP and CP201 in muscle transcriptomic patterns, in correlation with motor activity throughout the entire life span	*Adnp*$^{+/+}$ and *Adnp*$^{+/-}$ mice	Using quantitative RT-PCR, the *Adnp*$^{+/-}$ genotype in mice resulted in aberrant gastrocnemius muscle, tongue and bladder mRNA transcript expression, which was ameliorated by CP201 treatment.	A significant sexual dichotomy was revealed, coupled to muscle-, and age-specific transcriptional regulation. *Adnp*/CP201 regulated myosin light chain (*Myl*) in the gastrocnemius muscle, the language acquisition gene forkhead box protein P2 (*Foxp2*) in the tongue and the bladder-function linked, pituitary–adenylate cyclase activating polypeptide (PACAP) receptor PAC1 mRNA (*Adcyap1r1*) in the bladder. A significant age dependency was discovered, coupled to an extensive correlation to muscle activity (gait)	The results suggest a tight connection between Adnp and muscle activity throughout life, including (1) the acto-myosin muscle system (Myl2 and Myl9), (2) energy metabolism nicotinamide nucleotide adenylyl (NAD) transferase 1 (Nmnat1) (50), (3) speech acquisition Foxp1/Foxp2 tongue expression, (4) bladder activity feedback regulation (PACAP), and (5) multiple correlations with gait functions. Sexual dichotomy provides guidelines for better clinical design

cells (4), supporting the *Adnp*$^{+/-}$ mouse as a model predictive for *ADNP* heterozygous mutation deficiency in humans (6, 26). Furthermore, some children with *ADNP* syndrome show almost complete deletions of one allele (9), presenting a haploinsufficient loss-of-function phenotype (1, 9). Finally, there is a very high conservation of the *ADNP* gene between human and mouse (about 90% identity at the mRNA level) (8), and ADNP is critical for brain development in the mouse, like in human (23).

The *Adnp*$^{+/-}$ mouse model is representative of traits presented in children with *ADNP* syndrome as described before (22). The protective effect of CP201 was demonstrated by affecting animal traits that are equivalent to clinical symptoms in human patients with *ADNP* syndrome (20, 22). A summary of *in vivo* proof-of-concept studies in the *Adnp*$^{+/-}$ mouse model is provided in **Table 2** and expended below.

ADNP Deficiency in Mice Models the ADNP Syndrome

Results comparing synaptogenesis, dendritic spine formation, and immunohistochemistry of excitatory synapses in the *Adnp*$^{+/-}$ mice to human *ADNP* syndrome MRI data have been collected (22, 33, 34). These results demonstrate parallels between the *Adnp*$^{+/-}$ mouse and patients with *ADNP* syndrome, at multiple levels (developmental, behavioral, and motor). Furthermore, the mouse model allowed quantitation of excitatory synapse density in the hippocampus and motor cortex and evaluation of transcriptomic data, correlating molecular, anatomical, and functional consequences as described (22). These results establish CP201 *in vivo* target engagement and identify potential biomarkers, paving the way toward clinically advancing CP201 for the *ADNP* syndrome.

The data in *Adnp*$^{+/-}$ mice further demonstrate that hyperphosphorylation of Tau is decreased following CP201 treatment (20). This is in line with the findings of tauopathy in the human postmortem ADNP case and with mutated human ADNP reducing Tau–MT interaction, which is corrected/normalized by CP201 treatment (25).

Collectively, the data from *ADNP* syndrome mouse model demonstrate CP201 to be a promising therapeutic candidate for the treatment of children who suffer from this debilitating disease (**Table 2**).

It should be added that although the current review may seem limited in cellular and animal models, a previous book chapter summarized CP201 (NAP) *in vitro* and *in vivo* pharmacology up to 2017. This previous report includes dozens of our own investigations, as well as independent research in versatile disease models corroborating the proposed efficacious mechanism of action (46).

CLINICAL STUDIES

CP201 has not been previously approved for the treatment of the ADNP syndrome; however, clinical trials for other indications have been conducted [progressive supranuclear palsy (PSP), mild cognitive impairment (MCI), and schizophrenia] (42).

CP201 was previously referred to as AL-108, developed by Allon Therapeutics and subsequently licensed by Coronis Neurosciences from Ramot at Tel Aviv University.

The legal owner of all Allon Therapeutics materials is Ramot. Previous clinical trials for IN administered davunetide by Allon include the following:

1. ClinicalTrials.gov identifier: NCT00422981—MCI
2. ClinicalTrials.gov identifier: NCT00505765—Schizophrenia
3. ClinicalTrials.gov identifier: NCT01056965—Tauopathies
4. ClinicalTrials.gov identifier: NCT01110720—PSP

Allon also conducted an IV administration trial:

ClinicalTrials.gov identifier: NCT00404014—MCI Following Coronary Artery Bypass Graft Surgery.

No significant side effects were reported. Minor side effects in a small minority of patients may have included some nasal discomfort (51), which could perhaps be associated with the application volume requiring repeated daily nasal administrations (52). In general, all studies have proven safety and tolerance of CP201 in hundreds of adult compromised patients. Efficacy was seen in enhancement of cognitive function and functional activities of daily living as reviewed (46).

Additional clinical studies have shown that ADNP levels correlate with disease status (cognitive impairments, and schizophrenia) and tauopathy as illustrated above, e.g., ClinicalTrials.gov identifier: NCT01403519—Innovative Biomarkers in Alzheimer's Disease and Frontotemporal Dementia: Preventative and Personalized (24, 53).

Current *ADNP* syndrome clinical trials feature natural history (e.g., ClinicalTrials.gov identifier: NCT01238250 and NCT03718936). Furthermore, ketamine is being tested in the *ADNP* syndrome patients ClinicalTrials.gov identifier: NCT04388774, and as noted above, risperidone treatment has shown some efficacy in a case study (29).

Coronis was granted an Orphan Drug Designation #DRU-2017-6243 by the US Food and Drug Administration (FDA) for the treatment of the *ADNP* syndrome with CP201. Coronis has further officially met with the FDA for a Pre-Investigational New Drug Application, paving the path to a CP201 clinical trial (54).

AUTHOR CONTRIBUTIONS

IG designed, led and orchestrated the writing, provided funding, designed experiments, analyzed the data of many of the cited articles, and wrote the final mini review.

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A Comparison of Children Born Preterm and Full-Term on the Autism Spectrum in a Prospective Community Sample

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Introduction: Previous research suggests children diagnosed with autism spectrum disorder (ASD or "autism") born extremely and very preterm face substantially delayed development than their peers born full-term. Further, children born preterm are proposed to show a unique behavioral phenotype, which may overlap with characteristics of autism, making it difficult to disentangle their clinical presentation. To clarify the presentation of autism in children born preterm, this study examined differences in key indicators of child development (expressive language, receptive language, fine motor, and visual reception) and characteristics of autism (social affect and repetitive, restricted behaviors).

Materials and Methods: One fifty-eight children (136 full-term, twenty-two preterm) diagnosed with autism, aged 22–34 months, were identified prospectively using the Social Attention and Communication Surveillance tools during community-based, developmental surveillance checks in the second year of life. Those identified at "high likelihood" of an autism diagnosis were administered the Mullen Scales of Early Learning and the Autism Diagnostic Observation Schedule.

Results: The children born preterm and full-term did not differ significantly in their fine motor, visual reception, expressive language, or receptive language skills. No significant differences in social affect and repetitive and restrictive behavior traits were found.

Discussion: The findings of this study differs from previous research where children diagnosed with autism born very or extremely preterm were developmentally delayed and had greater autistic traits than their term-born peers. These null findings may relate to the large proportion of children born moderate to late preterm in this sample. This study was unique in its use of a community-based, prospectively identified sample of children diagnosed with autism at an early age. It may be that children in these groups differ from clinic- and hospital-based samples, that potential differences emerge later in development, or that within the autism spectrum, children born preterm and full-term develop similarly. It was concluded that within the current sample, at 2 years of age, children diagnosed with autism born preterm are similar to their peers born full-term. Thus, when clinicians identify characteristics of autism in children born preterm, it is important to refer the child for a diagnostic assessment for autism.

Keywords: prematurity, preterm, autism spectrum disorder, child development, social development, restricted repetitive behavior

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INTRODUCTION

Two key areas of development characterize a diagnosis of autism spectrum disorder (ASD), hereafter "autism": differences in social-communication (e.g., eye contact and interest in peers) and restricted, repetitive patterns of behavior (RRBs; e.g., fixated interests and stereotyped motor movements) (1). For children born preterm, there is a risk of early markers of autism, such as atypical eye gaze and protodeclarative pointing (2), being misattributed to the long-term effects from their preterm birth (3), as these can also be observed in children born preterm who do not go on to be diagnosed with autism (4, 5), despite the higher than expected prevalence of autism in children born preterm (6). This has the potential to delay diagnosis and appropriate support.

As survival rates following preterm birth increase with medical advances, more becomes known about the developmental outcomes of children born preterm (7). Children are considered preterm if they were born prior to or during the thirty-sixth week of gestation and full-term if they were born between the thirty-seventh and forty-second weeks of gestation (8). In Australia, 8.50% of children are born preterm (9). This is comparable to the estimated rate of 8.60% for developed regions and lower than the world-wide average estimated rate of 11.10% (10). There are several classifications for preterm birth based on gestational age: extremely preterm (<28 weeks gestation), very preterm (28–32 weeks gestation), and moderate to late preterm (32–36 weeks gestation) (11). Moderate to late preterm births account for 84.70% of preterm births across the world (12). Across the classifications for prematurity, children born preterm have a higher likelihood for developmental difficulties, such as having a neurodevelopmental disability (13) or meeting Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria for any mental health disorder (6, 14). Although there is no single known cause, several factors may increase the likelihood for preterm birth, such as multiple gestation, maternal ethnicity, and maternal age (15). A gene by environment interaction process is likely in the etiology of preterm birth (3).

By comparing children born preterm to children born full-term, Johnson and Marlow (16) identified and described a "preterm phenotype" characterized by a distinct pattern of behaviors. The preterm phenotype is believed to result in higher rates of attentional, cognitive, and socio-emotional difficulties that can be evident across the lifespan (16, 17). These difficulties have been attributed to reduced intrauterine development of the nervous system and complications typically associated with preterm birth (3). Atypical early life experiences [e.g., over-stimulation from the neonatal intensive care unit (NICU) environment] associated with preterm birth may contribute to differences in early brain development (18). While those born preterm are often described as needing to "catch up" to those born full-term, these structural differences typically continue throughout childhood development into adulthood (19). This suggests that, rather than being delayed, brain development after preterm birth has its own trajectory (16, 19).

Research on development after preterm birth largely focuses on extreme and very preterm birth; however, a "dose effect" (17) can be observed across prematurity, in which likelihood for developmental concerns is inversely associated with gestational age. The effects of moderate to late preterm birth, where the impact of dose effect would be weakest, can be observed throughout childhood and adolescence. For example, at 2 years of age, children born moderate to late preterm were twice as likely to have a neuromotor or sensory impairment when compared to children born full-term (13). In a meta-analysis of seventy-four studies, children and adolescents born preterm had significantly lower full scale and performance intelligence quotients than their full-term counterparts (20). A dose effect was observed, with effect sizes ranging from medium and large for children born extremely preterm, reducing to small effects for children born moderate to late preterm (20). Interestingly, children born moderate to late preterm were not significantly different than their peers born full-term on verbal intelligence (20), indicating that some differences in development from preterm birth are less clear, or even undetectable, for children born moderate to late preterm.

The association between autism and preterm birth has been investigated to better understand the potential implications of preterm birth on social development. For children under the age of three, there is an estimated prevalence of 7.00% for autism in children born preterm (21). This is substantially higher than population estimates of autism for children in that age group with an estimated prevalence in the United States at 0.02% (22) or Sweden at 0.80% (23). It has been suggested that, as preterm brain development has its own trajectory (16, 19), autism may manifest differently in children born preterm (3, 24). Some evidence for this hypothesis can be found when examining cerebellum development in children on the spectrum born preterm (25–27). Further, many risk factors for autism are common characteristics of preterm births, such as low birth weight (28, 29), birth complications, more days in spent in hospital following birth (30–32), maternal infection (33), and being born small for gestational age (29). There is emerging evidence for a relationship between preterm birth and autism diagnoses, with prevalence rates of autism having an inverse relationship with gestation age (6). This suggests the importance of examining autism in preterm populations across the different categories for preterm birth to understand the dose effect across development. The relationship between preterm birth and autism is further complicated by the hypothesis that children born preterm with subtle autism traits are misdiagnosed, as their atypical behavior is attributed to their preterm birth rather than a neurodevelopmental condition (3, 16). Common markers for autism include gaze aversion and inconsistent or lack of social smiling; both behaviors that can be observed in infants born preterm with and without autism (34–36). Furthermore, children born preterm are more likely to have visual and/or hearing impairments (37, 38), which may result in atypical eye contact or response to name and subsequently a false positive screen for autism (39). Thus, developmental difficulties related to preterm birth further entangle the presentation of development after preterm birth with autism.

Attempts have been made to identify early markers for autism specific to preterm populations with mixed results. One study found that 9 month old (corrected age) infants born preterm who showed typical eye contact and gaze behavior were more likely

to be screened as "high likelihood" for autism using screening measures (40). Corrected aged is used for children born preterm to account for their expected development had they been born full-term and is calculated subtracting the number of weeks born preterm from the child's chronological age. This finding was surprising as atypical eye contact and gaze are normally reliable markers for autism in young children (41–43). However, another study using a matched sample of NICU infants on the autism spectrum and not on the autism spectrum found infants later diagnosed with autism displayed expected patterns of atypical eye contact as early as 1 month (corrected age) when compared to infants not on the autism spectrum (44). Given the inconsistent results, it is unclear if children on the spectrum born preterm show the same key early markers as children on the spectrum born full-term and how these markers relate to children not on the spectrum born preterm.

Currently, there are no clear indicators of autism specific to children born preterm. However, retrospective (45) and prospective (42) studies have reliably identified early markers of autism in infants and toddlers in the general population. Specifically, atypical social-communication behavior can accurately differentiate between children on the spectrum and not on the spectrum in the first years of life (41, 43), whereas the presence of RRBs does not clearly distinguish autism from other developmental differences, such as global developmental delay (41, 43). As a result, developmental surveillance with an emphasis on early social-communication behaviors, rather than RRBs, has been shown to be effective in identifying children at an increased likelihood for autism (2). This is beneficial, as a reliable diagnosis can be made by 2 years of age (46) with early identification and diagnosis having positive impact on future development as compared to later diagnosis (47–49).

Barbaro et al. (2, 42, 50–53) used social-communication markers to develop a universal tool to monitor infants and toddlers for autism, the Social Attention and Communication Surveillance (SACS) tool. The SACS tool and its revised version (SACS-R) (53), were developed for use in community settings to prospectively identify children between 12 and 30 months of age who display a pattern of atypical behaviors indicating a higher likelihood of autism. Development is monitored at 6-month intervals based on age-expected behaviors. A strength of the SACS tool is in its positive predictive value (the probability that children with a positive screening test truly have autism) of 81.00–83.00% (42, 53) between 12 and 24 months of age, which is higher than other commonly used autism screening tools for young children, such as the Modified Checklist for Autism in Toddlers (M-CHAT) (54) with a positive predictive value of 6.00% (55) and the Ages and Stages Questionnaire with a positive predictive value between 26.70 and 30.30% (56). While there have been previous studies in preterm children using the M-CHAT, discussed in further detail below, thus far no studies have targeted infants and toddlers on the spectrum born preterm who were identified using the SACS.

In addition, few studies have explicitly examined differences between preterm and full-term groups with an autism diagnosis. Two studies considering the impact of preterm birth on social-communication presentation found greater autism behaviors for children born very or moderate to late preterm (57, 58). Five year old children on the spectrum were identified to have a specific weakness in social reciprocity compared to their peers born full-term on the Autism Diagnostic Interview-Revised (ADI-R) (59). However, no differences were found for the same domain on the clinical observation measure of the Autism Diagnostic Observation Schedule (ADOS) (57, 60). An additional study using the Revised Behavior Summarized Evaluation Scale (61), another observational measure, found no significant differences for social-communication behaviors in young children on the spectrum born moderate to late preterm (62). Differences in the other key criteria for autism diagnosis, RRBs, has not been well researched between preterm and full-term groups. Using the ADOS and ADI-R, no differences were found in RRB presentation for 5 year old children on the spectrum born preterm and full-term on either measure (57). Another study focusing on children born moderate to late preterm at 5 years of age used the Repetitive and Restrictive Behavior Scale (63), which includes four subscales: sensorimotor stereotypies, reaction to chance, restricted behaviors, and modulation insufficiency. No significant differences were found on any of the subscales between the preterm and full-term groups (62). While there is consistency in the findings of these two studies, presentation of RRBs at earlier stages of development for children on the spectrum born preterm has yet to be examined.

As with research on non-autistic children born preterm, previous research on children on the spectrum born preterm indicates that these children are more likely to have delayed development than their term-born peers. Several studies have compared preterm and full-term groups that were identified at "high likelihood" for autism using autism screening measures. These studies found that children born preterm had lower overall development scores across cognitive, language, and motor developmental profiles than their term-born peers, with medium to large effect sizes (64, 65). When considering older children and adolescents on the spectrum, those born preterm are more likely to be non-verbal compared to those born full-term indicating that differences in cognitive development can be identified by 3 years of age (62, 66). Identifying potential differences in developmental profiles would be useful in identifying additional needs that children born preterm may have as a group, though no known studies thus far have examined this.

Clinical uncertainty pertaining to diagnosis of autism in preterm populations could impact the care provided to these children and, consequently, their development. Concerningly, a meta-analysis found that the median age for diagnosis in children born preterm was 5.7 years of age (21) while the average age for diagnosis in Australia (67) and the United States (68) are both 4 years of age. Potentially, being born preterm may delay assessment, diagnosis, and the opportunity to access early supports that can improve developmental outcomes.

To date, no known studies have compared children on the spectrum born preterm and full-term within a prospectively identified, community-based sample. This study aimed to identify differences in developmental profiles and autistic trait presentation in children on the spectrum born preterm and full-term aged 22–34 months who were identified from a

community-based sample. It was hypothesized that children on the spectrum born preterm would have lower developmental quotients than children on the spectrum born full-term for receptive language, expressive language, fine motor, and visual reception. Further, when comparing children on the spectrum, it was hypothesized that those born preterm would have greater autistic presentation than those born full-term for social communication on clinical observation measures. Due to the limited number of studies that have investigated differences in RRB presentation between children on the spectrum born preterm and full-term, no hypotheses were made for this young sample.

METHOD

Participants

Participants were drawn from two existing prospective, community-based, studies: the SACS (42) and SACS-R (53). Between the two studies, 35,732 children from Victoria, Australia were monitored between 11 and 30 months of age using the SACS tools, resulting in 357 children identified at "high likelihood" for autism.

Of these, 218 children underwent diagnostic assessment at 2 years of age. After excluding children whose gestational age or birth weight was unknown ($n = 3$ preterm, $n = 55$ full-term), one twin born preterm (to retain independence of observations), one child born preterm with an incomplete assessment, the final sample included twenty-two children born preterm and 136 children born full-term with an autism diagnosis, aged 22–34 months at the time of assessment. Of the children born preterm, one (4.50%) was born very preterm, with twenty-one (95.50%) born moderate to late preterm; no child was born extremely preterm. Approximately half of the children born preterm ($n = 11$) were born in the thirty-sixth week of gestation (see **Table 1**).

TABLE 1 | Number of children born in each week of gestation within the preterm and full-term groups.

Classification	Number of weeks gestation	Number of children
Very preterm	31	1
Moderate to late preterm	32	2
	33	2
	34	2
	35	3
	36	11
Full-term	37	11
	38	30
	39	35
	40	35
	41	19

Measures

The SACS (42) and SACS-R (53) are universal, community-based screening tools for monitoring children between 11 and 30 months of age to identify those with a "high likelihood" of autism. Trained raters mark whether a child displays typical or atypical behavior against several items, the number and content of which differs at each age as the items are based on developmental expectations. Each assessment has five "key items" for autism and a number of "non-key" items, as identified in Barbaro and Dissanayake (2). Children who are rated as having atypical behavior on at least three of the "key items" for their age group are deemed at "high likelihood" for autism. The SACS and SACS-R tools both have overall positive predictive values of 81.00–83.00%, negative predictive value of 99.00%, sensitivity of 82.00–84.00%, and specificity of 99.00–99.50% for identifying children on the spectrum between 12 and 24 months of age, and an inter-rater reliability of 0.90 (42, 53).

Parents/caregivers completed a demographic questionnaire, reporting on characteristics of their family, education level, culture, occupation, income, and language/s spoken at home. The demographic questionnaire in the SACS-R study had additional questions on whether siblings or other family members had a diagnosis of autism. Information about the child's birth was recorded via the demographics questionnaire, notes provided by maternal and child health (MCH) nurses, documentation from families during their visit, or in photocopies from the "My Health, Learning and Development Record" birth record books provided to families in Victoria when their child is born. Birth and development information is recorded in these books by hospital and MCH nurses.

The Mullen Scales of Early Learning (MSEL) (69) was used to examine developmental profiles for children. This task-based measure for children aged between 3 and 68 months of age includes subscales of fine motor, visual reception, receptive language, and expressive language skills. In the current study, the MSEL subscales had excellent internal reliability ($\alpha = 0.75–0.91$). Further, the MSEL has been validated for use with young children with an autism diagnosis with excellent construct validity between 0.84 and 0.92 (70). Per procedure for the MSEL (69), corrected age was used when the child's chronological age was under 24 months and chronological age used thereafter. Developmental quotients for subscales were calculated by dividing scale age equivalents by the child's chronological or corrected age and multiplying by one hundred.

The ADOS is a semi-structured, standardized, play-based assessment with modules administered based on the child's age and language development. Module 1 of the ADOS-Generic (ADOS-G) (71) was used in the SACS study, as appropriate for the children's age and language development. In the SACS-R study, children aged between 12 and 30 months completed the ADOS-Toddler Module (ADOS-T) (72) or the ADOS-2 Modules 1–2 (60) were administered as appropriate for their language level. Items are coded between zero and two, with higher scores indicating greater autism traits. A Cochrane review of the ADOS-G, ADOS-T and ADOS-2 modules found a summary sensitivity of 0.94 and specificity of 0.80 in preschool aged children (73).

To allow for comparisons between the different ADOS versions and modules, algorithms for calibrated severity scores (CSSs) were created for social affect, RRBs, and overall severity, using the method proposed by Hus et al. (74), Gotham et al. (75),

and Esler et al. (76). Higher CSSs indicate greater autism traits, ranging from zero to ten. However, the possible range of scores for RRB CSSs is zero or between five and ten, skipping numbers one to four. These algorithms had internal reliability coefficients ranging from acceptable (ADOS-G; $\alpha = 0.68$) to excellent (ADOS-T and ADOS-2 all modules; $\alpha = 0.73$–0.91) within this study. The process to calculate CSSs is frequently used in autism research to allow comparisons across editions and modules of the ADOS (57, 77).

Procedure

Ethics approval from the La Trobe University Human Ethics Committee was obtained for the SACS (Project 06-94) and SACS-R (UHEC13-001) prior to data collection. An application for this secondary data analysis was approved prior to commencement (HEC-19209).

Across both studies, MCH centers from nineteen local government areas (LGAs) across Melbourne, Victoria took part, with five LGAs taking part in both studies. The MCH service provides caregivers with a schedule of free consultations with MCH nurses for ten "key ages and stages" of development in the first 6 years of life (78). Nurses from MCH attended a half-day workshop on identifying early behavioral signs of autism (42, 53). Children attending routine MCH appointments in Victoria, Australia were subsequently screened using the SACS or SACS-R tools at all scheduled 12-, 18-, and 24-month "key ages and stages" appointments between September 2006 to September 2008 for the SACS study (42) and June 2013 to June 2018 for the SACS-R study (53).

All children who were identified at "high likelihood" for an autism diagnosis were invited to attend developmental assessments at the University's Child Development Unit at six-monthly intervals to track their development over time. Parents provided informed consent for their child's assessment, MCH records, and photocopies made from their "My Health, Learning and Development Record" books to be used for the SACS/SACS-R research and future studies. At the developmental assessment for children at 2-years of age, parent/caregiver and child measures were completed in tandem, with one clinician administering the ADOS and MSEL to the child while another clinician interviewed the parent(s) or caregiver(s). An assessment report was provided for the family after each appointment.

Preliminary Analyses

Prior to analysis, assumptions were tested. The level of measurement assumption was met as dependent variables were continuous. As children were tested independently and one child from a pair of twins was removed from analysis, the assumption of independence of observations was met. Normality was assessed using visual inspection of histograms, skewness z-scores with magnitude >0.5, and Kolmogorov-Smirnov and Shapiro-Wilks Tests of Normality. The assumption of normality was violated for all MSEL developmental quotients (except receptive language) and ADOS RRB CSSs due to negative skew. When cell sizes are ≥ 20, multivariate analyses of variances (MANOVAs) are robust against violations to normality, so no transformations were made (79). Two multivariate outliers were detected from the full-term group and were not removed as they accounted for $<5.00\%$ of the participants in that group (80).

TABLE 2 | Correlations between gestational age, birth weight, Mullen Scales of Early Learning developmental quotients, and Autism Diagnostic Observation Schedule calibrated severity scores.

Variable	BW	VR DQ	FM DQ	RL DQ	EL DQ	SA CSS	RRB CSS
GA	0.68**	0.00	0.07	−0.03	−0.04	0.08	0.05
BW	–	0.01	0	0	−0.01	0.06	0.01
VR DQ		–	0.70**	0.66**	0.64**	−0.36**	−0.24**
FM DQ			–	0.46**	0.43**	−0.33**	−0.19*
RL DQ				–	0.77**	−0.41**	−0.24**
EL DQ					–	−0.28**	−0.16*
SA CSS						–	0.23*

*GA, gestational age; BW, birth weight; DQ, Mullen Scales of Early Learning developmental quotient; CSS, Autism Diagnostic Observation Schedule calibrated severity score; VR, visual reception; FM, fine motor; RL, receptive language; EL, expressive language; SA, social affect; RRB, repetitive and restricted behavior. *$p < 0.05$ (2-tailed). **$p < 0.01$ (2-tailed).*

Assumptions required for MANOVAs were tested to examine the MSEL development quotients and ADOS CSSs. Box's Test was not significant, indicating the assumption of variance-covariance matrices was met. Levene's Test was not significant for any of the MANOVAs, indicating that the assumption of equality of variances was met.

Statistical Analyses

Pearson's correlations were used to identify relationships between gestational age, birth weight, and the dependent measures. To identify differences in birth characteristics between the preterm group and full-term group, *t*-tests and Fisher's Exact Test were used. Fisher's Exact Test was used instead of chi-squared tests when the assumption of minimum cell frequency was not met, specifically, ≥ 10 cases per cell for 2x2 tables and ≥ 5 cases per cell for 2x3 tables. Given Fisher's Exact Test with tables larger than 2x2 is not available within the Statistical Package for Social Sciences (SPSS) (81), Fisher's Exact Test (2-tailed) with Freeman-Halton extension for 2x3 tables was calculated using VassarStats (82).

To examine group differences between the preterm and full-term groups, a MANOVA was used to examine the MSEL developmental quotients and another MANOVA for social affect and RRB ADOS CSSs. To determine whether a difference in age between the preterm and full-term groups affected the main results, an MANCOVA was perform on MSEL developmental quotients and ADOS CSSs.

RESULTS

Correlations were calculated to determine the strength of the relationships between gestational age, birth weight, and the outcome measures (see **Table 2**). While the correlation between birth weight and gestational age was significant, neither were significantly correlated with any of the outcome measures. Correlations between the MSEL developmental quotients were

TABLE 3 | Differences in birth characteristics and demographics of preterm and full-term groups.

	Preterm		Full-term					
Continuous variables	***n***	***M* (*SD*)**	***n***	***M* (*SD*)**	***t***	***df***	***p***	***d***
Age (months)	22	27.32 (2.72)	136	26.01 (2.53)	2.24	156	0.03	0.50
Gestational age (weeks)	22	35.17 (1.42)	136	39.37 (1.23)	14.61	156	<0.001	3.18
Birth weight (g)	22	2,395.91 (609.44)	136	3,485.27 (493.00)	9.30	156	<0.001	1.97
Categorical variables	***n***	**%**	***n***	**%**		**Fisher's Exact Test *p***		
Sex						0.768		
Males	19	86.4	110	80.9				
Female	3	13.6	26	19.1				
Birth Weight <2,500 g						<0.001		
No	14	63.6	136	100.0				
Yes	8	36.4	0	0.0				
Size for gestational age						0.167		
Small	6	27.3	17	12.5				
Average	13	59.1	103	75.7				
Large	3	13.6	16	11.8				
Complications at birth						<0.001		
Yes	18	81.8	42	31.6				
No	4	18.2	91	68.4				

Equal variances assumed for t-tests. n, number of participants; M (SD), Mean (Standard Deviation).

significant, with weak to large positive correlations (83). The ADOS social affect and RRB CSSs were significantly and positively correlated with moderate strength (see **Table 2**).

Children in the preterm group had significantly lower gestational age and birth weight and were significantly more likely to have been born small for gestational age and have a complication at birth than children in the full-term group (see **Table 3**). Children in the preterm group were significantly older than children in the full-term group in chronological age. After controlling for chronological age, the results of main analyses remain the same (see **Supplementary Table 1**).

No significant differences were found between the preterm and full-term groups on any of the MSEL development quotients for visual reception, fine motor, receptive language, and expressive language between children on the spectrum born preterm and full-term. Further, there were no significant differences in behavior presentation using the ADOS CSSs for social affect and RRBs (see **Table 4**).

DISCUSSION

Early years presentation of autism was examined in children born preterm and full-term who were prospectively identified at "high likelihood" for autism from the community. The hypothesis that children on the spectrum born preterm would have more delayed development than children on the spectrum born full-term was not supported as no significant differences were identified in visual reception, fine motor, receptive language, and expressive language developmental quotients. Further, the hypothesis that children on the spectrum born preterm would have greater social-communication presentation than children on the spectrum born full-term was not supported, with no significant group differences found.

The non-significant differences across key indicators of child development between children on the spectrum born preterm and full-term were not consistent with previous literature on older children and adolescents between 3 and 18 years of age, where delayed development was observed for those born preterm with a diagnosis of autism as compared to their peers without an autism diagnosis (62, 66). Specifically, previous literature identified verbal development as being delayed and was identified in children as young as 3 years of age (62, 66). In the current study using a sample of 2-year-old children, these differences were not identified. Similar results were found for children born preterm who were identified at "high likelihood" for an autism diagnosis using screening tools (28, 65). Further, previous findings using typically developing samples have suggested that children born preterm have substantially delayed development when compared to children born full-term (20, 84).

The inconsistency of findings regarding child development after preterm birth in the current study with previous literature may be attributed to the young age of the children in this sample (22–34 months) as compared to previous studies that had included children between 3 and 18 years of age (62, 65, 66). As previous research has not yet included children within toddler age with a diagnosis of autism, it is possible that differences in development may not become apparent until the child reaches an older age. While differences were found for young children who had screened at "high likelihood" without a diagnosis of autism in previous studies (28, 65), comparing them to children with a diagnosis of autism may be problematic due to the high rates of false positives when using autism screening tools in

TABLE 4 | MANOVA results for differences between preterm and full-term groups on Mullen Scales of Early Learning developmental quotients and Autism Diagnostic Observation Schedule calibrated severity scores.

	Preterm			Full-term						
	n	*M*	*SD*	*N*	*M*	*SD*	*F*	df_1, df_2	*p*	Λ	η_p^2
MSEL DQ	22			136			0.74	4, 153	0.556	0.98	0.02
VR		73.61	12.12		78.27	17.99					
FM		80.90	12.20		86.00	16.37					
RL		57.51	22.93		58.66	27.71					
EL		62.92	25.73		62.91	23.00					
ADOS CSS	22			136			0.17	2, 155	0.85	1.00	0.002
SA		6.05	1.96		6.32	2.22					
RRB		7.00	1.80		6.99	2.18					

n, number of participants; M, mean; SD, standard deviation; F, F-statistic; df, degrees of freedom; Λ, Wilk's Lambda; η_p^2, partial eta square; MSEL DQ, Mullen Scales of Early Learning developmental quotient; VR, visual reception; FM, fine motor; RL, receptive language; EL, expressive language; ADOS CSS, Autism Diagnostic Observation Schedule calibrated severity score; SA, social affect; RRB, restricted, repetitive patterns of behavior.

preterm groups (56, 85). Extrapolating development of children who have screened at "high likelihood" for autism to children with a diagnosis of autism may be misleading due to the other potential explanations for a child born preterm being identified at "high likelihood" without a full developmental assessment, as was conducted in the current study.

The finding that children on the spectrum born preterm and full-term did not differ on social communication behavior presentation was not consistent with previous studies using parent report measures, where children on the spectrum born preterm were shown to have greater social-communication behavior presentation (57, 58). It is possible that more subtle differences in social-communication behaviors could be unpacked using a measure with subscales within the domains or an item-by-item analysis. Another explanation for the findings of the current study on social-communication for populations of children on the spectrum born preterm and full-term being inconsistent with previous literature may be the young sample. As with developmental profiles, it is possible that differences in behavioral presentation do not emerge until children are at an older age. While Movsas and Paneth (58) included children as young as 4 years of age in their study, the mean age of their participants was 10 years (58)—much older than the current sample's mean age of 26.20 months. Another study found differences between children on the spectrum born preterm and full-term at 5 years of age; however, only children who were born very preterm but not at a low birth weight (<1,500 grams) were excluded from this study (57). The non-significant finding of the current study is consistent with other literature using children born moderate to late preterm (57, 62). When examining samples of 5-year-old children on the spectrum born very (61) and moderate to late preterm (66), no significant differences were found on social-communication behavior presentation. This is not surprising, given 95.50% of the preterm group of the current study were born moderate to late preterm.

This study also examined potential differences in RRB presentation between children on the spectrum born preterm and full-term. The current study builds upon emerging evidence that no differences exist in this domain. Previously, no differences were found for 5 year old children on the spectrum born preterm and full-term on parent report (57) and clinical observation measures (57, 62). The sample in the current study included children who were younger than those used in these previous studies, where the youngest participants were aged 3 years in Brayette et al. (62) and 5 years in Chen et al. (57). Therefore, the findings that children on the spectrum born preterm and full-term do not differ on RRB presentation extend to earlier in development with the current sample.

Instability in patterns of autism screening have been observed in children born preterm between 8 and 18 months of age (86). In that study, half of the children born preterm who were identified at "high likelihood" for autism at 18 months of age using the M-CHAT had not been previously identified at 8 or 12 months of age (86). Further, several children suddenly no longer screened positive at eighteen, despite previously screening positive at 8 and/or 12 months of age (86). When the children were 3 years old, only one child born preterm and no children born full-term was diagnosed with autism (65). These findings may point to some children born preterm having a "sudden onset" of behaviors while others have a sudden decrease at an older age (86). It is possible that instability of RRB presentation could explain the inconsistent findings in the literature at different age groups. The diagnostic inclusion for the current study was based on the child's most recent diagnosis (had they attended subsequent follow up appointments) rather than the diagnosis received at their first appointment. Furthermore, diagnoses were based on gold standard developmental assessments and clinical judgement, rather than the presence or absence of behaviors at the age of diagnosis. Thus, while the trajectory for autistic traits seems to be difficult to predict using screening measures in preterm population, the continued developmental surveillance was advantageous in ensuring their diagnoses were accurate.

Previous studies have largely recruited from clinics and/or university hospitals, where samples typically consist of families who have concerns about their child's development (87). As parents are less likely to be aware of the subtle differences

in development that may indicate that a child is at "high likelihood" of an autism diagnosis, children who attend clinics following parental concerns may have more challenging autism traits or other developmental concerns than those identified by trained clinicians. Further, when seeking advice on these subtle differences, clinicians who are unaware of the relationship between preterm birth and autism may assure parents that many of these behaviors, such as atypical eye contact (34) or toe-walking (88), are behaviors common for children born preterm without autism. Thus, autism is not considered as a potential explanation for behavior and the child is not referred to a full developmental assessment. It would not be until a child is showing a pattern of behavior which more clearly indicates autism as an explanation for behavior, that a full developmental assessment is considered. In contrast, the current study was a community-based sample where all children within the community were monitored for autism, rather than only those whose parents have concerns. This difference in sampling could account for the inconsistency between the findings of previous literature and the current study, due to the comparison of children with potentially more subtle developmental differences in both preterm and full-term groups.

Another potential explanation for the inconsistent findings across the literature may be the dose effect in the preterm phenotype, where children with lower gestational ages face more developmental difficulties (17). Previous studies (62) and the current study, which have involved children born preterm with higher gestational ages, have not detected differences in behavioral presentation; similar findings can be observed across preterm phenotype literature (3, 20). Alternatively, it may be that a subset of children born moderate to late preterm are susceptible to developmental difficulties, rather than all children born between moderate to late preterm (89). Further, Sansavini et al. (90) note that many children born extremely preterm, where occurrence of developmental difficulties would be greatest and easiest to detect due to the dose effect, are found to have development within the normal distribution for development (90). As many children born preterm have developmental scores within the range of children born full-term, differences become difficult to detect. The findings of the current study further suggest that even within children who have been diagnosed with autism, their general development is similar to children with an autism diagnosis born full-term—expanding the literature on similarities in development for children born preterm and full-term.

The preterm phenotype suggests that autism in preterm populations may have inherently different etiology than autism in full-term populations (3, 16). Children born preterm were identified at "high likelihood" and diagnosed using criteria based on full-term groups. Potentially, current diagnostic criteria may not accurately represent autism in preterm groups or difficulties might emerge later in life than in full-term groups (86). As a result, the prevalence of autism in preterm populations may be under- or over-estimated. Thus, participants in this study might reflect those with patterns of autistic traits that reflect "typical" autism for full-term populations, which may not accurately represent "typical" autism in preterm populations. While this is a limitation to the study, it is also a limitation to this area of research. Until differences in the presentation of autism in children born preterm and full-term are identified (or ruled out), using the diagnostic criteria based on full-term populations is unavoidable.

This study had several strengths in its unique contributions to an area of research in the preterm phenotype. Firstly, it was the first study to investigate developmental differences and behavioral presentation in children on the spectrum born preterm and full-term, aged 22–34 months. Development of children on the spectrum born preterm has yet to be examined in children at this age, with most studies focusing on children with a diagnosis aged 5 years or older. In younger samples, the children tend to have been identified at "high likelihood" for autism without a confirmed diagnosis, which becomes problematic due to low predictive value and instability of the screening tools used in those studies (55, 56, 86). The use of a young sample is well aligned with the current focus for autism research of early identification and support in autism (91). Secondly, the use of the SACS tools to prospectively identify children on the spectrum in the community may have allowed for more subtle presentations of autism to be detected compared to studies using other screening measures, where these children may have missed. Third, this is one of few studies using a community-based sample of children born preterm and full-term. Use of samples of children born extremely and very preterm from single hospitals are common in this research area, limiting the generalizability of their findings from children born moderate and late preterm, who comprise the majority the preterm population; thus, the use of community-based samples increases the ecological validity of the findings. Finally, as prior research has primarily focused on extremely and very preterm populations, the inclusion of the moderate to late preterm population helps fill the gap for an underserved group of children in the premature phenotype research area.

Although there are notable strengths of this study, it is not without limitations. Firstly, although rates of children born preterm identified with the SACS tools were consistent with population rates of preterm birth, a low number of children born very preterm and no children born extremely preterm were involved in the study. This did not allow for a detailed examination of the dose effect of the preterm phenotype on the outcome measures, as lesser developmental differences from moderate to late preterm birth may obscure larger differences from extreme and very preterm birth. Secondly, while the age range of the current sample was small relative to other studies, very subtle differences in development within this age group may not have been captured. However, measures that account for age and developmental norms were chosen to counteract this, resulting in children being compared based on their expected level of development. Third, children in the preterm group in the current sample were significantly older than children in the full-term group in chronological age. However, when the data were analyzed with age as a covariate, the results remained the same indicating that the difference in age did not significantly affect the results. Lastly, as children who did not have known gestational ages were excluded from the study, fewer preterm

children were excluded from the study ($n = 3$) than children born full-term ($n = 55$). This discrepancy likely occurred as more care is taken to record gestational age and birth weight for children born preterm than children born full-term. Gestational age and birth weight have a much more relevant impact on development for children born preterm and the accuracy of these figures becomes more important than for a child born full-term. However, one demographic difference (paternal education) was found between the children who were excluded due to missing gestational age or birth weight when compared with children who were included in the current study (see **Supplementary Table 2**). As no other demographic differences were identified, and there was incomplete data on paternal education, this indicated that those included and excluded were largely similar to each other.

In future, researchers may wish to further explore the differences in RRB presentation and preterm groups using other measures that further break down RRBs into subscales for more detailed analysis. As this study did not examine gross motor performance, it is possible that children on the spectrum born preterm were unable to perform gross motor-based RRBs due to other developmental difficulties, which may have been a confounding factor in the lack of differences between the preterm and full-term groups on the RRB measure. A more detailed examination into other predictors of autism, such as birth weight, and their effect on development and behavioral presentation, may be useful in unpacking inconsistent findings on preterm autism literature. Additionally, no detailed data were available regarding participants experiencing neonatal complications, which could also account for future developmental difficulties. Further, an examination into behaviors and characteristics of children born preterm, with and without autism, would give further insight into the boundary between the preterm phenotype and autism in preterm populations. Lastly, future research using longitudinal study designs could examine the trajectories of behavioral presentation within preterm populations to determine whether, and at which age, differences become apparent.

Previous literature paints a picture of children born preterm having many additional needs due to developmental difficulties and delays. This picture may lead clinicians to expect and look for more challenging characteristics of autism when assessing a child born preterm, overlooking those presenting with more mild developmental differences and behavioral presentation. Using a community-based sample of children identified prospectively, it was found that children on the spectrum born moderate to late preterm did not differ in development and behavioral presentation from their peers born full-term, when assessed at the age of 2 years. As current autism research has largely focused on identifying children at younger ages and providing support as early as possible, the findings of the current study suggest that clinicians should consider autism as a potential explanation for behaviors that are often presumed to be due to preterm birth, particularly for children who were born moderate to late preterm. As these results indicate that the autism phenotype is similar for moderate to late preterm and full-term children, clinicians should not change their clinical approach of diagnosis and treatment for a child presenting with the characteristics of autism simply due to their preterm birth. While further research is required to replicate and extend these findings, it is possible that many 2-year-old children on the spectrum born moderate to late preterm whom clinicians meet in the community will have similar needs to their term-born peers. Still, individual differences in development should not be overlooked, particularly for children born preterm who are more likely to face additional developmental difficulties.

As autism research moves to improving early identification, these findings have practical implications for clinicians who may overlook autism as an explanation for behavior due to expectations for greater developmental differences in children born preterm. Further, these findings may provide reassurance to families, who may have concerns for their child's support needs and outcomes after an autism diagnosis.

DATA AVAILABILITY STATEMENT

The data analyzed in this study are subject to the following licenses/restrictions: the original contributions presented in the study are included in the article/supplementary material. The data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study. Requests to access these datasets should be directed to Josephine Barbaro, j.barbaro@latrobe.edu.au.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by La Trobe University Human Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JL conducted the analyses, contributed to the interpretation of the results, and drafted the initial manuscript. RJ and JB provided supervision to JL, assisted with study design, data analysis and interpretation, and reviewed drafts. MY assisted with the study design and interpretation of results. MG assisted with data analysis and interpretation of results. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.597505/full#supplementary-material

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Autism Screening in Early Childhood: Discriminating Autism From Other Developmental Concerns

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Early identification of autism, followed by appropriate intervention, has the potential to improve outcomes for autistic individuals. Numerous screening instruments have been developed for children under 3 years of age. Level 1 screeners are used in large-scale screening to detect at-risk children in the general population; Level 2 screeners are concerned with distinguishing children with signs of autism from those with other developmental problems. The focus here is evaluation of Level 2 screeners. However, given the contributions of Level 1 screeners and the necessity to understand how they might interface with Level 2 screeners, we briefly review Level 1 screeners and consider instrument characteristics and system variables that may constrain their effectiveness. The examination of Level 2 screeners focuses on five instruments associated with published evaluations in peer-reviewed journals. Key criteria encompass the traditional indices of test integrity such as test reliability (inter-rater, test-retest) and construct validity, including concurrent and predictive validity, sensitivity (SE), and specificity (SP). These evaluations reveal limitations, including inadequate sample sizes, reliability issues, and limited involvement of independent researchers. Also lacking are comparative test evaluations under standardized conditions, hindering interpretation of differences in discriminative performance across instruments. Practical considerations constraining the use of such instruments—such as the requirements for training in test administration and test administration time—are canvassed. Published Level 2 screener short forms are reviewed and, as a consequence of that evaluation, future directions for assessing the discriminative capacity of items and measures are suggested. Suggested priorities for future research include targeting large and diverse samples to permit robust appraisals of Level 2 items and scales across the 12–36 month age range, a greater focus on precise operationalization of items and response coding to enhance reliability, ongoing exploration of potentially discriminating items at the younger end of the targeted age range, and trying to unravel the complexities of developmental trajectories in autistic infants. Finally, we emphasize the importance of understanding how screening efficacy is dependent on clinicians' and researchers' ability not only to develop screening tests but also to negotiate the complex organizational systems within which screening procedures must be implemented.

Keywords: autism screening, early childhood, level 2 screeners, STAT, biscuit, ADEC, SORF, RITA-T

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INTRODUCTION

One focus for autism researchers from various disciplinary backgrounds (e.g., genetics, neuroscience, psychiatry, psychology, virology) has been the identification of markers of the condition that can be detected reliably in infancy and early childhood, and incorporated into screening measures for identifying children showing early signs. For many reasons (1), this is a difficult task. Yet, researchers have persisted, motivated perhaps by two considerations. First, it is widely accepted that the optimum developmental outcomes for individuals with autism will be facilitated by implementing appropriate intervention strategies from an early age (2, 3). Second, established and comprehensive diagnostic instruments such as the Autism Diagnostic Observation Schedule (ADOS) (4), the Autism Diagnostic Observation Schedule—Toddler Module (ADOS-T) (5) which is designed specifically for children younger than 30 months, and the Autism Diagnostic Interview-Revised (ADI-R) (6) are not practical for screening purposes due to their lengthy administration time and demanding administrator training. It is important to stress, of course, that comprehensive assessment using such instruments should be conducted when screening measures indicate the possibility of an autism diagnosis, thereby enabling clinicians to confirm any diagnosis and identify any co-occurring conditions that may underlie any symptoms thought to be associated with autism.

Researchers' efforts have now resulted in various screening devices that focus on behavioral markers of autism and specifically target its early detection. Screening instruments for very young children fall into one of two categories: Level 1 screeners used primarily in large-scale screening to identify children in the general population whose developmental trajectory may indicate autism, and Level 2 screeners designed specifically to differentiate young children with autism from those with other developmental concerns. Here we first outline criteria for evaluating these instruments. The behaviors targeted and the rationale for their inclusion are then examined. After a brief review of Level 1 screeners which is important for understanding their relationship to Level 2 screeners, we provide a detailed evaluation of Level 2 screeners. We highlight some practical issues with these measures and examine recently developed short forms, their development motivated by the desire to offer more congenial instruments for practitioners. In so doing, we highlight the potential for misleading conclusions that can arise regarding the discriminative capacity of different instruments. We conclude by identifying major priorities for future research.

CRITERIA FOR EVALUATING ASD SCREENERS

An initial consideration is the source of the screening information. Some screeners rely upon the reports of parents or caregivers about the child's behavior, reports that might be obtained via an interview or checklist completion. Others involve observation of the child's behavior as they engage in a live (or video recorded) interaction with the parent and/or examiner. Both approaches have limitations. For example, parents may not be sensitive to the occurrence, deviation or absence of particular behaviors that may point to developmental concerns (7, 8) or, in response to their child's functioning, may have developed compensatory strategies to minimize perceived deviation or to involve their children in various social interactions (9). If the screening relies upon parents' retrospective reports, other issues may arise should the parent not accurately recall certain behavioral patterns or when they were detected. Observational measures are often labor-intensive and may be insensitive to the behaviors of interest because they only capture a limited behavioral sample due to the duration of the testing session, the responsiveness of the child at the time of observation or the dynamics of the interaction between the child and the parent or clinician. A tightly controlled direct comparison of these approaches with the same children is extremely difficult given the difficulties associated with producing comparable operationalizations of items for each approach. Given the potential biases that may influence parental reports, researchers have generally argued for the superiority of observational measures, although some research runs counter to that view. Moreover, under some conditions, parents may observe clinically significant behaviors sooner than clinicians (10, 11). Note, however, that in these studies, parents were reporting behaviors of younger siblings of an older child with autism and, thus, may have been sensitized to the behaviors.

Another consideration relates to the age and developmental levels of the children sampled in the construction and evaluation of the screener. For example, the effectiveness of screening children below 3 years of age, when using instruments evaluated with children beyond 3 years, is obviously unknown. And, even if the screener were developed using samples of very young children, it is important to establish that the specific behavioral patterns targeted as indicators of potential developmental concerns have some longer term predictive validity of an autism diagnosis, rather than simply being atypical behaviors that are still within the boundaries of a typical developmental trajectory. The developmental levels of children sampled are also important. For example, instruments evaluated using samples from clinics accessing a preponderance of children with relatively severe developmental concerns may have shown strong discriminative performance and yet not be so effective at discriminating children with more subtle symptomatology or autism specifically.

The practicalities of screening are also important. That there may be practical constraints on screening implementation is indicated by findings that, despite recommendations from organizations such as the American Academy of Pediatrics that all children be screened at 18 and 24 months (12), reported rates of screening of children in this age range among pediatricians and physicians vary widely—for example, from 22% (13) to more than 85% (14). Some obvious constraints on screening are the administration time, lack of familiarity with and training in both the administration and scoring of the screener, and system variables associated with making post-screening referrals and following up outcomes (15, 16).

A fourth criterion is the instrument's reliability. Inter-rater reliability and test-retest reliability are important considerations when evaluating any psychometric instrument, but they warrant emphasis in this context for two reasons. First, very young children are likely to show considerable intra-individual variability in reactivity or responsiveness during interpersonal interactions. Second, as will become apparent when we discuss the specific types of behaviors that these instruments are trying to assess, there is considerable potential for individual observers—whether they be parents or experienced clinicians—to vary in their evaluations of whether the presence, or absence, of certain behaviors meet criterion for passing or failing a test item.

The issue of how effectively the instrument is measuring the construct of interest is an important fifth criterion. The items should converge on the focal construct(s) or dimension(s) as evidenced by the pattern of relationships between items. Concurrent validity should be suggested by meaningful correlations with extant measures and the instrument should differentiate children diagnosed with and without autism. Ideally, the measure should also predict maintenance (or absence) of diagnosis as the child develops.

Finally, Level 2 screeners should discriminate children with autism from those with other developmental disorders (e.g., language disorders, intellectual disabilities, other neurodevelopmental disabilities). This discrimination is typically evaluated using a signal detection theory (SDT) approach which offers several informative indices for establishing specific test cutoff scores to indicate likely presence of autism. For example, for any given cutoff on the test, sensitivity (S_E) refers to the proportion of children among those known to have the condition who screen positive. Specificity (S_P) refers to the proportion of children among those known *not* to have the condition who screen negative. In SDT parlance, S_E denotes the "hit" rate and $1–S_P$ indicates the "false alarm" rate. If the cutoff score is varied across the range of possible test scores, a range of operating points is obtained that summarizes the diagnostic performance of the test. Plotting these points produces a Receiver Operating Characteristics (ROC) curve which plots S_E values against $1–S_P$ values (i.e., hits against false alarms) across the range of possible cutoff values for the test. An area under the curve (AUC) statistic can then be used to assess the test's ROC performance (i.e., its discriminative performance). A test with an AUC = 0.5 is providing no predictive information whereas an AUC = 1.0 indicates a test that is providing perfect discrimination. In other words, when tests (or items) are contrasted via ROC analysis, the higher AUC denotes the more discriminating test (or item). Although the AUC is a commonly used statistic for evaluating discriminative performance, in our evaluation of Level 2 screeners we discuss potential problems that may be associated with its use. We emphasize that any assessment of the discriminative performance of an instrument must take into account considerations of sample size and statistical power if reliable conclusions are to be drawn about issues such as the most appropriate subset of prospective items to include in an instrument, the instrument's capacity to discriminate autism from other developmental conditions, and the comparative performance of different instruments.

THE TYPES OF BEHAVIORS ASSESSED BY SCREENING INSTRUMENTS

Although there is a to-be-expected overlap across instruments with respect to the behaviors addressed, there are differences between screeners. One reason for such differences is an historical one, with measures obviously shaped to some degree by the specific DSM (DSM-IV, DSM-IV-TR, DSM-5) (17–19) or the International Classification of Diseases [ICD-10, (20)] criteria operating when they were developed. Another reason relates to the length of the test, with more items providing opportunities to capture a wider range of behaviors or more subtle variations on particular types of behavior—objectives that must be traded off against that of having a screener that may be widely adopted because it is more efficient to administer. A third reason is apparent from a quick scan of the rather generic formal criteria. For example, items tapping a criterion for Autistic Disorder (AD) such as "Impaired social interaction, including … failure to reciprocate socially or emotionally" (DSM-IV-TR) or, for Autism Spectrum Disorder (ASD), "Sustained and widespread deficits in social communication and interaction, spanning the areas of social-emotional reciprocity" (DSM-5) can obviously be instantiated in various ways.

Perusal of the published studies describing content development for different tests reveals other influences. One factor is the test developer's clinical diagnostic experience and knowledge, which inform judgments about items that might best discriminate young children with autism from developmentally matched age peers (21). Other influences described by test developers are parents' retrospective reports of their child's behavior and behavioral observations coded from parents' home videos of their child (22), and research findings that highlight potential behavioral differences between children diagnosed with autism and typically developing (TD) children (22–24).

The reliance on these various influences as part of item development is not surprising if one attempts to interpret the diagnostic criteria in the context of the behavioral development of very young children. Consider, for example, DSM-5 criteria such as "Sustained and widespread deficits in social communication and interaction, spanning the areas of …developing and maintaining relationships" or "restricted and abnormally intense interests." Their generic nature means that, although experienced clinicians might find it relatively easy to specify likely behavioral characteristics of children aged 4–5 years and older who don't meet specific criteria, their task will be more challenging when dealing with infants aged 9–24 months.

Rather than describing all behaviors from the numerous existing screeners, and showing how they link to DSM criteria operating when they were developed, we simply provide an overview of behaviors captured in four of the five Level 2 screeners described in more than one published evaluation and scrutinized in a later section—the Screening Tool for Autism in 2-Year Olds [STAT; (21)], the Baby and Infant Screen for Children with aUtIsm Traits–Part 1 [BISCUIT-Part 1; (25)], the Autism Detection in Early Childhood [ADEC; (22)], and an updated version of the Systematic Observation of Red Flags

[SORF; (26)]—and indicate how they link to specific DSM-IV-R and DSM-5 criteria (see **Table 1**).

The key things to highlight from **Table 1** are the following. First, most items from the four measures sampled in **Table 1** illustrate (at least) one of the diagnostic criteria. Thus, instruments reflecting the DSM-IV-TR address impairments in social interactions (e.g., in non-verbal behaviors, peer relationships and sharing with others, social reciprocity) and communication (e.g., language delay, conversing, stereotyped language, play behavior), and repetitive behaviors and interests (e.g., intense restricted interests, rituals and repetitive behaviors, preoccupations). DSM-5 driven behavioral foci include deficits in social communication and interaction (e.g., social reciprocity, non-verbal social communication, relationships), and repetitive behaviors and interests (e.g., motor behaviors, insistence on sameness, rituals and intense interests, sensory sensitivities).

Second, different measures frame items differently and differ in their operationalization of the target behaviors and the way in which they access the information. Moreover, the importance placed on each behavior is reflected in the number of items probing that behavior. Nevertheless, a scan of **Table 1** reveals a high degree of similarity between many items from the different tests that reference the same domain.

Third, the distribution of items across the different domains varies across measures, with the following quite pronounced differences. The ADEC has a higher proportion of its items aligning with DSM-IV-TR's non-verbal behavior domain (A1-a) and the DSM-5's social reciprocity domain (A1) than the other measures. The STAT is almost exclusively comprised of items compatible with DSM-IV-TR's sharing enjoyment (A1-c) domain. Unsurprisingly, items tapping repetitive and stereotyped behaviors and interests are more prominent in the post-DSM-5 measures (26), although they are certainly not absent from earlier measures (22, 24). Ultimately, of course, the number and proportion of items are not the key issues. Rather, the major considerations are whether the operationalization of items and data collection method ensure reliable measurement, the items discriminate as intended, and this discrimination spans the age range that early screeners are designed to target, and they have some predictive validity (i.e., the diagnosis is maintained).

LEVEL 1 SCREENERS

The focus for Level 1 screeners is large-scale screening of young children in the general population to identify potential concerns with the child's developmental trajectory. In the last two decades there has been a proliferation of Level 1 screeners, including (inter alia) well-documented instruments such as the Checklist of Autism in Toddlers [CHAT; (27)], the Checklist for Early Signs of Developmental Disorders [CESDD; (28)], the Early Screening of Autistic Traits Questionnaire [ESAT; (29)], The First Year Inventory [FYI; (30)], the Infant-Toddler Checklist [ITC; (31)], the modified Checklist for Autism in Toddlers [M-CHAT; (32)], and the Social Attention and Communication Study [SACS; (33)].

These measures differ in many respects. Here we provide just a few examples to highlight the extent of the differences. The number of items ranges from 4 on the pre-screening component of the ESAT to 63 on the FYI. Although Level 1 screener content reveals similar types of behaviors across instruments, the item list often differs depending on the age of the child being evaluated (28, 33). Items tapping similar aspects of behavior are operationalized with what appear to be varying degrees of precision, and coding might involve a simple yes/no or a rating on a Likert-type scale. For example, the CESDD requires observers reporting on the child's eye contact to tick the box "lack of eye contact" if the child's behavior is qualitatively different from their peers. In contrast, for the SACS, the observer is asked, "Has the child spontaneously made eye contact with you during the session? If not, interact with the child to elicit eye contact. Does he/she make eye contact with you?" and the observer is trained to identify and record whether the behavior is atypical (or typical). These different degrees of specification may or may not produce different profiles for the same behavior. For both instruments, observers completed a training workshop involving systematic instruction, video demonstrations of behaviors, etc. (28, 33).

Different instruments have also used different types of administrators, including physicians (e.g., ESAT pre-screener items), clinical psychologists (e.g., ESAT), child care workers (e.g., CESDD), maternal and child health-care center nurses (e.g., SACS), and parental reports (e.g., FYI, ITC). Thus, even with appropriate training, administrators are likely to differ with respect to clinical expertise, understanding of behavioral criteria, extent of exposure to the child, understanding of normative behavior patterns and so on. Moreover, the use of screening, and the likelihood of children receiving subsequent comprehensive diagnostic assessments, will also vary depending on whether the screening is embedded within a broader community health program (e.g., SACS) or is reliant on parents electing to volunteer.

Several broad observations have been made in evaluations of these instruments. First, the S_E of some instruments (e.g., CHAT, ESAT) appears to sub-optimal (27, 34). Second, some instruments (e.g., CESSD, CHAT, ESAT,) appear to generate relatively high rates of false positives (27, 28, 34). Third, where S_E and S_P appear to be more impressive (e.g., ITC), they may be overinclusive, not differentiating children with autism from those with language or other developmental delays (31). Also, the effectiveness of these instruments appears less impressive below 12 months (31) and is enhanced by repeated screening from 8 to 24 months (33).

Evaluation of Level 1 screeners is constrained by the fact that comparisons of different instruments' performance with the same samples have been scarce. Dereu et al. (35) reported comparisons of the CESDD (completed by child-care workers) with several parent completed measures—the ESAT, M-Chat (32), and the Social Communication Questionnaire [SCQ; (36)]—and concluded that they showed similar discriminative capacity. However, Dereu et al. noted a range of issues that

TABLE 1 | Items from STAT (21), ADEC (8), SORF (26), and BISCUIT-Part 1 (24) linked to DSM-IV-TR and DSM-5 criteria.

	Test items			
DSM version/ criteria	**STAT (11 items)**	**ADEC (16 items)**	**SORF (22 items)**	**BISCUIT—Part 1 (54 items)**
DSM-IV-TR (2000): Autistic disorder				
A1-a Non-verbal behaviors		• Gaze monitoring • Reciprocity of smile • Use of gestures • Eye contact • Reaction to common sounds • Nestling • Anticipating social advances	• Warm expressions • Reduced facial expressions • Eye gaze to faces • Non-verbal communication	• Understand cues or gestures • Reads non-verbal cues • Too few or too many gestures • Gives subtle cues or gestures • Appropriate face expressions • Body posture and gestures • Use of facial expressions • Eye-to-eye gaze • Maintains eye contact • Displays range of appropriate facial expressions • Use of non-verbal communication
A1-b Peer relationships			Interest in objects over people	• Peer relationships • Make and keep friends • Social interactions with same age • Socializes with other children • Development of social relationships • Plays appropriately with others
A1-c Sharing enjoyment	• Play: turn taking • Requesting: snack • Requesting: bubbles • Directing attention: balloon • Directing attention: puppet • Directing attention: toys • Directing attention: noisemaker • Directing attention: rattle • Motor imitation: car • Motor imitation: drum • Motor imitation: hop dog	• Joint attention • Task switching • Imitation	• Sharing interests • Showing and pointing	• Motivated to please others • Shares interests with others
A1-c Social reciprocity		• Response to name	• Response to name • Using hand as tool • Reciprocal social play	• Interest in social games • Participates in games • Interest in other conversation • Understand appropriate jokes, figures of speech • Responds to others' cues • Make believe play • Responds to another's distress • Expects others to know their thoughts • Recognize emotions of others • Isolates self
A2-a Spoken language		• Delayed language	• Directed consonant sounds	• Use of language to communicate • Verbal communication • Communication skills • Language development
A2-b Conversation skills				• Use of language in conversations with others • Communicate effectively
A2-c Stereotyped language			• Repetitive speech	• Saying words/phrases repetitively

(Continued)

TABLE 1 | Continued

	Test items			
DSM version/ criteria	**STAT (11 items)**	**ADEC (16 items)**	**SORF (22 items)**	**BISCUIT—Part 1 (54 items)**
A2-d Play	• Play: doll play	• Functional play • Following verbal commands • Pretend play	• Clutches objects—or A3(d)	• Pretend play
A3-a Restricted interests			• Ritualized behavior • Excessive interest • Sticky attention	• Restricted interests • Limited number of interests • Restricted interests and activities • Curiosity with surroundings
A3-b Rituals/routines		• Ritualistic play and stereotyped behavior (& distress over change)	• Distress over change	• Upset if change in routine • Needs reassurance if things don't go to plan
A3-c Repetitive movements		• Ritualistic play and stereotyped behavior	• Repetitive movements	• Abnormal movements of whole body • Repetitive movements for no reason • Repetitive hand or arm movements
A3-d Preoccupation			• Repetitive use of objects • Fixation on object parts • Clutches objects—or a2(d)	• Fascination with spinning objects • Odd routines or rituals • Preoccupation with object parts
Not applicable			• Sensory aversion • Sensory interest	• Intellectual abilities • Age appropriate adaptive skills • Reaction to sounds/sights • Prefers food of certain texture/smell • Reactions to normal sounds • Reactions to normal lights
DSM-5 (2013): Autism spectrum disorder				
A1 Social-emotional reciprocity		• Response to name • Gaze monitoring • Joint attention • Following verbal commands • Reciprocity of smile • Task switching • Delayed language • Imitation	• Response to name • Showing and pointing • Interest in objects over people • Sharing interests • Reciprocal social play	• Interest in social games • Interest in other conversation • Shares interests with others • Isolates self • Use of language to communicate • Verbal communication • Communication skills • Language development • Use of language in conversation with others • Communicates effectively
A2 Non-verbal social behavior		• Eye contact • Nestling • Anticipating social advances	• Warm, joyful expressions • Reduced facial expressions • Eye gaze directed to faces • Using hand as a tool • Non-verbal communication • Fixation on object parts • Sticky attention	• Understand cues or gestures • Reads non-verbal cues • Motivated to please others • Responds to others' cues • Too few or too many gestures • Gives subtle cues or gestures • Body posture and gestures • Recognize emotions of others • Use of facial expressions • Eye-to-eye gaze • Maintains eye contact • Displays range of socially appropriate expressions • Use of non-verbal communication

(Continued)

TABLE 1 | Continued

DSM version/ criteria	Test items			
	STAT (11 items)	**ADEC (16 items)**	**SORF (22 items)**	**BISCUIT—Part 1 (54 items)**
A3 Developing relationships	• Play: turn taking • Play: doll play • Requesting: snack • Requesting: bubbles • Directing attention: balloon • Directing attention: puppet • Directing attention: toys • Directing attention: noisemaker • Directing attention: rattle • Motor imitation: car • Motor imitation: drum • Motor imitation: hop dog • Requesting: snack • Requesting: bubbles	• Functional play • Pretend play		• Peer relationships • Make and keep friends • Social interactions with same age • Socializes with other children • Development of social relationships • Plays appropriately with others • Participates in games • Understand appropriate jokes, figures of speech • Appropriate facial expressions • Make believe play • Responds to another's distress • Expects others to know their thoughts
B1 Repetitive motor behaviors, use of objects or speech		• Ritualistic play and stereotyped behavior	• Repetitive use of objects • Repetitive body movement • Clutches objects • Fixation on object parts	• Fascination with spinning objects • Restricted interests • Abnormal movements of whole body • Preoccupation with object parts • Repetitive movements for no reason • Repetitive arm or hand movements • Saying words/phrases repetitively
B2 Insistence on sameness, adherence to routines		• Ritualistic play and stereotyped behavior (& distress over change)	• Ritualized behavior • Distress over change	• Odd routines or rituals • Upset if change in routine • Needs reassurance if things don't go to plan
B3 Restricted and intense interest			• Excessive interest	• Limited number of interests • Restricted interests and activities
B4 Hyper- or hypo-sensitivity to sensory stimuli		• Response to everyday sounds	• Sensory aversion • Sensory interest	• Reaction to sounds and sights • Reactions to normal sounds • Prefers food of certain texture/smell • Curiosity with surroundings • Reactions to normal lights
Not applicable			• Directed consonant sounds	• Intellectual abilities • Age appropriate adaptive skills

The STAT was developed in the era of the DSM-IV, prior to the DSM-IV-TR but, given it has been subjected to several evaluations between 2000 and 2020, its inclusion aids the demonstration of these links. Also, there is no attempt in this table to represent all criteria or the various contingencies that must be met (e.g., number of criteria met from each domain, when symptoms emerged, symptom severity) to meet the relevant DSM criteria.

clouded the interpretation of their findings, not the least being that the comparison sample contained children who had previously screened positive on the CESDD or had language delay.

The Level 1 screener literature suggests several broad messages. First, multiple screens in the first 2 years may improve both S_E and S_P by accommodating the variations in individuals' developmental trajectories. Second, a positive screen at a young age ideally should be followed up with either a subsequent screen to ascertain whether (a) the result may have been a false positive (followup with a Level 1 or 2 screener), and (b) the child is showing signs of autism or other developmental issues (followup with a Level 2 screener or comprehensive developmental assessment). Likewise, it would be ideal for negative screens to be followed up given the damage that may accrue when false negatives delay referrals for more comprehensive assessments. However, the issues associated with achieving such outcomes are, of course,

complex and involve a variety of organizational and socio-political considerations—in addition to having discriminating test items. For example, the attainment of broad Level 1 screening is likely to be facilitated by the availability and accessibility of a measure that can be administered quite quickly (i.e., relatively few items), is not dependent on highly intensive training or specialist expertise, and can be scored quite easily. The likelihood of such a measure being widely adopted will depend on it being embedded within a community health assessment program that has strong and sustained governmental support and extremely broad outreach, such as that documented by Barbaro and Dissanayake (33). Realizing that objective may be more difficult than designing the measure.

Third, the Level 1 screening literature highlights the fact that, even if widespread adoption can be achieved, major obstacles to realizing the benefits that could flow from a positive screening outcome while the child is young are still likely to exist. For example, Barbaro and Dissanayake (33) found that only about 50% of the at-risk infants identified by the screening progressed to the comprehensive developmental unit associated with the research team (although the others may have followed other pathways). Issues associated with parental compliance with subsequent followups have been noted in some, although not all, other studies (28). Testimony to the importance of recognizing that there are likely to be important "system" variables constraining early detection is provided by Oosterling et al.'s (37) study. It revealed a lower mean age of autism diagnosis and higher proportions of diagnoses before 36 months in an experimental (compared with a control) region that integrated training of professionals in recognizing early signs, the use of a Level 1 screener accompanied by a specific referral procedure, and the availability of a multidisciplinary diagnostic team. The importance of these system variables was reinforced by an examination of the sustainability of that program. A cessation of funding and staffing support, that undermined staff training in recognizing early signs and using the referral protocol, saw the beneficial effects on early detection no longer sustained (38).

LEVEL 2 SCREENERS

The content of Level 2 screening instruments may often look similar to that of Level 1 screeners but the focus in instrument development and evaluation differs. Whereas, Level 1 screeners are probing in the general population for a potentially problematic developmental trajectory that may suggest the child is showing early signs of autism, and may indeed differentiate autism from other conditions, Level 2 screeners are designed to differentiate young children with ASD from those with other developmental disorders or concerns. Numerous Level 2 screeners have been described in the last two decades. Here we focus on five instruments that have been subjected to psychometric scrutiny involving either a large sample or several different samples, with the evaluation outcomes published in refereed journals. We review each of these instruments—the STAT, BISCUIT–Part 1, ADEC, SORF, and RITA-T. Other widely used instruments not specifically targeting very young children—for example, the Childhood Autism Rating Scale [CARS; (39, 40)]—are not considered in detail.[1]

The Stat

The STAT was first described in an unpublished manual [see (21)] as a brief interactive measure that did not demand language comprehension and could be used by health-care workers and related professionals. A number of published evaluation studies have emanated from laboratories led by researchers from four different US institutions and from one Taiwanese university (21, 43–48).

The original target age range was 24–36 months, but it has subsequently been evaluated with children as young as 12 months. As shown in **Table 1**, it includes 12 social-communicative items administered in a play-based interaction session of about 20 min duration. The items deal with "negative" symptoms, that is, the absence of behaviors, with responses on each scored as pass/fail giving rise to both an area and a total score. Scoring criteria are set out in an instructional manual, with various options for training and certification in the use of the STAT available online.

Samples for the aforementioned studies were recruited from various sources including multidisciplinary evaluation centers, speech and hearing centers, research projects recruiting siblings of autistic individuals, children referred to an early intervention program from a community Level 1 screening program, etc. Total sample sizes (i.e., depending on the specific diagnostic categories captured, AD; Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS); ASD, developmentally delayed (DD); language impaired, no ASD) varied from 33 to 382 (median = 104). Depending on the study, diagnoses against which these measures were validated were based on DSM-IV, DSM-IV-TR or DSM-5 criteria, sometimes using only a clinician diagnosis (21) but generally supplemented with standardized measures such as the ADOS, ADI-R, and other measures, with diagnosticians blind to STAT outcome in at least five of the published studies.

Whether the administrator was blind to referral reasons and diagnosis of the child in the studies cited above is sometimes clear (21, 43, 46, 48), but not always (45). Administrator training is usually carefully described, but inter-rater reliability data are presented meticulously in some papers (44, 46), quite vaguely (i.e., difficult to interpret) in some (43, 48) and not at all in others (45)—although reference to other published reliability data (46) or the meeting of online training criteria may be present (44, 47). Test-retest reliability data have been presented (47) but are seldom provided in subsequent studies using different samples and administrators.

Concurrent validity was demonstrated via the high agreement between the STAT's classification of a child as high or low risk for autism and their ADOS-G classification (46). The STAT's capacity to differentiate children with autism from other developmental

[1]Note that although the CARS has been criticized for over diagnosing infants (41), others argue that it may be useful for distinguishing autism from other developmental disorders as early as two years [e.g., (42)].

disorders is illustrated by the S_E and S_P data reported in the seven evaluation studies cited above. Using the optimal STAT cut-off score identified within each of these seven studies, and the total score for the full scale, S_E ranged from 0.47 to 0.93 (median = 0.86) and S_P ranged from 0.69 to 0.86 (median = 0.80).

Several other findings from these studies should be noted. First, Stone et al. (47) reported substantially better S_P when they removed the youngest infants (12–13 months) from their 12–24-month sample. Second, Stone et al. (46) found that the STAT did not reliably classify children with a PDD-NOS diagnosis, suggesting that the detection of milder autism spectrum symptomatology may be less likely. Third, although the predictive validity of a positive screen at a very young age is a relevant consideration when evaluating these instruments, published data on this are (to our knowledge) limited. In Stone et al. (47), the average lag between screen and diagnosis was 15 months ($SD = 5$). Wu et al. (48) reported S_E and S_P of 0.86 and 0.71 (or 0.70 and 0.79, depending on the optimal cutoff adopted) given an average lag to diagnosis of 18.6 months ($SD = 1.1$). Thus, based on the screening information provided by the STAT, the likelihood that children will continue to meet diagnostic criteria throughout childhood, or the likely severity of the condition, cannot be estimated. Of course, if the child's positive screen were followed (as would be desirable) by an evaluation using a standardized diagnostic tool such as the ADOS or the ADI-R that, when administered beyond 2 years, suggests a stable diagnosis at least up to 9 years (49), the issue of the screener's predictive validity is less relevant at least for that child.

The Biscuit–Part 1

The BISCUIT-Part 1 was designed by Matson et al. [(25), cited by (50)] as an informant interview with the child's primary caregiver, with the interview conducted within the framework of a state-funded early intervention program in Louisiana (USA). Several published evaluation studies have emerged from the Louisiana team (24, 50–53). Note, however, that each article examines different psychometric properties of the same instrument apparently drawn from the same referral base and thus could be viewed as components of a single psychometric evaluation. To our knowledge, evaluations by research teams at other sites have not yet emerged.

The age range of the sample targeted in the BISCUIT-Part 1 evaluations is 17–37 months. In most of the published evaluations, the number of items included was 62. Items captured all domains of the DSM-IV-TR and DSM-5 (**Table 1**) and were selected based on DSM-IV-TR and ICD-10 criteria, clinician observations and research findings. Items were read aloud to a child's parent or primary caregiver, each accompanied by age-appropriate qualifiers (not listed in the papers cited above). Test administration time is listed as approximately 20–30 min, depending on the child's characteristics. The response format involves a three-point Likert-type scale describing whether, on each item, the child compares to a TD peer as 0 (not different; no impairment), 1 (somewhat different; mild impairment), or 2 (very different; severe impairment). Assessors were credentialed in one of several relevant disciplines and received a day's training that included information on autism, the scales, practice, and so forth.

Children tested were in a state-funded service for families of infants with a developmental delay or another condition likely to produce some delay. Sample sizes varied depending on the focus of the study (e.g., depending on the specific diagnostic categories included) and, compared to other screener studies, were typically much larger, ranging from several hundred to over 3,000. In some published studies the sample was obviously the same; in others the sample is described as a rolling sample, with children being progressively added to the cohort as time passed. Thus, some children appear in multiple studies, though the proportions doing so are unclear.

All diagnoses of AD or PDD-NOS were made by an experienced clinical psychologist in the field who was blind to BISCUIT scores. The diagnoses were based on clinical judgment using some combination of the DSM-IV-TR algorithm for AD, DSM-IV-TR descriptors for PDD-NOS, M-CHAT scores, and developmental profiles on the Battelle Developmental Inventory-Second Edition (54). It is unclear if this combination was available for each diagnosis and, if not, what proportion of diagnoses depended on each indicator. Nevertheless, in two of the papers cited above where another psychologist also provided a diagnosis, inter-rater agreement for diagnoses based on subsets of cases ($N = 97$ and $N = 203$) was impressively high, $k = 0.98$ and agreement = 98.7% (24, 52).

Reliability data for the BISCUIT are scarce. When examining the original list of items, Matson et al. (50) report item-total correlations and inter-item correlations suggestive of a coherent item set. Also reported is coefficient alpha—now heavily criticized as an index of reliability (55, 56). Moreover, at least in the "BISCUIT" papers cited above, neither test-retest nor inter-rater reliability data for the BISCUIT (as distinct from diagnosis) are reported.

Validity data for the measure were presented more thoroughly. Examination of the factor structure highlighted three factors—deficits in socialization, restricted interests and repetitive behaviors and communication—that align with characterizations of autism symptomatology (24). Convergent validity was suggested by robust correlations with the M-CHAT and the socialization skills domain of a standardized index of developmental functioning (51). The optimal cut-off score suggested by the authors for differentiating autism from PDD-NOS gave rise to S_E and S_P of 0.84 and 0.83, respectively (52), with the corresponding values for PDD-NOS vs. no diagnosis being 0.85 and 0.86. More recently, with a larger sample, S_E and S_P were examined for different age levels. For children aged 17–23 months, the optimal cut-off scores for differentiating autism from PDD-NOS produced S_E and S_P values of 0.80 and 0.81; the corresponding values for differentiating PDD-NOS and non-ASD related atypical development were 0.93 and 0.76 (53). No predictive validity data have been reported.

The ADEC

The ADEC was first described in a published test manual (22) (Note that two of the authors of the current paper were involved

in the ADEC's development). One objective was to detect pre-verbal behaviors that predict the development (or emergence) of AD in children under 3 years. The second was to operationalize these behaviors so precisely that clinicians with minimal expertise in autism could readily learn to administer the instrument within 10–15 min. Subsequent published evaluation studies have been led by researchers from four different Australian and US institutions (15, 57–62) with a further study conducted in Mexico using a Spanish translation (63).

The ADEC includes 16 items from the domains of disturbances in interacting with others and objects, stereotyped and repetitive movements, and bizarre responses to environmental stimuli (**Table 1**). Items are administered in a play-like interaction session involving the child, the tester and a parent or caregiver. Item development was guided by (a) core deficits suggested by the broad literature, (b) retrospective parental reports (8), and (c) analysis of home videos of infants subsequently identified with autistic disorder, developmental or language delay, or TD (64). The operationalization of these behaviors, scoring criteria for each item (0 = appropriate behavior, 1 = somewhat inappropriate, 2 = clearly inappropriate behavior) and lists of examples and non-examples are precisely specified in the manual, together with specific administration instructions. Thus, for example, a score of 1 might be received by a child who did not perform the behavior when required but at some other stage performed it spontaneously, or only displayed the behavior on some of the required attempts to elicit the behavior.

Extensive piloting of the instrument, often involving children older than 3 years (e.g., $M \approx 40$ months), with a confirmed diagnosis of AD, other disability or TD, is described in the published manual and indicated impressive inter-rater and test-retest reliability, concurrent validity with the CARS, CHAT, and ADI-R, and promising diagnostic discrimination, S_E and S_P. Other promising reliability and validity pilot data were provided in a study using a Spanish translation (63), but there were too few children under 3 years to allow any decisive conclusions regarding the instrument's merits for use in the target population.

Samples in the published ADEC studies cited above were recruited from state and privately funded centers for children referred with various developmental concerns, a university-based research center, a child development clinic of a large pediatric clinic hospital, and via general advertising. Total sample sizes (i.e., depending on the specific diagnostic categories captured, AD, PDD-NOS, ODD, TD) varied from 53 to 270 (median = 112).

The key studies we discuss in this section are Nah et al. (61), Hedley et al. (59) and Nah et al. (60), with the latter's sample overlapping with Nah et al. (61). In Nah et al. (61), children's ages ranged from 12 to 36 months, with mean ages in months for the various sub-samples of 29.4 (AD), 28.2 (PDD-NOS) 24.1 [other Developmental Disorder (DD), and 23.5 (TD). In Nah et al. (60), children's ages ranged from 12 to 36 months, with mean ages in months for the various sub-samples of 29.4 (AD), 28.2 (PDD-NOS) 24.1 (DD), and 23.5 (TD). Hedley et al.'s (59) children ranged from 14.3 to 36.9 months ($M = 28.7$, $SD = 5.4$); autistic children ranged from 19.2 to 36.9 months and the DD group from 15.9 to 36.8 months. A best estimate clinical (BEC) diagnosis was made by an experienced clinician who was blind to ADEC outcomes and used information from a variety of sources including the ADOS and ADI-R—but not the ADOS Toddler (5) or the ADI-R Toddler (65) designed specifically for children below 24 months]. Hedley et al.'s (59) diagnoses were based on DSM-5 criteria. The two earlier studies used DSM-IV criteria, with independent confirmatory diagnoses reported for 77.5% of participants with autism or DD diagnoses. The remaining ADEC studies cited above (15, 62) are discussed in a later section dealing with a brief version of the ADEC.

The test manual (22) reported impressive inter-rater agreement for a subset of the sample, with similar values of 0.98 (61) and 0.95 (59) reported subsequently. Test-retest reliability, after an average interval of 54.5 days, was 0.72 (61), but has not been reported in other studies.

The validity analyses reported in these studies yielded relatively consistent patterns. Nah et al. (61) conducted a factor analytic examination of the ADEC which indicated a one-factor (social communication) solution. In the same study, concurrent validity was indicated by robust correlations between ADEC total score and the sub-scales of the ADOS and ADI-R (except for the restricted and repetitive behaviors sub-scale of the latter). Hedley et al. (59) also reported strong correlations with ADOS-2 sub-scales and with the CARS. Both studies found that the ADEC predicted diagnostic status, whether it was DSM-IV or DSM-5. Nah et al. (61) also demonstrated diagnostic validity with ADEC total scores discriminating AD, PDD-NOS and DD, with this pattern prevailing even after controlling for non-verbal IQ and adaptive behavior, and in a subgroup aged 24 months or younger. In a similar vein, Hedley et al. (59), confronted with a sample of autistic children of a lower developmental level than both the DD and no diagnosis comparison groups, matched infants on age, adaptive behavior and developmental quotient and reported that the ADEC still discriminated the autistic children from those with other developmental disabilities. Nevertheless, as previously noted (61), given the relatively low developmental levels of their AD sample, further work is needed to clarify the discriminative capacity of the ADEC when used with more developmentally advanced autistic children.

For both the full AD and DD samples, and for AD and DD samples matched on age, non-verbal IQ and adaptive behavior, Nah et al. (61) examined S_E and S_P for the following contrasts: AD disorder vs. DD, and AD vs. DD + TD. For the full samples, using the optimal, and the manual recommended, total score cutoffs, S_E and S_P for these two contrasts were 1.0 and 0.77, and 1.0 and 0.89, respectively. For the matched samples, the corresponding values the were 1.0 and 0.74, and 1.0 and 0.90, respectively. In other words, S_P was lower when TD children were excluded from contrasts. In Hedley et al. (59), whose sample included no TD children—but, unlike Nah et al. (61), were selected using DSM-5 criteria—S_E and S_P at the corresponding cutoff were 0.93 and 0.64.

It is interesting that the accuracy of screening clinicians' ADEC judgments about the presence or absence of autism was related to their judgmental confidence. Clinicians with experience in screening rated their confidence in their screening

test judgment on a scale ranging from 0% (not confident at all) to 100% (absolutely certain) (58). Confidence was predictive of a subsequent accurate diagnosis only when it was in the range of 70–100%. Lower confidence ratings predicted accuracy of diagnosis at no better than chance levels. Subsequent research with samples sufficiently large to enable a more precise examination of the confidence-accuracy relationship may indicate how best to exploit experienced assessors' confidence judgments about the ADEC screen result when planning subsequent followup assessments.

Finally, predictive validity data are available from two studies, although in both cases the sample sizes are very small. Nah et al. (60) presented ADEC, and CARS, screening data for 55 children, 67% of whom were participants in Nah et al. (61). Comprehensive diagnostic assessments were conducted at the initial and two followup assessments, with assessors blind to the child's outcomes on the ADEC and CARS. The initial assessment occurred at 19–42 months ($M = 33.5$, $SD = 5.6$), with the sample comprising 51 who received a BEC DSM-IV-TR AD diagnosis, 2 with PDD-NOS and only 2 with non-autism spectrum disorders. Followups occurred 2 and 6 years later, though the latter followup was only available for 22 children. Both measures predicted an autism diagnosis at 2 years but not at 6 years, although combining individuals diagnosed with AD and PDD-NOS resulted in most children retaining their diagnosis after 6 years. The ADEC was also able to predict symptom severity at 6 years. Dix et al. (57) also reported ADEC data for a small sample of 53 children ($M = 32.2$ months, $SD = 8.4$) referred for developmental concerns or autism risk and subsequently followed up 49–97 months later. Diagnoses were made jointly by an appropriate multidisciplinary team using DSM-IV-TR criteria and including the ADOS and ADI-R, with age at diagnosis ranging from 22 to 65 months ($M = 41.2$, $SD = 9.2$). At the followup, 60% had received an ASD diagnosis and 36% had developmental delays. S_E and S_P were 0.88 and 0.62, respectively.

The SORF

A published version of the SORF appeared in Wetherby et al., (66), with teams (led by Dow, a US researcher) subsequently reporting formal evaluations of a modified form of the SORF as a Level 2 screener (26, 67). The original version comprised 29 items based on DSM-IV criteria, with item content later modified to include 22 items based on DSM-5 criteria. The two evaluation studies (26, 67) studies examined the screening performance of the SORF with infants ranging in age from 16 to 24 months when applied in conjunction with a video-recorded administration of Wetherby and Prizant's Communication and Symbolic Behavior Scales (68), and coded by individuals without expertise in autism. Note that the first author of the Dow et al. evaluation studies (26, 67) has indicated that the two samples overlapped (exact degree not specified), with some children being coded for the SORF based on both clinic observations (26) and home observations (67). The degree of overlap has potentially implications for the likely generality of the findings.

The social communication and restricted and repetitive behavior domains of the DSM-5 are each represented by 11 items. The children were recorded interacting with their parents in a range of activities in their home environment for 1 hour. Undergraduate coders, blind to diagnosis, received training on diagnostic features of autism and the coding system, then watched a 20 min (26) or 1 h (67) recording of the interview. Using a 0–3 response scale (0 indicated absence of relevant concern, 3 indicated the greatest level of severity or concern), they rated the presence/absence of behaviors referred to by the items—see Wetherby et al.'s (66) Appendix A for detailed descriptions—that are atypical/typical of TD children. Inter-rater reliability was indicated by intraclass correlation generalizability coefficients for the total composite score for the best performing 17 items (26) and 6 items (67) of 0.86 and 0.75, respectively. No test-retest reliability data were reported in these papers.

Samples were recruited to a longitudinal, prospective study of autism and communication disorders. The children were referred via primary care screening and met the criteria that the SORF and a diagnostic assessment had been completed between 16 and 24 months. In Dow et al. (26), children's mean ages were 20.8 months for each of the autistic, DD and TD sub-samples; the corresponding values in Dow et al. (67) were 20.7, 20.3 and 20.4 months, respectively. The autistic and DD children did not differ significantly on the non-verbal development quotient measure used; importantly, the sub-samples performed at a higher developmental level than those reported using the same developmental scales in the STAT and ADEC evaluations of Stone et al. (46), Nah et al. (61), and Hedley et al. (59). BEC diagnoses in both Dow et al. (26, 67) studies were based on a combination of sources including the ADOS-T, developmental and adaptive behavior scales, and the video-recorded observation session.

Outcomes for the validity analyses were similar in the two studies. Both studies reported diagnostic validity with the respective composite (and other summary) scores discriminating the autistic and non-autistic children. Using the optimal composite scores, S_E and S_P for the contrasts of autistic and non-autistic children were 0.80 and 0.78 (26) and 0.77 and 0.72 (67), respectively. No predictive validity data were provided.

The RITA-T

Our discussion of the RITA-T is much briefer than that of the preceding instruments because it needs to be viewed as being at an early stage of evaluation. The two main data-based publications are a small sample study reported by US-based researchers, Choueiri and Wagner (23), and a larger-sample study recently described by Canada-based researchers, Lemay et al. (69). The measure involves 9 semi-structured and play-based activities that are administered and scored in a 10-minute session, with very modest administrator training requirements. The samples ranged in age from 18 to 36 months.

We have included this measure because of two interesting features. First, the activities, or items (23), which cluster exclusively under domains A1 and A of the DSM-IV-TR and DSM-5, respectively, are operationalized a little differently to items in other measures. Second, S_E and S_P at the optimum score cutoffs identified are promising, reported as 1.00 and 0.84 (23) and 0.97 and 0.71 (69). Although these features of the studies should pique researchers' interest, there are several reasons why the findings should be regarded as preliminary.

First, in neither study was a measure such as the ADOS administered to all at-risk children; nor were diagnoses independently confirmed. Second, the inter-rater reliability protocol lacks clarity and no test-retest data were reported. Third, in the large-sample study (Lemay et al.), all children had been referred to the clinic with ASD concerns and diagnosing clinicians could have accessed the RITA-T administrators' clinical observations. Fourth, Choueiri and Wagner's conclusion that their ASD and DD sub-samples were of comparable developmental levels is not justified, despite their reporting non-significant differences between the samples. Those contrasts were clearly under-powered, and the descriptive statistics strongly suggest that developmental level should have been controlled in analyses. Moreover, the samples were too small to match on developmental level as was done, for example, by Nah et al. (61). Further, in Lemay et al., no data are provided on developmental level for their ASD and non-ASD groups.

Level 2 Screeners: A Summary

What should we conclude regarding the utility of these screeners? Some researchers—perhaps all—not surprisingly, appear to favor their own measure. For example, Dow et al. (26, 67), while acknowledging important unanswered questions about the SORF, appear to be leaning toward the superiority of the SORF on the (reasonable) basis that the items reflect DSM-5 criteria and their sample is younger, a little bit larger, and of a higher developmental level than those in some of the other papers they cite. We, however, are unsure because the various evaluation studies differ in so many potentially important respects: sourcing of the samples (e.g., primary health care referrals vs. clinical samples) and the likely prevalence and severity of individuals at risk; sample sizes; ages and developmental levels of individuals sampled; rigor and reliability of diagnoses; whether the individuals were classified according to DSM-IV-TR or DSM-5 criteria; precision of operationalization of target behaviors; quality of rater training; reliability of the raters and stability of those ratings, and so on. The instruments also differ in terms of the very important criterion of whether they have been subjected to any evaluations by researchers beyond the laboratory or clinic of the test developers, let alone in different countries and cultures. And although the S_E and S_P indices suggest variations in the discriminative performance of the instruments, who knows which of the many variables mentioned above contribute to those variations. Maybe these apparent performance differences reflect differences in the overall package of items or the testing methodology—but perhaps they just reflect one or more of the other factors listed above. The picture is so complicated that, although all the measures offer potential, we are not prepared to make an unequivocal case for the superiority of any of them in terms of discriminative performance.

Each measure is deficient in one or more of the following respects. Although independent evaluations (i.e., from other labs or clinics) have appeared in refereed journals for the STAT, ADEC, and RITA-T, this is not yet the case (to our knowledge) for the SORF or the BISCUIT. Large sample sizes—obviously an issue in research with autistic individuals—at all ages within the range of interest and encompassing different developmental levels is an issue for all instruments (except the BISCUIT). Demonstrations of inter-rater reliability and the stability of the screening measure—at least in the refereed publications—are not always apparent, or the protocols are vaguely described; nor is the independence of screening outcomes and diagnoses always unambiguous. And, even though the published papers on the STAT, ADEC, and SORF report reliability data systematically, we question whether sufficient attention is being paid to reliability issues. The various dimensions of test reliability not only have implications for the discriminative performance of a test in any individual study but, given the nature of the decisions made on the basis of these screening instrument outcomes in individual cases, they are also potentially of great significance for the young children and their families. Thus, a reasonable question to ask is whether those reliability levels that are cited as acceptable, or even impressive, for research purposes should be considered adequate when critical decisions are being made that are likely to shape the lives of an individual child and their parents. Complicating this issue specifically with respect to test-retest reliability is the heterogeneity of the condition and the way in which it unfolds. Consequently, although test outcomes should be stable over short test-retest intervals, fluctuations might be expected after longer intervals, thereby emphasizing the importance of multiple assessments. Finally, the relatively small sample sizes in all studies make it difficult to determine whether the (likely heterogenous) "other developmental disorder" sub-samples that are such an important part of Level 2 evaluations effectively represent the different conditions whose symptoms are perhaps most likely to be confused with ASD.

Taken together, such concerns highlight that missing from the field is any systematic comparison of these measures under consistent conditions, with substantial sample sizes, and adequately capturing the diversity of other potentially confusable developmental disorders. One sensible way to achieve such objectives would, therefore, be cooperation between researchers (something that is becoming much more prevalent in many areas of science). Of course, even if all those conditions were met and one measure appeared to outshine the others, it does not mean it should necessarily be the go-to measure. As we noted earlier when discussing Level 1 screeners, these instruments are used in diverse organizational systems to guide delivery of assessment and intervention services. Thus, for example, the availability of comprehensive followup assessment services and the known effectiveness of particular intervention programs are likely to influence judgments about the specific age and developmental levels that a screener should be targeting. Moreover, in some contexts an elaborate, time-consuming screening process conducted by professionals may be readily accommodated; in others the way forward may be via a speedier process routinely administered by professionals with minimal training. Or, the particular assessment and intervention context may well shape how S_E and S_P are prioritized and, hence, whether clinicians rely upon the optimum score cut-offs identified in the published evaluations or favor a different cut-off that increases S_E at the expense of S_P (or vice versa).

The importance of these system variable considerations is suggested by the difficult to resist observation that many screener

projects look like the work of an enterprising clinic, laboratory or individual, with only the STAT and the ADEC (among Level 2 screeners) thus far revealing evidence of long term and significant cross-institutional followup. Access to the requisite samples is obviously one limiting factor. And in most research areas, turning scientific research into specific and influential practical outcomes can be extremely difficult. Organizational imperatives will likely vary from region to region, state to state, and country to country. The ability to negotiate a way through those imperatives may prove to be far trickier, and ultimately much more significant, than any subtle differences in screener efficacy. Consequently, we need to identify and understand potential organizational and cultural constraints and to be able to make clear economic and socio-political cases for the advantages of screening. Inevitably this will involve providing precise information on costs and benefits—both economic and social—for the children, their progress to adolescence and adulthood, as well as for their families and service providers.

In addition to the organizational considerations that are likely to shape decisions about the selection of an appropriate screener, its implementation, the interpretation of the test outcomes(s), and subsequent decisions about referral for further assessment and intervention, there are some important practical issues that we review in the next section.

SOME PRACTICAL ISSUES

Many of the practical barriers to the adoption of screeners and their effective use have already been canvassed widely in the literature, including a comprehensive and concise overview (16). Some of these barriers have already been mentioned in our concluding remarks on Level 1 screeners and are equally apposite here. There are, however, three issues that we wish to emphasize, two of which have been covered in some form elsewhere in the literature or briefly mentioned in earlier sections.

The first issue is that a screen (or diagnosis)—negative or positive—at a very young age should not be seen as a single-point event given developmental trajectories may vary in unpredictable ways. Although the stability of diagnoses at around 18–24 months appears to be high across different sample types, an early negative screen does not guarantee that autism symptoms will not emerge at some later date. One study, for example, with a sample of children at familial risk, reported high diagnostic stability at 36 months for children detected at 18 and 24 months and, yet, nearly half of the sample were not identified at 24 months but were diagnosed at 36 months (70). In a similar vein, longitudinal data from later-born siblings of children with autism revealed that, despite multiple negative assessments in the preschool years, some met criteria for autism when reassessed in the 5–9-year age range (10). The authors suggested that, in some children, autism symptoms may continue to evolve after only showing quite subtle signs at younger ages. Others have reported that some children who showed regression (loss of skills) as they approached 24 months had previously appeared to be developing typically (71). Similarly, a false positive for autism on a single early screening test should not then be regarded as a guarantee of a typical developmental trajectory, as researchers have demonstrated that significant proportions of such children are likely to be at risk for various other developmental disorders that should be the focus of systematic observation and assessment (72, 73). Of course, it is also possible that an individual's trajectory may change if they are screened and subsequently exposed to some systematic intervention program that perhaps moderates their ASD symptomatology or influences the manifestations of other (potentially confusable) developmental conditions. In sum, as we noted when discussing Level 1 screeners, ongoing monitoring and assessment of children who appear at risk has the potential to aid the early detection of symptomatology of autism and other developmental disabilities. Moreover, a focus of such monitoring should be on younger siblings of children already diagnosed with autism given the heightened level of risk (16).

The second issue is one that has assumed prominence given the spread of Covid-19. The widespread distribution of the virus and its devastating consequences have had significant implications for those seeking and delivering health care services. First, with communities in isolation for extended periods it seems possible that the likelihood that parents would seek professional advice when they suspected developmental issues with their children would have fallen. Second, the accessibility of face-to-face services likely diminished. Under such conditions, telehealth services become critically important, just as they are for people who live in remote regions.

Researchers have taken up the challenge in the areas of assessment (74) and intervention (75), although it is still "early days" in terms of delivering assessment using this approach. For some Level 2 screeners the adaptations required will be less demanding than for others. For example, the administration of the SORF (67) involved video recording of parents interacting with their child in a variety of activities during a 1-hour session, with coding done by research assistants using the video. For measures that involve a more structured interaction between assessor or parent child (e.g., the ADEC), the use of telehealth assessments that may by necessity need to be parent-led will need to be evaluated to ensure fidelity of administration and reliability of the assessment. Nevertheless, at face value it would be surprising if these objectives were not achievable.

The third issue relates to parameters of the instrument that might lend it to being readily integrated into existing assessment frameworks. Within many primary care and organizational contexts, the likelihood of Level 2 screeners being used when appropriate may be enhanced by the availability of a measure that can be administered and scored in a timely manner—that is, few items and a brief administration time—and is not dependent on highly intensive training or specialist expertise. These practical considerations may also be particularly relevant in resource scarce contexts and where few professionals have expertise with comprehensive diagnostic techniques (e.g., developing countries).

Considerations of this nature have spawned interest in the development of short forms of Level 2 screeners. We consider these short forms here in some detail for two reasons. First, they obviously meet the practical efficiency criterion for instrument evaluation that we outlined earlier. Second, our examination of

their performance will highlight an area of concern associated with evaluating the discriminative performance of different measures, a concern that has wider implications for the ongoing evaluations of Level 2 screeners and their item content.

Short Forms

Researchers have published evaluations of short forms of two of the Level 2 screeners discussed in the previous sections: the ADEC and the SORF. In each case the evaluations have involved examining the performance of the best performing subset of the full item set in discriminating children with autism from other developmental concerns. In other words, the children were originally assessed using the full measure. The data for the best performing 6 items of the SORF have already been discussed (67). We do not discuss it further here because scoring the 6 items still involved a 1-hour video observation session. It will be interesting to see how that measure performs if based on a much shorter observation sample that reduces overall administration time significantly. In contrast, administration time for the full ADEC is only 10–15 min, with the short form discussed below taking less time.

Two studies have explored possible short forms of the ADEC (15, 62). Nah et al. (15) had 270 children aged 12–36 months, 197 of whom were part of Nah et al.'s (61) sample: based on BEC DSM-5 diagnoses, there were 106 (ASD), 86 (non-TD), and 78 (TD), with mean ages of 28.7, 23.1 and 23.5 months, respectively. Inter-rater reliability between two trained and experienced clinicians blind to the other's diagnosis was high ($k = 0.96$).

Nah et al. (15) specifically targeted a five-item version of the ADEC that could be administered and scored within 5 min. Those items—response to name, reciprocity of smile, joint attention and social referencing, following verbal commands, and use of gestures—were the items that yielded the highest AUC when comparing the autism and non-TD group, and together they formed the brief ADEC, or BADEC. The optimal cut off score yielded S_E =.81 and S_P =.78 (for ASD vs. non-TD) and 0.91 and 0.81 (for ASD vs. non-TD + TD). For the full ADEC, the corresponding S_E and S_P values for the former contrast were 0.87 and 0.84. The BADEC also demonstrated concurrent validity with the ADOS and ADI-R, and diagnostic validity with non-verbal developmental quotient controlled. Although the latter finding suggests the BADEC is detecting autism and not simply low cognitive functioning, we again emphasize that more work is needed to address the performance of the BADEC with autistic children who are at more advanced developmental levels.

Nevill et al. (62) replicated Nah et al. (15) using a US clinical sample [previously described in Hedley et al. (59)]. The sample included 110 children aged 14–36 months ($M = 28.8$), 49 with a confirmed ASD diagnosis and 61 without ASD. As in Nah et al. (15), diagnostic validity with non-verbal developmental quotient controlled was reported for total score on the best performing 5 ADEC items, and strong correlations with the ADOS and CARSs emerged. The best performing five item cutoff score (albeit with a more stringent cutoff than in Nah et al.) that optimized the S_E-S_P balance produced S_E and S_P indices of 0.77 and 0.86, respectively.

Nevill et al.'s best performing five items were not identical to those found by Nah et al. (15). To identify the best performing five items, each research team conducted ROC analyses and then simply selected the items that produced the highest AUC values (i.e., best discriminated ASD from non-ASD). Three items were common to both studies: following verbal commands, response to name and reciprocity of smile. For Nah et al., the remaining two items were use of gestures and joint attention and social referencing; for Nevill et al. they were gaze monitoring and task switching. Nevill et al. suggested the different outcomes perhaps reflected sample characteristics and that the careful approach might be to use all seven items.

These two studies suggest that a short form of the ADEC—whether it be a five- or seven-item version—might constitute a useful practical addition to the range of Level 2 screeners because of ease of administration and coding. Before advocating a specific short form version based on these two studies, however, we speculate on a critical issue that those item comparisons have brought to the fore—the use of AUC to evaluate test performance—one that has significant implications for all evaluations of Level 2 screeners and their item content.

Using AUC to Evaluate Discriminative Performance

As is common practice, in the two short form studies discussed above the discriminative performance of the various ADEC items making up the short forms was evaluated by calculating area under the curve (AUC). For both of these short forms (15, 62)—as has been the case in numerous other evaluations of the diagnostic merits of screener items and tests—AUC values were contrasted without the use of any inferential test to determine whether the AUC differences are meaningful.

We suggest that interpretation of AUC differences when assessing the discriminative performance of different items or, indeed, different tests needs to be on a firmer footing than that provided by an eye-balling of AUC values (76), as was the case in the two short form studies discussed above (15, 62). Whether inferential testing reveals meaningful AUC differences will obviously be dependent on sample sizes. Given that in much of the Level 2 screening research sample sizes are modest, substantial AUC differences will be required to detect meaningful differences between items or measures. However, one cannot simply infer based on sample size whether two AUCs are likely to differ significantly because (a) the correlation between items will also affect the significance of the difference between two paired AUCs and (b) the correlation between items will not necessarily be stable.

In sum, truly meaningful AUC comparisons will be possible in contexts where the samples are sufficiently robust for the detection of reliable AUC differences. Therefore, in the absence of some data simulations that vary sample sizes and inter-item correlations while maintaining the AUCs reported by Nah et al. and Nevill et al., we are not prepared to arbitrate on which of the two subsets of ADEC items might provide better discriminative performance. We also emphasize that these considerations are relevant more generally for comparative evaluations of the discriminative performance of individual items and tests.

ADVANCING THE FIELD

In this section, we highlight four issues that we believe are priorities for future research, some of which have already

been foreshadowed. The most important objective should be to devise and execute an approach to collecting sufficiently large sample sizes, originating from diverse referral sources, to allow realistic appraisals of Level 2 item and instrument discriminative capacity right across the 12–36 month age range. Only then will researchers be able to compare with authority the contributions of different items, the performance of the instruments as a whole, the capacity of observers to reliably code behaviors that are operationalized in different ways, and the stability or test-retest reliability of different items and measures. Realizing this objective will be a complex task and likely will only be achievable with multi-site approaches to study design and data collection. Such an approach may provide a number of benefits. It would help overcome a major limitation of existing research: namely, the limited evaluation of measures beyond the confines of the clinics and labs of the developing clinicians and the samples they are able to access. In so doing it might help the field progress beyond its current dependence on clinical judgments based on poorly operationalized and ever-changing DSM criteria, relying instead on standardized and meticulously operationalized instruments that provide a universal protocol for the diagnosis of autism.

A collective large-sample approach might also expedite dealing with reliability issues about which we can, at present, only speculate. It seems trite to say that it is highly desirable for items to be operationalized in ways that permit (with standardized training) perfectly consistent administration, interpretation and coding, but we cannot help but think that a focus on these issues that goes beyond the reliability criteria generally considered acceptable for research purposes might reap rewards. We wonder, for example, if we were to take multiple random draws of five or six items—with each item operationalized precisely and able to be scored with very high reliability—from a larger pool of well-targeted items (as in the short forms discussed) and administer them to very large samples, whether we might discover that the specific item content turned out to be less important than the precision of measurement.

A second objective should be to continue the search for item content, or behavior, that is discriminating and/or particularly so at certain age levels. Different lines of research have focused on non-social behavioral indicators that may be discernible prior to the emergence of social communicative deficits. A recent comprehensive review of such research (77) examined observable non-social behaviors in domains such as attention, visual processing, motor development, and repetitive and stereotyped behavior, exploring possible differences between younger siblings of children with ASD vs. siblings of TD children. Although the findings within many domains were non-significant or mixed, in some domains the balance of evidence suggested differences by around 12 months between siblings at elevated risk for ASD when the outcome was either later emergence of ASD or typical development. Impairments in the former group were detected in domains such as disengagement of attention (from a stimulus already engaged), motor development, repetitive interests and behaviors, and atypical sensory behaviors that, to date, have generally received less attention in screeners.

Examination of item content of current Level 2 screeners shows that they generally include items that tap into the just mentioned behavioral dimensions in some form, although more so if developed based on DSM-5. The challenge for the development of screeners, of course, is having items that are amenable to administration in a relatively brief testing session with the child and/or parent. The sometimes quite sophisticated paradigms that researchers have developed to probe specific processes and behaviors in different non-social domains may—given their measurement precision, their use of repeated trials and so on—be able to reliably detect fine behavioral impairments that are predictive of emergent ASD far better than their more "clumsy" equivalents that are typically seen in screener items. Although such paradigms can provide excellent research insights and may be able to be adapted for subsequent comprehensive assessments, translating them into a screener item that in one or two trials will produce a stable and discriminating measure will often be more difficult. Consequently, at least when it comes to probing some behaviors, it is likely that a considerable research effort will be required to bridge the gap required to translate a sophisticated lab based technique or measure into easily administered clinical test items.

A third focus for future research that has enormous implications for screener development should be trying to unravel the developmental trajectories of ASD in young children. We have highlighted research illustrating the problems that can arise when single-point screening of children deemed at risk is the norm. Although screening at several points in the early years is likely to reduce such problems, decisions about when to screen, screener content, or when to refer for more thorough assessment or intervention would be much better informed if we had a comprehensive understanding of how and when the condition may unfold. There exists a substantial body of research from various disciplinary areas that has contributed to our current understanding of developmental patterns (78). A challenge for clinicians interested in refining screeners will be to keep abreast of that literature, a literature that almost certainly will continue to burgeon, and to integrate the diversity of findings to achieve a coherent understanding of the development paths of ASD. Most, if not all, of the above suggestions apply equally to the refinement of Level 1 screeners.

Finally, at the risk of being way too repetitive, we conclude with a reminder that screening, and all the associated decisions about the measures used, any subsequent assessment, intervention and so on, occur within complex organizational systems and structures. Understanding the constraints those systems impose and how to shape them must be an ongoing focus.

AUTHOR CONTRIBUTIONS

NB drafted the manuscript in consultation with RY. CL drafted the section on AUC. RY and CL commented on the manuscript. All authors contributed to the article and approved the submitted version.

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Pragmatic Profiles of Toddlers With Autism Spectrum Disorder at the Onset of Speech

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Using speech to communicate pragmatic functions is challenging among individuals with Autism Spectrum Disorder (ASD). Given the role language plays in developing everyday skills, we traced the unique pragmatic profile of early words, seeking comparison to typically developing (TD) toddlers at similar lexical stages. Twenty-four mother-toddler dyads participated (9 ASD and 15 TD). Dyads were video recorded when toddlers reached a productive lexicon of 40–70 words. These recordings were captured three times during naturalistic interaction and at two consecutive visits with a 2-month interval. Seven thousand three hundred seventy-six productions were analyzed and classified into four communicative intentions (Declaratives, Requests, Objections, and Non-Communicative speech). ASD toddlers were delayed in the emergence of words compared to TD toddlers, with a greater within-group variability (median 28 months, IQR 24.5–35, median 17 months, IQR 17–18, respectively, $p < 0.001$). In both groups, the most common communicative intention was Declarative. However, the percentage of Declaratives was higher among TD toddlers across visits compared to ASD toddlers. In both groups, most productions were directed toward the communicative partner, but ASD toddlers used Non-Communicative speech more often than TD peers. Non-Communicative speech gradually decreased over time. We conclude that while TD toddlers begin to talk with an already-established knowledge of the main communicative functions of words, ASD toddlers seem to have only a partial understanding and gradually improve communicative use as they expand their lexicon. These findings bear theoretical and practical implications for early intervention in ASD. We suggest that communicative profiles are affected by individual characteristics and by the interaction style.

Keywords: autism spectrum disorder, toddler (MeSH), early language, development, pragmatics, communicative intention, naturalistic interaction

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INTRODUCTION

Language learning in young children with Autism Spectrum Disorder (ASD) is characterized by great variability (1, 2). Quite often, toddlers with ASD exhibit delays in the emergence of first words in comparison to age-mates with typical development (TD) (3–5). Both differences and similarities have been reported between ASD children and language-matched TD children, regarding word composition profiles (6), the application of various mechanisms for language learning (7–10), and the association between language production and development in other domains (11). Therefore,

the question of whether ASD toddlers follow the typical path to acquiring a language cannot be answered unequivocally. However, there seems to be an agreement that the use of language to communicate in a manner sensitive to the context, otherwise known as pragmatics, is impaired throughout the Autistic Spectrum. Such pragmatic deficits are present even among highly verbal or high functioning individuals with ASD (11, 12). Research on pragmatic deficits focuses either on nonverbal communicative skills of pre-verbal toddlers with autism (e.g., pointing) (13) or on higher-level pragmatic abilities such as carrying conversation (14). Research on the way ASD toddlers use their early words to communicate is still lacking.

The transition from pre-verbal communication to spoken language seems to be impaired in ASD children. TD infants use gestures and vocalizations a few months before their first birthday to convey basic communicative intentions such as simple requests, protests, and declaratives. These nonverbal means are prerequisites for the learning of shared, conventional meanings of words (15). As words appear, they serve similar communicative functions to those represented by the nonverbal means and address similar referents (16). Of note, the typical transition from pre-verbal communication to speech does not occur in toddlers with ASD who demonstrate restricted use of gestures and pre-verbal productions (17, 18). The matter of how ASD toddlers use their first words for conveying their communicative intentions has yet to be explored.

As the essence of pragmatics is using speech in context, we suggest that communicative intentions should be assessed within the setting of naturalistic interactions. It is agreed that a familiar context and a familiar partner have an optimal influence on the toddler's communicative functioning; thus, we believe that direct observations of dyads' naturalistic interactions are a vital tool for corroborating findings from questionnaires (19, 20).

When attempting to predict which communicative intentions are expressed via early words, it seems reasonable to assume a developmental course similar to that found in nonverbal communication. Several studies referred to the referential deficit, stating that gestures serving requests and protests (e.g., reaching) are relatively similar in the ASD population to those of the TD. On the other hand, lack of declarative (e.g., showing objects), is one of the core features observed in toddlers with ASD (13, 21, 22). If one assumes a continuity between pre-verbal and verbal means for communication, it would be reasonable to expect that the majority of early words would convey requests rather than declaratives. In addition, taking into account that difficulties in cooperation and sustaining interaction are common in ASD, one could also assume that the proportion of words used for protesting would be greater among toddlers with ASD than among TD toddlers at a similar lexical level. Last, given the well-documented tendency for speech for the self, we assumed that this proportion of "Non-Communicative Speech" would be greater in toddlers with ASD than in TD toddlers at similar lexical levels (23). The goal of the present study was to classify the communicative intentions that toddlers with ASD express at the onset of speech and to compare the distribution of communicative intents in this group to that of TD toddlers at similar lexical levels. In addition, we examined the trajectory of early words learning in the two groups.

METHODS

Participants

All participants came from monolingual Hebrew-speaking typical families (one mother and one father). ASD toddlers were recruited via advertisements in developmental centers and early intervention programs. Toddlers received a diagnosis of ASD (DSM-5) by an experienced multidisciplinary clinical team in one of the public developmental centers in Israel. The diagnosis of ASD was confirmed using the Autism Diagnostic Observation Schedule (17). TD toddlers were recruited by advertisements in social media. Children diagnosed with genetic and chronic medical conditions were excluded from both groups. Normal hearing status was confirmed for all participants.

To exclude minimally verbal toddlers, we set an age limit of 48 months for ASD participants. A limit of 24 months was set to exclude language delays in the TD control group.

Of 36 dyads initially screened, 12 were excluded due to medical conditions, being bilingual, having a non-typical family structure, having a vocabulary exceeding the criterion or, in the case of TD toddlers, having any form of atypical development.

The final ASD sample included nine toddlers (seven boys, two girls).

Cognition was evaluated with either Bayley or Mullen examination. Six children scored extremely low IQ and were defined by DSM 5 (18) as requiring very substantial support. The other three children needed substantial support while one of them scored low average IQ and two scored average. During the period of the study all nine ASD participants were enrolled in rehabilitation daycare centers. Treatment typically consisted of 12–14 weekly hours of therapy including parental guidance, speech, occupational, and psychologic therapy in an "eclectic" approach (24, 25). The final TD sample included 15 toddlers (13 boys, two girls). A comparison between the two groups showed no differences in parental educational level or socio-demographic background variables.

Tools and Measurements

A Playing Kit

To create a unified context as a base to comparison of the free play environment, we provided a kit of toys. The kit included age-appropriate toys such as wooden puzzles, pop-up puppets, bubbles, balloons, a book, and a doll house which typically provide opportunity for mutual play and function as communicative temptations (26–28).

Hebrew Communicative Development Inventory-Words and Gestures (HCDI- WG)

Each child's expressive level was evaluated using the Hebrew standardized version of the McArthur-Bates Communicative Development Inventory (MBCDI-WG). This report consists of lists of early gestures and words, each classified to either "understands" or "produces." This tool demonstrated high

TABLE 1 | Definitions for pragmatic categories.

Communicative intention	Definition	Example
Request	A verbal production addressed to the partner in order to receive a desired object or to perform an action	Child hands the mother a closed box, saying "open!"
Declarative	A verbal production intended to share knowledge or to get the partner's approval/ attention	Child points at a picture of a cow and says "Moooo"
Objection	A verbal production intended to stop or prevent an undesired event	Child says "no" while pushing the object or nodding upon being offered an object
Non-communicative speech	A verbal production that does not appear to be addressed to a communicative partner, may serve as self-stimulatory or practice	Child appears distant or disconnected, makes no eye contact, and recites a poem.
Others	A verbal production that was not necessarily addressed to another person or its communicative intent is unclear.	The child makes an unintelligible verbal production ("ARGH") and it cannot be determined whether it is a communicative act

reliability in all questionnaire components with alpha Cronbach ranging from $\alpha = 0.65$ to $\alpha = 0.98$ (29).

Classification of Early Words Into Communicative Intentions

All verbal productions of each child during dyadic interaction with his/her mother constituted the data set for the present study's analysis. Productions were analyzed providing they were either sound effects (e.g., "MEOW" for cat) or any verbalization that consisted of at least a single syllable (e.g., "BA" for "Bu-bah" = a doll). Crying and shouting were excluded from the word production analysis.

Given the lexical stage, we set the code for classifying communicative intents to address four exclusive categories: Requests, Declaratives, Objections, and Non-Communicative productions, and a fifth "other" default category. **Table 1** presents the operational definitions for each communicative intention.

Interact ® Software

In order to perform the pragmatic analysis of intentions, we utilized a computerized software (INTERACT ® software 14th edition), which enables frame by frame encoding of simultaneous verbal and nonverbal behaviors of the toddlers and their mothers. A timeline of behaviors was determined within the highly compressed interaction, making it possible to track changes in both form and frequency of each verbal production, including its antecedent and subsequent behaviors by both partners in the interactions.

Procedure

The study was approved by the local hospital and the university Institutional Review Boards. Parents signed an informed consent form. Direct observations in the homes of each child took place on three occasions with a 2-months' interval between any two visits. Shortly prior to entering the study, mothers tracked the productive lexicon of their child using the HCDI-WG questionnaire. The first visit was scheduled when the toddler's expressive lexicon reached 40–70 different words. The questionnaire was refilled in proximity to the second and third visits, thus reflecting the accumulating lexicon of the child. In each visit, dyads were video recorded during a 30-min free play session. Mothers were instructed to play with their child as they would normally while using the toy kit as much as possible. A sample of the first uninterrupted 15 min of each play session was coded. Each toddler's verbal production was transcribed alongside with the nonverbal behaviors that accompanied it using the INTERACT software. A timeline of behaviors was formed, thus enabling the analysis of contingencies and other relationships among the toddler's verbal productions and non-linguistic and linguistic behaviors. The present study focused on verbal productions.

Judging Communicative Intentions and Reliability Measures

Since parents are experienced in judging communicative intentions conveyed by toddlers' verbal productions, we recruited a mother to conduct the pragmatic analysis of the video recordings. To keep judgment as intuitive as possible, the mother-judge was introduced to the main communicative categories and was assured that the manifestation of each intention may vary in form and appear in various contexts. She was asked to watch two recordings along with the first author and to raise her concerns or questions. In order to examine the reliability, another mother with similar demographic characteristics was similarly trained using the same protocol.

Reliability regarding the pragmatic intentions of word productions was carried out by the two independent judges who coded 12% of the complete data set. The samples were derived from nine different participants from both groups and different visits (I, II, III). The calculation yielded Cohen's Kappa values of 0.71 and 0.87 for the ASD and TD groups, respectively.

Statistical Methods

Descriptive statistics were used to profile the scores of the questionnaires in each group. Continuous variables were summarized using means ±SD or median with interquartile range (IQR) and compared between groups using the Mann Whitney test. Categorical variables were summarized with counts and percentages. Agreement between raters was evaluated using Cohen's Kappa.

Repeated measures ANOVA was applied to evaluate the differences in communicative intentions over time and between groups and to evaluate the differences in the relative proportion of the communicative intentions.

P-values at 0.05 or below were considered significant.

Analyses were carried out using SPSS version 25.0, released 2017 (IBM Corp, Armonk, NY, USA).

RESULTS

Chronological Age for Achieving the 40–70 Words Entry Criterion

As expected, the age by which the entry criterion was achieved was significantly higher among the toddlers with ASD (median = 28 months, IQR 24.5–35) in comparison to that of the toddlers with TD (median= 17 months, IQR= 17–18) ($p < 0.001$). Among the ASD group, the variability as expressed by the IQR was significantly larger (10-fold) as compared to the TD group ($p < 0.001$). Eighty-seven percentage of the toddlers with TD already possessed an expressive lexicon of 40–70 words by the age of 18 months, while none of the toddlers from the ASD group achieved this criterion before the chronological age of 20 months.

Rate of Accumulating New Words

The number of expressive words derived from the HCDI – WG is presented in **Figure 1** for the two groups at each of the three visits.

Both groups significantly expanded the size of their expressive vocabulary ($p < 0.001$) and did so at a similar rate: an increase by 2.5 times from the first to the second visit and by 3.9 times on the third visit. There were no significant differences between groups regarding the course of expanding the vocabulary ($p = 0.652$). A high variation was found in both groups.

Verbal Communicative Intentions' Trajectory

A total of 7,376 verbal productions was collected from the two groups over the 15 min sample of the three visits. As mentioned, each verbal production was exclusively classified into one of the five communicative categories. However, since the total number of verbal productions per 15 min sample increased from one visit to the next, we measured the proportion (%) of each category out of the total number of all verbal productions. **Figure 2** presents the trajectory of those proportions (the category "others," showing low values, was omitted from this figure).

To identify the contribution of the group and the visit on each communicative intention a repeated measure ANOVA model was applied yielding the following findings:

- Declaratives were the most prominent intention, significantly higher than all other communicative intentions ($p < 0.05$). This was the case for both groups and at all visits, with the exception of the first visit in the ASD group: while the proportion of Declaratives was higher than the Non-Communicative speech, this difference was insignificant ($p = 0.224$). The proportion of Declaratives remained similar during all three visits ($p_{time} = 0.305$). However, Declaratives were significantly higher among the TD group in comparison to the corresponding proportion in the ASD group across the three visits ($p_{group} = 0.012$).
- The proportion of Requests remained similar during all three visits with no significant differences between the groups ($p_{time} = 0.820$, $p_{group} = 0.107$).
- The proportion of Objections remained fairly low and stable during all three visits with no significant differences between the two groups ($p_{time} = 0.181$, $p_{group} = 0.199$).
- The proportion of the category of "others" remained similar during all three visits with no significant differences between the groups ($p_{time} = 0.557$, $p_{group} = 0.942$).
- Both groups used Non-Communicative speech. However, and as expected, the proportion of this category was significantly higher in the ASD group in comparison to the TD group ($p_{group} = 0.001$). Even though this category remained higher in ASD in comparison to TD across all three visits, we found an interaction effect ($p = 0.012$). In the ASD group, the Non-Communicatives decreased from 34.2 to 25.2% after 2 months and further decreased to 22.2% after 4 months, while in the TD group the proportion of this category remained stable and below 13% in all three visits.

DISCUSSION

The results of this study suggest both similarities and differences between toddlers with ASD and TD toddlers at similar lexical levels, regarding word onset, the rate of lexical growth, and the use of words for communicative purposes. As a group, toddlers with ASD reached the milestone of 40–70 words considerably later than TD toddlers. Moreover, the variability was higher in the ASD group as compared to the TD group, supporting previously-published research in other languages (1, 30). While toddlers with ASD were late in producing first words, we found that they accumulated new words at a similar pace to their TD peers. Smith et al. (31) reported that some participants with ASD demonstrated a rapid rate of vocabulary accumulation. Indeed, our results indicate that some toddlers with ASD, once passing an initial barrier, may proceed at a similar pace to their TD peers.

Our detailed analysis of the pragmatic intentions expressed via words revealed that Declaratives, rather than Requests, predominated the verbal productions of both groups. Participants in the two groups used their early words to comment on their surroundings or to name objects more often than to request them. Relatively speaking, the prevalence of Declaratives was higher in the TD group than in the ASD group.

Bearing in mind the difficulties toddlers with ASD have with cooperating in general and in sustaining interactions, we expected to identify a higher frequency of words expressing Objections, but this was not the case; low levels of words expressing Objections were found in both groups. Plumet and Veneziano (14) suggested that not the rate of oppositional episodes but rather the way they were handled distinguishes between children with ASD and language matched TD. A similar frequency of requests was found in both groups. This result is in accord with Paparella's findings (13) regarding similarities between toddlers with ASD and TD toddlers in the frequency of nonverbal requests.

The predominance of Declaratives and the low level of Objections may be the result of the naturalistic nature of the dyadic interactions during which our data was collected.

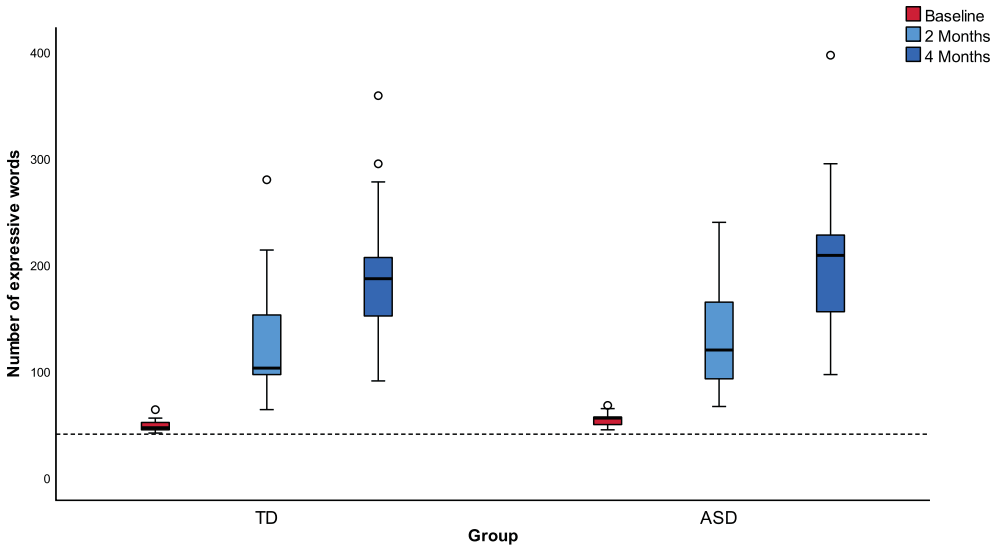

FIGURE 1 | Mean numbers of expressive words at each visit in the two groups (TD and ASD).

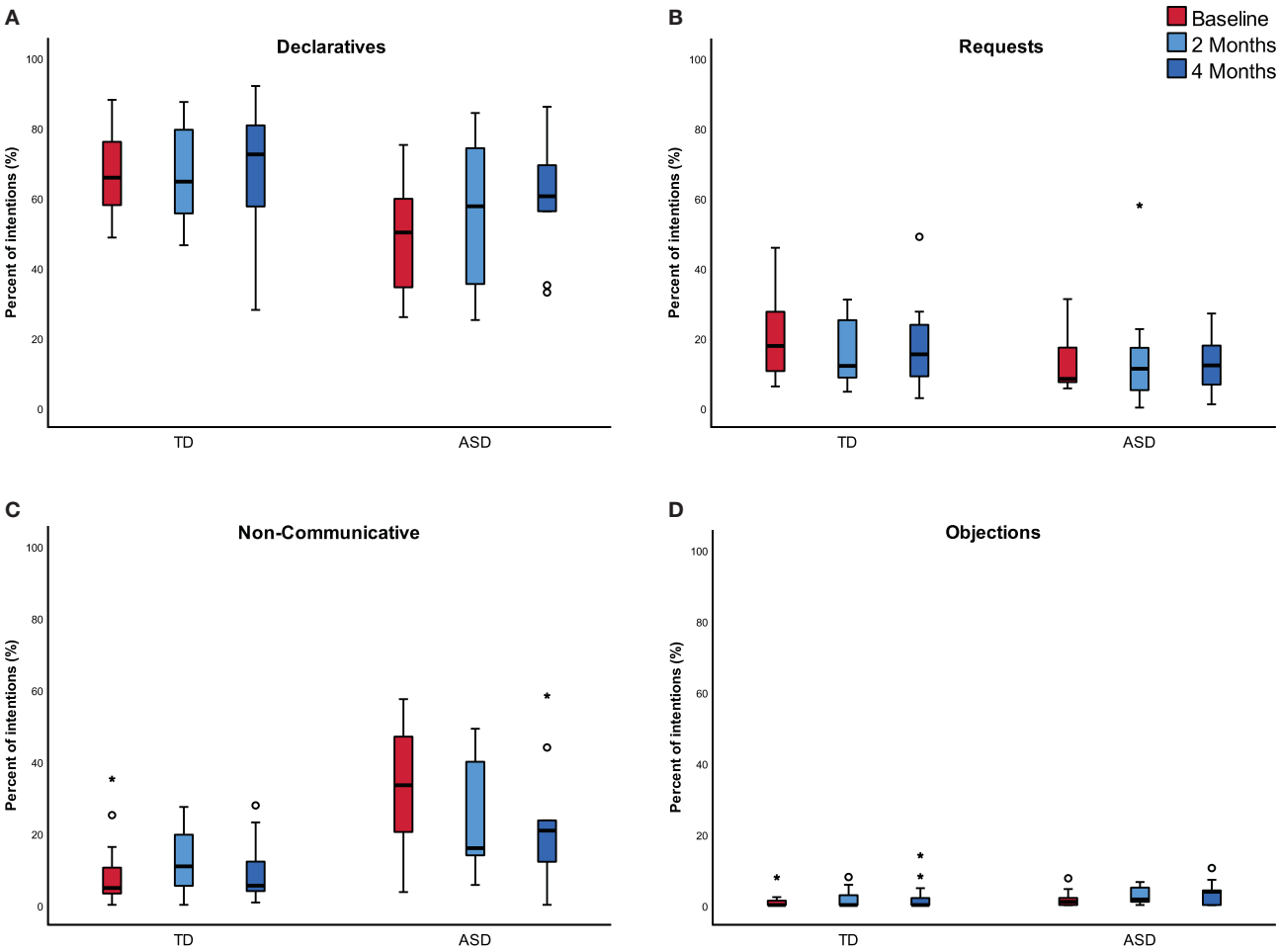

FIGURE 2 | Proportion of communicative intentions in each group across three visits. In **(A)** declaratives, **(B)** requests, **(C)** non-communicative, and in **(D)** objections.

Such style is thought to minimize potential conflicts and lower the need for request since items are available for the child's use. Therefore, it promotes sharing thoughts using Declaratives (32–34).

Alternatively, Requests and Objections, both serving imperative functions, are essentially different than Declaratives. To declare, attaching a label to objects will suffice. Thus, toddlers with ASD who are not necessarily impaired in mapping labels to objects may find the expression of this function relatively easy. However, aversive situations or needs may pose strong cognitive, emotional, and communicative demands, leaving toddlers with ASD unable to verbally express what they want or to ask for the termination of unwanted events. Thus, they may apply nonverbal means or withdraw from the interaction. Highly verbal children with autism have been described to have troubles using behavioral means including speech in diverse contexts (14). In addition, preference toward nonverbal means for resolving conflicts and achieving one's needs is actually rather common at early stages of typical language development, despite having adequate verbal means (35). In other words, the similar proportion of Requests between the two groups may be a byproduct of either the early stage or specifically the tendency of children with ASD to express Requests via nonverbal means. Further studies should explore whether preference toward nonverbal means in toddlers with ASD is restricted to early lexical stages or still characterizes later stages.

Perhaps the most interesting finding regarding the ASD group concerns their prevalent use of speech for non-communicative purposes, a phenomenon characteristic of this population (23). Uttering words with little communicative intention stands in sharp contrast to Objections, Requests, and Declaratives, which are addressed toward another person. As we expected, the proportion of Non- Communicative speech was greater in the ASD group than in the TD group throughout the three visits. While in TD toddlers the level of Non-Communicative speech remained low and steady, in our ASD group we noticed a gradual decline in this category as words accumulated. In other words, the number of productions addressed to the other in the ASD group rose from one visit to the next, though it did not quite reach the corresponding high level of communicative speech that TD toddlers achieved upon study entry.

The finding that TD toddlers use their speech mainly for communication comes as no surprise, as they convey communicative intentions via gestures and vocalizations for a prolonged period of time and so by the time they start uttering words, they are already proficient with the basic functions words may serve. Toddlers with ASD, on the other hand, arrive at a similar lexical point with limited pragmatic abilities and seem to hold on to a self-stimulatory function of speech. Only later, while expanding their active lexicon, do they gradually shift their speech to mainly use it for communicative purposes, though never completely abandoning the self-stimulatory function. Overall, the findings of the present study suggest that single word counts do not suffice when attempting to describe the linguistic profiles of toddlers with autism, as Dromi suggested previously (36). While their lexical acquisition could be described as a simple lag, when we examined the communicative functions of the words, a distinct pragmatic profile rose.

ASD toddlers' ability to develop an active lexicon and expand it is of great interest to language learning theorists. All toddlers face "the mapping mission," trying to attach labels to objects and need to learn the extension range of different words in their native tongue (37, 38). The extent to which this process relies on social-pragmatic cues and whether grasping the pragmatic functions of speech is, indeed, the driving force of language development are still under debate (39). Past research has shown that toddlers with ASD tend to ignore social cues that signal the speaker's intentions while learning new words (7). Our findings add to this literature from the expressive perspective. Despite only having a partial understanding of what speech may be used for, toddlers with ASD were able to obtain an initial expressive lexicon. Such accomplishment may challenge the necessity of a fully intact pragmatic mechanism as a prerequisite to developing speech.

Our study's findings bear interesting clinical implications. First, the elevated levels of Declaratives may indirectly support the notion that the presence of specific pragmatic functions are highly influenced by contextual variables such as interaction style. Thus, whenever increasing the frequency of certain intentions is warranted, manipulating interaction from a directive to a facilitative style should be considered (32, 33).

Second, it should be emphasized that Requests and Objections enable the child to accomplish his needs and control his environment. Lack of appropriate verbal Requests and Objections is often associated with behavioral problems (40, 41). It is possible that some ASD toddlers substitute requesting and objecting by Non-Communicative means, hence the relative paucity of verbal requesting in our study. If further supported, it may highlight the need to teach appropriate requesting and objecting skills (36, 41).

Another clinical implication rises from the emerging ability of a naïve judge to accurately classify early word productions into the main pragmatic categories, with little training and achieving fairly good inter-rater reliability. It is possible that when the classification system is kept simple, referring to the broad categories of intentions, parents as well as other non-professionals may be able to attend to the pragmatic profiles instead of merely focusing on semantic development. With pragmatic deficits being one of autism's hallmarks, such ability seems promising.

LIMITATION OF THE STUDY

The present study can be defined as a pilot study with relatively few participants with ASD. The standardization of our sample was based on their linguistic abilities rather than on their cognitive and severity scores. Future larger scale studies should control for differences in cognition and severity.

Two groups of participants with ASD were under-represented: minimally verbal ASD toddlers (not achieving our word criterion by 48 months) (42) and toddlers with ASD who develop

an active lexicon on time ("Autism language normal") (43). The latter group is often diagnosed at a later age, thus less available to research at such early stages. Further studies are warranted to explore whether those two groups display different early pragmatic profiles than the ones described in the present study.

In the current study we focused solely on verbal productions. Further studies call for analyzing verbal as well as nonverbal communication and the interaction between the two modes.

Since the pragmatic profile may be influenced by interaction variables, we suggest further studies addressing diverse elicitation techniques and contexts.

In the current study, toddlers with ASD were assessed as a group. Future studies may further define the association between an individual pragmatic profile and the rate of progress in expressive words.

CONCLUSION

Our findings provide evidence that early lexical development among ASD toddlers, while delayed, shares both similarities and differences with respect to their pragmatic pattern as compared to TD toddlers. It is worth noting that lexicon may sometimes be achieved despite the lack of complete understanding as to the communicative nature of speech. Possibly among ASD toddlers, such insight is gained only later as their lexicon expands.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Loewenstein Rehabilitation Medical Center Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AO and AM-B planned the study, performed it, analyzed it, and wrote it. ED planned the study, overviewed its performance, analyzed it, and wrote it. SG took part in performing the study, took part in its analysis, and reviewed the manuscript. All authors revised the final manuscript and approved it.

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Diagnosing Autism Spectrum Disorder Without Expertise: A Pilot Study of 5- to 17-Year-Old Individuals Using Gazefinder

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Atypical eye gaze is an established clinical sign in the diagnosis of autism spectrum disorder (ASD). We propose a computerized diagnostic algorithm for ASD, applicable to children and adolescents aged between 5 and 17 years using Gazefinder, a system where a set of devices to capture eye gaze patterns and stimulus movie clips are equipped in a personal computer with a monitor. We enrolled 222 individuals aged 5–17 years at seven research facilities in Japan. Among them, we extracted 39 individuals with ASD without any comorbid neurodevelopmental abnormalities (ASD group), 102 typically developing individuals (TD group), and an independent sample of 24 individuals (the second control group). All participants underwent psychoneurological and diagnostic assessments, including the Autism Diagnostic Observation Schedule, second edition, and an examination with Gazefinder (2 min). To enhance the predictive validity, a best-fit diagnostic algorithm of computationally selected attributes originally extracted from Gazefinder was proposed. The inputs were classified automatically into either ASD or TD groups, based on the attribute values. We cross-validated the algorithm using the leave-one-out method in the ASD and TD groups and tested the predictability in the second control group. The best-fit algorithm showed an area under curve (AUC) of 0.84, and the sensitivity, specificity, and accuracy were 74, 80, and 78%, respectively. The AUC for the cross-validation was 0.74 and that for validation in the second control group was 0.91. We confirmed that the diagnostic performance of the best-fit algorithm is comparable to the diagnostic assessment tools for ASD.

Keywords: autism spectrum disorder, school-age children, adolescent, Gazefinder, machine learning, Japan

INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by atypicality in social communication, and restricted and repetitive behaviors. A recent epidemiological study from Japan reported that the prevalence of ASD is higher than 3% in the general population at the age of 5 years (1). ASD affects the quality of life across the lifespan of the affected individual (2). Various early intervention practices have been developed, some of which are effective and promising (3). Timely intervention is key to better outcomes (4), for which accurate diagnostic assessment is prerequisite.

Regardless of the clinical significance of diagnosis, professionals face challenges inherent to diagnostic assessments of ASD. The first challenge lies in the nature of the diagnosis. As there are no well-established biophysiological diagnostic tests, diagnosis is made solely based on behavioral assessment of children. However, the development of such signs in children is not stable along the time course of diagnosis and can change as they grow. The complexity of social engagement in children generally increases from month to month, especially during a younger age. Furthermore, complex social interactions are affected by the children's cooperativeness, which is further influenced by physical conditions such as hunger and fatigue, as well as their moods and tempers (5–7). Furthermore, no single behavioral sign or trait sufficiently points toward the diagnosis. Therefore, a single diagnostic assessment is usually insufficient to confirm the diagnosis of ASD. To overcome this challenge, standardized tools for diagnosing ASD have been developed, including the Autism Diagnostic Observation Schedule, second edition (ADOS-2) (8) and the Autism Diagnostic Interview-Revised (9), which are widely accepted in research and clinical settings because of their high reliability and validity. However, the use of these tools leads to the second challenge because an interview with these tools followed by the *post-hoc* assessment takes considerable time. Moreover, interviewers need to have the required expertise and undergo training sessions beforehand. Unfortunately, the costs associated with the use of the above methods have restricted the clinical availability of the tools. A recent Australian study reported that only a small proportion of children were assessed using these tools when parents raised concerns over the possibility of their children having ASD, and a "wait-and-see" approach was advised instead (10). This has likely happened in Japan as well, where only 32% of children confirmed to have a diagnosis of ASD at 5 years had had a history of clinical diagnosis of ASD until the fifth birthday (1). Many children with ASD are left undiagnosed and are not provided appropriate interventions even at school age.

Owing to these two challenges that clinicians face in the diagnostic assessment of ASD, models that balance the quality and accuracy of assessment with timeliness and ease are desired (11). To meet this demand, biological/physiological biomarkers have been tested in several studies.

Among them, atypical eye gaze patterns in children with ASD have been tested to determine whether they can serve as candidate markers. Recent advances in eye gaze studies rely heavily not only on advances in technology, but also on the fact that eye gaze patterns reflect both biological and behavioral aspects of ASD (11). Eye gaze patterns are under genetic control (12), and a lack of eye gaze onto human faces measured by eye-tracking devices can be a reflection of lack of eye contact with the examiner, a well-known behavioral diagnostic marker of ASD (13). A recent systematic review pointed out that the effect size resulting from the atypicality of eye gaze patterns in individuals with ASD can have standard deviations (SDs) as large as 0.5 (14, 15). The most consistent finding in these review articles is that individuals with ASD spend looking at the non-social stimuli for longer durations than at the social stimuli, human faces in particular (*social paradigm*); the contrast was independent of age, sex, intelligence quotient (IQ), and other conditions. This is consistent with the social processing theory of ASD (14). This atypical eye gaze pattern was more specifically tested in a preferential viewing paradigm, in which individuals, particularly young children with ASD stare preferentially at geometric figures than at human figures (*preferential paradigm*) (16, 17).

Considering the relative ease of measurement and the biophysiological significance of eye gaze, the atypical eye gaze patterns measured with automated eye-tracking devices can serve as diagnostic markers. In preliminary attempts involving young children, the diagnostic validity and test-retest reliability of eye gaze measurements have been supported (16, 17). However, to the best of our knowledge, no study has tested individuals with ASD in a wide age range. The lack of knowledge is particularly evident among school-aged children and adolescents. Furthermore, previous studies have used eye-tracking devices not specifically developed for individuals with ASD. Since some individuals, especially of young ages, cannot cooperate in keeping their eyes on the monitor, the quickest calibration without any intentional cooperation of the child and minimal duration of stimulus movies should be implemented to validate these attempts in clinical settings. To address this, we have attempted to establish specific eye gaze patterns as a biophysiological markers predicting the diagnosis of ASD, using an eye-tracking system called "Gazefinder" in a broad age range of study participants (18, 19). Novel devices and software were designed, including a calibration movie (5–7 s) and stimulus movies. In the stimulus movies, two paradigms were adopted to test the diagnostic predictability of ASD: the social paradigm and the preferential paradigm. The application of Gazefinder to children does not require any expertise, and it takes <2 min to obtain an output (18, 19). Thus, this system is anticipated to fulfill current demands in ASD diagnosis.

In this study, we propose a computerized diagnostic algorithm for ASD using Gazefinder, implemented with social and preferential paradigms in individuals aged 5–17 years. To realize this, we conducted a multisite study to create a computerized best-fit diagnostic algorithm with satisfactory sensitivity and specificity, and validated it in two ways.

METHODS

Participants

Two hundred and twenty-two individuals aged between 5 and 17 years were enrolled by physicians at seven research

sites and affiliated clinics at Hamamatsu University School of Medicine, Hirosaki University, University of Fukui, Chiba University, Saga University, Kanazawa University, and Tottori University during a 6-month period beginning on 25 February 2018. The seven university clinics are located in small or middle-sized cities and metropolitan areas throughout Japan. All clinics play pivotal roles in providing services for children and adolescents with developmental disabilities in the context of child and adolescent psychiatry and/or pediatric neurology. The reasons for enrolling the participating individuals were as follows: (1) They were previously suspected by psychologists, speech therapists, physicians, or school teachers as having ASD, including "autism" and "Asperger disorder, or (2) they self-nominated to participate in response to the web-based advertisement and have never been suspected to have developmental disorders such as ASD, attention-deficit hyperactivity disorder (ADHD), and learning disabilities. All the participating individuals and their parents were of Japanese ethnicity.

All the legal guardians (i.e., parents in this study) of the participants provided written informed consent, and the participating individuals provided informed assent orally. The study protocol was approved by the ethics committees of the seven research sites and conformed to the tenets of the Declaration of Helsinki.

Measurement

Clinical Evaluation, Screening, and Diagnosis

The initial clinical evaluation by a board-certified psychiatrist or pediatrician included face-to-face behavioral assessment and collection of the developmental history, physical morbidity, and history of medication. This clinical evaluation was followed by screening for ASD using the Pervasive Developmental Disorders Autism Society Japan Rating Scale [PARS, a questionnaire for parents, 57 items (20)], the Strength and Difficulty Questionnaire [SDQ, a questionnaire for parents, 25 items (21)], and the Social Responsiveness Scale in Japanese, second version [SRS-2, a questionnaire for parents, 65 items (22)]. ADHD was screened using the ADHD Rating Scale [ADHD-RS, a questionnaire for parents, 18 items (23)]. General cognitive ability was assessed as indexed by the IQ with the Wechsler Intelligence Scale for Children-fourth edition (WISC-IV) for 215 (97%) individuals, or with the Tanaka-Binet test (Japanese version of the Stanford-Binet Test) for four individuals (2%), or as indexed by developmental quotient (DQ) with the Kyoto Scale of Psychological Development by trained clinical psychologists for three individuals (1%), depending on the participants' mental age. An IQ or DQ lower than 70 was defined as general cognitive delay. An IQ of lower than 70 in WISC-IV is an indication of 2 SD below the population average. The comparability of the IQs derived from the Tanaka-Binet test was tested with the IQ derived from WISC-III, the prior version of WISC-IV (24), and the comparability of the DQs derived from the Kyoto Scale of Psychological Development was tested with the Tanaka-Binet IQ (25).

After the screening tests and assessment of general cognitive abilities, individuals exhibiting positive results to any one of the three screening tests for ASD (PARS, SDQ, SRS-2) were assessed to have a diagnosis of ASD using the ADOS-2 (a semi-structured, play-based observational assessment tool involving interaction with the child and observation of the activities proposed to the child, 11–16 diagnostic algorithm items depending on module) (8), or the Autism Diagnostic Interview-Revised-Japanese Version (ADI-R-JV, a semi-structured interview for parents, 93 items) (9) conducted by trained clinical researchers. In addition, all the participants were assessed to have a diagnosis of ADHD using Conners 3 Japanese version (Conners 3, a questionnaire for parents, 108 items) (26). We did not use both ADOS-2 and ADI-R-JV in our study because of the limited availability of examiners for these tools who had established research reliability with the developers of the instruments. Among 102 individuals with a positive screening result, 81 individuals were assessed only with ADOS-2, nine individuals were assessed only with ADI-R-JV, and 12 individuals were assessed with both ADOS-2 and ADI-R-JV. The remaining 120 individuals were not assessed with ADOS-2 or ADI-R-JV because of negative results in the screening tests.

Apparatus

Gazefinder, a system in which a set of devices to capture eye gaze patterns and stimulating movie clips are equipped in a personal computer (PC) with a 19-inch monitor (1280 × 1024 pixels), manufactured by JVC Kenwood Co. Ltd. (Yokohama, Japan), was chosen to measure eye gaze patterns. The technical features of this system have been described in previous studies (18, 19, 27, 28). In brief, corneal reflection techniques enable the device to calculate eye gaze positions on the PC monitor as (X, Y) coordinates in pixel units, at a frequency of 50 Hz (i.e., 3,000 eye gaze positions detected per minute). Using the device, the (X, Y) coordinates provide information regarding where a participating child exactly looked at in the movie clip every 1/50 s when the movie clips were shown to the child on the monitor. The participants were asked to sit in front of the monitor to retain the distance between the face and the monitor at approximately 60 cm. Before the diagnostic measurement of the eye gaze patterns, the calibration of the eye gaze position started automatically and took 5–7 s to complete. The calibration was judged as successful if the child looked at no less than three of the five regions of the calibration movie clip. Otherwise, the calibration movie clip restarted. This movie clip contains a black background with a circular region covered with animated animals, moving from the center to the four corners of the monitor consecutively. After the calibration, 12 short movie clips were automatically presented as experimental stimuli in a fixed order. Between the movie clips, we inserted short attention-grabbing movie clips (2.0 s) to set the eye gaze position at the center of the monitor, prior to the stimuli presentation appearing next. This insertion also canceled out the post-images of the movie clip shown previously. The total time of the movie sequence was 95 s, including time for non-stimulus movie clips.

Movie Clips as Stimuli

Figure 1A presents the 12 movie clips used as stimuli. The social paradigm represented in three movie clips were as follows: a

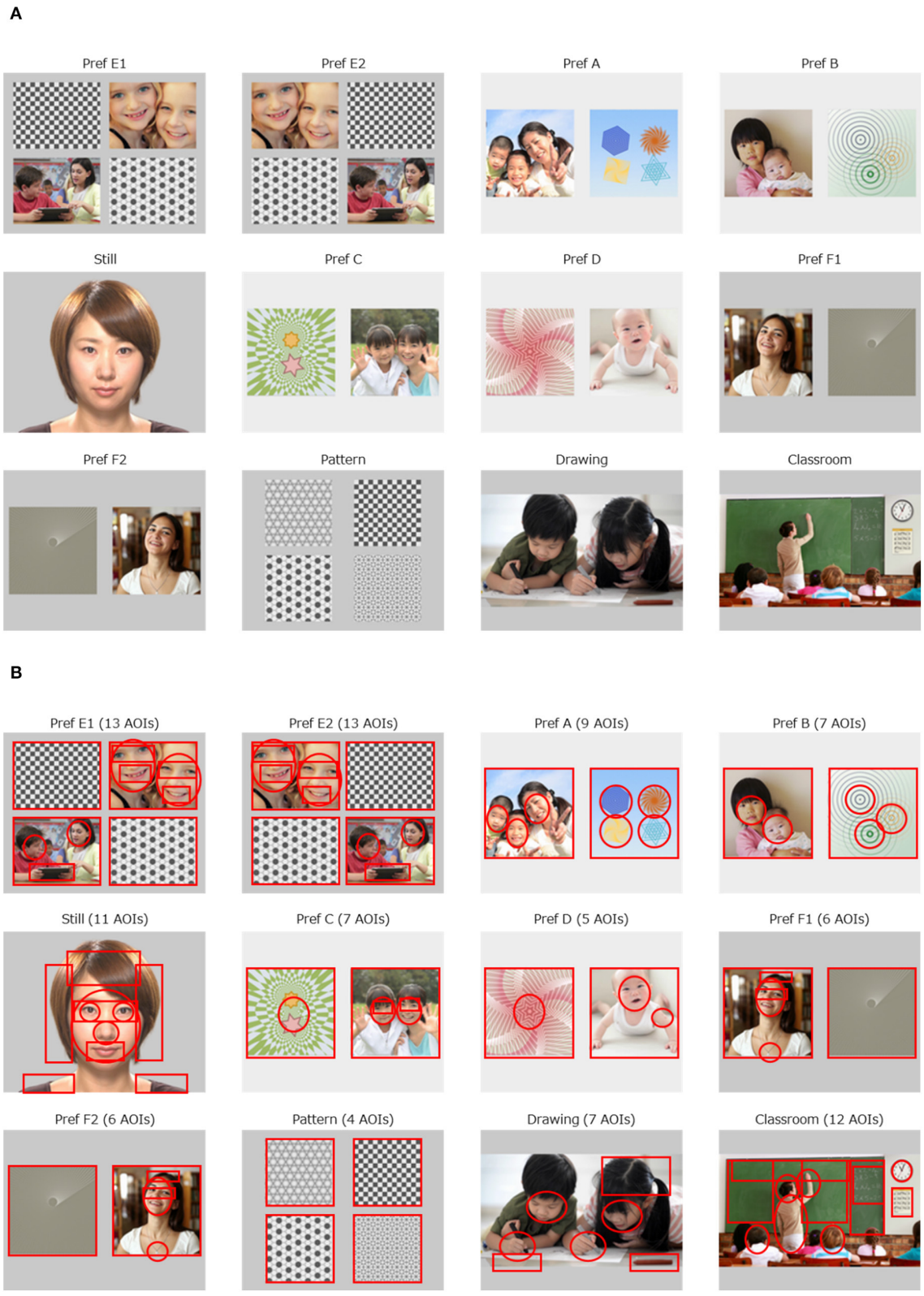

FIGURE 1 | Movie clips implemented with Gazefinder, and the AOIs. **(A)** The 12 movie clips. **(B)** 100 AOIs embedded in 12 movie clips.

still face of a young amateur actress ("Still," 4.5 s), two children drawing pictures cooperatively ("Drawing," 7.0 s), and a teacher teaching a math class in a classroom ("Classroom," 11.0 s). The preferential paradigm was represented in nine movie clips, in which the visual field on the monitor was divided into two or four regions of equal size, with human figures or objects in parallel. The preferential paradigm movie clips consisted of one movie clip of four graphical patterns ("Pattern," 7.0 s), and of eight movie clips with human figures with geometric patterns aligned side by side ("Pref A," 5.0 s, "Pref B," 4.5 s; "Pref C," 5.0 s, "Pref D," 4.5 s, "Pref E1," 5.0 s, "Pref E2," 4.5 s, "Pref F1," 6.0 s, "Pref F2," 5.5 s). The duration of these movie clips was set identical to that used in our previous studies. The remaining movie clips were kept as short as possible while preserving their context.

The rationale for creating the movie clips was as follows. As for the social paradigm, there is sufficient evidence to support the fact that a decreased gaze fixation on human faces, especially on eye regions on the monitor is associated with a diagnosis of ASD, regardless of age or IQ (14, 29). We have previously reported that fixation onto the eye region decreases in individuals with ASD at ages of 10 years and older (18). In addition, a decreased amount of gaze fixation onto human figures in social scenes has been reported consistently in individuals with ASD (14). Furthermore, it has been suggested that the quantity of the social content (e.g., the number of human figures presented in the scene) as well as its quality (e.g., human interaction) is related to the amount of reduction in gaze fixation in individuals with ASD (14, 15). Thus, the duration of gaze fixation is measured to contribute to the likelihood of ASD diagnosis, probably in accordance with the number of human figures (2 in "Drawing" and 8 in "Classroom"). Therefore, we presented two children drawing pictures while interacting in the movie clip "Drawing". As for the preferential paradigm, we have previously confirmed that human figures were more preferentially looked at than non-social figures in typically developing individuals compared to individuals with ASD (18). To control the spatial preference (e.g., adherence to the right half of the visual field) that may be present in some participants with ASD (30), two sets of movie clips were duplicated ("Pref E1" vs. "Pref E2," "Pref F1" vs. "Pref F2"), but the allocation of the targets (human figures) were exchanged horizontally, and inserted as different movie clips. In the other four sets of movies, the appearance of the side (left or right) of the target (human figures) was balanced.

Quantification of Eye Gaze Patterns

Through the sequence of the 12 movie clips, we defined 100 areas of interest (AOIs) in circles or squares on each move clip, specified with x and y axes on the monitor (**Figure 1B**). We calculated two types of eye gaze indices: the AOI rate score and the AOI count score. The AOI rate score was defined as the percentage of gaze fixation time allocated to each AOI divided by the duration of each movie clip. The AOI rate score was between 0.0 and 1.0 and represents the focus on the object in a dose-response manner (i.e., the higher the AOI rate score, the more intensively the child focused on the object). The AOI count score was a representation of the presence (or absence) of a fixed gaze over each AOI, regardless of the duration of the eye gaze. The possible AOI count score was 0 or 1 and reflects the presence of eye gaze on the AOI, irrespective of possible distractions occurring due to the child focusing on another AOI because of knowledge-driven prediction (31). This is likely to emerge among older individuals. This distraction also occurs when a human agent on the monitor acts as if she/he looked at the individual in front of the monitor (32). As such, we expected that the AOI count score would be more suitable for older individuals. We calculated both the AOI rate scores and AOI count scores separately for all 100 AOIs. In addition, we also calculated two different methods for AOI rate scores applied to the 100 AOIs. The first is to calculate the AOI rate scores of the first 1.0 s and the other is to calculate the AOI rate scores of the first 2.0 s. The intention for this was to generate more attributes with diagnostic value. Specifically, young individuals with ASD have been reported to pay less attention to faces during the initial viewing period (33). As a result, 300 sets of calculation for AOI rate scores and 100 sets of calculation for AOI count scores were applied.

Analysis

Strategy for Creating and Validating the Diagnostic Algorithm

Selection of Participants

We selected participants to generate a training dataset to realize the computerized diagnostic algorithm, and an independent dataset for the validation. We first excluded 57 participants from the following analyses because they had a diagnosis of ADHD ($N = 29$), general cognitive delay ($N = 24$), or a clinical diagnosis of epilepsy ($N = 4$). The intention was to minimize the neurophysiological heterogeneity of the subjects included in the dataset, except for the difference between ASD and typical development (TD). The remaining 165 participants were divided into three groups: ASD, TD, and the second control group. The ASD group ($N = 39$) consisted of individuals with a diagnosis of ASD confirmed with ADOS-2 or ADI-R and with a negative screening result for ADHD. The TD group ($N = 102$) consisted of individuals fulfilling negative screening results for both ASD and ADHD. The ASD and TD groups were primarily used as the source of the best-fit computerized diagnostic algorithm and the training and test datasets to check the validity of the best-fit diagnostic algorithm. The second control group consisted of two types of individuals: (1) those with a diagnosis of ASD with a positive screening result for ADHD, and (2) those without a confirmed diagnosis of ASD but with a positive screening result for ASD (the screening result for ADHD can be either positive or negative). The second control group served as an independent sample to validate the diagnostic predictability of the best-fit diagnostic algorithm.

We further divided the ASD and TD groups according to age. Although the social paradigm was assumed to be age-independent (14), the preferential paradigm was reported to distinguish ASD from TD individuals, particularly when the subjects were 10 years and older (18). Considering these, both the ASD and TD groups were divided into younger (<10 years) and older (10 years and older) groups, respectively. We set the age of 10 as the breaking point as in the previous study (18) and

because of the statistical reason that 10 was the median age of the individuals in the ASD and TD groups.

Extracting Indices (Candidate Attributes)

We calculated the 300 sets of AOI rate scores and 100 sets of AOI count scores for all participating individuals. To extract indices to be included in the best-estimate diagnostic algorithm, the mean values for both AOI rate scores and AOI count scores were compared between the ASD and TD groups for the younger and older age bands. When we found indices that were significantly ($p < 0.05$) associated with the ASD diagnosis or had an effect size (Cohen's *d*) of 0.5 or larger, we retained these as *candidate attributes*, the indices to be included in the next step. To this end, we extracted four sets of candidate attributes based on the AOI rate scores and count scores of the younger and older individuals. To minimize unnecessary weights and to avoid overfitting resulting from choosing multiple AOIs out of one region on a movie clip, we chose only one attribute with the largest effect size out of the candidate attributes that were calculated on the same AOI. This rule also applies to the three AOI rate scores that share the same AOI (the AOI rate scores calculated from the first 1.0 s, from the first 2.0 s, and from the beginning to the end of the movie clip). In addition, to avoid including chance findings with large effect size, we dropped candidate attributes that were extracted from the AOIs with gaze fixation percentage of <20%, which corresponds roughly to a duration of 1.0 s or longer for most movie clips.

Creating the Best-Fit Diagnostic Algorithm

We first created four diagnostic algorithms, Algo #1 (the AOI rate scores for the younger individuals), #2 (the AOI rate scores for the older individuals), #3 (the AOI count scores for the younger individuals), and #4 (the AOI count scores for the older individuals), based on the four sets of candidate attributes combined (**Figure 2**). For each algorithm, the candidate attributes were either divided by the standard deviation, or dichotomized to 0 or 1 and summed, followed by a division by the number of the candidate attributes. These

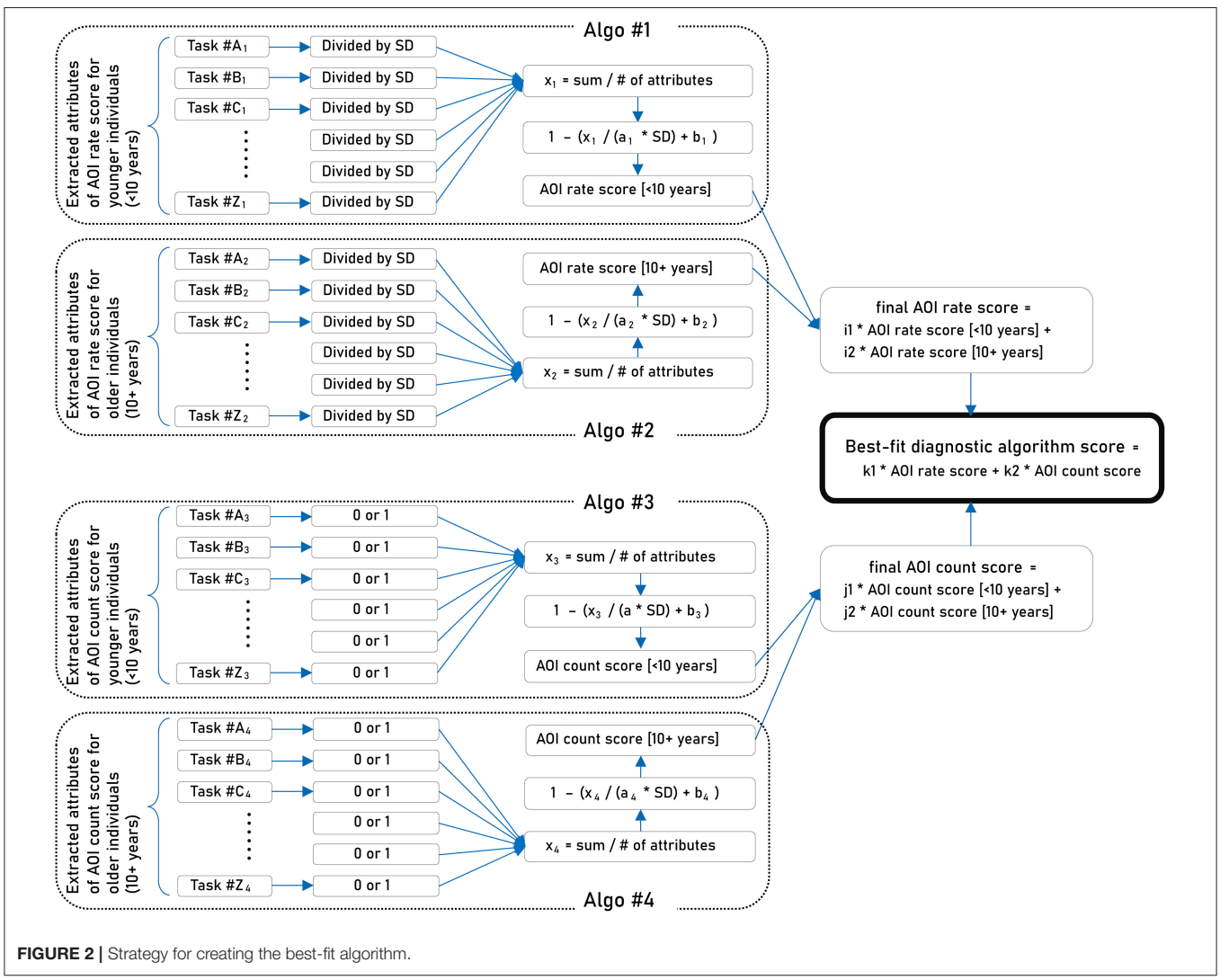

FIGURE 2 | Strategy for creating the best-fit algorithm.

values were fit for a sigmoid function that only took a value between 0 and 1. The next step was to merge Algo #1 and #2 to estimate the final AOI rate score, and to merge Algo #3 and #4 to estimate the final AOI count score. To merge two sigmoid functions, coefficients were estimated automatically to maximize the predictability of ASD diagnosis.

Next, we finalized the best-fit diagnostic algorithm using the two separately estimated algorithm-based scores: the final AOI rate score and the final AOI count score. Before merging the two scores, we chose one algorithm score of a better fit in the younger and older participants separately, to maximize the predictive validity. The fit was assessed with the area under the receiver operating characteristic (ROC) curve (AUC) for each set of comparisons. We then made the selected estimated scores merge smoothly as a continuum along the age range of the participants of 5–17 years (the best-fit algorithm). To merge the two sigmoid functions, coefficients were again estimated automatically to maximize the predictability of the ASD diagnosis. The best-fit algorithm score was made to take values between 0 and 1. In the following analyses, a value of 0.5 or higher of the best-fit algorithm score was assumed as an indicator of the individual under investigation having ASD.

Evaluation of the Diagnostic Performance (Cross-Validation)

We evaluated the general classification performance of the best-fit algorithm using the leave-one-out (LOO) method (34). We repeated the procedures described above, including extraction of candidate attributes and formulation of four separate algorithms to be merged into a single best-fit diagnostic algorithm, without the inclusion of one specific individual (LOO algorithm). The removed individual was tested for ASD based on the LOO algorithm. This procedure was iterated for all participating individuals ($N = 141$; cross-validation). We then drew the ROC curves and calculated AUCs for both the best-fit diagnostic algorithm, and votes of the 141 LOO algorithms were used to interpret whether the validity of the best-fit algorithm might have been compromised. To simplify the interpretation, we also calculated the sensitivity, specificity, and accuracy for the best-fit algorithm and for the votes of 141 LOO algorithms separately. The point where the sensitivity and specificity were extracted was set at the Youden J index (i.e., sensitivity + specificity – 1) was maximized (35).

Evaluation of the Diagnostic Performance in an Independent Sample

Using the second control group ($N = 24$), we checked the diagnostic validity of the best-fit algorithm independently. Since this group consisted of individuals with ASD with a positive screening result for ADHD ($N = 17$) and individuals with no diagnosis of ASD but with a positive screening result for ASD ($N = 7$), we applied the best-fit algorithm and checked whether the diagnosis predicted with the best-fit algorithm score complied with the real diagnosis using AUC, sensitivity, specificity, and accuracy.

Statistics

All statistical analyses were conducted using Stata version 15.1 and R version 3.6.2. To calculate AUC values with 95% confidence intervals, ROC-kit 0.91 was used for resampling. For comparison of two continuous variables, we carried out either the *t*-test or the Kruskal-Wallis test, depending on the distribution. To avoid missing any potential candidate attributes at the early stage of the analyses, we set 0.05 as the significance level.

TABLE 1 | Characteristics of the participants.

	TD	ASD	Second control	Statistics*
Number of subjects	102	39	24	
Age in years, mean (SD)	9.5 (4.0)	10.3 (4.0)	10.4 (3.6)	$F_{(2,162)} = 0.86$, $p = 0.42$
Male sex, number (%)	43 (42%)	30 (77%)	15 (63%)	$\chi^2_{(2)}$=14.6, $p = 0.001$
IQ/DQ, mean (SD)	104.1 (13.6)	94.5 (12.3)	98.7 (14.4)	$F_{(2,162)} = 7.64$, $p < 0.001$ TD>ASD
ASD screening [PARS total], mean (SD)	0.8 (1.1)	7.8 (5.2)	8.0 (7.0)	$F_{(2,162)} = 70.6$, $p < 0.001$ TD<ASD, TD<Second control
ADHD screening [ADHD-RS inattention], mean (SD)	3.3 (3.5)	7.9 (4.8)	11.7 (6.7)	$F_{(2,162)} = 42.1$, $p < 0.001$ TD<ASD, TD<Second control ASD<Second control
ADHD screening [ADHD-RS hyperactivity], mean (SD)	1.9 (3.0)	5.2 (4.4)	7.9 (6.2)	$F_{(2,162)} = 26.6$, $p < 0.001$ TD<ASD, TD<Second control ASD<Second control
Diagnosed as having ASD, number (%)	0 (0%)	39 (100%)	17 (71%)	$\chi^2_{(2)} = 142.9$, $p < 0.001$
Overall gaze fixation percentage, mean (SD)	92.1 (7.3)	89.0 (10.2)	91.2 (8.1)	$F_{(2,162)} = 2.24$, $p = 0.11$

**Statistically significant results after one-way ANOVA (F tests) were followed by group comparison with Bonferroni correction.*

RESULTS

Characteristics of the Participating Individuals

Table 1 shows the demographic and clinical characteristics of the participants. There was no significant difference in the mean age of the participants among the groups. Compared with the TD group, the ASD group showed significantly lower IQ and higher scores on the ASD screening scale (PARS total) and ADHD screening scales (inattention and hyperactivity subscales of ADHD-RS).

Overall Gaze Fixation Percentage (Success of Data Retrieval)

The bottom row of **Table 1** shows that the overall gaze fixation percentage values during the measurement using Gazefinder were not statistically different across the groups (92% in the TD group, 89% in the ASD group, 91% in the second control group). The lowest value was 47.4% of a child belonging to the ASD group, but this was the only record below 60%. Out of the 165 participants, 161 showed a value of 70% or higher.

Extracting Indices (Candidate Attributes)

As for the first steps to create the best-fit diagnostic algorithm, we extracted the four sets of the candidate attributes to be used in the algorithm. The candidate attributes are shown on the corresponding AOIs in association with the AOI rate scores in **Supplementary Figures 1**, **2** for the younger and older individuals, respectively, and in association with the AOI count scores in **Supplementary Figures 3**, **4** in the younger and older individuals, respectively.

TABLE 2 | Calculated values of the area under curve (AUC) and their 95% confidence intervals (CIs) for the proposed algorithms for younger and older participants using either AOI rate scores or AOI count scores.

	Subjects to be tested	AUC	95% CI
Algo #1: AOI rate score for the younger individuals	Younger individuals ($n = 80$)	0.83	0.72–0.90
Algo #2: AOI rate score for the older individuals	Older individuals ($n = 61$)	0.83	0.72–0.92
Final AOI rate score algorithm	Younger individuals ($n = 80$)	0.82	0.70–0.90
Final AOI rate score algorithm	Older individuals ($n = 61$)	0.82	0.69–0.92
Algo #3: AOI count score for the younger individuals	Younger individuals ($n = 80$)	0.74	0.63–0.83
Algo #4: AOI count score for the older individuals	Older individuals ($n = 61$)	0.88	0.78–0.95
Final AOI count score algorithm	Younger individuals ($n = 80$)	0.75	0.63–0.85
Final AOI count score algorithm	Older individuals ($n = 61$)	0.87	0.72–0.95

Creating the Best-Fit Diagnostic Algorithm

Table 2 shows the AUCs for Algo #1, 2, 3, 4, the final AOI rate score algorithms, and the final AOI count score algorithms. To select one algorithm for the two age bands, we found that the final AOI rate score algorithm fit equally for the younger and older individuals (0.82 vs. 0.82) and that the final AOI count score algorithm showed a better fit for the older individuals (0.75 vs. 0.87). For the younger individuals, we selected the final AOI rate score algorithm, and for the older individuals, we selected the final AOI count score algorithm. Merging the two algorithms along the age bands provided the best-fit diagnostic algorithm, of which the ROC curve is shown as a solid line in **Figure 3**, and the AUC was 0.84, as shown in the first row of **Table 3**. The sensitivity, specificity, and accuracy were 74, 80, and 78%, respectively. We also checked whether the best-fit diagnostic algorithm showed a good fit for the younger and older participants. The second and third rows of **Table 3** show that the AUC, sensitivity, specificity, and accuracy did not differ between the younger and older participants.

Evaluation of Diagnostic Performance

To show that the high accuracy of the best-fit diagnostic algorithm did not result from overfitting or from chance alone, the diagnostic performance of the best-fit algorithm was first

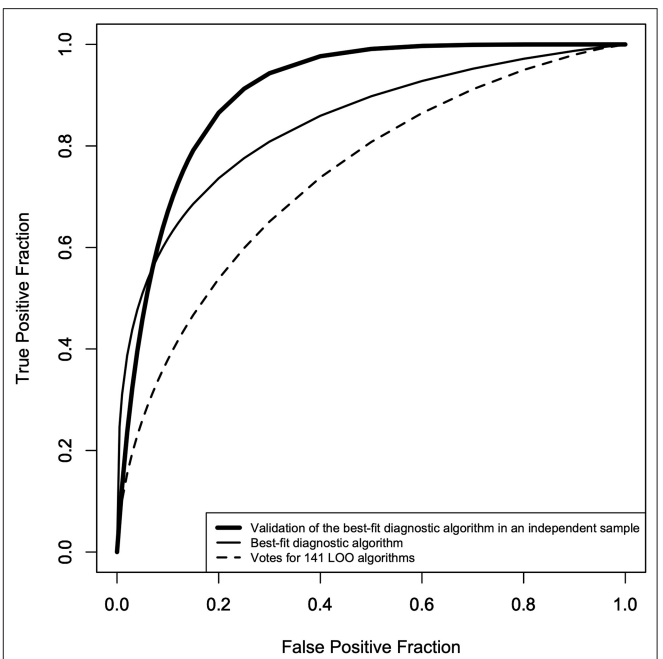

FIGURE 3 | ROC curves of the best-fit diagnostic algorithm* and the votes of the 141 LOO** algorithms. Solid line: ROC curve of the best-fit diagnostic algorithm (AUC = 0.84, sensitivity = 71%, specificity = 80%, accuracy = 78%), dotted line: ROC curve of the votes of the 141 LOO algorithms (AUC = 0.74, sensitivity = 65%, specificity = 70%, accuracy = 67%), thick line: ROC curve of the best-fit diagnostic algorithm in an independent sample (second control group: AUC = 0.91, sensitivity = 87%, specificity = 80%, accuracy = 88%). *The merged algorithm of the final AOI rate score algorithm for age <10 years, and the final AOI count score algorithm for 10 years and over. **Leave-one-out method to cross-validate the best-fit diagnostic algorithm.

assessed using the LOO method, the result of which is shown as a dotted line in **Figure 3** and in the fourth row of **Table 3**. The AUC was 0.74, which was smaller than the AUC of the best-fit algorithm, and the sensitivity and specificity were 65 and 70%, respectively. We also checked the diagnostic performance of the best-fit diagnostic algorithm in an independent sample (the second control group, $N = 24$). For the 24 participants in this group, we found an AUC of 0.91, with a sensitivity and specificity of 87 and 80%, respectively.

DISCUSSION

Using Gazefinder, we successfully created the best-fit diagnostic algorithm to discriminate school-aged and adolescent individuals with ASD from typically developing individuals of the same age range, with a sensitivity of 78% and specificity of 80%. The diagnostic performance was tested in two ways: one was a machine-learning procedure called the LOO method and the other was a test in a different, independent sample of the same age range. These two tests of diagnostic performance indicated acceptable to excellent discriminability.

The reported sensitivity and specificity of ADOS-2 (Module 3) were as high as 91 and 66%, respectively, in a large sample of German children with an average age of 10 (36). Similarly, the sensitivity and specificity of ADI-R in a small sample of Japanese children of age 5–9 were 92 and 84%, respectively, and of age 10–19 were 97 and 90%, respectively (9). The diagnostic accuracy of Gazefinder was not better than that of the standard diagnostic tools, but comparable. In addition, the diagnostic validity of Gazefinder was even better than the established screening tools that are available for a wide range of ages. For instance, the Social Communication Questionnaire (37) is a widely used tool available for a wide age range, although the sensitivity and specificity were 64 and 72%, respectively, in a sample of 1–25-year-old individuals (38). There are also a number of screening tools with reported sensitivities and specificities exceeding 80%, although these figures have not been cross-validated or tested in different datasets (39). Considering the applicability of the best-fit algorithm to a wide age range of the subjects, our validated data support the use of the best-fit algorithm in clinical settings as an available alternative to a range of screening tools for detecting ASD, particularly in terms of diagnostic performance.

In addition to the fact that the diagnostic evaluation using Gazefinder was completed within 2 min without any expertise, it is worth noting that a substantial majority of the participating individuals succeeded in completing the examination. Surprisingly, 161 (98%) of the 165 participants kept staring at the monitor for 70% or longer of the total duration of the examination. The four individuals with <70% of the total fixation time were diagnosed as having ASD. Among these four individuals, only two individuals were correctly predicted as having ASD (data not shown in tables). Apparently, the diagnostic accuracy may be decreased if we include individuals with <70% of the overall gaze fixation percentage. One explanation for this observation is that the lower overall gaze fixation percentage by itself may be predictive of ASD. This leads to an understanding that the accuracy of the best-fit algorithm might have been compromised when the overall gaze fixation percentage was lower than the specific cutoff, for example, 70%. Although the overall accuracy of the best-fit diagnostic algorithm was secured, we may set a threshold of 70% as the lowest percentage for securing algorithm-based diagnosis for clinical use, until firm conclusions are drawn.

Discussion of the Methodology

There was an initial possibility of potential overfitting due to the limited sample size in our results. This has been discussed in the context where the attributes outnumber the observations in machine-learning-assisted neuroimaging studies (40). We have made several attempts to overcome this shortcoming. First, to enhance the efficiency in creating a valid algorithm, we tried to increase the clinical homogeneity among the diagnostic groups. We extracted participants with ASD without any comorbid conditions and TD participants without any suspicions of ASD symptomatology. Second, we avoided building a multi-layered algorithm. Until recently, neural networks, and their applications such as in deep learning, the prominent feature of which is a combination of perceptrons aligned in multiple layers, has been used widely in literature. The major problem inherent to these techniques is overfitting, particularly if the sample size is small, when the network cannot learn itself (41). Therefore, we adopted a single-layered algorithm. Third, we adopted cross-validation using the LOO method (34). Cross-validation is required not only for checking the predictive validity, but also for achieving optimal

TABLE 3 | Diagnostic performance of the best-fit algorithm.

	Subjects to be tested	AUC	95%CI	Sensitivity**	Specificity**	Accuracy**
Best-fit algorithm	All ($n = 141$)	0.84	0.76–0.91	0.74	0.80	0.78
Best-fit algorithm	Younger individuals ($n = 80$)	0.82	0.70–0.90	0.78	0.70	0.76
Best-fit algorithm	Older individuals ($n = 61$)	0.87	0.72–0.96	0.73	0.87	0.75
Votes for 141 LOO algorithms*	All ($n = 141$)	0.74	0.64–0.82	0.65	0.70	0.67
Best-fit algorithm	Second control group ($n = 24$)	0.91	0.66–0.99	0.87	0.80	0.88

**Leave-one-out (LOO) algorithm: an algorithm developed with a procedure identical to that applied to develop the best-fit algorithm without the inclusion of one specific individual (LOO algorithms). The removed individual was tested for the diagnosis of ASD, based on each LOO algorithm. This procedure was iterated for all participating individuals, and the majority of the votes for the 141 LOO algorithm was used as the cross-validated predicted diagnosis (N = 141; cross-validation).*

***Sensitivity and specificity were calculated at the point on the ROC where the Youden J index (Sensitivity + Specificity – 1) was maximized.*

diagnostic performance (42). LOO is assumed to perform better than other cross-validation methods because the test data are secured not to be used in the training data to form an algorithm. Furthermore, we used a different, independent dataset (the second control group) to be tested with the best-fit diagnostic algorithm. It is worth noting that the independent second control group was a mixture of individuals with ASD with clinical signs of ADHD, and non-TD individuals with subthreshold signs of either ASD or ADHD or both. However, the diagnostic performance of this sample was not compromised. To this end, our validation processes have supported the robust predictive validity of the proposed best-fit diagnostic algorithm.

Limitations

Despite the fact that the predictive validity of the best-fit diagnostic algorithm was established, potential limitations of the findings should be acknowledged. First, we enrolled a relatively small sample of individuals of Japanese origin. Our stimulus movie was created to be used among non-Japanese people as well, and included actors of various ethnicities. Our findings may be better replicated in a larger sample with different cultural and biological settings. Second, we invented the diagnostic algorithm based on responsivity to social stimuli; however, this is only one aspect of the broad behavioral spectrum of ASD. Particularly, we have not established that our measure reflects the symptoms of restricted interest and repetitive behaviors (RRBs). Furthermore, the predicted diagnosis does not indicate the severity of the symptoms, as the diagnostic algorithm has been designed to monitor whether responsivity to specific stimuli was observed or not. Thus, Gazefinder has immense possibility for customization in the future. Third, we did not investigate whether the indices we collected were associated with clinical correlates, severity, or prognosis. In a previous study using eye-tracking devices, children with ASD who were more oriented toward social images were shown to have better language and higher IQ scores (16). The clinical applicability of Gazefinder can be further developed in this direction in the context of treatment monitoring. Fourth, we did not assess social anxiety symptoms. In a previous study, gaze avoidance was reported in adolescents with either social anxiety disorder or ASD, but delayed orientation to the eye regions was observed in the latter. We did not examine whether the reduced gaze fixation to the eye regions results from delayed orientation or from orienting in a direction outside the eye regions; this should be addressed in the future. Fifth, we did not assess the participants of our study with both ADOS-2 and ADI-R-JV; we assessed them with only one of these tools. Among 39 individuals with ASD in the analysis, five were assessed only with ADI-R, and two individuals among these were over 10 years. This may compromise the diagnostic accuracy because of higher likelihood of recall biases, although the number of such individuals is minimal. Sixth, we excluded individuals with ADHD and general cognitive delay. Although this exclusion will allow the algorithm to be more sensitive to ASD, the general clinical applicability of the diagnostic algorithm may be limited in clinical settings, where individuals with ASD are frequently comorbid with ADHD and/or cognitive delay. In our future study, we may include individuals with or without ADHD and compare them with individuals with ASD using the diagnostic algorithm. Finally, since we did not conduct full diagnostic assessments for screen-negative individuals, we might have overlooked ASD diagnoses in this group of individuals. However, this is unlikely since our thorough clinical assessments were conducted by trained psychiatrists or pediatricians, all of whom have an experience of joining clinical/research workshop of ADOS-G or ADOS-2 and some have established research reliability with the developers, followed by the consistent negative results for all the three screening tests for ASD.

Clinical Applicability

In typical community settings, individuals with ASD are expected to be diagnosed at certain stages during childhood (43). However, more than half of the children, adolescents, and young adults with confirmed diagnosis of ASD do not have a history of clinical diagnoses related to ASD, as was reported in a community survey in the last century from the US (44). This was reported a decade ago, although the situation appears to remain the same at present. A more recent study from Japan pointed out that only 32% of children confirmed to have ASD at 5 years of age had any history of neuropsychiatric/neuropediatric diagnosis until their fifth birthdays, meaning that more than half of the children with ASD are left undiagnosed at 5 years of age or even older (1). This may be due to the lack of appropriate chances to be screened, although general health checkups during childhood are a rule in most developed countries. The challenges inherent to the diagnostic evaluation of ASD, particularly in the community setting, should be resolved with ease and without expertise. We propose the computerized best-fit diagnostic algorithm implemented in Gazefinder as a solution to this.

Despite the diagnostic accuracy and convenience of the diagnostic algorithm, standard diagnostic procedures in clinical settings, such as ADOS-2, should not be replaced with diagnosis with Gazefinder. We assume that a diagnostic evaluation with Gazefinder is a mechanical one and thus should be followed with an expert clinical diagnosis. Presently, clinical evaluation may not be readily available in countries where trained manpower is limited, and mechanical diagnosis alone can result in a false sense of security among caregivers. However, in such countries, we can propound that Gazefinder can function as a screener and thereby reduce the burden on experts including pediatricians, child psychologists, and child psychiatrists. We should minimize the drawbacks and maximize the advantages of using Gazefinder in the future. In order not to give false sense of security when a false negative result is provided, the cutoff point should be adjusted to increase the sensitivity.

We have confirmed that the diagnostic performance of the best-fit algorithm is comparable to standard diagnostic tools and is even better than current screening tools for ASD. Diagnostic evaluation using Gazefinder is secured in more than 90% of the participants and adolescents. Thus, the proposed best-fit diagnostic algorithm is ready to be used in clinical settings and to be tested in clinical trials. We have drafted and submitted the protocol to the Japanese supervisory authorities, and currently, a clinical trial is under way. Clinical trials using Gazefinder to

establish diagnostic validity in countries other than Japan are also appreciated.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because sharing the data with the third parties not listed in the original protocol has not been approved by the Ethical Committees. Requests to access the datasets should be directed to Kenji J. Tsuchiya, tsuchiya@hama-med.ac.jp.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Hamamatsu University School of Medicine and Hospital, the Ethics Committee of University of Fukui, the Ethics Committee of Hirosaki University School of Medicine and Hospital, the Ethics Committee of Graduate School of Medicine and School of Medicine, Chiba University, the Ethics Committee of Saga Medical School Faculty of Medicine, Saga University, the Ethics Committee of Kanazawa University School of Medicine, and the Ethics Committee of Tottori University Faculty of Medicine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

KT, SH, THara, MN, MS, HK, YH, MM, MK, YM, and TK: study concept and design. KT, MS, TF, HK, YH, MM, MK, YM, and THarada: resources arrangement, clinical evaluation, and measurement. KT, SH, THara, MN, and TN: analysis and interpretation of data. KT, THara, and TN: drafting of manuscript. KT and TK: obtained funding. All authors contributed to critical revision of manuscript for important intellectual content and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.603085/full#supplementary-material

Supplementary Figure 1 | Candidate attributes* of AOI rate scores** among the individuals of younger than 10 years. AOIs outlined with red lines represent parameters with a positive effect (i.e., AOI rate score was higher in individuals with ASD) and AOIs outlines with blue lines represent parameters with a negative effect (i.e., AOI rate score was lower in individuals with ASD). *Attributes that were significantly ($p < 0.05$) associated with the diagnosis of ASD or had an effect size of Cohen's d with 0.5 or larger. **Percentage of fixation time allocated to the AOI divided by duration of each movie clip.

Supplementary Figure 2 | Candidate attributes* of AOI rate scores** among the individuals of 10 years and over. AOIs outlined with red lines represent parameters with a positive effect (i.e., AOI rate score was higher in individuals with ASD) and AOIs outlines with blue lines represent parameters with a negative effect (i.e., AOI rate score was lower in individuals with ASD). *Attributes that were significantly ($p < 0.05$) associated with the diagnosis of ASD or had an effect size of Cohen's d with 0.5 or larger. **Percentage of fixation time allocated to the AOI divided by duration of each movie clip.

Supplementary Figure 3 | Candidate attributes* of AOI count scores** among the individuals of younger than 10 years. AOIs outlined with red lines represent parameters with a positive effect (i.e., AOI count score was higher in individuals with ASD) and AOIs outlines with blue lines represent parameters with a negative effect (i.e., AOI count score was lower in individuals with ASD). *Attributes that were significantly ($p < 0.05$) associated with the diagnosis of ASD or had an effect size of Cohen's d with 0.5 or larger. **Presence (or absence) of countinous fixed gaze ofer the AOI; takes the value of either 0 or 1.

Supplementary Figure 4 | Candidate attributes* of AOI count scores** among the individuals of 10 years and over. AOIs outlined with red lines represent parameters with a positive effect (i.e., AOI count score was higher in individuals with ASD) and AOIs outlines with blue lines represent parameters with a negative effect (i.e., AOI count score was lower in individuals with ASD). *Attributes that were significantly ($p < 0.05$) associated with the diagnosis of ASD or had an effect size of Cohen's d with 0.5 or larger. **Presence (or absence) of countinous fixed gaze ofer the AOI; takes the value of either 0 or 1.

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The Weak Link: Hypotonia in Infancy and Autism Early Identification

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Background: Presenting symptoms and age specific differential diagnosis of Autism Spectrum Disorder (ASD), determine the age of initial assessment and the age of a definite diagnosis. The AAP recommends screening all children for ASD at 18 and 24 months followed by a comprehensive evaluation for children with developmental concerns. More recently it has been recommended that the evaluation should be performed at a younger age, with a diagnosis being made as early as the beginning of the second year of life resulting in earlier intensive intervention.

Objective: To assess early developmental milestones in a cohort of children diagnosed with Autism Spectrum Disorder (ASD) in order to find an objective and reliable early marker. We suggest that low muscle tone- hypotonia, is a sign that meets the above criteria of consistency and reliability and may be related to early diagnosis.

Methods: We compared age distributions of ASD diagnosis in the presence of hypotonia in a dataset of 5,205 children diagnosed at Keshet Center. One thousand, one hundred eighty-two children (953 males) were diagnosed with ASD and compared to other developmental diagnoses. Within the ASD cohort we further analyzed for gender and pre-maturity differences.

Results: In the presence of hypotonia, the mean age for ASD diagnosis was significantly lower (by 1.5 years for males and females) and this effect increased in children born at term as compared to pre-maturity.

Conclusions: Hypotonia is a recognizable marker of ASD and may serve as a "red flag" to prompt earlier recognition and neurodevelopmental evaluation toward an autism diagnosis.

Keywords: autism, infant, hypotonia, comorbidity, girls

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INTRODUCTION

The diagnosis of Autism Spectrum Disorder (ASD) is clinical. Presenting symptoms and age specific differential diagnosis determine the age of initial assessment and the age of a definite diagnosis. The American Academy of Pediatrics recommends screening all children for ASD at 18 and 24 months followed by a comprehensive evaluation for children with developmental concerns (1). However, more recently it has been recommended that the evaluation should be performed at a younger age, with a diagnosis being made as early as the beginning of the second year of life (2).

When a child receives a final diagnosis of ASD, an intervention program including intensive approach and parental guidance is implemented (3). Early intervention is paramount to improve the function and social participation of children with ASD (4). As such, an accurate identification of easy to recognize, measurable and reliable "red flags" is essential to improve outcomes in autism.

Since motor milestones are easy to recognize and measure, we suggest that low muscle tone- hypotonia, is a reliable early "red flag" to prompt ASD evaluation that could translate into an earlier diagnosis, intervention and possibly an improved outcome.

Early intervention is an umbrella term covering many different services funded by a variety of public and private sources. Available services are determined by each locality. Public Law 99-457, 1986 that was reauthorized in 1991 as PL 102-119 led to expanded services for young children with disabilities. Part C of the Individuals with Disabilities Act (IDEA) has assisted in developing comprehensive services that mandate a family directed approach (5). The main message that health-care providers convey to parents is that an early diagnosis warrants early professional services that are designed to promote the child's communicative, behavioral and social functioning development as well as assist him/her in acquiring better adaptive skills. An earlier ASD diagnosis prompts an earlier intervention, which will result in more effective improvements in the child's functioning (6). However, at times, an early diagnosis of ASD is made based on obvious and significant developmental deficits that are associated with more severe autism. In such cases, early diagnosis does not always assure a good prognosis (4).

Early signs and symptoms that can be recognized during the first year of life: By 4 months of age, babies should not only be crying but using other means of communicating their needs such as vocalizations and facial expressions (7, 8). Lack of evolution of body language and lack of modulation of eye contact should raise concerns. During the first 6 months, babies increase their motor control by incorporating movements to express their needs. Before learning how to crawl they reach the motor milestone of "working toward an object" by moving their bodies and limbs toward people and objects of interest. As infants learn about the reactions of others, they reach the social milestone of raising both arms in a request to be picked up (9). Infants that present with motor gaps such as head lag, low muscle tone (hypotonia), exaggerated or lack of response to sensory stimuli (such as noise or touch), should raise concerns and elicit a more extensive neurological evaluation (10, 11). Additional hints may present as overt motor asymmetries that do not improve with time. Minor inconsistent asymmetry involving asynchronous movements of limbs in infants is part of normal development. Consistent asymmetry should prompt a more extensive evaluation because movements should be synchronous until about age two with the development of the dominant hand (4, 12).

From 6 to 12 months of development motor control advances along with the emergence of a more extensive vocal repertoire such as razzing and babbling. The motor pathway is an indicator of maturation and it may serve as a sign for normal general developmental processes (11).

Following the rapid growth of non-verbal communication during the second year of life, the repertoire of motor gestures such as pointing, waving, nodding, clapping and more, should increase spontaneously after 1 year of age (13).

With the increase in motor activity and control, unusual behaviors may become more obvious. For example, hand flapping, walking in circles, lining objects, and a particular interest in spinning objects may be reported by parents early on. Unusual early motor movement patterns are common in infants that are subsequently diagnosed with ASD and may be an early sign of atypical behaviors (14–16). These patterns could be consistent asymmetric movements or milestones appearing earlier than expected, such as rolling from their belly onto their back. Other early signs include unusual motor interests such as holding a metal object instead of the usual transitional nappies and prolonged interest in mechanical objects such as spinning wheels (17).

Hypotonia

Hypotonia is defined as decreased muscle tone or floppiness with varying degrees of progression. It occurs in multiple neuromuscular, metabolic and genetic disorders and can be a sign of global developmental delay, that may pre-dispose to a cognitive disability (18).

The severity and progression of hypotonia varies with each child and with each diagnosis. For example, children with Down syndrome or with hypotonic cerebral palsy have non-progressive low tone hypotonia while those with neuromuscular disorders such as muscular dystrophy have progressive hypotonia that worsens with time. Hypotonia present in pre-mature infants may improve with maturity of the central nervous system or evolve to cerebral palsy (19).

Benign congenital hypotonia (BCH) is a diagnosis of exclusion, given to many children after workup has been exhausted. BCH is considered a non-progressive neuromuscular disorder that does not worsen but tends to improve with time and intervention. It may have a high familial incidence that may indicate BCH is of an autosomal dominant, genetic origin (20).

Hypotonic children may also have very flexible joints either in BCH or in syndromes presenting with hypotonia such as Down syndrome and Fragile X (21). Since the infant has poor head and axial control, this combination is associated with motor delay characterized by delayed sitting and late independent walking (22).

Hypotonia, Feeding, and Additional Influences on Motor Postural Control

Hypotonia may involve axial tone including neck muscles and the muscles around the mouth, influencing the infant's sucking and feeding abilities (21).

Positioning of the infant for feeding is a particular challenge for parents of hypotonic babies, as the child lacks head and chest control. These infants experience sucking, chewing and swallowing difficulties along with persistent drooling from the mouth. Posture control during feeding or breastfeeding may also influence eye contact and communication with the caregiver. In retrospect, feeding difficulties are common in children

subsequently diagnosed with autism and may persist for a long time (23).

Hypotonia may start prenatally, and the abnormal postures can lead to a neck deformity called torticollis, that develops in some children who hold their head to one side (20, 24).

Hypotonia may be associated with global developmental delay, either as a cause or a result of delayed milestones (21, 25).

Since hypotonia, hyperlaxity and motor delay can impair an infant's ability to explore his or her environment, the infant could ignore critical visual cues resulting in impaired learning and cognitive development (26).

Additional cues to atypical development in infancy are general movements of the infant and sleep- arousal patterns. General movements are a distinct movement repertoire carried out spontaneously without external stimulation and are seen in fetuses of 9 weeks gestational age until 21 weeks post-term. General movements are helpful in the early diagnosis of an impaired central nervous system such as the specific prediction of cerebral palsy (27) and they reflect impairments of brain areas involved in cognitive development (28). Measurements of GM and sleep are particularly important to assess in infants born prematurely.

Emphasis on Motor Development as a Key to Early Diagnosis

There is still a significant gap between "state of the art" research on autism and common practice as they relate to age of diagnosis. This gap varies in its magnitude between countries, among communities, and in relation to socioeconomic status. There is an established direct connection between early diagnosis, early intervention and subsequent outcomes, however, the path is not linear. Early diagnosis of ASD is made based on obvious and significant developmental deficits that may be associated with more severe autism, or the result of extremely observant parents. In the former, an early diagnosis does not always assure a good prognosis. The earlier the intervention, the more effective it is in improving functioning (6).

As such, accurate identification of easy to recognize, measurable and reliable "red flags" is paramount to improve outcomes in autism. We suggest that low muscle tone- hypotonia, is a sign that meets the above criteria of consistency and reliability and may indeed serve as an early "red flag" to prompt neurodevelopmental evaluation and autism screening.

We also postulate that early hypotonia may be present before birth, which can lead to the complicated deliveries, cesarean sections or general anesthesia that has been associated with Autism spectrum disorder (29–31).

MATERIALS AND METHODS

Participants

The present study is a dataset of 5,205 children (male = 3,346, female = 1,859) out of which 1,182 were children diagnosed with ASD with documented age of ASD diagnosis (male = 953, 81% of total ASD population, female = 229, 19% of total ASD population). Among 4,023 non-ASD children there were 2,393 (59%) males and 1,630 (41%) females. These differences of sex distribution reflect the male to female ratio of ASD as compared to other diagnoses. The participants included children from January 2010 to December 2018 who underwent a diagnostic process consisting of a neurodevelopmental and psychological assessment based on DSM-IV-TR or DSM-5 criteria (32, 33). Evaluations were performed at Keshet Center- a specialized, hospital-based tertiary developmental center.

Procedure and Measures

The study was approved by the hospital's IRB as part of a larger study predicting developmental disability including autism and intellectual level of children that were referred for a developmental evaluation (Helsinki approval 8458-11-SMC).

The clinical diagnostic process at Keshet Center is performed according to the national MOH guidelines (34). It includes a physical, neurological and a developmental exam performed by a physician specialized in pediatric neurology with special expertise in neurodevelopmental disabilities. Each child was additionally evaluated by a developmental or rehabilitation psychologist according to age. The Autism Diagnostic Observation Schedule—ADOS (35) was used to confirm the diagnoses. The physician also determined a Developmental Quotient (DQ) score based on the Denver Developmental Screening Test (DDST II) (36) up to age five, and performed a Clinical Adaptive Test/Clinical Linguistic and Auditory Milestone Scale (CAT/CLAMS) test up to age 3 years (37), in parallel to the formal psychological evaluation. Children with motor, fine motor and language delays additionally underwent a thorough evaluation for each of these areas, as performed by physical, occupational and language pathologists. In addition to the ASD diagnosis and the neurodevelopmental evaluation, all participants underwent standardized cognitive testing as part of their clinical evaluation. Specific instruments selected for cognitive testing were chosen according to the child's age and functioning level. Instruments used included the Bayley II scales (38), Mullen Scales for Early Learning (39), and cognitive tests: Stanford-Binet Fourth Edition (40), Wechsler Pre-school and Primary Scale for Intelligence—Third Edition (41), Wechsler Intelligence Scale for Children—Revised (42) Leiter-R (43) and Kaufman Brief Intelligence-Test (K-BIT) (44).

Statistical Analyses

We explored the relationship between developmental and motor comorbidities associated with ASD and their potential effect on the age of ASD diagnosis. Age distribution of initial ASD diagnosis was divided into age sub-groups. By using parametric and non-parametric multiple comparisons that incorporated intervening factors such as gender and pre-maturity, we identified a group of comorbidities (CM) that were consistently associated with a lower age of initial ASD diagnosis. Specifically, we made comparisons of ASD diagnostic age distributions and tested for significance in the presence of low muscle-tone indicators (hypotonia, torticollis, feeding issues) and other common co-morbidities (CM) groups (motor and global delays, Developmental Coordination Disorder, speech, and language difficulties).

Since a child may have more than one CM, it is more than likely that the overall number of subjects in all CM groups is higher than the actual cohort. In this kind of stacked data structure, it is more challenging to detect differences between subgroups, as each single data point (each individual) may be shared by more than one CM and therefore has the potential to expand over a wider age range.

Gender is a significant variable that may influence diagnosis and may present with different CM between males and females (45). We analyzed gender influences on age of diagnosis and on comorbidity.

Since motor delays including hypotonia are prevalent in infants born prematurely, and given the elevated rate of autism diagnosis linked to pre-maturity (46), we further analyzed variability in age of diagnosis as related to pre-maturity.

Tests Used

Parametric tests

Pearson Chi-Square: establish correlation and significance for the presence or absence of certain CMs with ASD.

ANOVA (analysis of variance): establish significant differences between certain CMs and ASD in the age of ASD diagnosis.

Non-parametric tests

Wilcoxon each pair comparisons (multiple comparisons)—compare between ages of diagnosis of ASD for each of the CMs.

Kolmagorov-Smirnov: compare between distributions of ages of ASD diagnosis at the presence or absence of a certain CM.

Gender and pre-maturity in the above tests were considered as intervening factors.

RESULTS

The initial cohort of 5,205 children comprised of 3,346 males and 1,859 females of which there were 1,476 children with ASD, 1,200 males with ASD and 276 females with ASD. Data that included age of initial diagnosis was available in 1,182 children with ASD.

Age of Initial Diagnosis

The ASD cohort of 1,182 children was comprised of 953 males and 229 females diagnosed initially between the age of 10 months and 12 years (M = 4.3 years, SD = 2.6). The age of ASD diagnosis was significantly different by gender with females being diagnosed at a younger age: males' mean age of ASD diagnosis was 4.4 years (±2.6 SD), while the mean age of ASD diagnosis for females was 3.8 years (±2.5 SD), [$F_{(1,1,181)} = 10.28$, $p < 0.01$]. The distribution of the ages of ASD diagnosis can be adequately described as a normal 3-mixture (M1 = 2.5, SD1 = 0.9 Pi1 = 0.50, M2 = 5.4. SD2 = 1.7, Pi2 = 0.42, M3 = 10.1, SD = 3 = 1.1, Pi3 = 0.08).

In order to address age specific comorbidities (CM), we divided the cohort to 3 sub-groups of ages, following the three means described in the 3-mixture distribution:

Category ≤2.5 years, n = 345, Males = 261, Females = 84 (24%)

Category ≤5.4 years, n = 504, Males = 404, Females = 97 (19%)

TABLE 1 | Comorbidities of the entire cohort by gender.

Comorbidities	Total	Males		Females	
		Non-ASD	ASD	Non-ASD	ASD
Developmental speech or language disorder	1,481	849	169	425	38
ADHD	1,403	703	388	252	60
Global delay (GD)	1,294	481	331	370	112
Behavioral/emotional	870	522	84	243	21
Intellectual disability (ID)	521	162	204	108	47
Fine motor difficulties	365	238	47	77	3
Communication	341	138	127	43	33
Hypotonia	333	148	23	155	7
Motor delay	243	150	6	87	0
Developmental coordination disorder (DCD)	206	105	50	46	5
Learning disability	206	102	41	55	8
Cerebral palsy (CP)	201	90	10	92	9
Motor impairment	163	92	19	48	4
Disorders of muscles/tendons (includes hypertonus, torticollis etc.)	155	85	4	63	3
Epilepsy	152	66	34	36	16
Feeding and eating disorders	75	35	9	28	3
Sensory motor integration difficulties	75	47	8	18	2
Anxiety	63	31	17	10	5
Sleep disorders	54	19	13	20	2
Stereotypic/involuntary movements	46	27	8	8	3

Category >5.4 years, n = 336, Males = 288, Females = 48 (14%)

Although the mixture proportion for age on the 2.5 years mean was the largest (50%), when we cut the categories by the mean values, the comparison became more strict, as it reduced the range of ages in that group (it will now encompass 30% of the overall population).

Of all the participants in the cohort diagnosed at the Keshet Center, there were multiple developmental diagnoses such as developmental speech and language delay, motor delay or disability, Global Developmental Delay (GDD), ADHD etc. All diagnoses present in more than 5 children in the cohort were listed in **Table 1**. The classification of primary vs. secondary diagnosis depended on if it was reached before or after ASD diagnosis. When a diagnosis such as GDD, motor delay or ADHD were present in a child before their ASD diagnosis, the diagnosis was considered primary, but when it occurred in a child with known ASD it was considered to be a CM or a secondary diagnosis.

Within the ASD group, the stacked dataset included numerous CM diagnoses such as 177 children with over

TABLE 2 | Comorbidities (CM) by age group of diagnosis.

CM subgroups	*N* % in sub group ≤2.5 Y	% in sub group ≤2.5 Y	*N* in sub group ≤5.4 Y	% in sub group ≤5.4 Y	*N* in sub group >5.4 Y	% in sub group >5.4 Y
Hypotonia	17	58.6%	7	24.1%	5	17.2%
Global delay (GD)	236	51.5%	178	38.9%	44	9.6%
Hypertonus	2	50.0%	2	50.0%	0	0.0%
Sleep disorders	5	35.7%	7	50.0%	2	14.3%
Feeding and eating disorders	4	33.3%	8	66.7%	0	0.0%
Sensory motor integration difficulties	3	30.0%	6	60.0%	1	10.0%
ASD	371	29.0%	550	43.00%	358	28.0%
Motor impairment	5	26.3%	11	57.90%	3	15.8%
Communication	31	25.0%	56	45.20%	37	29.8%
Stereotypic or involuntary movements/tics	2	22.2%	4	44.4%	3	33.3%
Developmental speech and language disorders	47	21.4%	111	50.5%	62	28.2%
Anxiety	4	21.1%	7	36.8%	8	42.1%
Epilepsy	9	18.4%	23	46.9%	17	34.7%
Emotional problems	11	15.5%	30	42.3%	30	42.3%
Fine motor difficulties	6	14.6%	19	46.3%	16	39.0%
Behavioral problems	4	13.3%	16	53.3%	10	33.3%
ADHD	74	13.3%	241	43.3%	242	43.4%
Intellectual disability (ID)	28	11.8%	111	46.8%	98	41.4%
Learning disability	4	11.1%	9	25.0%	23	63.9%
Motor delay	1	11.1%	6	66.7%	2	22.2%
Developmental coordination disorder (DCD)	2	3.8%	16	30.2%	35	66.0%
Cerebral palsy (CP)	1	3.1%	14	43.8%	17	53.1%
Torticollis	0	0.0%	3	100.0%	0	0.0%

5 diagnoses, 748 children with 2–4 diagnoses and 257 children with only an ASD diagnosis without any co-morbidities. For the purpose of investigating motor delays with respect to the age of ASD diagnosis, we decided to exclude children with severe physical co-morbidities, such as cardiac, gastrointestinal or other systemic disorders. We focused only on participants with neurodevelopmental and neuro-behavioral CM diagnoses which resulted in a reduced cohort size of 1,182 children. In order to further reduce the CM variability, we analyzed only CM that occurred in more than five children, other than those with hypertonus and torticollis who were included even if sparse, due to their connection to motor development.

Table 2 below describes the percentages of co-morbidities within each category of group of means.

When analyzing frequent occurring CM in the early diagnosis group (<30 months) the most frequent CM were: hypotonia, global delay (GD), sleep disturbances, hypertonus, feeding and eating issues.

Within the ASD cohort, mean values of the age of ASD diagnosis were graded lowest to highest amongst CM sub-groups (**Figure 1** shows the means and the 95% confidence interval of the mean for each diagnosis category). When CM were attached to age of diagnosis of ASD, the main CM that correlated with lower age of diagnosis were: GDD, hypotonia, hypertonus, torticollis, and feeding/eating disorder.

Within the subset cohort of ASD there were 29 children with hypotonia and 1,153 children without hypotonia and this diagnosis was the most frequent among early diagnosed comorbidities.

In view of the CM grouping according to age of ASD diagnosis, we examined the center's complete cohort of children for specificity and sensitivity of hypotonia and GDD diagnoses to ASD. Of the initial cohort of 5,205 children (3,346 males and 1,859 females) there were 1,476 children diagnosed with ASD (1,200 Males and 276 females). Of this population there were 303 children without ASD with hypotonia as a primary diagnosis, and 52 children with ASD and hypotonia.

Performing a Pearson chi square-test we did not find a significant difference for the ASD proportion in the presence of hypotonia (Pearson, chi square = 65.55, $p < 0.001$, for non-hypotonic children). Testing with gender as an intervening effect yielded similar results.

We further examined the proportion of children with GDD and hypotonia with or without ASD in the full cohort. There were 443 children with GDD and ASD, and 851 children with

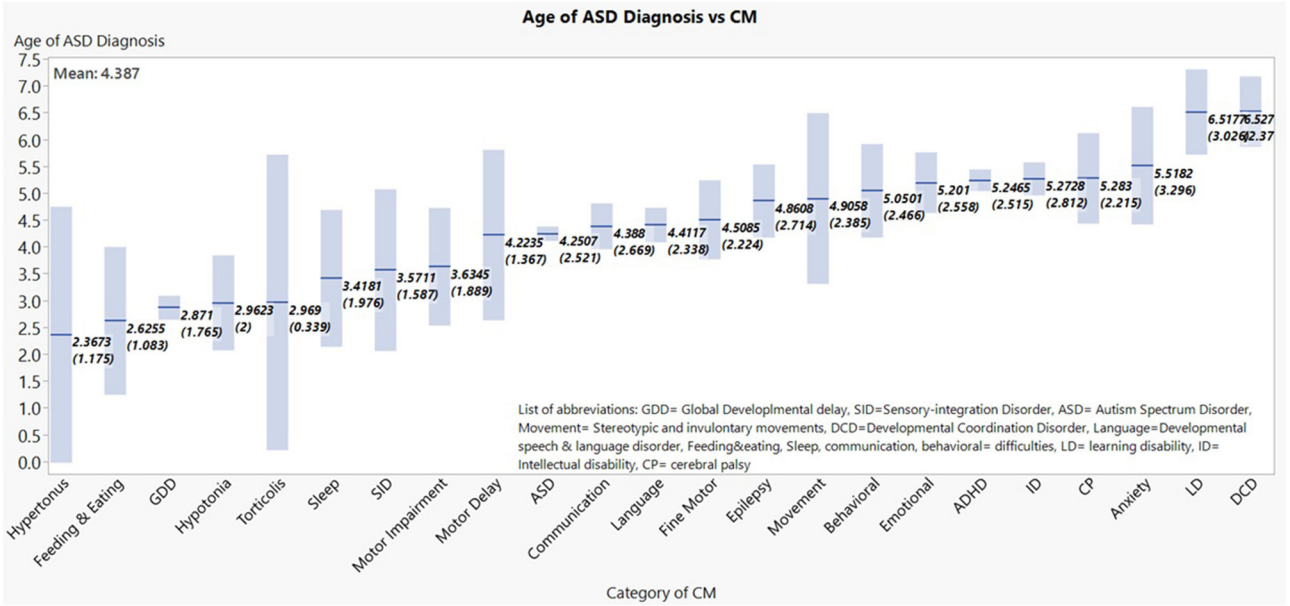

FIGURE 1 | Mean age of ASD diagnosis age by respective comorbitidy sub-group are shown in ascending order, with their respective 95% confidence levels. *The number of children in the graph is higher than the total number of children with ASD since each child may get more than one diagnosis.

GDD without an ASD diagnosis. There were 125 children with both hypotonia and GDD, while the number of children with GDD without hypotonia was 1,169 and children without GDD nor hypotonia were 3,703.

When examining GDD, we found that GDD correlated significantly with ASD (Pearson chi square-test 29.28, p<.001), and with hypotonia (Pearson chi square-test 30.61, $p < 0.001$).

Differences of Age of ASD Diagnosis Among Children With Hypotonia and Other Co-Morbidities

Comparisons of means using the Wilcoxon method for each pair between hypotonia and all other typical categories including a standalone ASD diagnosis, which showed that the only means differing non-significantly from hypotonia were GD, sleep, torticollis, hypertonus and feeding (hypotonia and sleep $Z = 1.36$, $p = 0.17$, torticollis with hypotonia $Z = 1.26$ $p = 0.21$, hypotonia and GDD $Z = -0.38$, $p = 0.71$, hypotonia, feeding, and eating $Z = -0.33$, $p = 0.74$, hypotonia and hypertonus $Z = 0.03$, $p = 0.98$). Hypotonia incidence was significantly linked to an early diagnosis when compared to all other CM. Therefore, an ASD early age of diagnosis cluster can be regarded with the above CMs: GD, sleep, feeding, torticollis, and hypertonus.

Over 50% of GM CM occurred more frequently within the ≤2.5Y ASD diagnosis age group, therefore we further explored the differences between GD and the remaining CMs. We observed that GM was not significantly different than hypotonia CM as it was associated with a diagnosis below 30 months. GD bared no significant differences to torticollis, hypotonia and hypertonus; thus forming a cluster of early ASD diagnosis indicators (GDD and sleep $Z = 1.35$ $p = 0.18$, GDD and torticollis $Z = 0.91$ $p = 0.36$, GDD and hypertonus $Z = -0.39$ $p = 0.70$, GDD and hypotonia $Z = -0.38$ $p = 0.71$ GDD, feeding, and eating $Z = -0.09$ $p = 0.93$).

Since hypotonia meets the criteria of a simple and relatively objective symptom, we analyzed it as a standalone CM.

In the presence of hypotonia the ASD initial diagnosis was significantly at lower age by nearly 1.5 Y in average [with hypotonia $M = 2.96$ years, SD = 2.0, without hypotonia $M = 4.41$ years, SD = 2.56, ANOVA result is F ratio (1, 3,405) = 9.23, $p < 0.01$]. Testing while assuming a non-parametric distribution resulted in similar conclusions (Kolmogorov-test, KSa = 2.48, $p < 0.001$).

Gender Differences

Testing for differences for age of ASD diagnosis in males as compared to females with vs. without hypotonia, resulted in significant differences for all between w/wo hypotonia groups without interaction between gender and hypotonia: Males with hypotonia $M = 2.95$ years, SD = 1.87, Males wo hypotonia $M = 4.49$ years, SD = 2.54, Females with hypotonia, $M = 3.01$ years, SD = 2.54, Females wo hypotonia $M = 4.07$ years, SD = 2.59. When testing separately using the Kolmogorov-Smirnov asymptotic test for gender w/wo hypotonia we found a higher significance for males in the presence of hypotonia vs. its absence when compared to females (Males KSa = 2.28 $p < 0.001$, Females KSa = 1.39, $p < 0.05$ calculated).

Influence of Pre-maturity

The age of ASD diagnosis in pre-term and in term children differed significantly with pre-term ASD being identified almost

1 year earlier than children born at term: Mean diagnostic age at term = 4.3 years, SD = 2.6, $N = 1{,}127$ mean diagnosis at pre-term = 3.5 years SD = 2.2, $N = 55$, using the Kolmogorov-Smirnov asymptotic-test—Ksa = 1.38, $p < 0.05$.

When testing within gender groups for pre vs. in term children we found that the age of ASD diagnosis for males born at pre-term was significantly lower than those of term males. For females the age of diagnosis was similar between pre-term and term:

In Term males age of diagnosis = 4.4 years SD = 2.6, $N = 915$, Pre-term males = 3.3 years, SD = 2.2, $N = 38$, Kolmogorov-Smirnov Ksa = 1.59, $p < 0.01$, for females Ksa = 0.63, $p > 0.05$.

Testing for differences in age of ASD diagnosis in the presence or absence of hypotonia when comparing by gestational age resulted in a lower age of diagnosis in the presence of hypotonia for all children, term and pre-term: pre-term with hypotonia $M = 2.2$ years, SD = 1.1 $N = 4$, pre-term without hypotonia, $M = 3.5$ years, SD = 2.2 $N = 55$, term with hypotonia $M = 3.1$ years, SD = 2.1, term without hypotonia $M = 4.3$, SD = 2.5 pre-maturity).

Testing the effect of hypotonia separately within in term vs. pre-term cohorts using the Kolmogorv-Smirnov asymptotic-test we found that for in term children the effect is highly significant (in term KSa = 2.35, $p < 0.001$) and for pre-term children it is not significant (pre-term Ksa = 0.73, $p > 0.05$).

DISCUSSION

When analyzing a large cohort of more than 5,000 children diagnosed at one tertiary center, more than a quarter of participants received a diagnosis of ASD. The male gender was more prevalent in the ASD group and the common diagnoses were delays in specific developmental areas such as motor and language as well as global developmental delay (GDD) and ADHD.

When assessing the age of diagnosis, we found a large spectrum ranging from <1 year to 12 years. Clearly there are significant differences expected between children diagnosed very early on such as below 2 years with children diagnosed in late childhood. This resulted in the emergence of three age groups of children according to the age range of their initial diagnosis. Though not significant, more girls were identified younger than in the older age group.

When additional developmental diagnoses occurred in conjunction with ASD, we considered those diagnoses as comorbidities. Most children with ASD had multiple CM, while only 21% had a diagnosis of ASD without additional diagnoses (47). If the same child had different comorbidities at different ages, we accounted for the age of diagnosis of their comorbidity. We found that hypotonia was detected more frequently in the younger group, making it a good marker for an earlier ASD diagnosis. In addition, other motor difficulties such as hypertonus and torticollis also occurred more frequently in the younger group, as well as eating and feeding problems. More than half of the group diagnosed with ASD below the age of 30 months had each of the motor diagnoses and one third had eating and feeding CM. Non-significant differences were localized around hypotonia, feeding, hypertonus, and torticollis, thus, forming a cluster of indicators that may characterize an early ASD diagnosis.

All of the additional CM that occurred early, may be related to abnormal motor development such as feeding which directly relates to neck and facial musculature (48, 49).

With respect to the first aim- we indeed proved that low muscle tone is a recognizable marker of ASD and its effect on lower age of diagnosis differs according to gender with a more accentuated influence on younger boys. Hypotonia in males can accelerate the age of ASD diagnosis by an average of 1.5 Y while for females, it will be accelerated by an average of 1 Y. Since motor difficulties and ASD diagnosis are prevalent in infants born prematurely and those infants are followed prospectively from birth, the mean age of ASD diagnosis was significantly lower in pre-mature children by almost 1 year, but only in males. An ASD diagnosis in females born pre-term did not differ from term girls, probably since pre-term females display "masking" signs such as common comorbidities that result in a delayed diagnosis (48).

The effect of hypotonia was not significant within the pre-mature cohort, probably due to cohort size differences, or the myriad of common comorbidities present in pre-mature infants (46).

The sample size including hypotonia is a limitation of the study, nevertheless it was sufficient and proven significant in the various statistical tests performed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sheba Tel-Hashomer Hospital's IRB. Helsinki Approval 8458-11-SMC. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LG: conceptualization, methodology, validation, writing—original draft preparation, writing—review and editing, supervision, and approval of final version. MD: writing—review and editing, supervision, and approval of final version. TG: data entry and approval of final version. RR: data entry, writing—original draft preparation, and approval of final version. SS: resources, writing—original draft preparation, project administration including ethics (IRB), and approval of final version. OL: methodology, data entry, data analysis, resources, writing—original draft preparation, and approval

of final version. MS: methodology, software, data analysis, validation, writing—original draft preparation, and approval of final version. All authors contributed to the article and approved the submitted version.

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Protein Biomarkers of Autism Spectrum Disorder Identified by Computational and Experimental Methods

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Background: Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder that affects millions of people worldwide. However, there are currently no reliable biomarkers for ASD diagnosis.

Materials and Methods: The strategy of computational prediction combined with experimental verification was used to identify blood protein biomarkers for ASD. First, brain tissue–based transcriptome data of ASD were collected from Gene Expression Omnibus database and analyzed to find ASD-related genes by bioinformatics method of significance analysis of microarrays. Then, a prediction program of blood-secretory proteins was applied on these genes to predict ASD-related proteins in blood. Furthermore, ELISA was used to verify these proteins in plasma samples of ASD patients.

Results: A total of 364 genes were identified differentially expressed in brain tissue of ASD, among which 59 genes were predicted to encode ASD-related blood-secretory proteins. After functional analysis and literature survey, six proteins were chosen for experimental verification and five were successfully validated. Receiver operating characteristic curve analyses showed that the area under the curve of SLC25A12, LIMK1, and RARS was larger than 0.85, indicating that they are more powerful in discriminating ASD cases from controls.

Conclusion: SLC25A12, LIMK1, and RARS might serve as new potential blood protein biomarkers for ASD. Our findings provide new insights into the pathogenesis and diagnosis of ASD.

Keywords: autism spectrum disorder, blood, protein, biomarker, computational, experimental

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INTRODUCTION

Autism spectrum disorder (ASD) is a complex neurodevelopment disorder characterized by impairments in social interaction and communication, as well as expression of restricted interests and repetitive behavior (1). These symptoms would be presented during the first 3 years of life. Boys are with four to five times higher risk of autism than girls (2). According to reports of 2015, ∼24.8 million people worldwide were affected by autism (3). In developed countries, the proportion of

children with autism increased from 0.67% in 2000 to 1.5% in 2017 (4, 5). Obviously, the number of patients with ASD is increasing year by year.

Genetic and environmental factors are generally acknowledged as important contributors to the pathogenesis of ASD (6). However, the exact pathological mechanism remains uncertain and there are no effective treatments for ASD. Studies show that early intervention with behavioral therapy at an early stage can improve patient social communication and reduce anxiety and aggression. Thus, it is critical to detect ASD at an early stage (7, 8). Currently, the clinical diagnosis of ASD is based on the fifth edition of the Statistical Manual of Mental Disorder (DSM-V) (9), which may lead to exclusion of autistic individuals with mild form. Therefore, there is a need to find useful and reliable biomarkers to assist the diagnosis of autism.

Blood is a potential source for disease biomarker discovery because it contains large numbers of proteins associated with the physiology or pathology of disease. Several studies have been performed to search for blood biomarkers of ASD. Smith et al. (10) found that the combination of glutamine, glycine, and ornithine amino acid dysregulation identified a dysregulation in amino acid/branched-chain amino acid metabolism with good specificity and positive predictive value in the ASD subject cohort. Momeni et al. (11) found three differentially expressed peptides in the heparin plasma of children with ASD. Ngounou Wetie et al. (12) reported that apolipoproteins (Apos) ApoA1, ApoA4, and serum paraoxanase/arylesterase 1 (PON1) were increased in the sera of children with ASD. Wu et al. (13) proposed a movement biomarker to characterize the neurodevelopment level, which could differentiate ASD subjects from typically developing individuals. Howsmon et al. (14) developed multivariate statistical models to distinguish children with ASD from controls based on the metabolic abnormalities. Oztan et al. (15) employed a multidimensional neuropeptide analysis and found low blood neuropeptide receptor might act as promising biomarker of disease presence and symptom severity in ASD. Recently, we found a protein pattern that could distinguish the plasma samples of autistic children from healthy controls (16). In addition, we identified 41 proteins as differentially expressed proteins in the peripheral blood mononuclear cells of autistic children (17); three of them, i.e., complement C3 (C3), calreticulin (CALR), and alpha-1-antitrypsin (SERPINA1), are common differential proteins in the plasma (16). Despite these advances, there are still no diagnostic biomarkers available for ASD nowadays.

It is generally known that the blood–brain barrier (BBB) plays an important role in the defense of the central nervous system by limiting harmful solutes, macromolecules, and cells circulating from the bloodstream into the brain. However, several studies have shown that dysfunctions of BBB had a relationship with pathogenesis of neurological diseases including ASD (18–21), suggesting that some ASD-related proteins might be secreted from brain into blood as potential biomarkers. In addition, Cui et al. (22) developed a computational method to predict whether a protein could be secreted from tissue into blood with a high accuracy. Therefore, it would be possible to apply this program on the proteins encoded by the ASD-related genes to predict some potential ASD-related proteins in blood.

In this study, we identified blood protein biomarkers for ASD through computational prediction combined with experimental verification. First, we identified ASD-related genes by analyzing brain tissue–based gene expression data of autistic patients and healthy controls collected from a public database. Then, we predicted whether the protein products of these genes could be secreted into blood as ASD-related proteins. Further, we made bioinformatics analysis and literature survey on these proteins, and then selected some ASD-related proteins for verification in plasma of children with ASD by ELISA analysis.

Abbreviations: ASD, autism spectrum disorder; BBB, blood–brain barrier; GEO, gene expression omnibus; SAM, significance analysis of microarrays; FDR, false discovery rate; DAVID, database for annotation, visualization and integrated discovery; GO, gene ontology; SVM, support vector machine; PPD, plasma protein database; PPI, protein–protein interaction; LENS, lens for enrichment and network studies of proteins; BP, biological process; CC, cellular component; MF, molecular function; HD, Huntington disease; ALS, amyotrophic lateral sclerosis; ROC, receiver operating characteristic; AUC, area under curve.

MATERIALS AND METHODS

Collection of Brain Tissue–Based Gene Expression Data of ASD Patients

The workflow used in this study is shown in **Figure 1**. Gene expression data of brain tissues from patients with ASD and healthy controls were collected from public database Gene Expression Omnibus (GEO) (23). One dataset, GSE28521 (24), was selected for data analysis according to the following criteria. First, it contains both brain tissue samples of ASD patients and healthy controls. Second, the number of samples for ASD and controls are larger than 10, respectively. There are 79 brain tissue samples obtained from 19 autistic patients and 17 healthy controls in this dataset. Among these samples, 10, 16, and 13 samples are from cerebellum, frontal cortex, and temporal cortex of autism cases, and 11, 16, and 13 samples are from corresponding tissues of controls, respectively. The average age of the patients and controls were 24 (ranged from 2 to 56) and 34.6 (ranged from 16 to 56), respectively. The ratio of male to female was about 14:5 and 16:1 in the patients and controls. Detailed information of these samples in this dataset can be accessed from the GEO database.

Identification of Differentially Expressed Genes for ASD

Generally, different tissues have different gene expression patterns. We investigated the differentially expressed genes of cerebellum, frontal cortex, and temporal cortex for ASD patients, respectively. A computational method, significance analysis of microarrays (SAM) (25), was employed to identify differentially expressed genes for ASD. A statistic delta was calculated for each gene in SAM, measuring how strong the relationship between gene expression and a response variable. R package "siggenes" was used to implement SAM analysis. To obtain the appropriate number of differentially expressed genes of ASD, delta was 1.2 and the false discovery rate (FDR) was 0.05 as cutoff.

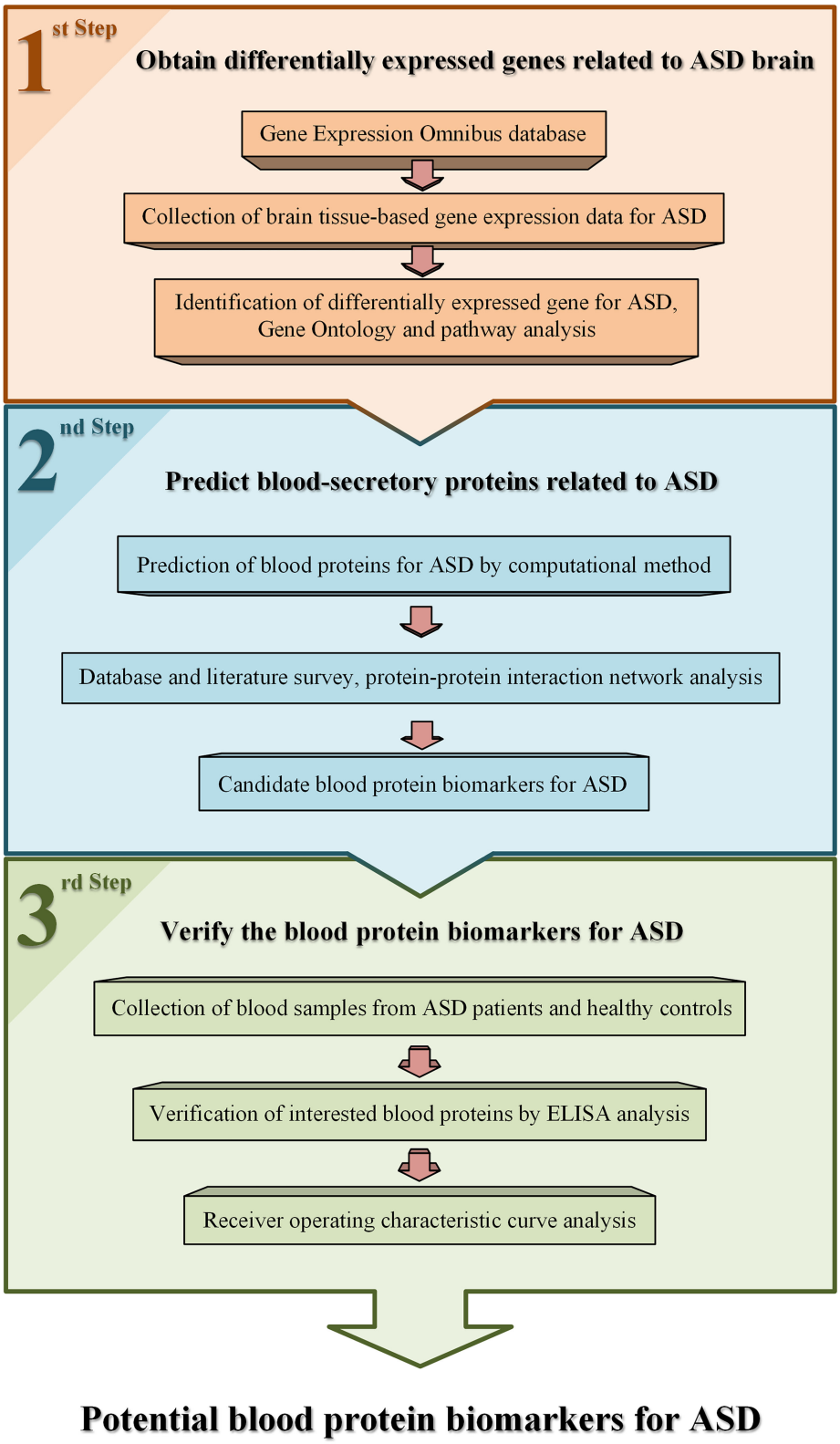

FIGURE 1 | An overview of the work flow used in this study.

To understand the functions of the differentially expressed genes, Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) (26) was used to conduct Gene Ontology (GO) annotation and pathway analysis on these genes. In addition, functional interaction network analysis was performed using ClueGO cytoscape plugin [GlueGO v2.5.7; (27)].

Prediction of ASD-Related Proteins in Blood

All differentially expressed genes of ASD were analyzed to determine whether their protein products could be secreted into blood by using a prediction program developed by Cui et al. (22). The main idea of the program is described as follows. Human proteins known to be blood secretory or not were collected from the published data to constitute the positive and negative training data, respectively. A list of protein features including sequence, structure, and chemical and physical properties was examined, and core features were selected according to their abilities in distinguishing the positive data from the negative. Based on the core features and training data, a prediction program for blood-secretory proteins was constructed by using support vector machine (SVM) (28) method.

In addition, to further determine whether these predicted proteins associated with ASD and presented in blood, we compared their genes with autism-associated gene database AutismKB (http://db.cbi.pku.edu.cn/autismkb_v2/) (29), and the proteins with plasma protein database (PPD, http://www.plasmaproteomedatabase.org/) (30), respectively. Moreover, protein–protein interaction (PPI) network analysis was conducted by using Lens for Enrichment and Network Studies of Proteins (LENS, http://severus.dbmi.pitt.edu/LENS/) (31) and Search Tool for the Retrieval of Interacting Genes/Proteins (String database, http://string-db.org/) (32).

Verification of Potential Blood Protein Biomarkers for ASD by Using ELISA

After the aforementioned prediction of blood-secretory proteins associated with ASD, we selected some potential protein biomarkers for ASD to validate according to the following criteria. First, we ranked these proteins according to the likelihood of protein secretion into blood derived from the prediction program. Then we compared their genes with autism-associated gene database AutismKB and the proteins with plasma protein database. Further, we made functional analysis and literature survey on these proteins. Based on this criterion, we selected six proteins for verification by ELISA. ELISA analysis was conducted on blood samples of children with ASD and healthy controls. The research protocol of this study was permitted by the Human Research Ethics Committee of Shenzhen University and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. A total of 40 subjects were recruited from Maternal and Child Health Hospital of Baoan between September 2017 and September 2018, including 20 children with ASD, and 20 age- and gender-matched healthy controls. The written consents were obtained from the caregivers of the participating children before this experiment. These patients were all diagnosed by a child neuropsychiatrist based on the criteria defined in the DSM-V (33). The male to female ratio was 4:1. The average age was 4.7 for patients and 4.5 for controls. The control cases had no known neurological disorders. There were no significant differences in weight, height, or body mass index (BMI) between the autistic children and healthy subjects. Blood samples (5 ml) were collected with EDTA-coated plastic tubes in the morning and then centrifuged at $3{,}000\times g$ for 10 min at room temperature. The supernatants were divided into aliquots and stored at -80°C until further analysis.

For ELISA analysis, the protein concentration was measured by a commercial ELISA kit (Uscn Life Science, Wuhan, China) according to the manufacturer's instructions, and then normalized by the total protein concentration determined by bicinchoninic acid (BCA) protein assay kit (Beyotime, Jiangsu, China). G-test (34) was used to detect the outliers in the normalized data. GraphPad Prism 5 software (GraphPad Software, San Diego, California) was applied to make statistical analyses on the concentrations of protein in ASD patients vs. healthy controls by using t-test with p-value <0.05 as cutoff.

RESULTS

Identification of Differentially Expressed Genes in Brain Tissues of ASD

There were 283 probes (3 up-regulated, 280 down-regulated) and 142 probes (3 up-regulated and 139 down-regulated) identified differentially expressed with FDR <0.05 as cutoff in the frontal cortex and temporal cortex of ASD, respectively. There were no differentially expressed probes found in the cerebellum of ASD. After combining the up- and down-regulated probes identified in the frontal and temporal cortex of ASD, six probes (corresponding to six genes) and 373 probes (corresponding to 358 genes) were differentially up- and down-regulated in cortex of ASD, respectively (**Supplementary Tables 1**, **2**).

To assess the functions of these differentially expressed genes, GO annotation and pathway analyses were conducted by using DAVID database. A total of 72 GO terms and 18 pathways were significantly enriched by these genes with p-value <0.05 as threshold (**Supplementary Tables 3–6**). The enriched GO terms include 31 biological processes (BP), 19 cellular components (CC), and 22 molecular functions (MF). The top 10 enriched terms of BP, CC, and MF are shown in **Figures 2A–C**. BP analysis showed that they were involved in tricarboxylic acid (TCA) cycle, neurotransmitter transport, ATP hydrolysis coupled proton transport, synaptic vesicle exocytosis, canonical glycolysis, exocytosis, response to calcium ion, glycolytic process, actin and cytoskeleton organization, etc. CC analysis showed that they were associated with mitochondrion, myelin sheath, synaptic vesicle membrane, mitochondrial matrix, neurofilament, postsynaptic density, and synaptic vesicle. MF included ATP binding, calcium ion binding, syntaxin binding, etc. In addition, pathway analysis showed that some metabolic

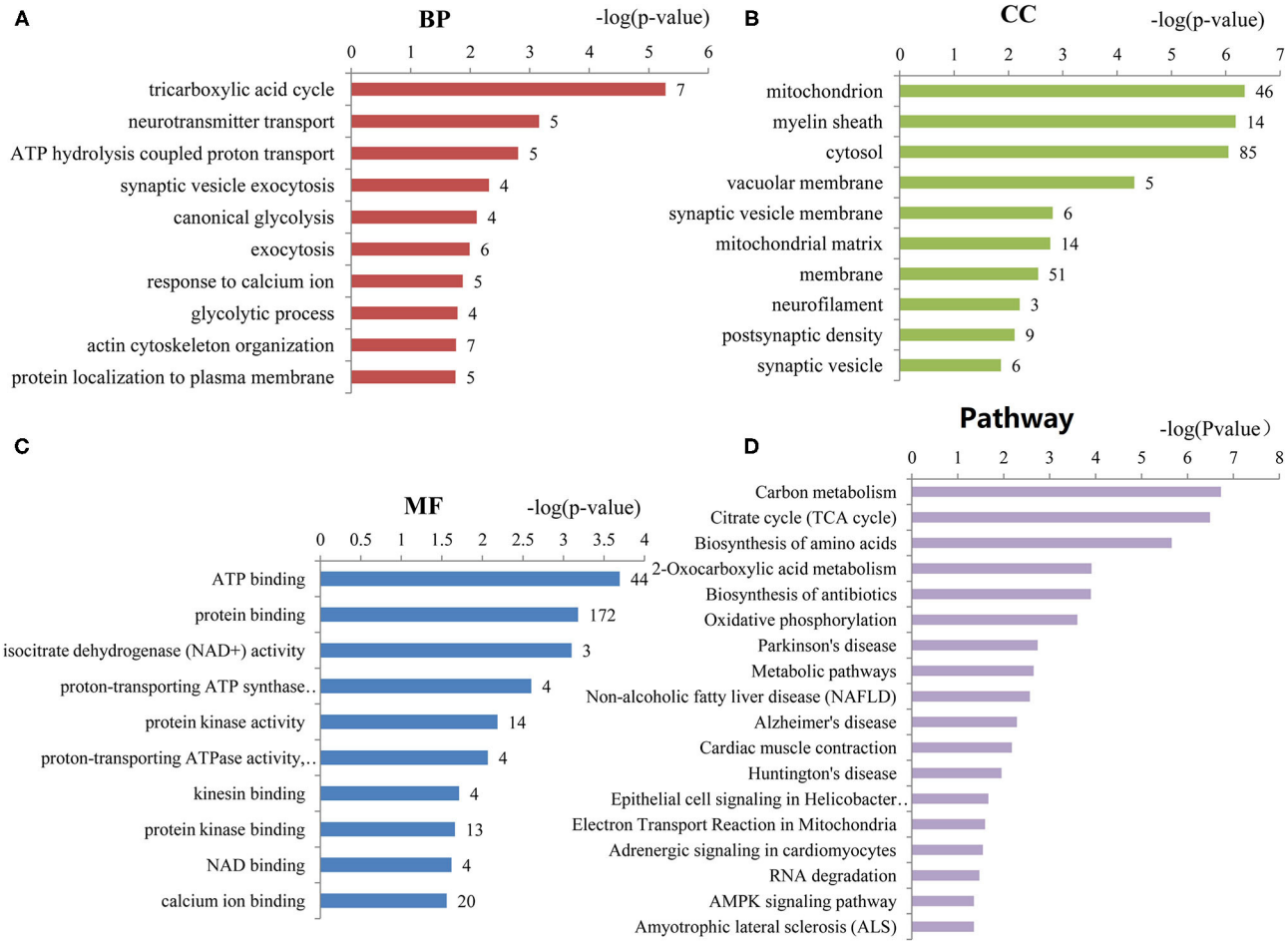

FIGURE 2 | The top 10 enriched GO terms of biological processes, cellular components, and molecular functions. **(A)** Biological processes (BP) enriched by the differentially expressed genes of ASD. **(B)** Cellular components (CC) enriched by the differentially expressed genes of ASD. **(C)** Molecular functions (MF) enriched by the differentially expressed genes of ASD. The number of proteins associated with each category is presented at the end of each bar. **(D)** The pathways enriched by the differentially expressed genes of ASD. The number of proteins enriched in each pathway is at the end of each bar.

pathways were enriched, including carbon metabolism, TCA cycle, and oxidative phosphorylation (**Figure 2D**). Interestingly, pathways of Alzheimer's disease (AD), Huntington disease (HD), and amyotrophic lateral sclerosis (ALS) were also enriched by these genes. Similarly, the enrichment of BP and CC on these genes by ClueGO cytoscape plugin showed that they were mainly associated with glucose catabolic process, proton-transporting two-sector ATPase complex, catalytic domain, Schaffer collateral-CA1 synapse, and vesicle-mediated transport in synapse (**Figure 3A**, **Supplementary Table 7**). Pathway analysis showed that they were mainly involved in the citric acid (TCA) cycle and respiratory electron transport, mitochondrial protein import, and glycolysis and gluconeogenesis (**Figure 3B**, **Supplementary Table 8**).

Prediction of ASD-Related Proteins in Blood

Based on the differentially expressed genes of ASD, we applied a computational program developed by Cui et al. (22) on these genes to predict whether their protein products could be secreted from tissue into blood. Subsequently, 59 proteins encoded by down-regulated genes were predicted to be blood-secretory proteins, suggesting that they might act as ASD-related proteins in blood (**Supplementary Table 9**).

To examine whether the predicted blood-secretory proteins were present in blood, we compared these proteins with proteins in PPD (30) and found that 43 proteins were in common (**Figure 4A**, **Supplementary Table 9**). To further determine whether these predicted proteins were associated with ASD, we compared their corresponding genes with autism-related genes listed in the AutismKB (29) and found that 55 genes were reported associated with ASD except *TTC13*, *RARS*, *THOC5*, and *ATPAF1* (**Figure 4B**, **Supplementary Table 9**). There were 40 overlapping blood-secretory proteins between PPD and AutismKB database (**Figure 4C**, **Supplementary Table 9**). After literature survey on the four genes, we found that *RARS* and *THOC5* had been reported related to brain development (35, 36).

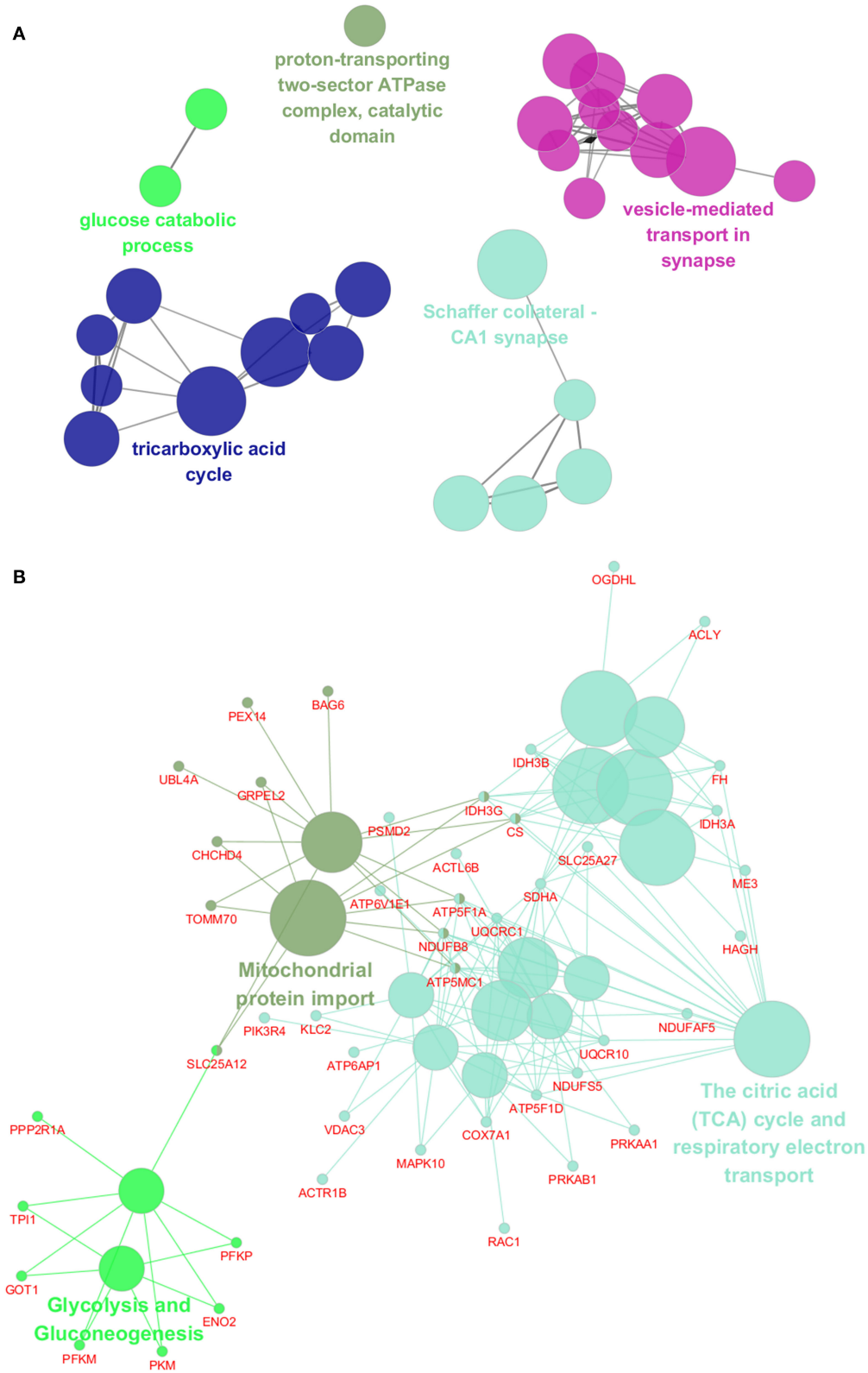

FIGURE 3 | Functional interaction network analysis of the differentially expressed genes. **(A)** The differentially expressed genes were mapped to the GO categories, including biological processes and cellular components by using ClueGO cytoscape plugin. **(B)** The differentially expressed genes were mapped to the KEGG pathway, REACTOME pathway, and Wiki pathway by using ClueGO cytoscape plugin.

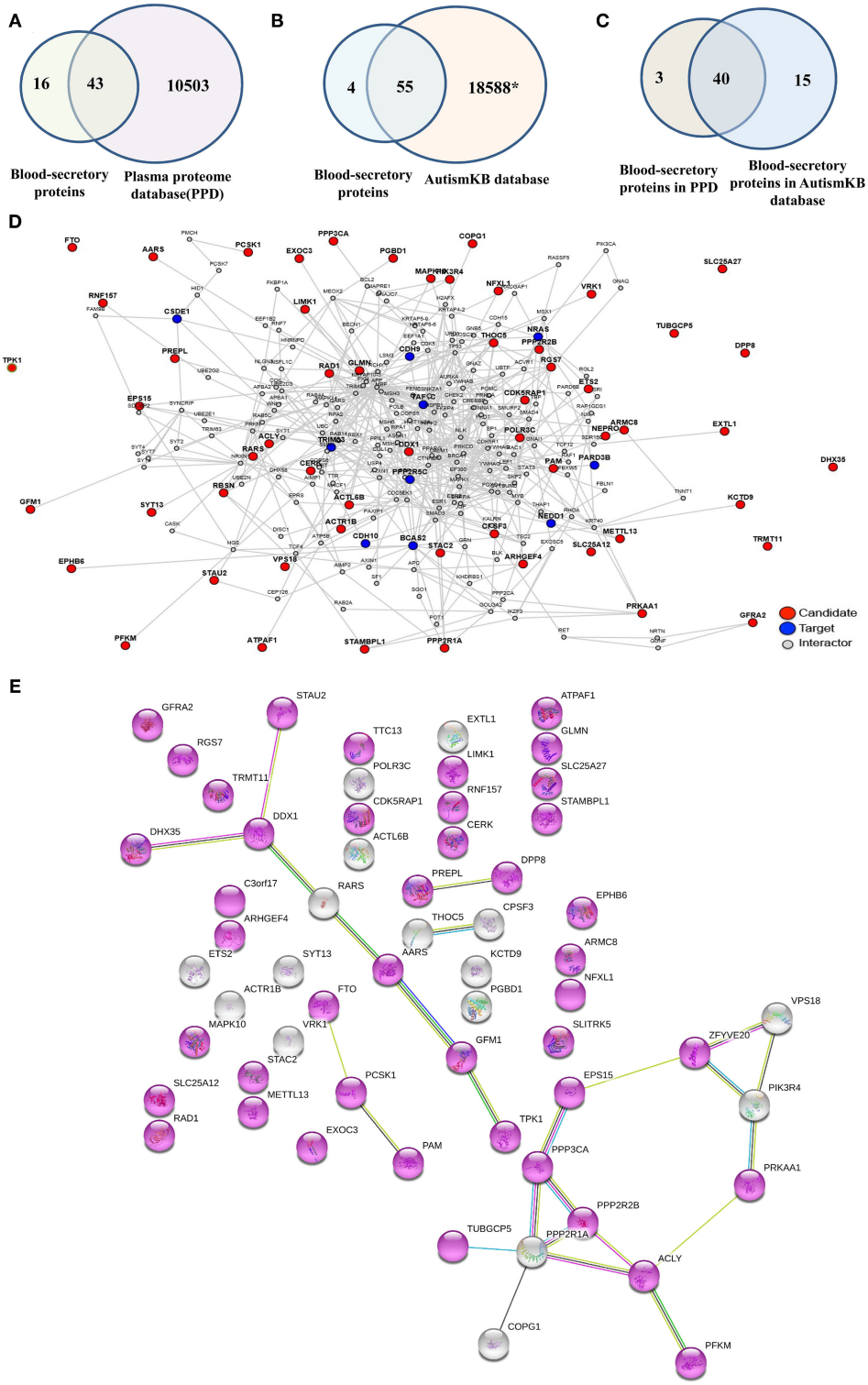

FIGURE 4 | Database survey and protein–protein interaction network analysis on the 59 blood-secretory proteins. **(A)** Compared with plasma protein database. **(B)** Compared with ASD-related database (AutismKB). *The AutismKB database contains 1,379 genes, 5,420 copy number variants (CNVs)/structural variations (SVs), 11,669 single-nucleotide variations (SNVs)/insertions and deletions (InDels), and 172 linkage regions associated with ASD. **(C)** The blood-secretory proteins overlapped in plasma protein database and AutismKB database. **(D)** The protein–protein interaction network of these 59 blood-secretory proteins. The predicted blood-secretory proteins are shown as red nodes and autism pathology-related proteins are shown as blue nodes. **(E)** UniProt keywords were enriched by using String database. Gene with a node color of purplish red, whose Uniprot keyword is alternative splicing.

TABLE 1 | Six predicted blood-secretory proteins selected for validation in this study.

No.	Gene	UniProt ID	Protein[a]	Function[b]
1	*RARS*	P54136	Arginine–tRNA ligase, cytoplasmic	Protein synthesis, inflammatory
2	*ACTL6B*	O94805	Actin-like protein 6B	Transcriptional activation and chromatin remodeling
3	*ARHGEF4*	Q9NR80	Rho guanine nucleotide exchange factor 4	High levels in the brain and is involved in cell migration and cell–cell adhesion
4	*PRKAA1*	Q13131	5′-AMP-activated protein kinase catalytic subunit alpha-1	Cellular energy metabolism, cell growth and proliferation, phosphorylation
5	*SLC25A12*	O75746	Calcium-binding mitochondrial carrier protein Aralar1	Calcium ion binding, amino acid metabolism
6	*LIMK1*	P53667	LIM domain kinase 1	Stimulates axonal outgrowth and may be involved in brain development

[a]*Except RARS did not match the two databases (i.e., AutismKB and plasma protein database), all the other proteins were matched. However, RARS has important functions and may be implied in the pathogenesis of ASD.*
[b]*Functional information obtained from UniProt database (https://www.uniprot.org/).*

In order to understand how these predicted proteins were involved in the pathogenesis of ASD, we conducted a PPI network analysis on these proteins by using a web tool of LENS. **Figure 4D** shows the network, which was constructed by 59 blood-secretory proteins (red nodes) input as candidate proteins and 15 proteins known related to autism pathology (blue nodes) provided as targeted proteins. From the network, we found that most of these blood-secretory proteins were connected with the targeted proteins except SLITRK5, TPK1, SLC25A27, DHX35, DPP8, TTC13, TUBGCP5, FTO, and TRMT11. Interestingly, String database analysis showed that 43 proteins might be associated with alternative splicing (**Figure 4E**).

Verification of the Potential Protein Biomarkers for ASD by ELISA

Based on the possibilities of proteins secreting into blood and their functions, six proteins were selected for validation in blood samples of children with ASD and healthy controls, including arginine-tRNA ligase, cytoplasmic (RARS), actin-like protein 6B (ACTL6B), 5′-AMP-activated protein kinase catalytic subunit alpha-1 (PRKAA1), calcium-binding mitochondrial carrier protein Aralar1 (SLC25A12), LIM domain kinase 1 (LIMK1), and rho guanine nucleotide exchange factor 4 (ARHGEF4) (**Table 1**). As shown in **Figure 5**, five proteins, RARS, ACTL6B, PRKAA1, SLC25A12, and LIMK1, were significantly down-regulated in plasma samples of ASD, which were consistent with the expression changes of their corresponding genes mentioned previously. Even though the expression level of ARHGEF4 was not significantly down-regulated in autism samples, it was still expressed lower in autism samples compared with controls.

To evaluate the performance of these five proteins in distinguishing samples of ASD from healthy controls, receiver operating characteristic (ROC) curve analyses were carried out on protein concentrations measured by ELISA. **Figure 6** shows that SLC25A12 has the most discriminative ability with the area under curve (AUC) of 0.976 (sensitivity 100%, specificity 88.2%), and the AUCs of LIMK1 and RARS are 0.898 (sensitivity 94.7%, specificity 75.0%) and 0.862 (sensitivity 84.2%, specificity 81.2%), respectively. The remaining two proteins ACTL6B and PRKAA1 are with AUCs of 0.793 and 0.768, respectively.

DISCUSSION

ASD is a neurodevelopment disorder that has affected the health of millions of people. However, the pathogenesis of ASD is poorly understood and there are no reliable diagnostic biomarkers currently. In this study, we identified the potential blood protein biomarkers for ASD by a new strategy of computational prediction in conjunction with experimental validation, which could provide a more effective and specific way for biomarker discovery in blood (37).

First of all, 364 differentially expressed genes were identified for ASD based on transcriptome analysis. Functional enrichment analysis showed that these genes were mainly involved in BPs of TCA cycle, neurotransmitter transport, and synaptic vesicle exocytosis; CCs of mitochondrion, myelin sheath, and synaptic vesicle membrane; actin and neurofilament cytoskeleton organization and synapse; MFs of ATP binding, calcium ion binding, and syntaxin binding; and pathways of metabolic, nervous system diseases, and alternative splicing, which are all known to be associated with the pathophysiology of ASD.

From the aforementioned functional analysis, it could be speculated that the mitochondria, myelin sheath, synapses, and cytoskeleton of neurofilaments are impaired in the brains of children with ASD. Previous studies have reported that mitochondrial dysfunction seemed to be the most prevalent metabolic disease associated with ASD (38, 39). Mitochondrial dysfunction could lead to metabolic changes. Here, the metabolic abnormalities include carbon metabolism, TCA cycle, oxidative phosphorylation, glycolysis, and gluconeogenesis, which are consistent with previous published research (40–42). In addition, changes in myelin sheath, and actin and neurofilament cytoskeleton have been reported associated with ASD (43–47). In agreement with previous research (24, 44, 45, 47–51), the genes associated with pre-synaptic and post-synaptic proteins, synaptic vesicles, and neurotransmitter transport were observed as significantly changed in ASD subjects vs. controls. Furthermore,

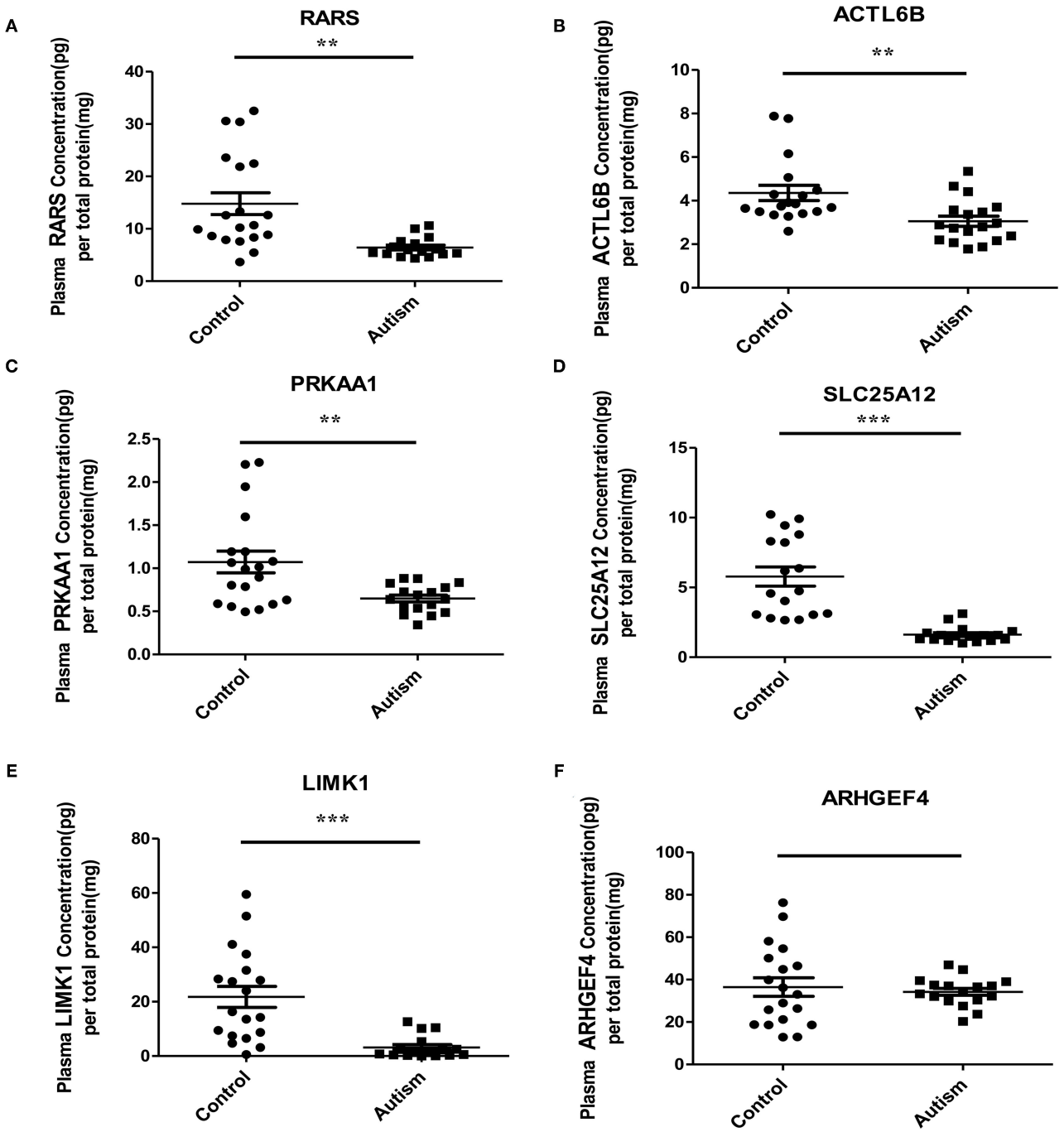

FIGURE 5 | Verification of the potential blood protein biomarkers for ASD by ELISA. $^{**}p < 0.005$; $^{***}p < 0.0005$.

it has been reported that differential alternative splicing was observed in ASD brains and blood (24, 52, 53), and the unfolded protein response and altered endoplasmic reticulum (ER) stress have also been reported to be associated with ASD (17, 54, 55). It should be a concern that these factors are interrelated with each other. Dysfunction of mitochondria might cause impairment of synaptic function, and both of them are related to neurological diseases such as AD, schizophrenia, and so on (42, 56). Alternative splicing has been reported to be related to the expression of synaptic-related genes in ASD (57). Mutations linked to ASDs in synaptic proteins such as NLGN3, CASPR2, and CADM1 might lead to ER stress conditions (58).

After functional analysis and literature survey, six proteins were selected for verified in plasma samples of ASD, and five

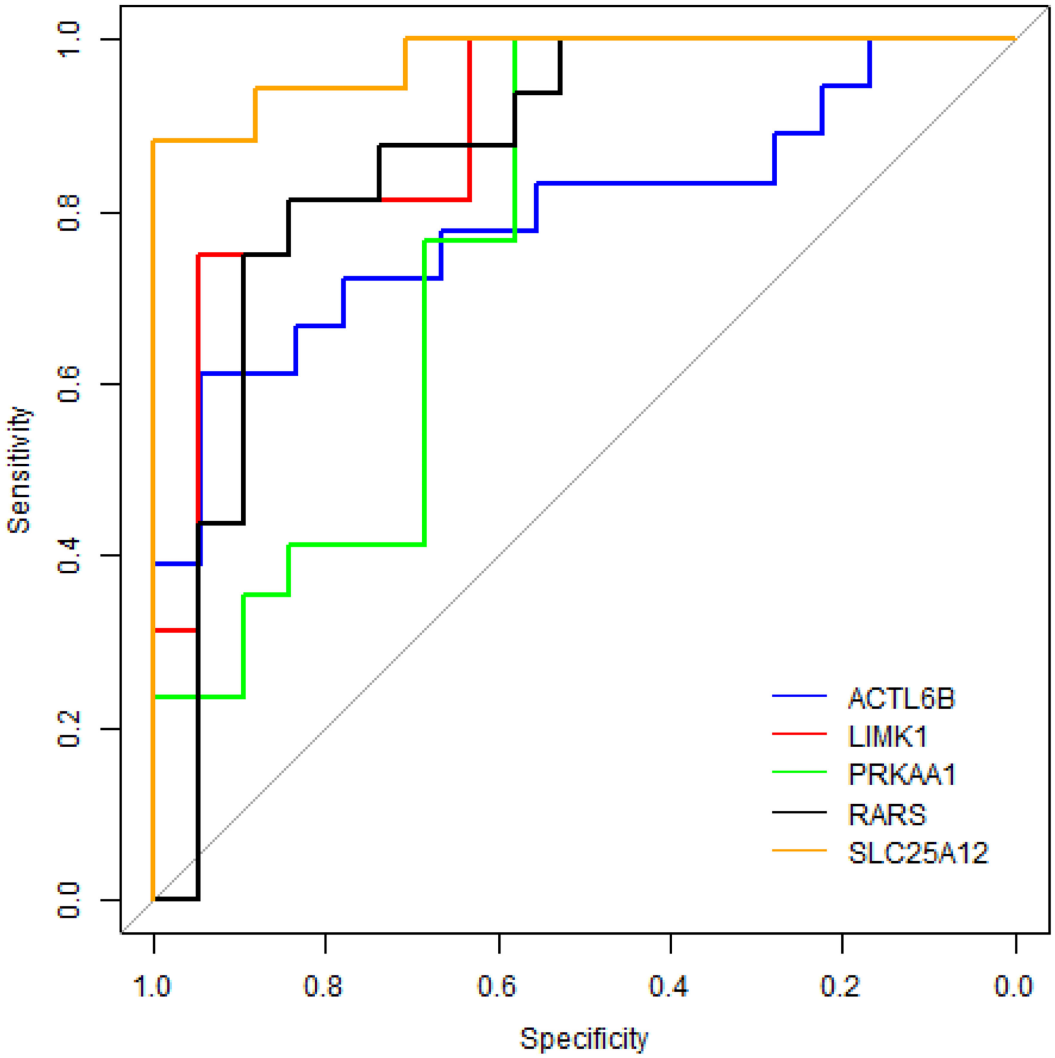

FIGURE 6 | ROC curve analyses on five differentially expressed proteins in plasma samples of ASD patients. The blue line represents protein ACTL6B, the red line is LIMK1, the green line is PRKAA1, the black line is RARS, and the orange line is SLC25A12.

were successfully verified, including RARS, ACTL6B, PRKAA1, SLC25A12, and LIMK1. Among them, RARS acts as an enzyme essential for RNA translation and plays an important role in myelination (35). *ACTL6B* was identified as a candidate risk gene for ASD with functions of neuron-specific chromatin remodeling and neurodevelopment (59, 60). PRKAA1, a catalytic subunit of protein kinase A (PKA), plays a key role in regulating cellular energy metabolism. It was found that regression in ASD might be associated with decreased PKA-mediated phosphorylation of proteins and abnormalities in cellular signaling (61). PRKAA1 has also been reported in several studies related to autism and/or ASD including linkage studies (62–64), NGS *de novo* mutation studies (65), and genome-wide association studies (66). *SLC25A12* has been proposed as a candidate gene for ASD due to its important role in mitochondrial function and ATP synthesis (67). Some research showed that single nucleotide polymorphism in *SLC25A12* might significantly contribute to ASD risk (68, 69). Increased evidence suggests that it may play a critical role in the pathogenesis of ASD (69–72). In particular, it has been reported that SLC25A12 is associated with autism of the Han Chinese in Taiwan (70). Meanwhile, it is worth noting that *SLC25A12* is with the highest "evidence score" in the AutismKB, indicating that it is closely associated with ASD. Moreover, *LIMK1* stimulates axonal outgrowth and involves in neurodevelopment and synaptic plasticity (73, 74). It has been reported to be related to ASD (24, 63). Furthermore, *ARHGEF* has been reported to be associated with copy number variants (CNVs) in children with ASD (75–77). Here, although it has no significant difference between the cases and the control group, the trend was in line with expectations and a larger sample size might be needed to verify the change.

ROC curve analyses showed that the AUCs of SLC25A12, LIMK1, and RARS were larger than 0.85, indicating that they are more powerful in distinguishing samples of ASD from healthy controls and might serve as new potential protein biomarkers for ASD in blood. As far as we know, this is the first study to investigate blood protein biomarkers for ASD through such a strategy. Proteins SLC25A12, LIMK1, and RARS were first reported here as new potential blood protein biomarkers for ASD. Clearly, these findings are needed to be confirmed on large number of samples.

In conclusion, the combination of computational prediction and experimental validation was used to identify blood protein biomarkers for ASD. A total of 364 differentially expressed genes were found in ASD, out of which 59 genes were predicted that their protein products could be secreted into blood as candidate ASD-related blood proteins. After functional analysis and literature survey, six proteins were selected for experimental validation and five were successfully verified in the plasma samples of ASD. ROC analysis showed that SLC25A12, LIMK1, and RARS are more powerful in differentiating ASD samples from controls and might serve as new potential protein biomarkers for ASD in blood.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Research Ethics Committee of Shenzhen University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LS conceived and designed the study and revised the article. FY performed statistical analysis, data interpretation, and drafted the first article. KZ and XL contributed to experimental validation and data analysis. CF and YG contributed to acquisition of blood samples and performed correlation analysis. JN coordinated the study design and article revision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.554621/full#supplementary-material

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An Epigenetically Distinct Subset of Children With Autism Spectrum Disorder Resulting From Differences in Blood Cell Composition

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Background: Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder that often involves impaired cognition, communication difficulties and restrictive, repetitive behaviors. ASD is extremely heterogeneous both clinically and etiologically, which represents one of the greatest challenges in studying the molecular underpinnings of ASD. While hundreds of ASD-associated genes have been identified that confer varying degrees of risk, no single gene variant accounts for >1% of ASD cases. Notably, a large number of ASD-risk genes function as epigenetic regulators, indicating potential epigenetic dysregulation in ASD. As such, we compared genome-wide DNA methylation (DNAm) in the blood of children with ASD ($n = 265$) to samples from age- and sex-matched, neurotypical controls ($n = 122$) using the Illumina Infinium HumanMethylation450 arrays.

Results: While DNAm patterns did not distinctly separate ASD cases from controls, our analysis identified an epigenetically unique subset of ASD cases ($n = 32$); these individuals exhibited significant differential methylation from both controls than the remaining ASD cases. The CpG sites at which this subset was differentially methylated mapped to known ASD risk genes that encode proteins of the nervous and immune systems. Moreover, the observed DNAm differences were attributable to altered blood cell composition, i.e., lower granulocyte proportion and granulocyte-to-lymphocyte ratio in the ASD subset, as compared to the remaining ASD cases and controls. This ASD subset did not differ from the rest of the ASD cases in the frequency or type of high-risk genomic variants.

Conclusion: Within our ASD cohort, we identified a subset of individuals that exhibit differential methylation from both controls and the remaining ASD group tightly associated with shifts in immune cell type proportions. This is an important feature that should be assessed in all epigenetic studies of blood cells in ASD. This finding also builds on past reports of changes in the immune systems of children with ASD, supporting the potential role of altered immunological mechanisms in the complex pathophysiology of ASD. The discovery of significant molecular and immunological features in subgroups of individuals with ASD may allow clinicians to better stratify patients, facilitating personalized interventions and improved outcomes.

Keywords: ASD, DNA methylation, epigenetics, granulocytes, blood cell proportion

INTRODUCTION

Autism spectrum disorder (ASD) is a heritable and prevalent neurodevelopmental disorder that is usually defined by impairments in cognition, communication and social interaction as well as by restrictive and/or stereotypical repetitive behaviors (1). Despite intense research efforts during the past decade, no definitive biological or clinical markers for ASD have been identified (2–4). This can be partly explained by the highly heterogeneous nature of ASD, both clinically and etiologically, which represents one of the greatest challenges in studying the molecular basis of ASD. The genetic underpinnings of ASD are mainly ascribed to different genetic variants such as rare copy number variations (CNVs), single-nucleotide variants (SNV) and *de novo* mutations that have been identified in ∼10–20% of individuals with ASD (5–7). While hundreds of ASD-associated genes have been identified that confer varying degrees of risk, no single gene variant accounts for >1% of all ASD cases (8–10). Despite its strong genetic component, several lines of evidence suggest that environmental factors and epigenetic mechanisms may contribute to ASD etiology; however, the molecular mechanisms underlying their contributions to the development of ASD are still unclear (11, 12). Epigenetic marks, including DNA methylation (DNAm), are involved in the programming of cellular differentiation and development; it is therefore plausible that the dysregulated DNA methylation patterns caused by genetic and/or environmental factors may permanently disrupt biological pathways involved in normal brain development (13).

There is direct evidence from case-control studies showing altered targeted and genome-wide DNAm and histone acetylation in multiple tissues of affected individuals, supporting a role for epigenetic dysregulation in the development of ASD (14–18). Of note, many ASD-risk genes function as epigenetic regulators, i.e., chromatin remodelers, histone modifying enzymes and transcriptional regulators (19–22).

Many epigenetic studies have investigated ASD-associated DNAm signatures in brain tissue, in order to identify epigenetic alterations potentially causative of or mechanistically related to ASD (23–25); however, they are seriously constrained by small sample sizes and the use of autopsy-derived tissue that may be confounded by post-mortem effects on epigenetic marks. Several candidate gene-based studies revealed DNAm alterations at ASD-risk genes such as *SHANK3*, *OXTR*, *EN2*, and *MECP2* in multiple brain regions (15, 17, 18, 26). Further, genome-wide screens of DNAm in the brain of individuals with ASD have identified inconsistent differences at a variety of genomic sites; the differentially methylated CpGs were mainly associated with genes enriched in synaptic function and immune response (16, 24). Although there is sufficient evidence for immune dysregulation in individuals with ASD, immune-related genes are not among those that contain loss of function variants in next-generation sequencing studies of autistic individuals, further reinforcing the evidence for the involvement of epigenetic mechanisms in the dysregulated immune system detected in brain samples of ASD-affected individuals.

Easily accessible tissues such as blood are often used in epigenetic studies for biomarker discovery in lieu of target tissues that are difficult to access, such as brain. Recent studies have identified specific peripheral blood DNAm signatures for each of 35 neurodevelopmental/ASD syndromes caused by pathogenic variants in genes that encode epigenetic regulators (27–30). There are a very limited number of genome-wide epigenetic studies that examined DNAm changes in peripheral blood of individuals with ASD (31, 32). These studies found inconsistent evidence for DNAm alterations associated with ASD, likely due to small sample sizes ($n < 100$) and a specific focus on twin pairs with a lack of extension to the general population. A recent study by Andrews et al. (33) performed a large case-control meta-analysis of blood samples in autistic patients. This study, despite a

Abbreviations: ADI-R, autism diagnostic interview revised; ADOS, autism diagnostic observation schedule; ANXA1, ANNEXIN A1; ANKRD22, ankyrin repeat domain 22; ASD, Autism spectrum disorder; SB-5, Stanford Binet Intelligence Scales; CHD7, Chromodomain-helicase-DNA-binding protein 7; CHD8, Chromodomain Helicase DNA Binding Protein 8; CNV, Copy number variant; CpG, Cytosine- phosphate- Guanine; CUL3, Cullin 3; DMR, Differentially methylated region; DNAm, DNA methylation; FDR, False Discovery Rate; G/L Ratio, granulocyte/lymphocyte ratio; GO, Gene ontology; MET, MET protooncogene; NK, Natural killer cell; NSD1, Nuclear Receptor Binding SET Domain Protein 1; PCA, Principal component analysis; REB, Research ethics board; SFARI, Simons Foundation Autism Research Initiative; SHANK2, H3,multiple ankyrin repeat domains 2; SNV, single nucleotide variant; VABS-II, Vineland Adaptive Behavior Scales; WAS, Wechsler Abbreviated Scale of Intelligence; WISC, Wechsler Intelligence Scale of Children.

higher sample size ($n = 796$), found no association between ASD and DNAm at genome-wide significance as no single CpG site achieved statistical significance at a Bonferroni correction level. However, they reported seven CpG sites that achieved suggestive statistical significance for association with ASD with consistent and stronger effects at the same sites among brain samples (33). Of note, these results were obtained from individuals of different ethnic backgrounds which can influence epigenetic changes as a potential confounding factor (34). Therefore, we reduced the ethnic heterogeneity by collecting the majority of samples from the same ethnicity in the present study.

In this study, we overcame previous limitations by investigating genome-wide DNAm in a large ASD ($n = 265$) cohort to identify blood-derived differentially methylated sites, as compared to control subjects. We hypothesized that, given the suggested role of epigenetics in ASD molecular etiology, epigenetic modifications could act as a useful biomarker that may contribute to the underlying etiology of subsets of patients with ASD. Our results demonstrate that DNAm alterations defined an epigenetically distinct subset of ASD cases that differentiate them from other ASD cases and controls. Notably, these observed DNAm differences were significantly associated with shifts in blood cell composition. Gene ontology analysis of the genes overlapping the differentially methylated CpG sites identified functions relevant to known pathophysiological mechanisms underlying ASD such as immune dysfunction, highlighting the biological significance of our DNAm signals.

METHODS

Research Participants

Participants of this study were selected from existing ASD cohorts: the Province of Ontario Neurodevelopmental Disorders (POND) Network, the Simons Simplex Collection (SSC), the Autism Speaks MSSNG project and the Genome Diagnostics Laboratory at The Hospital for Sick Children (SickKids). Participants were enrolled in studies approved by the Research Ethics Boards of the respective institutions (Holland Bloorview Kids Rehabilitation Hospital, Toronto; The Hospital for Sick Children, Toronto; McMaster Children's Hospital, Hamilton; Queen's University, Kingston; Western University, London) and informed consent was obtained from participating subjects and/or their parents or guardians.

ASD study cases consisted of individuals aged 1–18 years with a primary clinical diagnosis of ASD of undefined etiology; to that end, we excluded syndromic ASD cases carrying previously identified pathogenic variants with a known effect on DNA methylation, including variants in Chromodomain Helicase DNA Binding Protein 8 (*CHD8*), Chromodomain-helicase-DNA-binding protein 7 (*CHD7*), Nuclear Receptor Binding SET Domain Protein 1 (*NSD1*), and16p11.2 deletions (21, 28, 30). Clinical diagnoses were confirmed using the Autism Diagnostic Interview-Revised (ADI-R) (35) and Autism Diagnostic Observation Schedule (ADOS) (36) or ADOS-2 (37) by clinical staff formally trained on all measures. The neurotypical control samples matched for age- and sex were selected from a collection available in our laboratory, and the SSC sample. Individuals in the control group were recruited using physician/parental screening questionnaires. The majority of individuals included in this study are of Caucasian descent (**Supplementary Table 1**). No significant differences were found between the case and the control group in terms of age and sex. The description of the study sample can be found in **Table 1**.

DNAm Array Processing

DNA samples from whole blood were sodium bisulfite converted for all ASD cases and controls using the Qiagen EZ DNA Methylation kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The modified genomic DNA was then hybridized to the Illumina Infinium HumanMethylation 450 BeadChip array to interrogate over 485,000 individual CpG sites in the human genome, at The Center for Applied Genomics (TCAG), SickKids Research Institute, Toronto, Canada. The distribution of the samples on the arrays was randomized between cases and controls. Samples were run on arrays in a total of nine batches, with each batch containing ASD cases and controls.

The *minfi* Bioconductor R package (38) was used to preprocess the array data and generate Beta [β]-values from the raw intensity measures. Preprocessing included standard quality control metrics in *minfi*, including density plot, median intensity QC plots, and control probe plots. All samples passed quality control as previously described (28, 30). Methylation data were then filtered by removing probes exhibiting low detection *p*-value > 0.05 in more than 25% of the samples, cross-reactive probes, probes located on sex chromosomes, probes targeting CpG sites within 10 bp of a single-nucleotide polymorphic sites (SNPs) with a minor allele frequency $> 1\%$, probes with raw beta $= 0$ or 1 in $> 0.25\%$ of samples, and non-CpG probes; a total of 427,137 probes were retained after filtering based on these criteria for normalization and downstream differential analysis. Normalization with background subtraction was then performed using Illumina control probes. The resulting β values represent percent DNAm, ranging from 0 to 1 corresponding to an unmethylated to a fully methylated CpG site.

Differential DNAm Analysis Between ASD Cases and Neurotypical Controls

Linear regression was performed using the *limma* package (39) to identify statistically significant differentially methylated CpG sites between all ASD cases ($n = 265$) and controls ($n = 122$), accounting for covariates including age, sex, batch and estimated cell-type proportion. Blood cell type proportions were estimated using Houseman's algorithm and the Bioconductor packages *minfi* and *FlowSorted.Blood.450k* (40). Given that these cell type proportions are highly correlated, only monocyte, granulocyte, and natural killer (NK) proportions were included in the regression model (**Supplementary Table 1**). The remaining cell types were highly correlated with granulocyte proportion ($r > 0.6$, p-value < 0.05). We computed the false discovery rate (FDR) using the Benjamini-Hochberg method (41). A significant difference in DNAm between ASD cases and controls was called for each CpG site that met the cutoffs of FDR adjusted

TABLE 1 | Demographic characteristics for ASD cases and neurotypical controls.

Sample groups	Control ($n = 122$)	Full ASD sample ($n = 265$)	DNAm-based ASD subset ($n = 32$)	Remaining ASD sample ($n = 233$)
Sex	N	N	N	N
Male	84	220	27	193
Female	38	45	5	40
Age (years)	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
	12.20 ± 4	8.82 ± 4	7 ± 4.50	9.10 ± 4
DNA collection site	N	N	N	N
TCAG (POND/MSSNG)	6	220	26	194
SSC	30	43	6	37
Genome Diagnostics Lab (SickKids)	–	2	–	2
Weksberg Lab (SickKids)	86	–	–	–
Cell type proportion	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
B cell	0.10 ± 0.03	0.11 ± 0.04	0.16 ± 0.04	0.11 ± 0.04
CD4T	0.17 ± 0.04	0.20 ± 0.06	0.31 ± 0.06	0.18 ± 0.05
CD8T	0.10 ± 0.03	0.11 ± 0.04	0.16 ± 0.05	0.10 ± 0.04
Granulocytes	0.51 ± 0.10	0.47 ± 0.10	0.31 ± 0.05	0.50 ± 0.10
Monocytes	0.10 ± 0.02	0.08 ± 0.02	0.05 ± 0.02	0.08 ± 0.02
NK	0.05 ± 0.04	0.04 ± 0.04	0.02 ± 0.04	0.04 ± 0.04
G/L ratio	1.38 ± 0.50	1.11 ± 0.50	0.48 ± 0.10	1.20 ± 0.50
Clinical Measures		Mean ± SD	Mean ± SD	Mean ± SD
ADI_R: Communication domain verbal total		16.52 ± 4.60 ($N = 200$)	16.50 ± 4.30 ($N = 22$)	16.60 ± 4.60 ($N = 178$)
ADI_R algorithm total scores		43.10 ± 11 ($N = 110$)	41 ± 11 ($N = 10$)	43 ± 11 ($N = 100$)
ADOS: Communication + Social Interaction total score		13.60 ± 4.60 ($N = 168$)	14.30 ± 4.10 ($N = 17$)	13.50 ± 5 ($N = 151$)
ADOS: Social Affect total + Restricted and Repetitive Behavior total score		16.5 ± 6.10 ($N = 52$)	19 ± 5.40 ($N = 9$)	16.10 ± 6 ($N = 43$)
VABS-II: Communication Standard Score		78 ± 16.5 ($N = 123$)	80 ± 16 ($N = 14$)	76.50 ± 18 ($N = 109$)
IQ-Scale (FSIQ score)		80 ± 30 ($N = 129$)	80 ± 30 ($N = 114$)	83.60 ± 27 ($N = 15$)

NK, Natural killer cell; G/L, Granulocyte/Lymphocyte.

p-value < 0.01 and an effect size of 5% mean methylation difference ($|\Delta\beta| > 0.05$).

Functional and Genomic Enrichment of Differentially Methylated CpG Sites

For genomic enrichment analysis, the differentially methylated CpG sites were submitted to GREAT 4.0.4 (Genomic Regions Enrichment of Annotations Tool) (42) using a maximum near gene extension of 10 Kb and a hypergeometric FDR q-value < 0.01 for significance. Enrichment of the gene lists in each Gene Ontology (GO) term was defined against the background set of all probes that remained in the data after *minfi* probe filtering ($n = 427{,}137$). Terms with two or more gene hits were reported. We also compared our differentially methylated genes with known SFARI Gene ASD-risk genes (https://gene.sfari.org/) to further understand the biological relevance of our DNAm signal. In addition, we compared the genomic distribution of differentially methylated CpG sites to the background set of probes for relation to CpG island and overlapping enhancer region using a hypergeometric test (p-value < 0.05).

Identification of Differentially Methylated Regions

To identify significantly differentially methylated regions (DMRs) that are associated with ASD, we used the Bioconductor bumphunter package. The bumphunting design matrix accounted for the potential confounding effects of sex, age, batch, and blood cell-type proportions (estimated monocyte, granulocytes, and NK proportions). The analysis identified consecutive CpGs no more than 0.5 kb apart with an average regional methylation difference $|\Delta\beta| > 5\%$ between cases and controls. Statistical significance was established using 1,000 randomized bootstrap iterations. The resulting DMRs were post-filtered to retain only those with p-value < 0.05 across the DMR and a length (number of consecutive CpGs) of a least three sites. To further enhance stringency, we considered DMRs comprising

at least one CpG from the differentially methylated CpG sites identified between ASD cases and controls, as described above.

Comparisons of Blood Cell Composition in Sample Groups

As described above, relative proportions of underlying blood cell type were estimated using the Houseman method. We assessed our groups for possible differences in these proportions; the blood cell types measured included, B cell, CD4T, CD8T, monocyte, granulocytes, and NK. These blood cell types were those on which the Houseman method was originally trained, using peripheral blood leukocyte subtypes purchased from AllCells®, LLC (Emeryville, CA) or sorted cells from whole blood using negative and positive selection of surface antibodies (B-lymphocytes: CD19+; CD4 T-lymphocytes: CD3+, CD4+; CD8 T-lymphocytes: CD3+, CD8+; monocyte: CD14+; granulocytes: CD15+; natural killer: CD56+) (40). We also calculated the granulocyte/lymphocyte ratio (G/L ratio), which is a common indicator of inflammatory response. As well, the relationship between age and cell type proportion and G/L ratio was evaluated in the sample groups using the Spearman's rank correlation coefficient (r) and p-value <0.05.

TABLE 2 | Clinical measures analyzed in our ASD cohort.

Scale	Subscale	The age range analyzed
WASI, WASI II, WISC IV or SB-5	Full Scale Intelligence Quotient	6–18 years 1–6 years
ADI-R	Communication domain verbal total	2–18 years
	Algorithm total scores [in three domains: social interaction, communication, and restricted repetitive behavior (RRB)]	2–18 years
ADOS	Communication + Social Interaction total score	2–18 years
	Social Affect + Restricted Repetitive Behaviors total score	2–18 years
VABS-II	Communication Standard Score	1–6 years

The age range represents the group of ages administered to each clinical measure. WAS, Wechsler Abbreviated Scale of Intelligence; WISC, Wechsler Intelligence Scale for Children; SB, Stanford Binet Intelligence Scales.

Identification of Genetic Variants Associated With ASD

In our cohorts, we interrogated genetic variation at 366 candidate genes, selected based on the reported SFARI Gene association with ASD. These candidate genes were classified as "Category 1" (high confidence) and "Category 2" (strong candidate) by SFARI Gene at the time of manuscript submission. All the genetic variants were obtained through whole-exome and whole-genome sequencing data using the Genotypes and Phenotypes in Families (GPF) tool (https://gpf.sfari.org/) and MSSNG database (http://www.mss.ng/researchers) for individuals who underwent genome sequencing for investigation of ASD. For each gene, the mode of inheritance was specified, and rare non-synonymous variants were prioritized. For these genes, only *de novo* variants considered as likely pathogenic or pathogenic with minor allele frequency $< 1\%$ were retained as advised by the guidelines from the American College of Medical Genetics-Association for Molecular Pathology (ACMG-AMP). We then evaluated whether pathogenic *de novo* variants within ASD risk genes differed in frequency between the ASD subset and the remaining ASD cases.

To further investigate genetic factors involved in ASD, we assessed variants genome-wide (i.e., not limited to SFARI genes), including single nucleotide variants (SNVs), indels, and copy number variants (CNVs), in individuals with ASD from MSSNG ($n = 203$) and SSC ($n = 39$).

Individuals in MSSNG were sequenced using either Complete Genomics or Illumina (HiSeq 2000 or HiSeq X) platforms. SNVs, indels, and CNVs were detected from Complete Genomics samples as previously described (5), and the variants were lifted over from hg19 coordinates to hg38 coordinates for further analysis. For individuals sequenced on Illumina platforms, read alignment (hg38) and SNV/indel detection were performed using the Sentieon pipeline (43). Individuals in SSC were sequenced on the Illumina HiSeq X platform. SNVs and indels were downloaded from SFARI Base (https://www.sfari.org/resource/sfari-base). SNVs and indels from both cohorts were annotated using a custom ANNOVAR-based pipeline (44). CNVs were detected in both MSSNG (Illumina samples only) and SSC using a previously-described workflow (45) involving the algorithms ERDS (46) and CNVnator (47). High-confidence *de novo* SNVs and indels were detected using DeNovoGear (48) as previously described (5). *De novo* CNVs were identified as those that were detected by both ERDS and CNVnator, that were rare [$<1\%$ frequency in MSSNG parents and 1,000 Genomes Project (49) population controls], and that were not detected by ERDS or CNVnator in either parent.

We compared our ASD groups for possible differences in the number of *de novo* SNVs/indels per individual, and frequency or type of variants (for SNVs and indels: stop gain/ splice site/ frameshift; for CNVs: deletion/duplication).

Examination of Risk Factors and Clinical Phenotypes

See **Table 2** for descriptions of all clinical measures.

We assessed risk factors previously shown to be associated with an increased risk of ASD. These included assisted reproductive technology (ART), gestational age, maternal smoking, parental age at the time of birth, and proband sex. Clinical phenotypes were measured by the following scales: Vineland Adaptive Behavior Scales (VABS-II); ADIR and ADOS as indicators of ASD severity and symptomatology. IQ was assessed using the appropriate scale as determined by the child's age: the Wechsler Abbreviated Scale of Intelligence (WASI, WASI-II), the Wechsler Intelligence Scale of Children-IV (WISC-IV), or the Stanford Binet Intelligence Scales, 5th ed (SB-5) (FSIQ score used for each test). These measures, risk factors

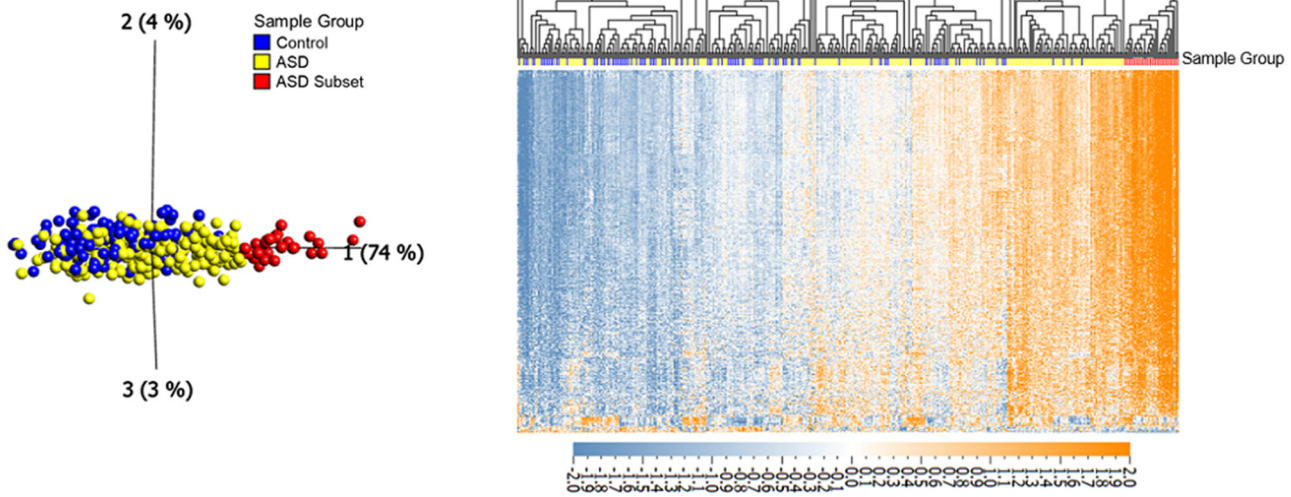

FIGURE 1 | Differential DNAm at 400 in ASD ($n = 265$) and neurotypical controls ($n = 122$) reveals an epigenetically unique subset of ASD cases ($n = 32$). **(A)** Principal component analysis performed on 400 CpGs (FDR adjusted p-value < 0.01 and $|\Delta\beta| > 5\%$), with axes representing first three principal components. **(B)** Corresponding heatmap hierarchical clustering using Eucledian distance metrics. Orange indicates high DNAm, and blue gray indicates low DNAm, normalized for visualization (mean = 0, variance = 1). Samples labeled with red and yellow represent the ASD subset and the remaining ASD cases, respectively, blue samples represent controls.

and clinical data were assessed against DNAm in all samples for which they were available, namely ASD cases recruited from the POND network, MSSNG and SSC. Of note, these clinical features were measured in the same study visit as tissue sample collection, or within a 7-month period.

RESULTS

Identification of DNAm Alterations Associated With ASD

To investigate DNAm alterations associated with ASD, we compared genome-wide DNAm in blood from individuals with heterogeneous ASD ($n = 265$) to sex- and age-matched, neurotypical controls ($n = 122$) at 427,137 CpG sites (**Table 1**). We identified 400 significantly differentially methylated CpG sites across the genome at an FDR adjusted p-value <0.01 and $|\Delta\beta| > 5\%$ (5% methylation difference) as illustrated by a volcano plot in **Supplementary Figure 1** and **Supplementary Table 2**. Ninety seven percentage of these CpG sites exhibited higher methylation levels (with DNAm differences ranging from 5 to 14%) in ASD cases as compared to controls. Principal component analysis (PCA) run on the 400 differentially methylated sites showed a gradient of DNAm values starting from a group of mostly controls to a separate subset of ASD-affected individuals that was epigenetically distinct from both controls and remaining ASD cases (n =32; **Figure 1**; **Table 1**). This observation was supported by hierarchical clustering of DNAm values, with this DNAm gradient going from a cluster comprised of mostly controls where the majority of differentially methylated CpGs are hypomethylated to the distinct ASD subset where the majority of these sites are hypermethylated. No significant differences were found between the ASD subset and the remaining ASD cases in terms of age and sex (Mann-Whitney p-values >0.05); both groups of ASD, the subset and remaining cases, included individuals from all three cohorts (POND, MSSNG and SSC; see **Table 1** and "Research participants" subsection of **Methods** for details). Moreover, running an additional *limma* regression model with DNA collection site included as an additional covariate did not alter the results; of the 400 significantly differentially methylated sites, 381 CpGs remained significant and could still differentiate the ASD subset from the remaining ASD cases (results not shown).

The majority of the differentially methylated CpG sites mapped to promoter regions or gene bodies of 159 RefSeq genes (**Supplementary Table 2**). The genomic distribution of the 400 CpGs was compared with that of the background test sites. We found a significantly higher proportion of CpGs located in open sea (72 vs. 35%; p-value <0.05) and a depletion of island CpGs (0 vs. 32%; $p < 0.01$) (**Figure 2A**). Moreover, the differentially methylated sites were found to be enriched in enhancers, as compared to the full 427,137 CpG sites (49 vs. 23%; p-value <0.05) (**Figure 2B**).

Functional Enrichment of Differentially Methylated CpG Sites

We performed gene ontology analysis of the 400 differentially methylated sites using GREAT 4.0.4 to assess enrichment of common biological processes, molecular functions,

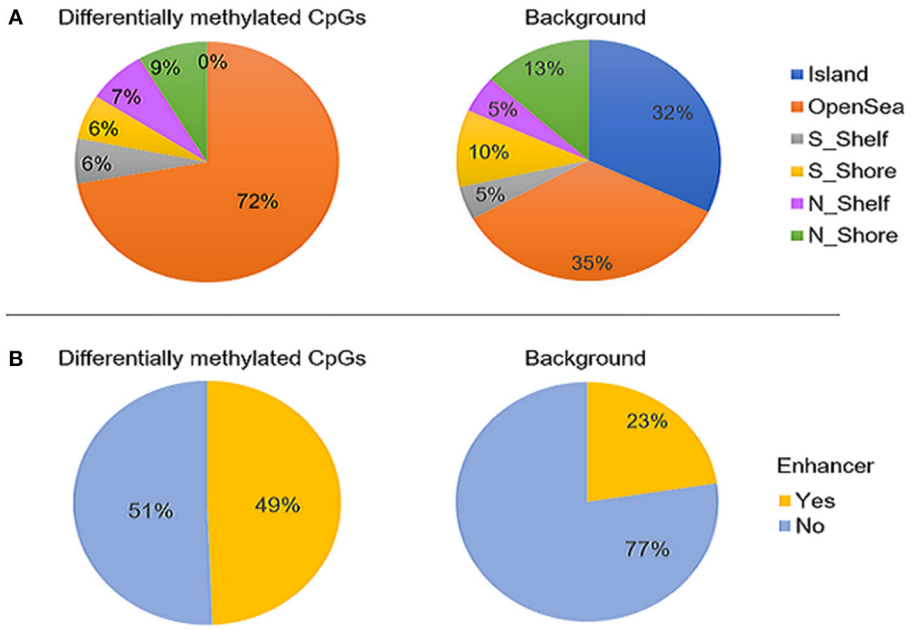

FIGURE 2 | The genomic distribution of the 400 differentially methylated CpG sites identified between ASD cases ($n = 265$) and controls ($n = 122$, left) compared to the background set of all probes that retained after probe filtering ($n = 427{,}137$) (right). **(A)** proportion CpG sites in relation to CpG islands and **(B)** proportion of CpGs overlapping enhancer regions. The differentially methylated sites were found to be significantly enriched in open sea and enhancers (p-values < 0.05) and depleted in CpG islands (p-value < 0.01). "Island" is CpG island; N_shore and S_shore are north (upstream) and south (downstream) shores, i.e. 2kb regions flanking island; N_shelf and S_shelf are north (upstream) and south (downstream) shelves, i.e. 2kb regions flanking island shores.

and cellular components of genes mapping to these CpG sites. GREAT identified 159 genes that overlapped the 400 CpG sites detected in our differential methylation analysis. We identified 27 GO biological processes assigned to the differentially methylated sites; the majority of them were related to immune function, such as immune and inflammatory response, in addition to enrichment for cellular secretion, as the top GO terms (hypergeometric FDR q-value < 0.01; **Supplementary Table 3**). Two GO cellular components met significance: granule membrane and inflammasome complex (**Supplementary Table 4**), while no molecular functions were significantly enriched.

Of the genes mapping to sites of differential methylation ($n = 159$), 26 are listed by SFARI as ASD-risk genes (**Supplementary Table 5**). These included genes encoding proteins of the immune and nervous systems, such as Cullin 3 (*CUL3* [MIM: 614496]), ANNEXIN A1 (*ANXA1* [MIM:151690]), SH3 and multiple ankyrin repeat domains 2 (*SHANK2* [MIM: 613436]) and MET protooncogene (*MET* [MIM: 164860]).

Identification of Differentially Methylated Regions

In addition to assessing each CpG independently, DMRs were evaluated, identifying regional DNAm differences. Significant DMRs were defined by $p < 0.05$, $|\Delta\beta| \geq 5\%$ and a length of at least three consecutive CpG sites, of which at least one had already been identified as a significant site in our differential methylation analysis (400 sites). Regional DNAm analysis identified 15 significant DMRs (**Supplementary Table 6**). As expected, these CpGs did not include those that mapped to open sea but rather higher density CpG regions including island shelves and shores. The longest DMR spanned 0.67 Kb and mapped to Galactoside-Binding, Soluble, 1 (*LGALS1*) which encodes Galectin-1 involved in regulating apoptosis, cell proliferation and cell differentiation (**Supplementary Table 6**).

Assessment of Blood Cell Type Composition in DNAm-Based Sample Groups

To investigate the relationship between the identified enrichment in immune function and inflammatory response associated with the ASD subset signature, we evaluated possible differences in estimated immune blood cell proportions between the three groups: the epigenetically unique ASD subset, the remaining ASD cases, and controls. Interestingly, we identified shifts in cell type proportions in the ASD subset as compared to the remaining ASD cases and controls (**Figures 3**, **4**). Namely, these individuals exhibited a significant increase in CD4T proportion (p-value < 0.01). In contrast, granulocyte proportion significantly decreased and accordingly, the G/L ratio was lower (p-value < 0.0001) in the ASD subset. We found significant differences in blood cell type composition between the remaining ASD cases and controls (**Table 3**). In addition, we ran 20 iterations of Mann-Whitney tests for each cell type on randomly sampled groups of $n = 32$ ASD cases (from the ASD cases excluding the DNAm-based subgroup; $n = 233$) vs. the remaining ASD cases, to ensure the

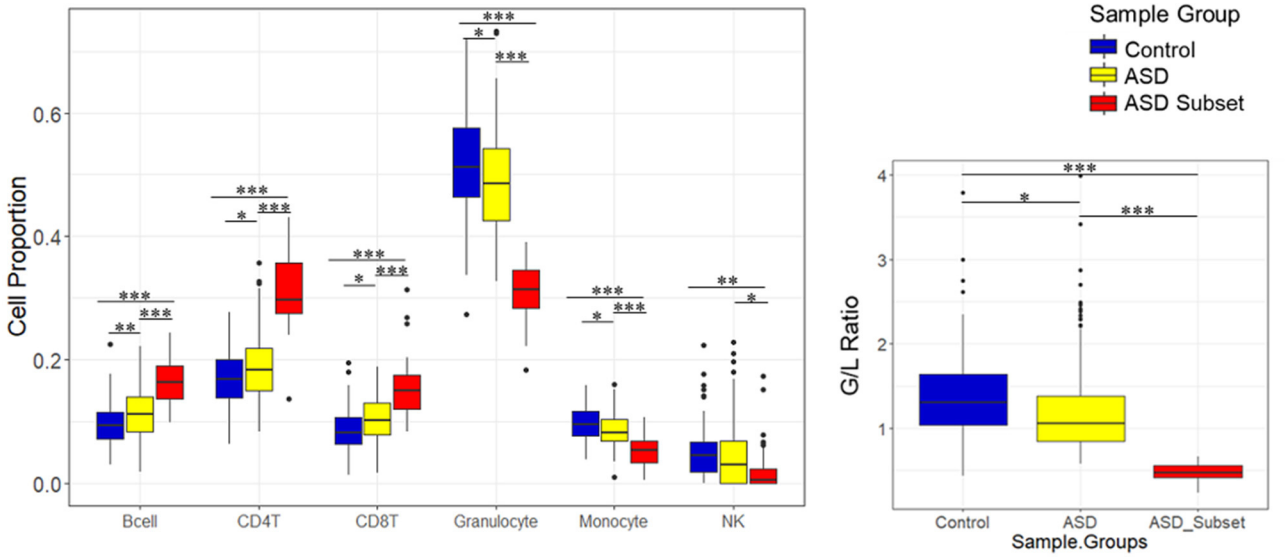

FIGURE 3 | Relative proportions of blood cell types in sample groups, as estimated by DNAm. Boxplots show immune blood cell proportions estimated by the Houseman method **(A)** and calculated granulocyte/lymphocyte (G/L) ratio **(B)**. Epigenetically unique ASD subset (red; $n = 32$), the remaining ASD cases (yellow; $n = 233$), and controls (blue; $n = 122$). ASD subset exhibited significant shifts in cell type proportions and the G/L ratio (p-value < 0.01) as compared to the remaining ASD cases and controls. Black bars with asterisk represent significant differences in estimated blood cell proportions between the groups (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

true differences held up to permutation testing. Only a single iteration produced a p-value < 0.05 (seen in monocytes) and no permutation analysis ed p-values approached neared those of the true associations (all p-values > 0.04; **Supplementary Figure 2**).

To investigate if the cell-type proportions was correlated with the age of the samples, we performed correlation analysis based on Pearson correlation coefficient (r) and a p-value <0.05. We found that in all the ASD cases, age was positively correlated with granulocyte proportion (ASD subset: $r = 0.43$, p-value $= 0.01$; the remaining ASD: $r = 0.35$, p-value < 0.001) (**Figure 5A**) and G/L ratio (ASD subset: $r = 0.45$, p-value $= 0.01$; the remaining ASD: $r = 0.37$, p-value < 0.001) (**Figure 5B**). A significant negative correlation was found between age and CD4T proportion in the ASD cases (ASD subset: $r = -0.4$, p-value $= 0.02$; the remaining ASD: $r = -0.2$, p-value $= 0.002$) (**Figure 5C**). In contrast, the cell type proportions, and the G/L ratio showed no correlation with age in control subjects ($r < 0.2$, p-value > 0.5).

Reassessment of Differential Methylation Associated With ASD After Removing the ASD Subset

To further investigate DNAm in our ASD cohort, we removed from our dataset the 32 ASD cases that were detected as unique both epigenetically and in blood cell composition and performed differential methylation analysis between the remaining ASD cases ($n = 233$) and controls ($n = 122$) using the same analytical methods, i.e., *limma* regression, covarying for age, sex, and estimated cell type proportion (granulocyte, NK, and monocyte). Linear regression analyses identified 77 significantly differentially methylated CpG sites with FDR adjusted p-value <0.01 and $|\Delta\beta| > 5\%$ (**Supplementary Table 7**). Notably, PCA continued to show a gradient of DNAm value tightly associated with granulocytes proportion and the G/L ratio across the ASD cases on PC1 (**Supplementary Figures 3**, **4**). As such, epigenetic differences between ASD cases and controls may be attributed either to underlying differences in blood cell composition or the identification of CpG sites that are blood cell type specific.

Identification of Genetic Variants Associated With ASD

We investigated 366 genes classified by SFARI Gene as high risk for ASD and looked for differences in pathogenic *de novo* variant frequency between our DNAm-based ASD groups. In the ASD subset, we identified six individuals (18%) with nine different *de novo* pathogenic variants at different genes (**Supplementary Table 8**). Only 34 individuals (14%) of the remaining ASD cases harbored *de novo* pathogenic variants (**Supplementary Table 9**); most of these were missense variants. Given that the ASD subset did not differ from the rest of the ASD cases in the frequency or type of high-risk variants, we, therefore, expanded our analysis to identify the genome-wide *de novo* pathogenic variants including SNVs, indels, and CNVs associated with ASD.

Likewise, no significant differences were detected between these two groups of ASD for the average number of *de novo* SNVs/indels per individual (average of 1.2 per individual in both groups), and frequency or type of SNVs and indels. However, in the larger ASD cohort, i.e., not the subset with unique DNAm, we

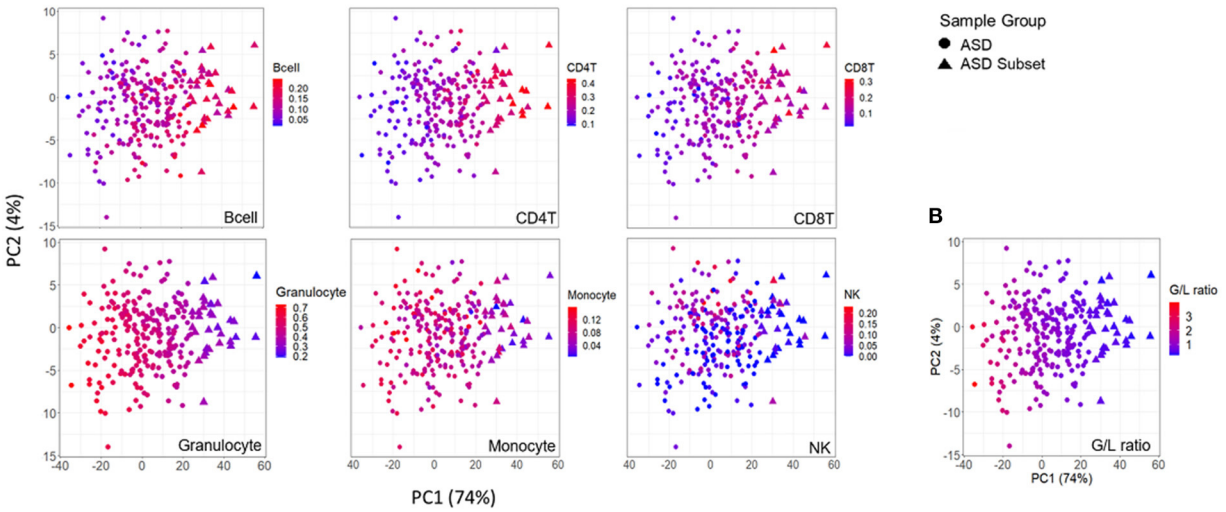

FIGURE 4 | Association between DNAm variation and cell type proportions across ASD cases. Scatterplots of first two principal components from principal component analysis (PCA) performed on 400 differentially methylated sites between ASD cases ($n = 265$), and controls ($n = 122$). **(A)** distribution of blood cell proportions [from left to right: B cells, CD4T cells, CD8T cells, granulocytes, monocytes and natural killer cells (NK)] and **(B)** granulocyte/lymphocyte (G/L) ratio across ASD cases. Samples plotted as triangles represent distinct ASD subset ($n = 32$) and circles represent remaining ASD cases ($n = 233$). Color of point indicates proportion of given cell type in each sample.

identified nine overlapping CNV regions of mostly duplications that were identified in more than one patient; The only common deletion identified was assigned to the gene Patched domain-containing protein 1 (*PTCHD1* [MIM: 300828]), which is a high -risk ASD gene (**Supplementary Table 10**).

Examination of Risk Factors and Clinical Phenotypes in DNAm-Based Groups

We evaluated the factors that increase the risk of ASD and behavioral phenotypes, comparing the epigenetically unique ASD subset to the remaining ASD cases. No significant differences were detected between these two groups of ASD cases for ART, gestational age, maternal smoking, parental age, and proband sex (t-test p-values > 0.05). Similarly, clinical phenotypes measured in the cohort did not differ significantly between individuals of the two groups of ASD. Namely, no significant differences were detected between these two groups of ASD cases for ADI-R (Communication domain verbal and algorithm total), ADOS (Communication + social interaction and social Affect + restricted repetitive behaviors) and VABS-II (Communication Standard), and IQ scores (FSIQ) (**Table 1**).

DISCUSSION

In this study, we sought to assess genome-wide DNAm alterations associated with ASD. We identified a subset of ASD cases that exhibit differential methylation patterns distinct from both controls and the remaining ASD group as well as significant shifts in cell type proportions, i.e., the granulocyte-to-lymphocyte ratio was significantly lower in the ASD subset than in the remaining ASD cases and controls. In the present study, beyond blood cell composition, we found no significant differences between the ASD subset and the remaining ASD cohort, including sex, age, genetic risk variants or clinical measures such as ADI, VABS and ADOS subscale scores. Furthermore, our study provides additional support for previously reported involvement of *SHANK2*, *ANXA1*, *MET*, *CUL3* and other genes in the pathophysiology of ASD. Our study suggests that at least one mechanism underpinning differential methylation between ASD cases and neurotypical controls is a difference in blood cell type proportion.

It is important to note that blood cell type proportion was estimated from DNA methylation. As such, it is possible that differences observed may be attributed to true changes in the blood cell composition or that DNA methylation alterations exist in the ASD subgroup at CpG sites used to estimate blood cell composition. A previous meta-analysis of methylation studies of ASD by Andrews et al. (33) reported in their patient demographics, significant differences in granulocyte and B cell proportions between ASD cases and control subjects parallel to those found in our study. These investigators found no single CpG to meet genome-wide significance using Bonferroni correction ($p < 1.12 \times 10^{-7}$) for the association between ASD and DNAm and did not interpret cell types differences in the discussion (33). Regardless, it is plausible that these methylation alterations may be indicative of altered immune function in the ASD subset. As well, this is not the first instance in which DNAm has been used to identify changes in blood cell proportion associated with a disorder; this has been reported in both asthma and systemic lupus erythematosus by Kong et al. (50). They showed that the proportion of DNAm alterations attributable to changes in cell type composition varies considerably in both

TABLE 3 | Immune blood cell composition comparisons between sample groups: the epigenetically unique ASD subset ($n = 32$), the remaining ASD cases ($n = 233$), and controls ($n = 122$).

Cell type composition	Comparison groups		
	ASD subset vs. ASD mean difference ± SE	ASD subset vs. Control mean difference ± SE	ASD vs. Control mean difference ± SE
B cell	0.05 ± 0.01***	0.07 ± 0.01***	0.02 ± 0.003**
CD4T	0.13 ± 0.01***	0.14 ± 0.01***	0.01 ± 0.005*
CD8T	0.06 ± 0.01***	0.07 ± 0.01***	0.01 ± 0.003*
Granulocytes	−0.20 ± 0.01***	−0.21 ± 0.01***	−0.01 ± 0.01*
Monocytes	−0.03 ± 0.004***	−0.04 ± 0.003***	−0.01 ± 0.002*
NK	−0.02 ± 0.01*	−0.03 ± 0.01**	−0.01 ± 0.004
G/L ratio	−0.72 ± 0.03***	−0.92 ± 0.05***	−0.18 ± 0.05*

NK, Natural killer cell; G/L, Granulocyte/Lymphocyte.
$^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

asthma and systemic lupus erythematosus, suggesting disease-specific cell subtype proportion changes contributing to DNAm alterations (50). Future studies using differential blood counts and DNAm-based blood cell estimates from the same blood draws are required to clarify this relationship.

Two additional findings in our study support the observed relationship between DNAm, immune cell type and ASD in our cohorts. The majority of the differentially methylated CpG sites were enriched for gene ontology categories implicated in immune and inflammatory response (**Supplementary Table 4**), which strengthens the scientific evidence that a dysregulated immune system is one of the contributing factors in ASD (51). Our findings are consistent with the methylation analysis in brain that demonstrates altered immune response in the cortical region of autistic cases, Brodmann area 10, correspond with epigenetic modulation of genomic regions relevant to several categories related to immune response, including inflammatory response to antigens and positive regulation of cytokine biosynthetic processes (16). As well, brain and blood transcriptome studies show that many of the genes exhibiting a higher variability in their overall expression pattern were related to the immune system in autistic individuals, indicating dysregulation in immune functions in ASD (52–55). A comparison between our differentially methylated sites and the transcriptomic data reported in gene expression studies showed overlap with two genes characterized by significant DNA hypermethylation in our ASD subset and decreased expression levels. The *ANXA1* gene, which encodes a protein that functions in adaptive immunity, was found to be upregulated in ASD individuals (56). As well, ankyrin repeat domain 22 (*ANKRD22*) overlapping three differentially methylated CpGs was found to be significantly downregulated in blood (57); this gene encodes a protein that specifically interacts with STING, a critical protein function in multiple anti-viral innate immune pathways (58). The prior evidence of hypermethylation of the immune-related genes correlating with decreased expression further supports the potential role of epigenetic regulation of the altered immune response associated with ASD. Further, when the genome-wide linear regression of ASD vs. controls was rerun with the ASD subset removed, the differentially methylated CpG sites were still strongly predictive of blood cell proportion.

We searched for overlap between differentially methylated sites in our blood DNAm and previous studies of DNAm in individuals with ASD. We found 23 overlapping CpG sites with DNAm signature detected in adult cortical regions (16); CpG sites overlapped notable genes such as syndecan-2 (*SDC2* [MIM:142460]), Dystonin (*DST* [MIM: 113810) and mediator complex subunit 12L (*MED12L* [MIM: 611318]); However, we did not find any overlap with the findings of blood DNAm studies (31, 32).

In recent years, there is emerging evidence and growing concern that a dysregulated or abnormal immune response may be involved in the development of some forms of ASD. Several lines of research have provided substantial evidence of immune dysregulation in subsets of individuals with ASD, including skewed inflammation responses, cytokines, and total numbers and frequencies of immune cells (59–61). The inconsistencies in previous research findings are marked by considerable variation in the prevalence of ASD by ethnicity/race, sex, geographic area, and level of intellectual ability. The heterogeneity of ASD is the source of much difficulty in study underlying pathophysiology, etiology and biomarkers of this neurodevelopmental disorder. This is especially apparent in genetic studies of ASD, in which rare SNVs and CNVs account for only a small proportion of ASD risk. Biological measures, such as DNAm or immune markers, which exhibit consistent changes across substantial subsets of individuals with ASD, may provide a new avenue for ASD research.

A number of limitations should be noted. As described above, cell types composition was not measured directly but rather estimated using specific CpG sites that exhibit blood cell-type specific DNAm patterns. While this method has been validated and is widely used, it is possible that DNAm levels at these CpGs sites were altered by factors other than cell type, causing skewed estimates. However, it is worth noting that cell type of origin is one of the strongest predictors of DNAm patterns. For example, DNAm patterns from a single tissue sampled from two individuals are more strongly

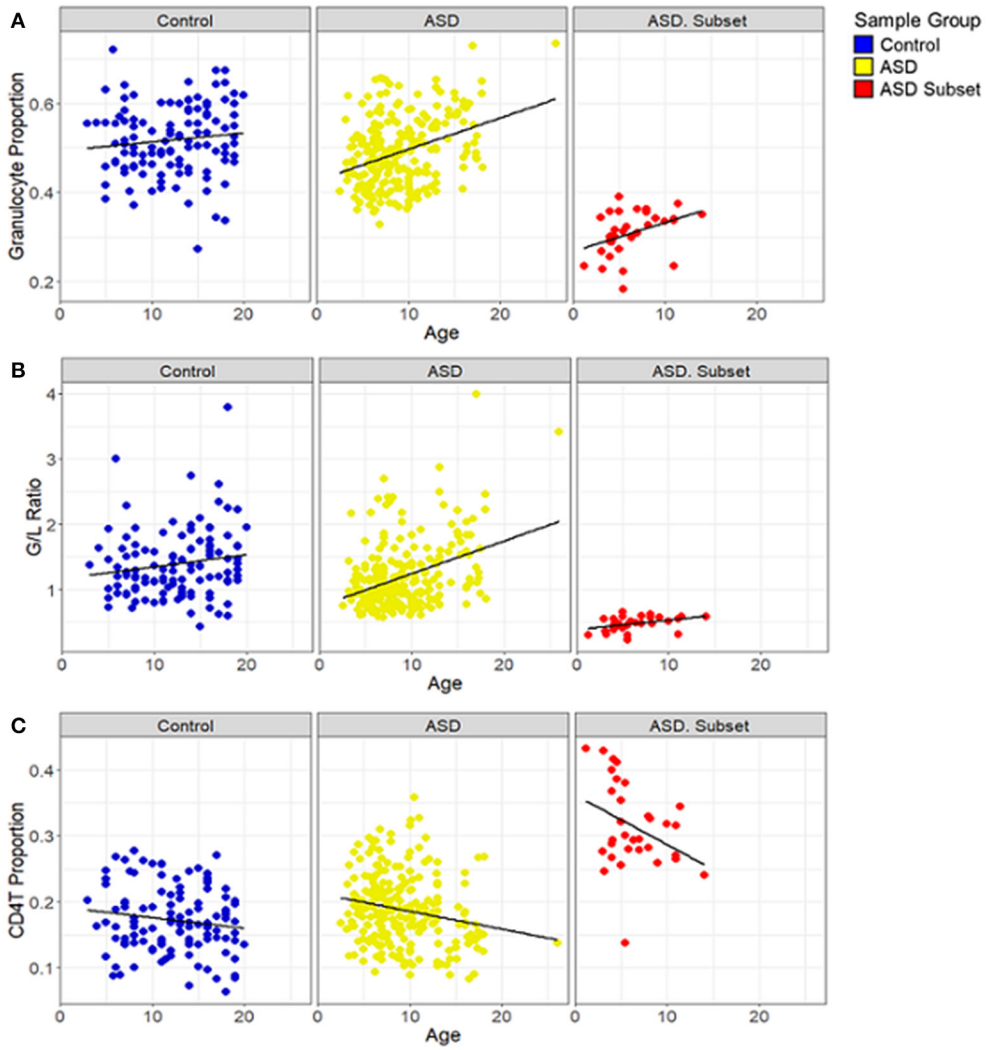

FIGURE 5 | Relationship between blood cell proportions and sample age in individuals with ASD. Box plots depict **(A)** granulocyte proportion, **(B)** granulocyte/lymphocyte (G/L) ratio and **(C)** CD4T proportion in samples plotted against age. ASD subset (red; $n = 32$), the remaining ASD cases (yellow; $n = 233$), and controls (blue; $n = 122$). In all both ASD groups, age was positively correlated with the granulocyte proportion (ASD subset: $r = 0.43$, p-value $= 0.01$; the remaining ASD: $r = 0.35$, p-value < 0.001) and the G/L ratio (ASD subset: $r = 0.45$, p-value $= 0.01$; the remaining ASD: $r = 0.37$, p-value < 0.001) and negatively correlated with CD4T (ASD subset: $r = -0.4$, p-value $= 0.02$; the remaining ASD: $r = -0.2$, p-value $= 0.002$); the remaining ASD: $r = -0.2$, p-value $= 0.002$). In controls, no significant correlation was found between age and the blood cell compositions.

correlated than patterns from a single individual in different tissues. Beyond this, risk factors and clinical phenotypes were available for only a subset of ASD individuals in this study, which may considerably reduce the statistical power to detect associations between DNAm and behavioral phenotype. This missing information is also important as it may have contributed to an unbalanced study design. The distribution of our nominal p-values suggested genomic inflation (see QQ-plot in **Supplementary Figure 5**), which is associated with inflated false positives. Although this can be result of an unbalanced study design or confounding factors that are not accounted for in the statistical model, it may also result from a strong association between DNAm and ASD status at a large number of CpGs sites (62). We propose that by testing the effect of systematic blood cell composition differences, as observed between our cohorts, on DNAm changes we expect broad, genome-wide differences with large effect sizes that would mimic genomic inflation. Furthermore, we accounted for important covariates, including technical factors, blood cell proportions, sex, age, etc. and only reported CpGs that met stringent significance threshold of FDR adjusted p-value < 0.01 and $\Delta\beta| > 0.05$. Nonetheless, this observation which can sometimes reflect genomic inflation does support the need for independent replication of these findings in future to better understand the relationship between ASD, blood cell composition and DNAm levels.

CONCLUSION

In summary, this study demonstrates a gradient of DNAm alterations across our ASD cases tightly associated with shifts in immune cell type proportions. Moreover, we report an epigenetically unique subset of ASD cases that exhibited a significant difference in immune cell type proportions, as compared to the controls and the remaining ASD cases. Our findings build on past reports of changes in the immune systems of children with ASD, supporting the potential role of altered immunological mechanisms in the complex pathophysiology of ASD. The discovery of significant molecular and immunological features in subgroups of individuals with ASD provides unique insight into the molecular pathophysiology of ASD that can help clinicians to better stratify patients, facilitating personalized interventions and improved outcomes. These results may lead to the hypothesis that immunological shifts may induce long-term changes through modulation of DNA methylation in genomic regions involved in the immune response, such as the hypermethylated regions observed in the subset of ASD cases in our data.

DATA AVAILABILITY STATEMENT

The datasets generated for this article are not readily available because the consents obtained did not cover open access/sharing of the data. Requests to access the datasets should be directed to Rosanna Weksberg, rweksb@sickkids.ca.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Board (REB), The Hospital for Sick Children, Toronto, Canada. REB number: 0019980189. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MJ and SGo analyzed and interpreted the data and wrote the manuscript. SC participated in study design and interpreted the data. BT generated genomic variant data. SS collected patient samples and performed genome-wide sequencing to identify variants. EK, MA, RN, SGe, JC, RS, SS, and EA are members of the executive committee of the POND Network, which provided patient cohorts and phenotype data. EG assisted with the interpretation of immune cell type data and provided input on study design. RW is the principal investigator and was involved in all aspects of the study. All co-authors edited and revised the manuscript.

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SUPPLEMENTARY MATERIAL

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SYSTEMATIC REVIEW
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Effectiveness of Virtual/Augmented Reality–Based Therapeutic Interventions on Individuals With Autism Spectrum Disorder: A Comprehensive Meta-Analysis

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In recent years, the application of virtual reality (VR) for therapeutic purposes has escalated dramatically. Favorable properties of VR for engaging patients with autism, in particular, have motivated an enormous body of investigations targeting autism-related disabilities with this technology. This study aims to provide a comprehensive meta-analysis for evaluating the effectiveness of VR on the rehabilitation and training of individuals diagnosed with an autism spectrum disorder. Accordingly, we conducted a systematic search of related databases and, after screening for inclusion criteria, reviewed 33 studies for more detailed analysis. Results revealed that individuals undergoing VR training have remarkable improvements with a relatively large effect size with Hedges g of 0.74. Furthermore, the results of the analysis of different skills indicated diverse effectiveness. The strongest effect was observed for daily living skills ($g = 1.15$). This effect was moderate for other skills: $g = 0.45$ for cognitive skills, $g = 0.46$ for emotion regulation and recognition skills, and $g = 0.69$ for social and communication skills. Moreover, five studies that had used augmented reality also showed promising efficacy ($g = 0.92$) that calls for more research on this tool. In conclusion, the application of VR-based settings in clinical practice is highly encouraged, although their standardization and customization need more research.

Keywords: autism spectrum disorder, virtual reality, rehabilitation, technology, augmented reality

INTRODUCTION

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by impairments in social communication and social interaction in conjunction with restricted, repetitive patterns of behavior, interests, or activities (1). Affecting 1 in 68, ASD is the most prevalent psychological childhood disorder with sustained long-term effects on the quality of life of these patients (2).

Although at present there is no particular accepted treatment for ASD, there is a growing consensus that appropriately targeted individualized behavioral and educational intervention programs [e.g., Treatment and Education of Autistic and Related Communication Handicapped Children (TEACCH) program, Early Intensive Behavioral program, Applied Behavior Analytic (ABA) program, Denver model, etc.] have the potential to positively impact the lives of individuals and their families (3–7). The increasing number of individuals with ASD together with the substantial achievements that have been made thus far by this behavioral rehabilitation programs has ignited a line of research aimed at developing several technologies with the focus on improving these programs (8). Some examples include robotics (9–11), interactive video modeling (12–14), mobile and touchpad devices (15–17), wearable training systems on Google Glass (18), and virtual reality (VR) (19, 20). Interestingly, individuals with ASD have shown special interest and adherence to computerized programs (21) and learning through it (22, 23). Moreover, the burden of many hours of training by a therapist can be alleviated by using technology-based training at home.

Among these technologies, VR has become one of the most promising tools to address the psychological needs of people with ASD in various settings. Since two decades ago, VR was introduced as an effective tool in the neurocognitive rehabilitation of patients with ASD (24). This effectiveness has been approved by a decade of research afterward practicing different types of VR configurations on patients with different levels of disorder (25, 26). Besides, some efforts could have possibly improved the application of VR technology in recent works by proposing consideration of psychological theories in task design (27) and highlighting particular features of VR configurations and human–VR interactions (28). VR reduces the social pressure on the patient and provides a realistic environment for more effective training and possibly reduces the needed training hours. Current studies cover a great range of training interventions, including training of social adaptation and communication skills (29–31); emotional skills (32–34); daily living skills such as shopping (35, 36), driving (37–39), and street crossing (40, 41); and cognitive functions (42–44).

VR is a human–computer interface, which by using computer graphics generates a multidimensional environment with multiple sensory channels that allow individuals to explore the virtual environment (VE) through visual, auditory, tactile, and sometimes even olfactory perception, creating an interactive and immersive experience for the user (45, 46). VR can be implemented in head-mounted visual display (HMD) systems, head and body tracking, CAVE (Cave Automatic Virtual Environment) automatic VEs or room-like displays, and other technologies. They can be used to create a realistic sense of "presence" within a computer-generated environment (47). Augmented reality (AR), which can be considered as another type of VR, is a real-time view of an existing world that is superimposed by some virtual data. Unlike VR technology that fully submerges people in an artificial environment avoiding the existing world, AR technology enhances the feeling by overlaying the computer-generated things over the real world (48).

VR training offers several advantages; perhaps the most important one is to provide a safe access to realistic environments that would be considered dangerous in the real world along with active participation in the virtual world. Furthermore, by providing flexibility in controlling the task complexity, reinforcement through repetition and real-time visual and auditory feedback, VR enhances enjoyment and thus improves learning quality through it. These favorable properties of VR have made it a viable tool to be used in training and rehabilitation (49, 50).

In the past decade, VR has served as an effective new treatment tool in different areas such as rehabilitation in post-stroke patients (51, 52), pain management (53), phobias, posttraumatic stress disorders, obsessive–compulsive disorders, anxiety and stress disorders (54), depression (55), attention-deficit/hyperactivity disorder (ADHD) (56), cerebral palsy (57), and of course, ASD.

Although during recent years, several systematic reviews have been conducted to evaluate the efficacy of technology application on training and teaching different skills such as communication and social skills (58, 59), academic skills (60), or information processing (61), only the contribution of Mesa-Gresa et al. (62) was focused on VR and autism as an evidence-based systematic review on the effectiveness of VR-based intervention in ASD. However, their study did not provide a statistical analysis of outcomes for different clinical targets; besides, the included population in their study was limited to children and adolescents.

To date of this study, there is only one meta-analysis on technology-based intervention such as computer games, interactive DVDs, shared active surfaces, and VR in patients with autism (63). Their study presented a comprehensive meta-analysis on the technology-based intervention used in ASD people; however, the type of technology used in their included studies was mostly based on computer gaming software. Since the time of that study, the number of studies applying VR technology for training patients with autism has witnessed a dramatic surge.

Hence, we have tried to conduct a comprehensive meta-analysis focused on the effectiveness of VR technology *per se* in the training and rehabilitation of patients with autism. To achieve this goal, we performed a systematic search for studies assessing this type of intervention on the ASD population and evaluated the effectiveness of VR training on different skills including social and communication, emotion regulation, daily living, and cognitive skills (CS). We evaluated and compared the effect sizes (ESs) of different skills' improvement to appraise the most influenced clinical targets.

MATERIALS AND METHODS

Study Identification and Selection

We systematically searched clinical and technical databases including PubMed, ERIC, Web of Science, PsycINFO, and IEEE following a comprehensive search strategy with the main search terms of *virtual reality*, *augmented reality*, *artificial reality*, *computer-simulated reality*, *virtual environment*, *virtual world*, *computer-simulated environment, mediated reality*, and *mixed reality* for intervention and the search terms of *autism spectrum*

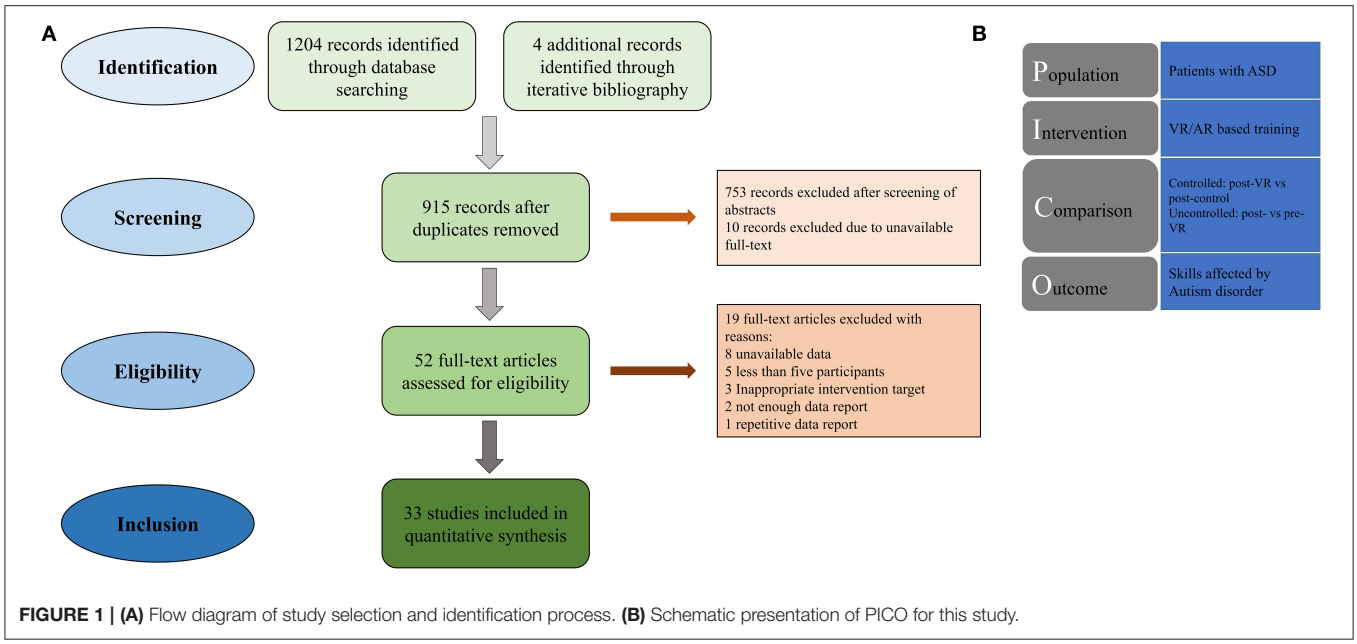

FIGURE 1 | (A) Flow diagram of study selection and identification process. **(B)** Schematic presentation of PICO for this study.

disorder, *pervasive children developmental disorder*, and *Asperger* for disorder, considering adjusted queries for each database. The detailed search strategy and search queries for each database can be seen in **Supplementary Material**. The initial search yielded a total of 1,204 articles. There was no limit on the publication date, and the search is updated until October 19, 2019.

After removing duplicate records, 915 articles remained for the preliminarily screening of titles and abstracts. Those studies presenting original work and discussed virtual or artificial realities for rehabilitation and training of the ASD population that were published in a peer-reviewed journal or peer-edited conference proceeding books were selected. Case reports, review articles, records that contained only an abstract, and records in non-English languages have been discarded. The articles left were 52 randomized clinical trials, cohort studies, and case series. The full texts of these articles were retrieved for more detailed consideration.

A full-text detailed review was done using the PICO (patient, intervention, comparison, and outcome) process (**Figure 1B**). The criteria for including studies in our meta-analysis were (1) participants of any age were diagnosed with ASD with a formal diagnostic tool; (2) intervention was conducted on an interactive VR-based setting; (3) the designed intervention aimed at improving skills related to the core symptoms or deficits of ASD; and (4) the same measured data were available on a control group as for the intervention group that measurements performed on the intervention group before undertaking the goal intervention or on a control group that did not receive the goal intervention; and (5) intervention outcomes were assessed by a quantitative measure that was similar for the intervention and control conditions. The studies that did not comply with these criteria were excluded. Along with those, nine records that contained incomplete and/or inaccurate outcome reports in its text or figures were contacted for further information (34, 35, 64–69). One of them responded (41) and thus included in the study. Besides that, five other articles were excluded for a very small sample size (<3) (34, 70–73) and three others for an unfavorable design of the experiment (e.g., single case reports) (32, 74, 75). In the end, 33 studies were proven to be eligible for entering the meta-analysis. The flow diagram of the study selection process is presented in **Figure 1A**.

All the studies were coded for the following items: definite diagnosis of disease, diagnostic tool, mean and standard deviation of age of participants, number of participants assigned and completed the course of intervention in the target and control groups, contributing factors and modalities that experimenters controlled for inclusion of study population, concomitant comorbidities with ASD in participants, type of intervention technology (VR or AR), technical details of intervention, experiment properties and intensity of intervention, design of experiment (uncontrolled or case–control), purpose of experiment, and outcome measures and their descriptions.

Coding and Defining Variables

ASD is a very heterogeneous disorder. Different patients may vary hugely in levels of deficit in different aspects of cognitive functionalities. Thus, most of the studies had attempted measuring multiple outcomes for assessment of therapeutic effectiveness. Dealing with this variability in study outcomes, we categorized them into four major categories: social and communication skills (SCS; e.g., social adaptation and interaction, communication, social reciprocity, social responsiveness, negotiation skills, theory of mind), emotion recognition and regulation skills (ERS; e.g., emotion expression, affect recognition, stress, and anxiety management), daily living skills (DLS; e.g., driving, shopping, street crossing, and job interview skills), and CS (e.g., attention and concentration,

reasoning and problem solving, executive function, language, and metacognition). By this means, we were able not only to determine the general effectiveness of VR training but also to distinguish different aspects of ASD-related disabilities in terms of benefit they receive from intervention.

There was a considerable number of trials in which outcomes were assessed by a measure that was mostly intuitive and specifically designed for that experiment (e.g., number of greeting with a friend in VE or subject performance in a face detection task, driving, cross walking, shopping task, or construction play task) rather than measures that are widely used in the field [e.g., Social Responsiveness Scale (SRS) score of social awareness, PEP-3 score affective expressions, Adaptive Behavior Assessment System score of leisure, etc.]. We classified these two types of measures as non-formal and formal, respectively, and considered it as a possible moderator of measured training effectiveness for each trial. Another presumed moderator was the type of technology used for intervention, namely, VR or AR, which are characteristically different in terms of design and application. To explore the effectiveness of the intervention at any age, we assumed four age categories of 4–8, 8–12, 12–16, and older than 16 years. Each trial fell into one of these categories based on their participants' mean age. In a considerable number of trials, patients had some concomitant comorbidity along with their main disorder, ASD. To see how much this comorbidity affected the results of the intervention, we considered the presence or absence of comorbidity as another moderator and compared the results of interventions when having or not having concomitant comorbidity. These four categorical moderators were defined for further subgroup meta-analysis. The trials in which full information regarding any of these moderators was not available were excluded from analysis for that moderator.

Subgroup meta-regression was also applied to three continuous moderator variables: number of intervention sessions, sex, and publication date. These variables were defined as the number of separate sessions or visits in which intervention was applied, male ratio (number male subjects divided by the total number of subjects), and the year of publication of the study, respectively.

Statistical Procedure

Similar to the majority of studies in the literature of training effectiveness, the pool of studies in our meta-analysis included a mixture of two major types of experiment designs, namely, controlled and uncontrolled designs. In controlled or independent-group design, one group received the training, and the other group served as a control. The difference between the groups on the outcome measure was used as an estimate of the treatment effect. On the other hand, in the uncontrolled or single-group pretest posttest design, each individual was measured before and after treatment, and the difference between the individuals' scores before and after it was used as an estimate of the treatment effect.

As the characteristic distinction between these two types of designs can lead to a significant difference in estimated ES and its precision, we opted for design-specific estimations proposed by Morris and DeShon (76) for each study. For uncontrolled studies, the repeated measure ES was calculated as the mean of change from pretest to post-test scores divided by its standard deviation, which is equivalent to the t statistic of paired t-test between two pre-test and post-test data. Then, the Standardized Mean Difference (SMD) for these trials was calculated as follows:

$$SMD\ (uncontrolled) = \frac{t}{\sqrt{n}} \tag{1}$$

where t represents t statistic, and n represents the number of participants. We used the t statistic values provided in the contents of articles whenever possible or calculated them from the exact pretest and posttest scores.

For controlled studies, the SMD at the posttest was calculated as follows:

$$SMD\ (controlled) = \frac{\mu c - \mu i}{SDpool} \tag{2}$$

where μc represents mean of the control group, μi represents mean of the intervention group, and $SDpool$ is calculated as follows:

$$SDpool = \sqrt{\frac{(Ni-1)*SDi^2 + (Nc-1)*SDc^2}{Ni+Nc-2}} \tag{3}$$

where Ni is the size of the intervention group, Nc is the size of the control group, SDi is the standard deviation of the intervention group, and SDc is the standard deviation of the control group at posttest.

For two controlled studies in which pretest data were available for both intervention and control groups, SMD was calculated as follows:

$$SMD\ (prepost\ controlled) = \frac{(\mu post_i - \mu prei) - (\mu postc - \mu prec)}{SDpre} \tag{4}$$

where $\mu post_i$ represents the mean of intervention group scores at posttest; $\mu prei$, the mean of intervention group scores at pretest; $\mu postc$, the mean of control group scores at posttest; $\mu prec$, the mean of control group scores at pretest; and $SDpre$ is again calculated with the following equation:

$$SDpre = \sqrt{\frac{(Ni-1)*SDprei^2 + (Nc-1)*SDprec^2}{Ni+Nc-2}} \tag{5}$$

where $SDprei$ and $SDprec$ represent the standard deviation of the intervention and control groups' scores at the pretest, respectively (77). All of the aforementioned calculations of SMDs were done in a way that ensures the highest precision in the estimation of each experiment's ES by the available information.

The final ES indicator, Hedges g, then defined as the product of the output SMD and small sample correction factor $C = \frac{3}{4^*df+1}$ where df is degrees of freedom. ESs were calculated and reported so that a positive sign represents an improvement in the target skill.

After the computation of ESs for each of the trials, we found that most of them reported more than one estimated value, which is called dependent nested ESs in the literature of meta-analysis. Assuming independence between estimated values for multiple outcomes in each study is usually trivial and thus obtaining a study-level ES by averaging the values within studies might lead to some useful information loss. Handling the dependency among ES estimates, three main methods have been proposed to date: multivariate meta-analysis, three-level meta-analysis, and robust variance estimation (RVE) (78). Multivariate meta-analysis is applied when one or multiple outcomes measured in each study are from a set of known and fixed outcomes across studies. The measured outcomes in our meta-analysis were highly variable from study to study, so we could not apply multivariate analysis. Because of the small sample size of the controlled trials, some of the estimated results of the three-level analysis were underpowered and unreliable, which would question drawn conclusions based on them. So, we opted for the third introduced method, RVE. It was shown that this method accommodates well the dependence arising from multiple sources simultaneously, including multiple measures and multiple treatment groups (78) and thus can be a felicitous choice for our study. Further details on the application of the RVE method on our data are described in *Results*.

According to the guidelines of Cohen (79), an absolute ES of 0.2–0.3 is regarded as a small effect, ∼0.5 as a medium effect, and from 0.8 on as a large effect.

Heterogeneity was assessed by Cochran Q, I^2, and τ^2 statistics. I^2 describes the percentage of variation in studies. The smaller the I^2, the lower the level of heterogeneity among estimated values. τ^2 statistic is also a measure of between-study variance of ESs. When Q statistic is very small, the estimated I^2 is not accurate in capturing the real heterogeneity (80). In these cases, τ^2 is more informative specifically when comparing among subgroups with low heterogeneity.

Publication bias was investigated by visual inspection of funnel plots looking for any clue of asymmetry plus Egger intercept test (81) to validate the conclusions.

All the analyses in the main text were done using customized scripts in MathWorks' MATLAB. Three-level meta-analysis was performed in R using an available R package (82).

RESULTS

Description of Studies

Thirty-three studies complied with the inclusion and exclusion criteria (see *Methods*) and entered into the meta-analysis.

The interventions were applied by a controlled experiment design in seven studies and by an uncontrolled design in 24 studies. There were two studies that recruited both types of controlled and uncontrolled designs (mixed-design) (83, 84). As these mixed-design studies included different participants in each design group, we treated them as separate uncorrelated trials. Doing so, we based our analysis on 35 independent trials obtained from 33 studies (The term *trial* refers to an independent design group consistently thereafter in this article). All in all, 540 ASD participants were included in this study, of which 360 belong to uncontrolled and 180 to controlled trials. There were also 156 ASD patients in the control arm of controlled trials who received neither VR-based nor conventional intervention.

In four controlled trials, the same outcomes were measured before (baseline) and after training in both the control and intervention arms. In the remaining five controlled trials, these measurements were done only after training, and there were no baseline data provided in any control or intervention group. Of 26 uncontrolled trials, three of them applied ABC measurement strategy in a way that outcomes were measured in three temporal phases: after the first session (pre), after the last session (post), and a while after completion of intervention (follow-up) (29, 44, 85). In the other trials, the measurements were performed before (pre) and after (post) interventions. In two trials (one from control and the other one from uncontrolled trials), measurement once was done after a non-VR conventional training, and it was repeated after VR-based target training (43, 86). The data in the first condition were labeled pre-intervention, and in the latter labeled post-intervention. A prior exposure to any type of training was neither recognized nor mentioned in the other studies. The identified pre-intervention and post-intervention data for each trial were used in computing ES statistics (see *Methods* for more detail).

The diagnostic tools used to integrate patients into the study were different across trials. For instance, in two trials, diagnosis was confirmed by the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (*DSM-IV*) (83, 85), in four other trials by Autism Diagnostic Observation Schedule (ADOS) (39, 42, 87, 88), in one of them by Social Communication Questionnaire (SCQ) (89), in the other two trials by SRS-II (90, 91), in the other 6 by *DSM-V* (41, 92–94), and in another one by Childhood Autism Rating Scale (CARS) (40). The diagnostic tool in the remaining 20 trials was not mentioned. Regarding stage/level of disease, three trials included patients with high-functioning ASD (HFASD) (90, 94, 95), one trial had patients with low-functioning ASD (LFASD) (92), two trials included Asperger or pervasive developmental disorder—not otherwise specified patients (31, 87), two other trials had patients with either HFASD or Asperger (96, 97), and one trial had patients with Asperger who received the intervention (98). The level of disease in the other 26 trials was not specified. Several trials considered controlling some contributing factors in the population of their study that could potentially impact the outcome of the intervention. For example, eight trials controlled the participants for IQ score (30, 41, 42, 85, 88, 94, 95, 99), two trials for SCQ score (44, 83), one trial for SRS-II score (39), one other trial for PEP-3 score of language and motor skills (92), and one trial for ASI score (86). In six uncontrolled and three controlled trials, patients had another concomitant comorbidity or disorder alongside their main disorder, ASD. Examples include some trials that had patients with a diagnosis of ADHD (42, 44), some other trials that included patients with phobia (83, 89), and finally other trials in which some patients had an intellectual disability or language disorder (40, 41, 86, 92, 93).

AR and VR were integrated into training paradigms through various tools and platforms in the 35 identified trials. From

these trials, five opted for AR and 30 for VR to deliver their intervention. They implemented most of these AR-based programs through smartphone, tablet, or desktop applications and platforms to augment three-dimensional (3D) visual features to the more simplistic features conventionally used in the training paradigms, making them more appealing and engaging for children with ASD (29, 43, 85, 98). There was one trial that used Google smart glasses equipped with blink sensors, gyroscope, camera, and display screen (44). In this trial, positive feedback was displayed on the screen whenever the participant could successfully gaze at the instructor's face and detect his/her emotion. Alternatively, most of the VR-based interventions were designed on immersive 3D VE settings in which audiovisual scenes were presented on the walls and ceiling of a room where the participant could fit in different characters in realistic social scenarios. There were also trials that training was based on a particular virtual agent that the participant could play and interact with it. Some VR interventions were also planned on desktop computers using commercial VR software and some of them on HMD devices, which are recently being more and more available and gaining popularity.

More details of included studies for uncontrolled and controlled trials are provided in **Tables 1**, **2**, respectively.

Meta-Analysis

In the 35 trials entailed in our meta-analysis, ES Hedges g were computed for 167 total number of outcome measures, in which 45 pertained to controlled and 122 pertained to uncontrolled trials. As ESs in two groups were significantly different from each other ($p = 0.0003$, unpaired t-test), we were not allowed to combine them into one group, and so we have done all further analyses separately for each of them.

To compute the study-level ESs, its variance, and also between-study variance, we followed the procedure described by Hedges et al. (102). Based on this procedure, an estimate of within-study correlation (ρ) is needed to compute other statistics. As this estimate could not be extracted from most of our included studies, we ran a sensitivity analysis by choosing various values for ρ ranging from 0 to 1 and then computed other statistics based on the chosen value. The results of sensitivity analysis are given in **Supplementary Table 1**. By this analysis, we inferred that the value study-level computed ES estimates are sensitive to the choice of ρ, but it was an ascending function of ρ (the larger the ρ, the larger the estimated study-level ESs). Therefore, to avoid any overestimation in computing summary ESs, we fixed the value of ρ at 0 and performed the analysis with this value. It is noteworthy to say that the procedure that we applied in this study is the most parsimonious one avoiding any overstatement of results, but in a realistic situation, the observed effectiveness might be larger as assuming the existence of some level of correlation between study outcomes seems to be a rational assumption. For each trial, we first computed study-level ESs for all of the outcomes irrespective of their category applying RVE procedure, which gave us overall ES of each study. Then, we repeated this procedure on estimated ESs of outcomes in each category of each study to obtain category-based ESs of that study.

Overall Effectiveness of VR Training

In the first step, we computed the overall ES for each trial. For nine controlled trials, summary effect size was at medium range ($g = 0.45$, $SE_g = 0.25$) with moderate heterogeneity ($I^2 = 49.5\%$ and $\tau^2 = 0.055$). Excluding one of the potential outliers with much larger ES ($g = 1.8$) (97) led to a bit smaller summary ES, but it was still at the medium range of effectiveness ($g = 0.38$, $SE_g = 0.2$) (**Figure 2**). For the 26 uncontrolled trials, a large positive ES was found ($g = 0.74$, $SE_g = 0.17$) with low heterogeneity ($I^2 = 2.5\%$ and $\tau^2 = 0.11$). Excluding one of the potential outliers with extremely large ES ($g = 4.8$) (85) led to similar results ($g = 0.736$, $SE_g = 0.17$) (**Figure 3**).

We have interpreted the results for overall effectiveness of studies with random-effects model of meta-analysis relying more on controlled trials because of their more robust experimental design.

Skill-Based Effectiveness of VR Training

Further, ESs were computed for each skill category (defined in *Methods*). In controlled trials, SCS was addressed in five trials, ERS in three trials, DLS in two trials, and CS in one trial. The effectiveness of VR training was weak for SCS ($g = 0.2$, $SE_g = 0.23$, $\tau^2 = 0.03$), weak to moderate for ERS ($g = 0.34$, $SE_g = 0.06$, $\tau^2 = 0.02$), and again very strong in DLS ($g = 1.37$, $SE_g = 0.18$, $\tau^2 = 1.12$). The single trial that addressed CS revealed weak to moderate effectiveness ($g = 0.37$, $SE_g = 0.002$). Regarding heterogeneity in these estimated summary ESs, a considerably large amount of between-study variance (τ^2) was observed in DLS category in both design groups, but it was relatively small for SCS, ERS, and CS, and it was small for ERS (compare the τ^2 values presented above) (**Figure 4**).

In uncontrolled trials, SCS had been addressed in 11 trials, ERS in 10 trials, DLS in nine trials and CS in seven trials. VR training led to medium to strong effectiveness in SCS ($g = 0.68$, $SE_g = 0.08$, $\tau^2 = 0.13$), medium effectiveness in ERS ($g = 0.46$, $SE_g = 0.05$, $\tau^2 = 0.07$), strong effectiveness in DLS ($g = 1.16$, $SE_g = 0.09$, $\tau^2 = 0.48$), and medium effectiveness in CS ($g = 0.45$, $SE_g = 0.02$, $\tau^2 = 0.03$) (**Figure 5**). Thus, while in other skills we observe promising effectiveness, the DLS is proven to be the most affected area as its strong effectiveness was consistent among both controlled and uncontrolled trials.

Analysis of Confounding Factors

As a sizable number of trials had not used any legitimate criteria for screening the participants undergoing intervention (e.g., IQ score, social responsiveness score, disease severity, etc.), we recomputed overall ESs for those trials that screened their population applying this kind of criteria to see how much our results would be biased by this potential confounding factor. In the population screened trials, the results for controlled trials were $g = 0.25$, $SE_g = 0.1$, $k = 7$, and $\tau^2 = 0.01$ and for uncontrolled trials were $g = 0.72$, $SE_g = 0.05$, $k = 12$, and $\tau^2 = 0.04$ and. These results in unscreened trials were $g = 0.8$, $SE_g = 0.05$, $k = 2$, and $\tau^2 = 0.35$ for controlled and $g = 0.73$, $SE_g = 0.2$, $k = 14$, and $\tau^2 = 0.18$ for uncontrolled trials.

Comparing these ESs among two screen groups, the results were not meaningfully different from each other in

TABLE 1 | Characteristics of included studies, uncontrolled trials.

References	Age	*N*	Type	Methodic details	Application details	Study purpose	Outcome measure				
	Mean (SD)						Name	Details	Skill	*g*	SE_g
Bai et al. (98)	6.8	12	AR	Playing with augmented toys in mirror AR display	N/S	Improve and learn pretend play and representation of pretense	Pretend play frequency	Play Observation Scale	CS	0.7	0.42
							Pretend play duration			0.94	0.47
							Constructive play frequency			1.42	0.59
							Constructive play duration			1.01	0.49
									Overall	**1.02**	**0.6**
Bernardini et al. (100)	N/M	19	VR	Playing game with VA	Several 10- to 20-min sessions in a week for 8 weeks	Help children acquire social communication skills	Response to social partner	SAP to assess socioemotional abilities of autistics	SCS	0.07	0.42
							Initiation to social partner			0.02	0.42
							Social behavior			0.02	0.42
							Sequences of social behaviors			−0.25	0.43
							Speech toward social partner			0.06	0.42
							Missed opportunities			0.81	0.54
									Overall	**0.12**	**0.55**
Chen et al. (85)	11.5	6	AR	ARVMS	Seven sessions	Facial expressions and emotions of others in social situations	Performance	Instructor assessment	ERS	4.81	2.94
									Overall	**4.81**	**2.96**
Didehbani et al. (42)	11.4 (2.7)	30	VR	Social scenarios in customized Second Life™ VE	Ten 1-h sessions	Enhance emotion recognition, social attribution, attention and executive function	NEPSY-2 affect recognition	Facial affect recognition	ERS	0.66	0.23
							EKMAN 60	Recognition of basic emotions		0.46	0.31
							Triangle total	Understanding of social intentionality	SCS	0.38	0.22
							Triangle intentionality			0.45	0.23
							Fluid reasoning	Selective attention and concentration	CS	0.52	0.27
									Overall	**0.44**	**0.41**
Ip et al. (99)	8.7	33	VR	School-related social scenarios in four-sided CAVE	28 sessions	Enhance social skills and coping skills while avoid unnecessary embarrassment	Eyes test	Emotion recognition	ERS	0.53	0.29
							Affective expression			0.68	0.31
							Social reciprocity	Social reciprocity	SCS	0.6	0.3
							PEP-3 overall	Social functioning and communication		0.76	0.32
									Overall	**0.64**	**0.45**
Josman et al. (40)	13.2 (3)	6	VR	Street crossing in VE computer program	Eight 10- to 30-min sessions	Teach street crossing skill	N of left looking at first crosswalk	Participant performance in VR software	DLS	1.72	1.16
							N of right looking at first crosswalk			0.58	0.63

(Continued)

TABLE 1 | Continued

References	Age	*N*	Type	Methodic details	Application details	Study purpose	Outcome measure				
	Mean (SD)						Name	Details	Skill	*g*	SE_g
							N of left looking at second crosswalk			0	0.53
							N of right looking at second crosswalk			0.37	0.57
							total N of left looking crossing the road			1.4	0.99
							total N of right looking crossing the road			0.19	0.54
							N looked left at crosswalk with traffic light			0.18	0.54
							N looked right at crosswalk with traffic light			0.45	0.59
							N of accidents at the crosswalk with traffic light			0.75	0.69
									Overall	**0.63**	**0.8**
Kandalaft et al. (87)	21.2 (2.7)	8	VR	Interacting with VA in second Life™ software	10 sessions	Enhancing social skills, social cognition, and social functioning	SP-total	Verbal and non-verbal emotion recognition by ACS-SP	ERS	0.89	0.56
							SP-affect			0.39	0.45
							SP-prosody			1.03	0.59
							SP-pair			0.59	0.48
							EKMAN 60	Theory of mind (ToM)	SCS	1.25	0.66
							Triangle			1.08	0.61
							SSPA	Conversation skills		0.32	0.44
									Overall	**0.79**	**0.64**
Ke et al. (95)	N/M	8	VR	3D virtual world designed by OpenSimulator	Average of 20.22 h, over 16–31 sessions	Enhance social skills	Responding	Performance evaluated by instructors	SCS	0.02	0.42
							Initiation			1.26	0.67
							Negotiation			1.61	0.78
							Self-identification			0.83	0.54
							Cognitive flexibility			2.09	0.96
									Overall	**1.16**	**0.77**
Kurniawan et al. (29)	N/M	12	AR	PECS-AR	N/S	communication ability	Communication ability score	Teacher's assessment	SCS	1.26	0.47
									Overall	**1.26**	**0.58**
Lamash et al. (36)	14.6 (1.8)	33	VR	Shopping training in VAP-S software	Five sessions	Shopping skills, executive cognitive and metacognitive skills	WebNeuro attention component	Evaluation of cognitive and meta-cognitive functis	CS	0.58	0.2
							WebNeuro executive function component			0.58	0.2
							WebNeuro verbal component			−0.38	0.19

(Continued)

TABLE 1 | Continued

References	Age	*N*	Type	Methodic details	Application details	Study purpose	Outcome measure				
	Mean (SD)						Name	Details	Skill	*g*	SE_g
							TOGGS accuracy	TOGGS, performance in shopping	DLS	1.5	0.27
							TOGGS time			0.62	0.2
							TOGGS redundancy			0.93	0.22
							TOGGS strategy usage			1.85	0.31
									Overall	**0.81**	**0.4**
Manju et al. (30)	4.6 (0.9)	5	VR	VE with scenes presented on wall	N/S	Social skills and attention	Likert score	Attention grasping	CS	2.39	2.07
							Likert score	Social interaction	SCS	1.6	1.47
									Overall	**1.99**	**1.82**
Maskey et al. (89)	11.2 (2)	9	VR	Exposure to fearful stimuli in VE	Four 20- to 30-min sessions	Reduction or treating specific phobia	SCAS-P	Children's Anxiety Scale parent score	ES	0.62	0.46
							SCAS-C	Children's Anxiety Scale child score		0.66	0.45
									Overall	**0.64**	**0.56**
Maskey et al. (83)	29.8	8	VR	Blue VR room	Four 20-min sessions of graded exposure	Treating phobia and anxiety	Anxiety BAI score	BAI	DLS	0.03	0.42
							Anxiety GAD score	GAD-7		−0.04	0.42
							Depression score	PHQ-9		0.32	0.44
							Quality of life (QoL) physical	WHOQOL-BREF questionnaire		−0.47	0.46
							QoL psychological	Addresses QoL		−0.03	0.42
							QoL social			−1.2	0.66
							QoL environmental			0.2	0.42
									Overall	**−0.17**	**0.58**
Miller et al. (101)	5.2	5	VR	HMD, Google cardboard	One session per week for 3 weeks	Improve air travel skills	Parent score	5-Point Likert score	DLS	0.98	1.03
							Researcher score			1.1	1.11
									Overall	**1.04**	**1.12**
Milne et al. (96)	10.5	14	VR	Interacting with VA	N/S	Teaching social skills and how to cope with bullying	Conservation skills	Performance in social scenarios measured by evaluators scoring	DLS	0.67	0.33
							Dealing with bully skills			1.09	0.39
									Overall	**0.88**	**0.49**
Nubia et al. (43)	6	5	AR	Pictogram recognition task	N/S	Improve attention process and appearance of verbal language	Attention process	No. of children successfully finished the attention task	CS	0.53	0.3
							Emergence of language			0.55	0.31
									Overall	**0.54**	**0.45**
Ross et al. (37)	18	46	VR	Driving simulation in VE	8–12 sessions	Improve attitude toward driving	DAS-PR positive attitude	Driving Attitude Scale–Parent Report	DLS	1.74	0.25
							DAS-PR negative attitude			1.07	0.19
									Overall	**1.41**	**0.4**

(Continued)

TABLE 1 | Continued

References	Age	*N*	Type	Methodic details	Application details	Study purpose	Outcome measure				
	Mean (SD)						Name	Details	Skill	*g*	SE_g
Saiano et al. (41)	24 (10)	6	VR	Street crossing and path following in VE representing a city	Ten 45-min sessions	Teaching of street crossing and path following skills	Caregiver score	Likert score questionnaire	DLS	1.85	1.23
							Parent score			0.92	0.76
							Speed	Subject performance in city surveying and street crossing		1.71	1.15
							Composite index			0.45	0.59
							Figural distance			0.75	0.69
							Path length taken			0.48	0.6
									Overall	**1.03**	**0.94**
Simoes et al. (93)	18.8 (4.5)	6	VR	Street crossing and bus taking in VE presented by HMD	Three 20 to 40-min sessions	Teaching bus-taking routines and effectively using bus for transformation	Action accuracy	Performance in bus taking	DLS	1.1	0.5
							Debriefing accuracy			1.8	0.69
							Global EDA	Stress level	ERS	0.66	0.66
							Bus EDA			0.81	0.72
							Streets EDA			0.51	0.61
									Overall	**0.98**	**0.72**
Stichter et al. (88)	12.6 (0.7)	11	VR	Social competence tasks in computer-generated 3D VE	31 sessions over a 4-month period	Enhance social competence	SRS total parent score	Social Responsiveness Scale	SCS	1.04	0.46
							SRS social awareness parent score			0.47	0.36
							SRS social cognition parent score			1.15	0.48
							SRS social communication parent score			1.26	0.51
							SRS social motivation parent score			0.75	0.41
							SRS total teacher score			0.53	0.37
							SRS social awareness teacher score			0.34	0.35
							SRS social cognition teacher score			−0.12	0.34
							SRS social communication teacher score			0.6	0.38
							SRS social motivation teacher score			0.34	0.35
							BRIEF global executive parent score	Behavior Rating Inventory of Executive Function	CS	0.68	0.39
							BRIEF behavioral regulation parent score			0.45	0.36

(Continued)

TABLE 1 | Continued

References	Age	*N*	Type	Methodic details	Application details	Study purpose	Outcome measure				
	Mean (SD)						Name	Details	Skill	*g*	SE_g
							BRIEF metacognition parent score			0.64	0.39
							BRIEF global executive teacher score			0.5	0.37
							BRIEF behavioral regulation teacher score			0.14	0.34
							BRIEF metacognition teacher score			0.33	0.35
							Reading in mind's eye	Student performance	ERS	0.17	0.34
							Faux pas stories			−0.35	0.35
							Strange stories			0.25	0.35
							DANVA	Child facial expression analysis		0.44	0.36
							Trail making: number letter switching	D-KEFS Delis–Kaplan executive functioning system	CS	0.17	0.34
							Design fluency: switching designs			0.62	0.38
							Design fluency: total correct designs			1.06	0.46
							Color–word interface: inhibition task			−0.03	0.34
							Color–word interface: inhibit/switch			0.16	0.34
							CPT-2 overall omission errors	Continuous performance test-II (CPT-II)		0.09	0.34
							CPT-2 overall commission errors			0.15	0.34
									Overall	**0.44**	**0.5**
Vahabzade et al. (44)	15 (3.4)	8	AR	Maintain gaze toward faces by AR smart glasses	One session	Improving gaze duration to faces and reducing ADHD symptoms	ABC-H score	Measure of ADHD symptoms	CS	0.72	0.51
									Overall	**0.72**	**0.61**
Wade et al. (38)	15.9 (1.3)	6	VR	Driving simulation in VE	Six visits of three driving sessions in 24 trials	Improve safe driving skills	Performance-based failures	Subject's performance	DLS	1.98	1.3
									Overall	1.98	1.34

(Continued)

TABLE 1 | Continued

References	Age	*N*	Type	Methodic details	Application details	Study purpose	Outcome measure				
	Mean (SD)						Name	Details	Skill	*g*	SE_g
Wade et al. (39)	15.3 (1.6)	8	VR	3D game driving simulator	Six 75-min sessions	Enhancing driving skills	Duration time	Performance	DLS	0.73	0.51
							No. of failures			1.27	0.67
									Overall	**1**	**0.7**
Yang et al. (94)	22.5 (3.9)	17	VR	VR-SCT computer program	Ten 1-h sessions	Emotion recognition training and ToM or sociocognitive skills improvement	ACS-SP emotion recognition	Social Perception	ERS	0.89	0.56
							ToM triangle test	ToM	SCS	1.08	0.61
									Overall	**0.99**	**0.67**
Yuan et al. (84)	9 (1.1)	36	VR	Social scenarios in four-sided CAVE	One 1-h session	Train emotional and social skills	PEP-3 affective expressions	Emotion expression and regulation	ERS	0.35	0.18
							PEP-3 social reciprocity	Social interaction and adaptation	SCS	0.64	0.19
									Overall	**0.5**	**0.38**
Zhao et al. (31)	12.4 (2.6)	12	VR	Social games in CVE	N/S	Motor skill and social interaction simultaneously	Completed pieces(/min) study 1	Performance in puzzle game	SCS	1.07	0.83
							Cooperative efficacy % study 1			0.76	0.7
							Total play time (s) study 1			0.9	0.75
							Word count of ASD subjects(/min) study 1			0.34	0.57
							Back-and-forth sentences(/min) study 1			−0.65	0.66
							Aggregate score study 1			0.76	0.7
									Overall	**0.53**	**0.8**

ABC-H, Aberrant Behavioral Checklist; ACS, Advanced Clinical Solutions; ADHD, attention-deficit/hyperactivity disorder; AR, augmented reality; ARVMS, Augmented Reality Video Modeling System; BAI, Beck Anxiety Inventory; BRIEF, Behavior Rating Inventory of Executive Function; CAVE, Cave Automatic Virtual Environment; CPT, continuous performance test; CS, cognitive skills; CVE, collaborative virtual environment; DANVA, Diagnostic Analysis of Non-verbal Accuracy; DAS-PR, Driving Attitude Scale–Parent Report; DLS, daily living skills; EDA, electrodermal activity; ERS, emotion regulation and recognition skills; GAD-7, Generalized Anxiety Disorder-7; N, number of participants; N/S, not specified; NEPSY, a developmental NEuroPSYchological assessment; NM, not mentioned; PECS, picture exchange communication system; PEP, psychoeducational profile; PHQ-9, Patient Health Questionnaire-9; SAP, SCERTS assessment protocol; SCAS_C, Spence Children's Anxiety Scale–Child Version; SCAS_P, Spence Children's Anxiety Scale–Parent Version; SCQ, Social Communication Questionnaire; SCS, social and communication skills; SRS, Social Responsiveness Scale; SSPA, Social Skills Performance Assessment; TOGGS, Test of Grocery Shopping Skills; VA, virtual avatar; VE, virtual environment; VR, virtual reality; VR-SCT, virtual reality social cognition training. The bold values represent overall effectiveness for each study.

TABLE 2 | Characteristics of included studies, controlled trials.

References	Age	Number		Methodic details	Application details	Study purpose	Outcome measure				
	Mean (SD)	NI	NC				Name	Details	Skill	*g*	SE*g*
Chen et al. (92)	4.9 (1.1)	11	11	3D virtual punctuation tutor	Three sessions	Improve speech	Consonants	Rated with linguistis	CS	0.36	0.45
							Vowels			0.38	0.45
									Overall	**0.37**	**0.5**
Ip et al. (33)	13.55	36	36	Social scenarios in half-CAVE	28, 30-min sessions	Improving emotion recognition, emotion expression and social reciprocity, social adaptive skills	Faces test	Emotion recognition	ERS	0.26	0.24
							Eyes test			0.14	0.24
							PEP-3 affective expressions	Emotion expression, regulation, and social reciprocity		0.44	0.24
							PEP-3 social reciprocity			0.47	0.24
							ABAS communication	Social adaptive skills	SCS	0.13	0.24
							ABAS community use			−0.64	0.25
							ABAS leisure			−0.24	0.24
							ABAS self-direction			−0.48	0.24
							ABAS social			−0.23	0.24
									Overall	**−0.05**	**0.34**
Lamash et al. (36)	14.58 (1.77)	33	23	Shopping training in VAP-S software	Five sessions	Improving shopping skills	TOGGS accuracy	Performance in shopping	DLS	1.02	0.29
									Overall	**1.02**	**0.38**
Maskey et al. (83)	10.8 (2)	16	16	Blue room VR	Four sessions	Reduce phobia in ASD patients with anxiety disorder	Target behavior rating	Rating of specific phobia change	ERS	1	0.39
							Total fearfulness	FSSC-R		−0.2	0.37
							Intense fears			−0.29	0.37
							Total anxiety score, parent	SCAS-P		0.21	0.37
							Total anxiety score, child	SCAS-C		−0.04	0.37
							Formal activity, diversity	CAPE: participation in a range of solitary and group voluntary activities		−0.14	0.37
							Formal activity, intensity			−0.1	0.37
							Informal activity, diversity			−0.24	0.37
							Informal activity, intensity			−0.28	0.37
									Overall	**0**	**0.44**
Smith et al. (91)	24.9 (6.7)	16	10	Being interviewed by VA in VR-JIT computer software	10 h	Improving job interviewing and vocational skills	Role-play performance total score	Standardized role-plays	SCS	0.52	0.43
							Job interview content score			0.39	0.43
							Hard worker			0.58	0.43
							Easy to work with/teamwork			0.32	0.42
							Sounding professional			0.25	0.42
							Negotiation skills			0.32	0.42
							Interviewee performance score	Training Experience Questionnaire by interviewee		0.49	0.43

(Continued)

TABLE 2 | Continued

References	Age	Number		Methodic details	Application details	Study purpose	Outcome measure				
	Mean (SD)	NI	NC				Name	Details	Skill	*g*	SE*g*
							Sharing things positively			0.73	0.44
							Sounding honest			0	0.42
							Sounding interested in job			0.26	0.42
							Comfort level			0.46	0.43
							Establishing overall rapport			0.35	0.42
							Job interview self-confidence rating	Self-confidence measure		0.61	0.43
									Overall	**0.4**	**0.48**
Smith et al. (90)	25 (6.9)	15	8	Being interviewed by VA in VR-JIT computer software	N/S	Improving job interviewing skills	Likert score	Self-confidence	SCS	0.82	0.48
							Weeks looking for a job			0.23	0.46
							Completed interviews			0.08	0.46
									Overall	**0.37**	**0.52**
Strickland et al. (97)	18.21 (1.03)	11	11	Being interviewed by VC in JobTIPS computer program	One session	Enhancing job finding skills	Response content scale	Content of the participant's responses	DLS	2.81	0.68
							Response delivery scale	Behaviors related to greetings and farewells		0.81	0.47
									Overall	**1.8**	**0.63**
Yuan et al. (84)	8.97 (1.1)	36	36	Social scenarios in four-sided CAVE	1,1-h session	Enhancing emotional and social skills	PEP-3 affective expressions	Emotion expression and regulation	ERS	0.54	0.24
							PEP-3 social reciprocity	Social interaction and adaptation	SCS	0.66	0.25
									Overall	**0.6**	**0.34**
Zhang et al. (86)	4 (1.21)	6	5	Quiver Vision augmented reality android app	20 weeks, two 15-min sessions per week	Enhance social skills	Social score	ASI disorder score	SCS	0.14	0.69
							Communication and language			0.14	0.69
							Anticipation and flexibility			−0.28	0.69
							Symbolization			−0.2	0.69
									Overall	**−0.05**	**0.73**

ABAS, Adaptive Behavior Assessment System; CAVE, Cave Automatic Virtual Environment; CS, cognitive skills; DLS, daily living skills; ERS, emotion regulation and recognition skills; FSSC-R, Fear Survey Schedule for Children—Revised; NC, number of participants in control group; NI, number of participants in intervention group; PEP, psychoeducational profile; SCAS_C, Spence Children's Anxiety Scale–Child Version; SCAS_P, Spence Children's Anxiety Scale–Parent Version; SCS, social and communication skills; TOGGS, Test of Grocery Shopping Skills; VA, virtual avatar; VAP-S, virtual action planning supermarket; VC, virtual character; VR-JIT, Virtual Reality Job Interview Training. The bold values represent overall effectiveness for each study.

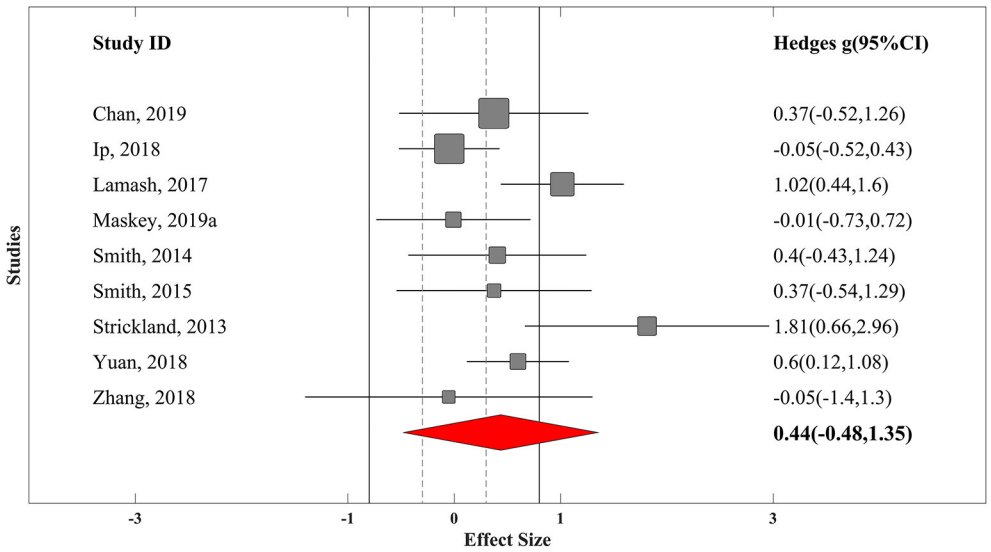

FIGURE 2 | Forest plot of overall effectiveness of VR training for controlled trials with 95% confidence interval. Solid vertical lines represent strong effect size boundary ($g = -0.8$ and 0.8), and dashed vertical lines represent weak effect size boundary ($g = -0.3$ and 0.3).

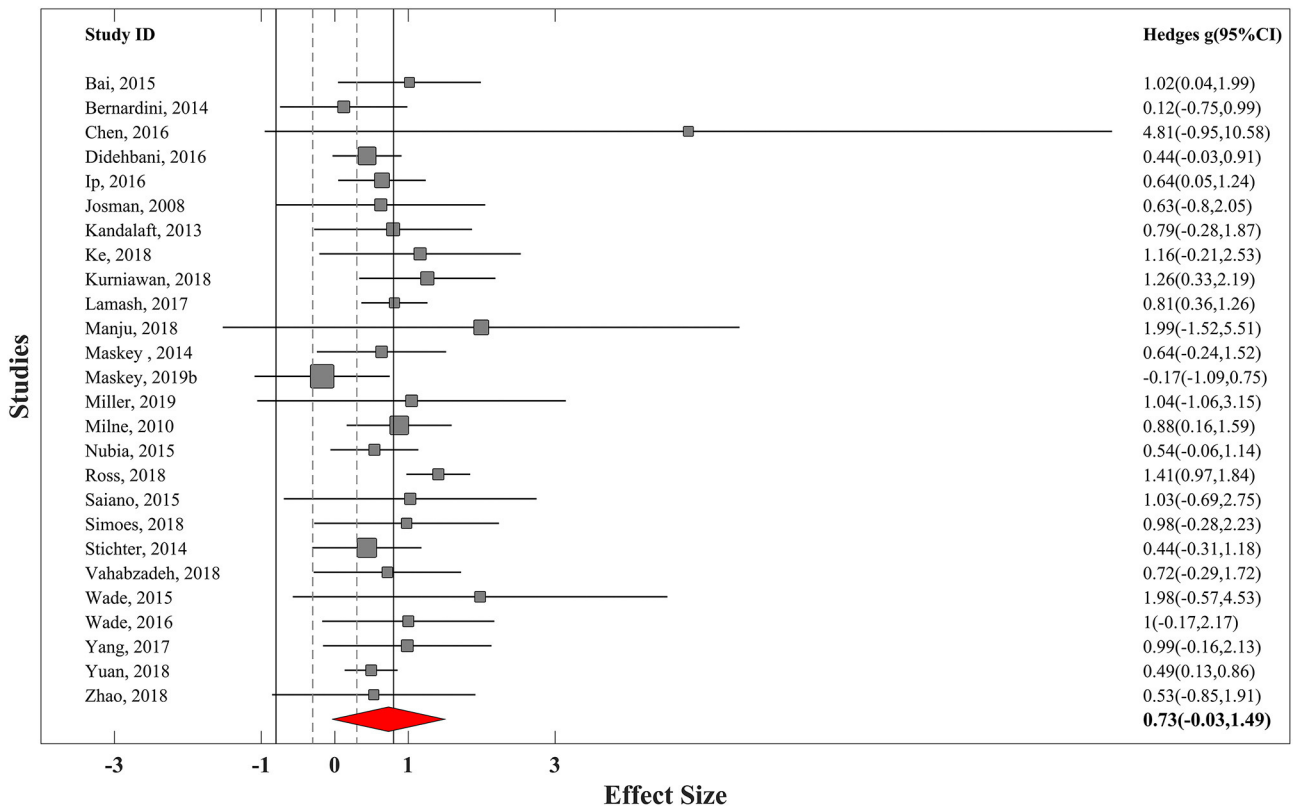

FIGURE 3 | Forest plot of overall effectiveness of VR training for uncontrolled trials with 95% confidence interval. Solid vertical lines represent strong effect size boundary ($g = -0.8$ and 0.8), and dashed vertical lines represent weak effect size boundary ($g = -0.3$ and 0.3).

uncontrolled trials unlike substantial decline from unscreened to screened trials in controlled interventions. Because of very small sample size of unscreened controlled trials, the results derived from them seem less reliable, although more cautions should be devoted to screening population before intervention.

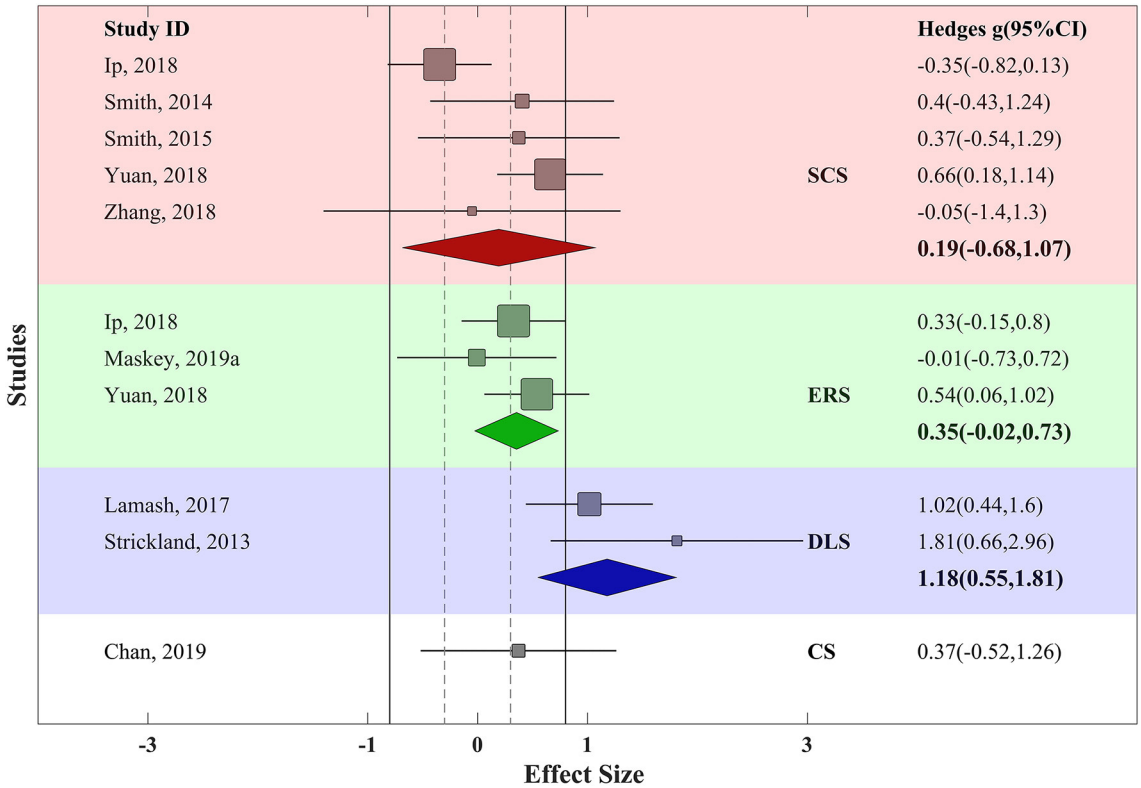

FIGURE 4 | Funnel plot for VR training effectiveness of both uncontrolled and controlled trials with pseudo−95% confidence interval. Red area represents SCS; green area represents ERS; blue area represents DLS; and yellow area represents CS. Solid vertical lines represent strong effect size boundary ($g = -0.8$ and 0.8), and dashed vertical lines represent weak effect size boundary ($g = -0.3$ and 0.3). Filled and empty circles represent Hedges g value of uncontrolled and controlled trials, respectively. Solid lines represent g with 95% confidence interval of uncontrolled trials and dashed lines represent g with 95% confidence interval of controlled trials.

Subgroup Meta-Analysis

The results of the subgroup analysis for controlled trials would be underpowered and misleading because of its small sample size, so the conclusions of subgroup meta-analysis and meta-regression are limited to the data of uncontrolled trials. By the way, the results of these analyses for controlled trials are available in **Supplementary Tables 4, 5**.

We performed a subgroup meta-analysis for the categorical moderators described in *Methods*. Results showed that the overall ESs that had been computed based on the data obtained from non-formal measures were somehow larger than those obtained from formal measures ($g = 0.93$ $k = 11$, and $\tau^2 = 0.2$ for non-formal and $g = 0.66$, $k = 15$, and $\tau^2 = 0.09$ for formal trials). This can be due to the customized measurements that suit the intervention design, less validity of measures, and susceptibility to the rater bias. Regarding the frequency of each measure's application for categories, most of them had applied formal measures except DLS, which application of non-formal measures was more frequent. In DLS, the summary ES was a bit larger for formal measures, although it was derived from only three trials ($g = 1.24$, $k = 4$, and $\tau^2 = 0.55$ for formal and $g = 1.06$, $k = 5$, and $\tau^2 = 0.28$ for non-formal measures). For the type of technology (VR or AR), AR interventions led to a larger overall summary ES ($g = 0.91$, $k = 5$, and $\tau^2 = 0.3$ for AR and $g = 0.71$, $k = 21$, and $\tau^2 = 0.1$ for VR). The most of AR interventions were applied for CS that showed more effective training in this skill than VR ($g = 0.72$, $k = 3$, and $\tau^2 = 0.15$ for AR and $g = 0.33$, $k = 4$, and $\tau^2 = 0.01$ for VR). Regarding intervention effectiveness for age categories, results showed that skill acquiring, in general, got better as the participants got older ($g = 0.8$, $k = 4$, and $\tau^2 = 0.13$ for ages 4–8 years; $g = 0.57$, $k = 7$, and $\tau^2 = 0.04$ for ages 8–12 years; $g = 0.84$, $k = 7$, and $\tau^2 = 0.09$ for ages 12–16 years; and $g = 0.85$, $k = 6$, and $\tau^2 = 0.36$ for ages >16 years). Skill categories followed the same trend as the strongest effectiveness observed in the age older than 16 years for all of them ($g = 0.98$, $k = 2$, and $\tau^2 = 0.3$ for SCS; $g = 0.46$, $k = 4$, and $\tau^2 = 0.03$ for ERS; and $g = 1.33$, $k = 3$, and $\tau^2 = 0.82$ for DLS for other age groups; **Table 3**). It is also noteworthy to say that effectiveness was relatively strong for CS in participants aged 4–8 years, which was the major outcome addressed by our included trials in this age group ($g = 0.77$, $k = 3$, $\tau^2 = 0.15$). These results point to a more favorable effect of VR interventions for older patients. Subgroup analysis for comorbidity revealed considerable decline in training effectiveness on ASD patients with concomitant comorbidity as $g = 0.77$ in 20 trials with $\tau^2 = 0.13$ in which patients did not have any specified comorbidity dropped to $g = 0.6$ in six trials with $\tau^2 = 0.03$ in which patients had some type of comorbidity alongside their main disease. This

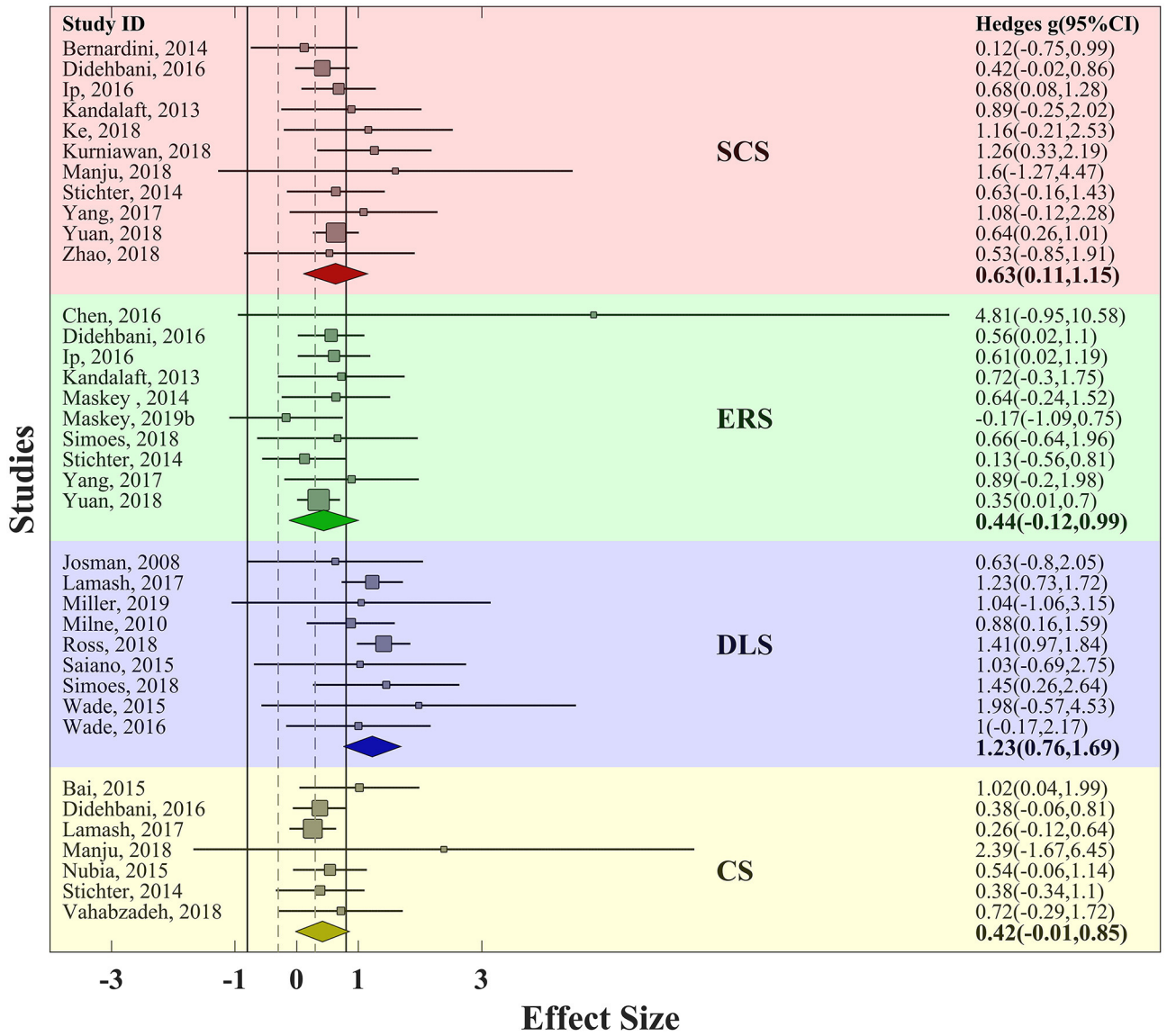

FIGURE 5 | Forest plot of skill-based training effectiveness for uncontrolled trials with 95% confidence interval. Red area represents SCS; green area represents ERS; blue area represents DLS; and yellow area represents CS. Solid vertical lines represent strong effect size boundary ($g = -0.8$ and 0.8), and dashed vertical lines represent weak effect size boundary ($g = -0.3$ and 0.3).

effect was even more sophisticated in controlled trials so that $g = 0.57$ in six trials with $\tau^2 = 0.08$ without specified comorbidity reduced to $g = 0.11$ in three trials with $\tau^2 = 0.03$ in which a comorbidity was diagnosed. The full *Results* can be seen in **Table 3**.

Meta-Regression

To see if there is any significant interaction between continuous moderators (number of sessions, sex, and publication date) and effectiveness of the intervention, we opted for univariate linear regression on weighted ESs as a function of each of these moderators for all designs and skill categories (**Table 4**). Significant relationship was found in publication date for overall (N = 122, beta1 = 0.4, $p = 0.02$) and DLS (N = 30, beta1 = 0.95, $p = 0.006$), which show that over time, intervention qualities have been likely to be improved as the technology has been advancing; there was no significant association between the number of sessions or gender and computed ESs in any of outcome categories.

Publication Bias

Visual inspection of the funnel plot (**Figure 6**) for both controlled and uncontrolled overall ESs pointed to a symmetrical funnel for both trials. To validate this conclusion statistically, we applied Egger regression intercept test. The test results corroborated the visual inspection by revealing no significant bias for uncontrolled

TABLE 3 | Subgroup meta-analysis results for type of measure, type of technology, and age moderators.

Subgroup	Category	*N*	*g*	SE_g	*Q*	τ^2
Formal measure	Overall	15	0.665	0.181	19.7	0.096
	SCS	8	0.604	0.044	3.3	0.107
	ERS	8	0.439	0.052	4.35	0.063
	DLS	4	1.236	0.04	0.701	0.552
	CS	5	0.366	0.007	1.716	0.017
Non-formal measure	Overall	11	0.931	0.097	5.192	0.203
	SCS	3	1.027	0.221	0.773	0.277
	ERS	2	0.957	0.101	1.916	0.241
	DLS	5	1.059	0.156	1.535	0.283
	CS	2	0.719	0.048	0.705	0.141
AR	Overall	5	0.912	0.097	3.975	0.304
	CS	3	0.72	0.039	0.705	0.151
VR	Overall	21	0.715	0.178	21.72	0.099
	SCS	10	0.627	0.047	4.024	0.102
	ERS	9	0.449	0.05	4.481	0.061
	DLS	9	1.155	0.093	3.242	0.477
	CS	4	0.334	0.004	1.173	0.013
Age 4–8 years	Overall	4	0.797	0.059	1.405	0.137
	CS	3	0.775	0.055	1.436	0.149
Age 8–12 years	Overall	7	0.572	0.024	3.457	0.045
	SCS	4	0.582	0.013	0.747	0.12
	ERS	6	0.462	0.028	3.969	0.089
	CS	2	0.377	0.001	2E-04	0.012
Age 12–16 years	Overall	7	0.847	0.06	1.406	0.091
	SCS	2	0.848	0.156	0.402	0.059
	DLS	4	1.11	0.1	1.129	0.295
	CS	2	0.339	0.008	0.704	0.021
Age >16 years	Overall	6	0.854	0.345	11.19	0.356
	SCS	2	0.982	0.041	0.055	0.299
	ERS	4	0.462	0.24	2.752	0.028
	DLS	3	1.331	0.039	0.284	0.825
Comorbidity present	Overall	6	0.608	0.019	1.104	0.033
	ERS	3	0.599	0.002	0.038	0.095
	DLS	3	1.077	0.361	0.771	0.16
	CS	2	0.455	0.011	0.387	0.043
Comorbidity absent or not reported	Overall	20	0.77	0.195	23.67	0.132
	SCS	10	0.744	0.101	4.763	0.143
	ERS	7	0.404	0.06	6.036	0.062
	DLS	6	1.193	0.059	2.208	0.553
	CS	5	0.45	0.03	3.252	0.03

AR, augmented reality; CS, cognitive skills; DLS, daily living skills; ERS, emotion regulation and recognition skills; g, Hedges g; N, number of trials; Q, Cochrane Q stat; SCS, social and communication skills; SE_g, standard error of g; VR, virtual reality.

and controlled trials [intercept= 0.27 ($p = 0.24$) for uncontrolled and intercept = 0.1 ($p = 0.88$) for controlled trials]. This implies that drawn conclusions are robust and reliable.

Comparing effectiveness of VR training with some of conventional behavioral programs that were addressed by three meta-analysis studies, we observed a comparable moderate effectiveness of our study with the most of clinical targets appraised by them. One exception was effectiveness of early intensive behavioral intervention on full-scale IQ of patients with ASD, which was proven to be strong ($g = 1.1$ of nine controlled studies). The other interesting finding was that TEACCH and ABA programs were not effective in improving daily living skills ($g = 0.34$ from 6 and $g = 0.14$ from 29 studies, respectively), while the effectiveness of VR training on this clinical target was very strong as it was observed in both controlled and uncontrolled trials. For full results on this part, see **Table 5**.

TABLE 4 | Metaregression results for number of sessions, sex, and publication date moderators.

Moderator	Skill	*n*	Slope	*p*
No. of sessions	Overall	122	−0.035	0.4912
	SCS	38	0.0086	0.8766
	ERS	27	0.0297	0.6552
	DLS	30	0.5425	0.3548
	CS	27	−0.148	0.188
Gender	Overall	122	4.5146	0.2318
	SCS	38	−2.162	0.8148
	ERS	27	6.6054	0.0935
	DLS	30	−0.25	0.9892
	CS	27	−18.7	0.1484
Publication date	Overall*	122	0.4021	0.0219*
	SCS	38	0.212	0.5024
	ERS	27	−0.237	0.4484
	DLS*	30	0.9515	0.0067*
	CS	27	0.7987	0.2738

**Significant values with $p < 0.05$.*
CS, cognitive skills; DLS, daily living skills; ERS, emotion regulation and recognition skills; N, number of outcomes in each group; SCS, social and communication skills.

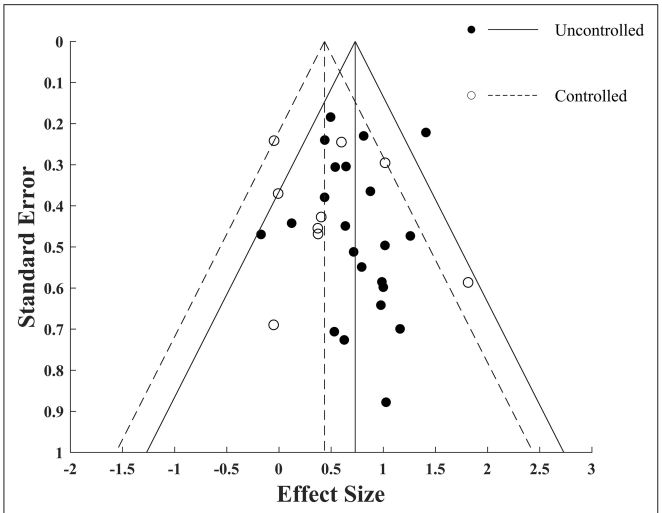

FIGURE 6 | Funnel plot for VR training effectiveness of both uncontrolled and controlled trials with pseudo 95% confidence interval. Filled and empty circles represent Hedges *g* value of uncontrolled and controlled trials respectively. Solid lines represent *g* with 95% confidence interval of uncontrolled trials and dashed lines represent *g* with 95% confidence interval of controlled trials.

DISCUSSION

We performed a systematic review and meta-analysis on the effectiveness of applying VR-based therapeutic interventions on the alleviation of deficits in ASD patients. Based on the results of 26 uncontrolled and nine controlled trials, we concluded that VR technology can be a viable tool for designing interventions aimed at enhancing and improving different skills in people suffering from ASD at any age. To our knowledge, this is the first meta-analysis focusing exclusively on the effectiveness of VR-based interventions for training ASD patients. Although there are some meta-analysis studies available on the same topic with various types of new technologies that may (46, 63) or may not (103–105) include VR, the number of trials that methodologically focused on VR was not large enough to draw rigorous conclusions around their efficacy. The increase in the number of VR interventions has been conducted recently; besides, its public availability has justified the need for this study. Overall moderate effectiveness of VR interventions that we observed in this study is in line with the results of previously mentioned studies. Our study shows moderate effectiveness ($g = 0.44$) of VR interventions based on controlled trials and strong effectiveness ($g = 0.73$) based on uncontrolled trials. Although the number of uncontrolled trials was conspicuously larger than controlled ones (26–9), a more credible design of controlled trials leads us to the point to claim moderate effectiveness of VR-based training in individuals with ASD.

Low heterogeneity in uncontrolled trials would provide further support for the conclusion drawn from these trials. Moderate heterogeneity in controlled trials cast doubts on interpretation of their results that could be explained by their relative small sample size (only nine trials comparing to 26 uncontrolled trials) and also the heterogeneity of control groups.

Further analysis of entailed categories of skills revealed relatively the same moderate effectiveness of intervention for SCS, ERS, and CS except daily living skills that outperformed other categories with promising large effectiveness in both design groups. This effect was proven to be consistent across different trials with different designs as heterogeneity was low for both of them. This finding can be specifically of interest because of the more reflective nature of DL skills, which means that they are gained and generalized in later stages of cognitive development, and ASD subjects are required to be trained for it similarly to their neurotypical counterparts. Unlike reflective skills, communication or emotional skills are more intuitive in the sense that they are generally gained in the early stages of development without any specific effort. It is also possible that the observed large effect originated from the larger mean age of participants in this outcome category comparing to other categories, which have given them superiority and dexterity in learning and practicing skills. Nevertheless, significantly larger effectiveness of VR training for DLS compared to others is persuasive enough for us to put forward the hypothesis that reflective skills hold more potential toward improvement by training than intuitive skills. Here we may have corroborated this hypothesis for VR-based training. On the other hand, medium effectiveness for communication, emotion, and CS may be due to the complex mental nature of these skills. As it is reflected in the recent systematic review (62), although these skills have been the center of attention in most studies, just partial improvements have been made. Knowing this, in future efforts, a more elaborated intervention design seems necessary to ascertain the effectiveness of VR training on these types of skills. Taken together, we encourage psychiatrists and educators of people with ASD to practice this type of technology with more focus on daily living skills.

TABLE 5 | Characteristics and effectiveness of three of the conventional rehabilitation programs.

References	Intervention	Publication year	No. of studies	Case number	Study design	Outcome measure	ES statistic	ES
Virues-Ortega et al. (3)	TEACCH	2013	13	172	Uncontrolled	Overall	Cohen *d*	0.47
			6	93		Eye–hand coordination		0.26
						Motor functioning		0.36
						Gross motor function		0.58
						Imitation		0.41
						Perception		0.4
			5	74		Communication skills		0.34
			6	81		Daily living skills		0.32
			5	74		Social functioning		0.64
			5	43		Cognitive functioning		0.41
			9	121		Verbal skills		0.36
			4	44		Maladaptive behaviors		−0.92
Eldevik et al. (4)	Early Intensive Behavioral	2013	9	153/105 (control)	Controlled	Full-scale IQ	Hedges *g*	1.1
						Adaptive behavior		0.66
Makrygianni et al. (5)	Applied Behavior Analytic interventions	2018	29	831	Uncontrolled	Intellectual abilities	Hedges *g*	0.74
						Communication skills		0.65
						Expressive-language skills		0.742
						Receptive-language skills		0.597
						Non-verbal IQ		0.463
						Adaptive behavior		0.422
						Socialization		0.444
						Daily living skills		0.138

ES, effect size estimate; TEACCH, Treatment and Education of Autistic and Related Communication Handicapped Children.

The results of our subgroup analysis are merely discussed on overall outcomes of uncontrolled trials. The small sample size of other subgroups precluded us from drawing a strong conclusion for them. The effectiveness of an intervention based on formal and non-formal measures was roughly similar and around moderate range, which indicates that our results might not be affected with the existence of non-formal assessments. Apart from that, cautious interpretation of informal measures should be considered, and it is possible that defined informal measures could be biased. The effectiveness of AR was similar and even a little larger than VR interventions. Although the sample size of the AR subgroup was relatively small, considering its low heterogeneity, the resultant conclusion on this subgroup can be reliable. This is particularly important because AR interventions can be conducted by means of AR-enabled mobile phones, which is ubiquitous nowadays providing more controlled interventions for larger populations of patients with ASD. The superiority of AR can be assigned to its simplicity in design and convenience of use compared to VR in which tasks are designed and applied in more complex environments with more parameters to understand and deal with. This simplicity can lead to a sooner and better engagement of participants in the task specifically for younger children.

The results of the subgroup analysis for age categories revealed that performance improves as the age gets larger. Particularly, it is important to note that this improvement is happening not only in daily living skills, which are reflective skills and later in development, but also in other intuitive areas, such as social skills and emotion recognition skills. This phenomenon may be induced by two factors. First, patients with autism presumably develop a kind of mechanism to overcome the deficits primarily caused by ASD, and so they assimilate to their milieu as they age. Second, older patients may have the advantage that they understand the task and VR environment better, and so they interact with it more efficiently, resulting in improved performance. In the first age category (4–8 years old), a notable relatively strong effectiveness was observed in CS, which was the only addressed area in this age group. Despite the small sample size, the relatively large ES was persuasive enough for us to consider it. This large effect may also be seen in other areas of SCS, ERS, and DLS; therefore, we encourage the scientific community to target their interventions on these areas too.

We observed a substantial decline in the effectiveness of training on patients who suffered from some sort of concomitant comorbidity along with ASD. This phenomenon was particularly interesting in controlled trials as observed moderate effectiveness of training on ASD patients without other comorbidity was completely vanished when concomitant comorbidity was taken into account. This alarms the future practitioners who are trying to improve skills in patients with ASD by means of VR interventions to carefully screen their target patients for having other concomitant comorbidity.

The effectiveness of training for HFASD patients was moderate to large, which was equivalent to its overall value

regardless of the level of the disorder. In most of the studies, whether composed of a combination of LFASD and HFASD participants or the level of disease was not specified, direct association between level of disease and effectiveness of intervention could not be derived. For this reason, we call for papers with more focus on defining the level and functionality of disease in included participants for better characterizing the target population of intervention.

The results of meta-regression revealed a significant correlation between publication date and VR training effectiveness, which can be interpreted under improvement in the design and conduction of VR interventions over time. Surprisingly, effectiveness was not influenced by the total number of intervention sessions. It is important to mind that the session's duration and its distribution over the course of intervention were unreported or highly heterogeneous among the trials, and therefore, the net number of sessions might not be a good representative for intensity and quality of intervention. For this reason, hesitant interpretations warrant caution, and more controlled interventions in terms of design, duration, and longevity are needed for more conclusive interpretations on this matter. The sex of participants was not a significant moderator of the results in our study as it is not seen in other studies of this kind.

Comparing the results of the current meta-analysis with those of more conventional training programs (**Table 5**), it is evident that VR-based training is at least as effective in most study endpoints as traditional programs. In addition, the more flexible and favorable nature of VR leads to more elaborate task designs, more enthusiasm in participants to do those tasks, and ultimately more accurate assessments of improvement. These factors together might result in more ecological validity of VR-based experiments and more reliability of their results.

The strong effectiveness of daily living skills (reflected in both controlled and uncontrolled trials) was achieved only through VR-based training, not conventional training. It is therefore sensible to use VR to design rehabilitation programs aimed at daily living skills in clinical practice. In the other clinical targets, a further improvement in the design and application of VR technology is still required.

Limitations and Recommendations for Future Research

Most of our included studies were uncontrolled pretest–posttest trials. It has been argued that these types of trials should be avoided in meta-analysis as the pretest and posttest scores are not independent of each other, and thus, accurate calculation of SMDs requires knowledge of correlation value between these two scores, which is not provided in most of the studies (106). Aside from that, perhaps due to differing epistemological bases of research being carried out in this broad domain, most of the studies have adopted this type of design for their intervention, which makes considering this massive body of data for analysis inevitable. Here, we have done all the calculations with the premise of independent pretest and posttest scores (zero correlation), which is subsequently leading to the largest pooled variance and thus the smallest possible value of computed ES. For this reason, we claim that our applied method is the most parsimonious one avoiding any overestimation in computing the ESs.

Although the number of participants in most studies was rather low, and so their estimations would not be adequately powered, its effect might be compensated by a considerably large number of included trials. Many trials had not screened participants for critical contributing factors that could affect the outcome. This issue seemed to be a challenge for our results. However, later analysis relieved this by showing that the summary ES of those trials that screened included population did not deviate drastically from those who did not perform this screening.

The type of VR technology applied by studies was diverse enough to prevent us from establishing a systematic relationship between the technology type and its effectiveness, so further studies are required to investigate such a connection. To our surprise, restricted and repetitive behavior, which is one of the core symptoms of ASD, was not addressed by any of studies, so more experiments are encouraged to be targeted in this area in the future works. Follow-up assessment of participants was performed in an only limited number of trials; therefore, the maintenance of treatment effects, although important, could not be assessed in our study.

CONCLUSIONS

The current findings support the effectiveness of VR training to improve ASD-related disabilities. The strong observed effectiveness for daily living skills could justify the application of VR interventions in clinical practice. For future research, the designed experiments need to be more controlled in terms of selection of participants, type and duration of intervention, and choice of a measurement tool, and finally, more efforts should be devoted to follow-up assessments carried out weeks or months after the end of the intervention to ensure that the effects of training are consolidated and maintained.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

A-HV and FA designed the study and wrote the protocol. BK, RK, and MR conducted literature searches and provided summaries of previous research studies. BK analyzed data and produced figures. BK and RK wrote the first draft of the manuscript. A-HV supervised the study. All authors contributed to and have approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.665326/full#supplementary-material

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SYSTEMATIC REVIEW
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Challenges on Diagnoses and Assessments Related to Autism Spectrum Disorder in Brazil: A Systematic Review

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Being a continental country, with over 210 million citizens, Brazil is similar to all of those who are part of the LAMIC (Low and middle income countries). It shows a big concentration of wealth, mainly in its south and southeast regions, as well as areas with immense poverty. In that sense, the health system also faces a huge amount of contrast. Inside University hospitals and facilities there are sophisticated tools and trained doctors prepared to assist in any kind of medical subject, including autism. But, unfortunately, at other times, the access to a good health system is made much harder. This results in many issues in the medical community, e.g., looking at the data regarding autism, there is a high average of the age of diagnosis. Another issue is the low number of professionals trained in ASD diagnosis and the few tools translated to Portuguese.

Keywords: austim, ASD, autismo spectrum disorders, diagnoses, diagnoses tools

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INTRODUCTION

Brazil is a continental country and is the fifth largest country in the world in territorial extension with over 210 million citizens (1, 2). Brazil is divided into five regions with different geographical, demographic, cultural, and financial characteristics (3), including different health indicators (4). Similar to other low- and middle-income countries (LMICs), Brazil shows a great concentration of wealth as well as areas with immense poverty. In this sense, access to the health system is also a great contrast (5).

In Brazil, the Single National Health System (SUS) (*Sistema Único de Saúde* in Portuguese) provides access to health services for all citizens. This system is organized regionally and is composed of services with different degrees of complexity. It is also financed and coordinated by various government agencies (6–8). The public health system is not always able to offer excellent care for the entire population, and the private health system only services 20–45% of the citizens. Some hospitals and clinics attend both systems, but, in certain fields, the private system is more complete with tools, experts, and resources that are not present in the public system (9, 10).

Autism spectrum disorder (ASD) is a complex neurobiological development disorder characterized by two main behavioral components: (1) difficulty with communication and social interactions and (2) restrictive and repetitive behaviors, interests, and activities (11). ASD is a disorder that manifests with a wide variety of symptoms in the cognitive, emotional, and neurobehavioral areas. Although the characteristics that compose it are well-defined, the great heterogeneity of findings in each individual makes its recognition challenging. The disorder

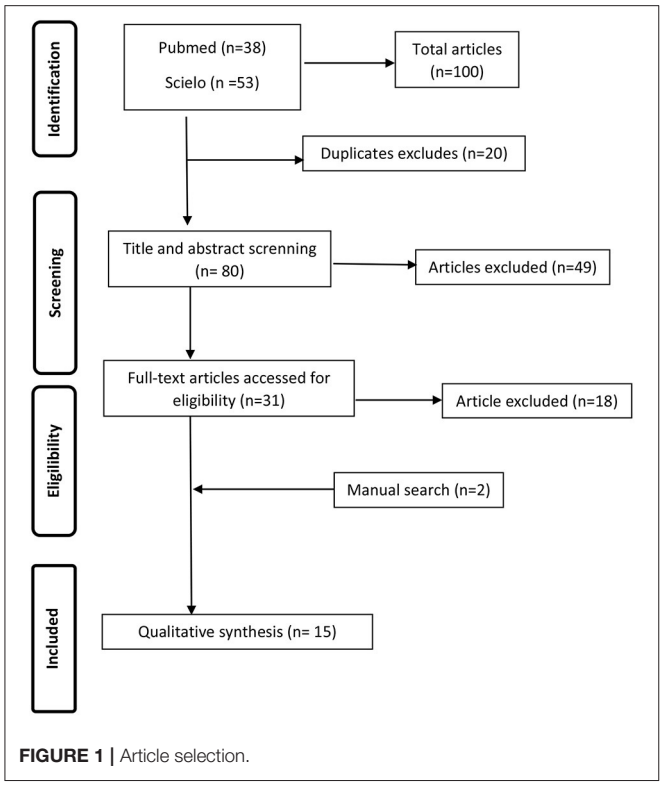

FIGURE 1 | Article selection.

encompasses extremely heterogeneous phenotypes, especially in the mildest cases of the spectrum, and the severity of central deficits varies greatly between patients (12–15).

The current prevalence points out that one in 54 children aged 8 years has ASD in the USA, and the ratio is higher in boys than in girls (4:1) (16). The prevalence of ASD makes it one of the most frequent neurological development disorders, representing a major public health concern, and it leads to high social and economic costs (17). There are no Brazilian studies that reliably estimate the prevalence of ASD; therefore, the federal government included the pathology in the 2020 national census (18). Current estimates show that about 1.5 million people have ASD in Brazil (15, 19, 20). A study analyzing the profile of children attended at the Child and Adolescent Psychosocial Care Center (Centros de Atenção Psicossocial Infanto-Juvenil [CAPSi]), from 2008 to 2012, showed that 23.6% of a total of 837,068 visits were related to developmental disorders (21).

In the absence of a biological marker, the diagnosis of autism remains a clinical decision (14, 22, 23), and instruments and scales are often used to aid in the diagnosis (14). Even in developed countries, studies describe the difficulty of early diagnosis, the parents' pilgrimage in different health services, and their discontentment with the diagnostic process (24–27). In addition, factors such as family income, residence in rural areas, ethnicity, child impairment, clinical presentation, and parental concern about initial symptoms are associated with later diagnosis (28–30). Therefore, it is expected that LMICs will have even greater difficulties in early diagnosis.

The diagnosis of ASD is based on a qualitative assessment of behavioral patterns and is directly influenced by the complexity and variability in the presentation of the disorder (e.g., levels of severity, association with intellectual disability, and other medical conditions). These characteristics have led to the development of a significant number of international instruments focusing on identification and early diagnosis (31, 32). Experience from high-income countries suggests that incorporating screening tools into routine healthcare visits can result in earlier and more accurate identification of children with developmental disorders, compared to only relying on clinical impressions (33). The use of ASD screening and diagnostic instruments in Brazil is still limited, representing an obstacle to the expansion of research in this field and to the improvement in the quality of health services. Although some instruments have been translated and validated, critical examination of the psychometric quality of these studies is still lacking in Brazilian publications (31).

OBJECTIVES

We aim to identify all data related to the tools and identification process of children and adolescents with ASD in Brazil.

REVIEW QUESTION

What data do we have related to the tools and the process of identifying children and adolescents with ASD in Brazil?

METHODS

We searched in PubMed (maintained by the United States National Library of Medicine at the National Institutes of Health), Scientific Electronic Library Online (SciELO), and Literatura Latino-Americana e do Caribe em Ciências da Saúde (LILACS) for systematic reviews about the tools and identification process for children with ASD in Brazil, and one article of 2010 was found. The authors performed a systematic review of the literature on the diagnosis of ASD in Brazil. The review follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) system of reporting (34). In regard to the article and abstract selection, the following inclusion criteria were used: (1) articles and abstracts published in the last 20 years; (2) articles that have at least one Brazilian researcher listed as an author; and (3) studies focused in identification/diagnosis of ASD.

The exclusion criteria included papers published more than 20 years ago, with no Brazilian authors listed, and whose languages were English and Portuguese. They also included articles of review, neurobiological and genetic bases, ASD comorbidities, epidemiological studies, phenotype and endophenotype studies, and intervention trials.

The articles were identified through a research of the major biomedical literature databases: PubMed, SciELO, and

LILACS. The following research terms were used, alone and in combination: "autism," "ASD," and autism spectrum disorder [Mesh]; "assessment," "diagnostic criteria," and diagnosis [Mesh]; "scale," "instrument," "tool," and "Brazil" [Mesh]; and "Brazilian." An eligibility assessment was performed independently in an unblinded standardized manner by three authors. Disagreements between reviewers were resolved by consensus.

RESULTS

After a systematic review, 15 articles were identified in the last 20 years (**Figure 1**). These articles are summarized in **Table 1**. We found seven validation studies, one before–after study, and eight cross-sectional studies.

Most studies are focused on the validation of scales and instruments and on the correlation of these with the diagnosis of ASD. Two studies stand out for us. The first led by Ribeiro et al. (46), which focuses on the barriers to early identification of autism in Brazil. This study shows us that the distance between the suspected diagnosis of ASD by parents and the formal diagnosis of the disease had an average delay of 3 years. Most mothers described their interactions with the doctorsas negative, and they felt discouraged to express their concerns again. The second was that of Bordini et al. (6), which shows that the training of health workers significantly increases knowledge about ASD. The number of patients referred to specialized ASD treatment centers has increased sixfold in just 4 months after training.

DISCUSSION

After the systematic analysis, it was found that there were few data on the TEA diagnosis process in Brazil and that most of the articles are related to the topic of translation processes and validation tools for Brazilian Portuguese.

It draws our attention to the fact that most of the difficulties encountered are late diagnosis, lack of training of health teams, problems of doctor–patient relationship, lack of knowledge about ASD, access to the health system, high cost of training professionals, and the high cost of tools for assessment of ASD patients. These problems are described in studies in other LMICs as can be seen in the paper of Ha et al. and blunt commentary of Ha et al. (48) and Durkin et al. (49).

Although the Brazilian mental health system is fully integrated with SUS (8, 50), unequal distribution of resources among different regions of Brazil and high levels of individual income inequality make access to care a significant challenge for many children and adolescents with mental health problems (7), the majority being ASD children who are not receiving specialized treatment (6).

There is a concentration of experts and services on some university sites, mainly in some south and southeast regions, which are the richest regions of the country. In addition, more than half of the doctors are concentrated in state capitals, where less than a quarter of the country's population lives (51). Inside universities and large hospitals, there are sophisticated tools and trained teams prepared to assist with any kind of medical diagnosis, including ASD. Unfortunately, access to the health system is made much harder in other situations. Some poor areas do not have trained and structured services to identify developmental delays, including ASD (7). It is estimated that only one in five children or adolescents in Brazil receive adequate mental care due to a shortage of specialized services, especially in the country's north and midwest regions (21, 52, 53).

The lack of professionals trained to recognize the manifestations of the disorder and the shortage of specialized services are associated with late ASD diagnosis (24). Even though the public health system should officially identify children with a developmental delay, some children (mainly the poorest) do not have access to skilled health services. Mandell et al. (54) showed that white children are diagnosed at 6.3 years old, while African American children are diagnosed at 7.9 years old, on average in the USA. These racial and ethnic differences in the age of diagnosis may be related to institutional factors, such as difficulties in a family's access to health services (30), although there are no similar studies in the Brazilian population. Based on other studies (55, 56), we believe that there is a similar reality in Brazil.

Although parents had concerns about their children's development, they had some obstacles until the diagnosis. Ribeiro et al., in an interesting work on the barriers of ASD diagnosis in Brazil, described the negative experiences of family members (56.3%) when reporting symptoms of autism to pediatricians and feeling discouraged to express their concerns again. Parents sometimes hear phrases from health professionals like "children should not be compared to each other" and "boys have a slower development rate" or they "are more agitated than girls" (46). Similar findings were reported in the UK and France, showing high rates of discontent with the diagnosis process. Even with parents suspecting that something was wrong with their children, it is not unusual for pediatricians to instruct them not to worry and to wait (26, 27). In Brazil, cases of 3- to 4-year-old children with a speech delay who are referenced to speech therapy without screening for ASD still occur. Interestingly, Machado et al. while evaluating the presence of signs of ASD in children referred to a hearing center to investigate hearing loss, despite the small sample, found signs for ASD (60%) more often than hearing loss (18%) (19).

Delays in obtaining an ASD diagnosis contribute to parental distress and postpone the start of therapeutic intervention, which, in turn, may affect the patients' long-term functional outcome and social adaptation (6, 25, 57, 58). Some advances have occurred, such as the promotion of early diagnosis by the Brazilian Academy of Pediatrics (59) and the release of guidelines for ASD diagnosis by the Brazilian Ministry of Health (60, 61). Unfortunately, validated protocols or screening algorithms for early ASD detection have not been implemented in most Brazilian public health facilities (15, 19, 20, 60), in spite of the World Health Organization (WHO) recommendation that LMICs should have an early ASD detection program (62). The insufficient information and clinical training of primary healthcare professionals, including scarce teaching about autism in medical schools, also contributes to the problem (6, 19, 63).

TABLE 1 | Selected articles.

References	Year	Title	Study	Finding/Summary
Barbosa et al. (35)	2015	Propriedades psicométricas da Escala de Responsividade Social 2 para Transtornos do Espectro Autista	Translation/validation	ERS-2 Portuguese version can be used as a screening tool; however, some items were not statistically consistent, especially those related to mild ASD.
Becker et al. (36)	2012	Tradução e validação da ADI-R (Autism Diagnostic Interview-Revised) para diagnóstico de autismo no Brasil	Translation/validation	Translated and validated by the ADI-R scale for Brazilian Portuguese.
Bordini et al. (6)	2014	Impact of training in autism for primary care providers: a pilot study	Before–after trial	The trained providers significantly improved their ASD knowledge after training in comparison with pre-training. Clinical practice also changed: 4 months after the training program, the providers had referred six times as many suspected cases of ASD to a specialized mental health service in comparison with the previous 4 months.
Losapio and Pondé (37)	2008	Tradução para o português da escala M-CHAT para rastreamento precoce de autismo	Translation/validation	Translated and validated by the M-CHAT scale for Brazilian Portuguese.
Machado et al. (38)	2016	Respostas parentais aos sinais clássicos de autismo em dois instrumentos de rastreamento	Cross-sectional study	Isolated points of the instruments Questionário de Indicadores de Risco para o Desenvolvimento Infantil (IRDI) and M-CHAT were unable to predict ASD in relation to the set of questions.
Machado et al. (19)	2016	Appropriateness of using autism spectrum disorders screening tools in a hearing evaluation service	Cross-sectional study	It assessed ASD signs in children referred to an audiological center to investigate hearing loss. Only 18% of the 43 children assessed had hearing loss, while 60% had ASD signs.
Marques and Bosa (39)	2015	Protocolo de Avaliação de Crianças com Autismo: Evidências de Validade de Critério	Cross-sectional study	Preliminary assessment of Protocolo de Avaliação para Crianças com Suspeita de Transtornos do Espectro do Autismo (PRO-TEA), suggesting that this instrument may assist in the ASD diagnosis.
Marteleto et al. (40)	2008	Administration of the Autism Behavior Checklist: agreement between parents and professionals' observations in two intervention contexts	Cross-sectional study	Verified the discrepancy in the responses in the Autism Behavior Checklist of parents and therapists of ASD children.
Duarte et al. (41)	2003	The CBCL and the identification of children with autism and related conditions in Brazil: pilot findings	Cross-sectional study	The Child Behavior Checklist (CBCL) scale was applied to ASD children, children with other psychiatric disorders, and healthy children. Scores on the "Thought Problems" and "Autistic/Bizarre" scales were related to cases of autism.
Galdino et al. (42)	2018	Evidence of validity of the Autism Mental Status Examination (AMSE) in a Brazilian sample	Cross-sectional study	The data suggest that this tool can be used for the screening of ASD.
Marteleto and Pedromônico (43)	2005	Validity of Autism Behavior Checklist (ABC): preliminary study	Translation/validation	It is a promising tool for identifying children with autism, especially with a cutoff point of 49.
Pacifico et al. (44)	2019	Preliminary evidence of the validity process of the Autism Diagnostic Observation Schedule (ADOS): translation, crosscultural adaptation and semantic equivalence of the Brazilian Portuguese version	Translation/validation	Translated and validated by the ADOS scale for Brazilian Portuguese.
Pereira et al. (45)	2008	Childhood autism: translation and validation of the Childhood Autism Rating Scale for use in Brazil	Translation/validation	Translated and validated the CARS into Brazilian Portuguese.
Ribeiro et al. (46)	2017	Barriers to early identification of autism in Brazil	Cross-sectional study	Family members of patients with ASD, describe the difficulties and delay in diagnosis.
Sanvicente-Vieira et al. (47)	2013	Revised Reading the Mind in the Eyes Test (RMET)—Brazilian version	Translation/validation	Translated and validated by the RMET scale, in both paper-and-pencil and computerized versions. The RMET is a well-accepted instrument for the assessment of theory of mind, an important component of social cognition.

Despite the relevance of this topic, the number of Brazilian scientific publications on the care of children with ASD from the perspective of their family members is still scarce (46, 64, 65), and the few existing studies have a small number of participants. It is noteworthy that there are few studies focused on the training of health professionals to identify children with ASD in a clinical practice, and there is also a lack of initiatives to guide education workers, such as kindergarten educators, to identify these children. Perhaps these initiatives are one of the keys to early identification of children with ASD.

Diagnosing ASD is a challenging task (66). The screening tools help to identify children who may have developmental delays, allowing their early referral to specialized centers. Some screening tools are used primarily in pediatric practices, while others are used by school systems or in other community settings. Diagnostic tools, although they cannot be used as a basis (the diagnostic process should include information from parents/caregivers and child observation and interaction along with the usage of clinical judgement), aid in diagnosis (38, 66–68).

There is a lack of consensus on which screening tools are most effective especially when the tools are used in cultures other than those in which they were developed, which occurs often in LMICs. Routine screening is an important first step toward addressing the need for services in LMICs, but high-quality tools take time to be conceptualized, developed, piloted, and validated, before implementation can happen (33). Most tools that help the diagnosis of autism are developed in English and need to be translated and validated for use in clinical practice in Brazil. We found many studies that made this process. Despite the success demonstrated in some papers, few tools were fully tested, and there is a great delay between their development and their validation for use in Brazil. As an example, Losapio and Pondé (37) translated the M-CHAT, a screening tool, in 2008, but the original article was published in 2001 (37, 69). Likewise, the CARS, which is so important in helping the identification of ASD children, was validated in the same year (2008) (45), 20 years after the original paper was published (70). In addition, health professionals make little use of these instruments in daily practice. Many parents of ASD children report that they visited many different health professionals in search of a diagnosis during their child's early years, but no specific ASD screening was performed (19, 71).

There is a lack of validated/translated tools to identify children with ASD in clinical practice, resulting in some initiatives to develop new tools that could be used in Brazil. However, this kind of work demands time and has obstacles, as we see in the work of Bosa et al. (72).

In summary, the difficulties encountered in Brazil do not seem to be very different from those encountered in other developing countries. According to Stewart and Lee (73), community-based screening was shown to be an effective method for identifying ASD in communities with limited clinical resources, and these studies offer the opportunity to identify individuals with symptoms across a wider spectrum.

Access to healthcare providers who are capable of diagnosing and treating individuals with ASD can be very limited in LMICs (73). An alternative to improve this situation is to invest in training primary healthcare workers and non-specialists. The WHO is conducting an alternative for the early intervention of children with ASD. This initiative is named the WHO Caregiver Skills Training (WHO CST) program and is designed to train people who are not specialists in the health field to perform health interventions aimed at delayed development in children, including ASD. This program is designed to use a combination of group sessions (e.g., community centers) and individual sessions in children's homes. The group session is tailored to teach the caregivers to carry out the necessary interventions for children with developmental disorders, while keeping the costs low. The session at home is held to adapt interventions to the individual needs of each child and family (74). This type of intervention is based on reviews that demonstrate that interventions performed by caregivers guided by non-specialists with ASD patients are effective (75, 76). The WHO CST program is currently undergoing field testing in more than 30 countries in regions throughout the world (77). Randomized studies are being carried out in Pakistan and Italy, and cultural adaptations are also being carried out for each community when necessary (74, 77–79).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed above participated independently in the selection of articles and review of the topic. After an initial selection, the content was reviewed together and the article presented here was elaborated. All authors contributed to the article and approved the submitted version.

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