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Etiology and treatment for children and adolescents with autism spectrum disorder

Edited by
Feiyong Jia, Tingyu Li, Wouter G. Staal
and Khaled Saad

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Etiology and treatment for children and adolescents with autism spectrum disorder

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Editorial: Etiology and treatment for children and adolescents with autism spectrum disorder

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Editorial on the Research Topic
Etiology and treatment for children and adolescents with autism spectrum disorder

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by social communication impairments and restricted, repetitive behavior (1). However, the etiology of ASD is under investigation and the treatment is still challenging. The current topic, therefore, aims to focus on the etiology and treatment of children and adolescents with ASD.

Etiology for ASD

The etiology of ASD is not incompletely understood, which can be interpreted as gene-environment interaction contributing to autism risk (2). The general consensus is that ASD is a collection of related disorders of different etiologies (1). Maternal and early developmental vitamin D (VD) may be associated with ASD (3). Muskens et al. found 75.9% of the children with ASD had VD deficiency. But The mechanism of VD in ASD is still unclear. Wang X. et al. reviewed current studies and found various factors (including abnormalities in neurotransmitters, immune disorders, etc.) may cause abnormal levels of nitric oxide (NO) during the critical period of brain development which was an important factor in the pathogenesis of ASD. VD can regulate the levels of molecules in the NO signaling pathway. Wang B. et al. hypothesized a potential mechanism that VD affects the pathogenesis and severity of ASD by regulating NO levels. Thus, monitoring maternal and early developmental NO levels may become an important strategy for ASD risk prediction in clinical practice in the future. Pichugina et al. compared salivary oxytocin (OT) concentration between ASD children with intellectual disability (ID) and children with ID. The results indicated that children with ID and ASD demonstrated a lower level of salivary OT concentration and a direct relationship between low salivary OT level and a high degree of severity of ASD. Research on the effect of OT on ASD with ID was limited. Further studies on the relationship between OT and ASD with ID are warranted. Chen et al. found children with ASD had an altered microbial

community structure of lower alpha diversity indices and higher Bacteroides and Faecalibacterium than children with ID or typically developing. The gut microbiota may modulate central nervous system activities through neural, immune, and endocrine pathways thereby affecting ASD. Further research is needed on the relationship between gut microbiota and the etiology of ASD. Qin et al. investigated the resting-state functional brain network characteristics of preschool children with ASD and found evident alterations in functional brain networks. Functional connectivity changed mainly in the default mode network (DMN), hippocampus, and parahippocampal gyrus. The study provided some evidence on understanding the brain functional pathogenesis of ASD.

Treatment for ASD

Children with ASD generally require comprehensive treatments including nonpharmacological and pharmacological treatments (4). The current evidence-based management of ASD in children relies primarily on behavioral treatments to modify the core symptoms of ASD. Parent-mediated intervention, delivered by trained parents is a potential treatment (5). Li et al. evaluate the effectiveness of an 8-week, online-delivered project Improve Parents as Communication Teacher (ImPACT) program for children with ASD and their parents in China during the COVID-19 pandemic. They have found social communication skills of children with ASD significantly improved and the parenting stress of parents decreased. This study provided evidence that parents' training delivered online may be a promising program to promote intervention accessibility when face-to-face training is unavailable. Gao et al. used Repetitive transcranial magnetic stimulation (rTMS) on the bilateral dorsolateral prefrontal cortex (DLPFC) of children with ASD and found the core symptoms and sleep problems improved significantly after two courses of intervention of rTMS. They also found that sensory abnormality mediated the improvement of rTMS on sleep problems of ASD. Current measures for managing sleep disorders in ASD are sleep education, sleep environment preparation, behavioral intervention, and drug treatment (such as melatonin) (6). The study provided new ideas for the intervention of sleep problems in ASD. Leuning et al. gave 21 adolescents with ASD a ten-session Eye Movement Desensitization and Reprocessing (EMDR) treatment on daily experienced stress and found EMDR treatment significantly reduced perceived stress as reported by the participants, and improves global clinical functioning. This study provided novel ideas for the treatment of core symptoms and experienced stress in adolescents with ASD. The stem cell is receiving attention as a potential therapy for ASD, but the efficacy and safety of stem cell is unclear (7). Qu et al. performed a meta-analysis of stem cell therapy for children with ASD and found that stem cell therapy might be safe and effective. However, the evidence was insufficient due to many factors, such as the small size of studies, varied injection routes, and doses of stem cells. There are still many problems with stem cell therapy for ASD, and a lot of work needed to be done.

Conclusions

The current work explores the etiology and treatment of ASD. These results support that ASD is a neurodevelopmental disorder with complex components. Vitamin D, OT, gut microbiota, and functional brain network may influence ASD through different pathways. Further research is still needed on the pathogenesis of ASD. Parent-mediated intervention is still important in clinical practice. There are various forms of parental training, and online teaching is a good choice in clinical practice. Other nonpharmacological treatments can serve as a supplement to behavioral interventions to improve core symptoms and comorbid problems of ASD.

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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REVIEW
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Autism Spectrum and Other Neurodevelopmental Disorders in Children of Immigrants: A Brief Review of Current Evidence and Implications for Clinical Practice

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Children of immigrants may have higher neurodevelopmental risks than those of non-immigrant populations. Yet, some evidence suggests that this group may receive late diagnosis, and therefore miss beneficial early interventions. Clinicians may misattribute symptoms of disorders to other social, behavioral or language problems. Likewise, there might be cultural differences in parents' likelihood of perceiving or reporting first developmental concerns to clinicians. Population-based standardized screening may play an important role in addressing ethnic inequalities in the age at diagnosis, although further research focusing on cross-cultural use is necessary. Once children are diagnosed, clinicians may rely on culturally sensitive procedures (translation services, cultural mediators) to increase the accessibility of interventions and improve adherence among immigrant families. In this brief review, we provide an overview about what is currently known about the epidemiology and risk factors of neurodevelopmental disorders, paying special attention to autism spectrum disorder (ASD), in children of immigrants and suggest the necessity of population-based screening and culturally sensitive care.

Keywords: developmental disabilities, autism spectrum disorder, child development, migration, neurodevelopment, migrant health, maternal and child health

INTRODUCTION

There is increasing evidence based on literature reviews that children of immigrants are at higher risk of neurodevelopmental disorders, particularly autism spectrum disorder (ASD) (1–4). Other neurodevelopmental disorders, such as attention deficit hyperactivity disorder (ADHD), intellectual disability, and specific learning disabilities, have been studied less frequently and results are less consistent, with some studies reporting higher risks and others reporting lower or equal risks (5–8). Some evidence suggests that children of immigrants may face delays in diagnosis and

increased difficulties in accessing appropriate health care (9–11). Especially in relation to ASD this is very concerning, as children of immigrants may be at risk of missing early interventions, which have been shown to be highly beneficial in terms of clinical prognosis (12). In this brief review, we provide a short overview on what is currently known about inequalities in neurodevelopmental health in children of immigrants and discuss implications for screening and clinical practice, paying special attention to ASD.

EPIDEMIOLOGY OF NEURODEVELOPMENTAL DISORDERS IN CHILDREN OF IMMIGRANTS

Increased risks of ASD in children of immigrants were first reported in Sweden, the UK, and Australia as early as the 1970 and 1980s (3, 13), and have since been confirmed in various countries (1–4). The main results are summarized in **Table 1**. This table does not present an exhaustive list of all studies on ASD in children of immigrants.

Although there is variability in the strength of the reported associations, systematic reviews have found support of increased risks of ASD in children of immigrant parents (1–4). Particularly maternal immigrant status has been reported as an environmental risk factor for ASD (23, 24). Parental immigrant status seems to be mainly associated with low-functioning ASD (20) and other ASD phenotypes characterized by intellectual disability and communication disorders (25). Indeed, studies reported generally lower risks of high-functioning ASD (20) and Asperger's syndrome (26) in children of immigrants (25, 27), as compared to the host population. Neurodevelopmental disorders other than ASD have been examined less frequently, and results are less consistent. When considering ADHD, divergent results have been found, partially due to differences in informants, as well as in the accessibility of mental healthcare across countries. In Finland, Lehti et al. found higher odds of ADHD in children of immigrants in a case-control study (5). Similarly, in Germany, immigrant parents reported more ADHD symptoms in their children (28) and stronger increases in symptoms over time (29). However, German children born to immigrants had lower odds of receiving a diagnosis of ADHD (7). In Sweden, only non-European immigrants reported more symptoms of ADHD in their children (30). In Denmark, teachers reported similar numbers of ADHD symptoms in children of immigrants and non-immigrants, whereas immigrant parents reported lower levels of symptoms among their children (31). In Belgium, adolescents with a migrant background tended to self-report fewer symptoms of ADHD than their non-immigrant peers (32), whereas no differences in self-reported symptoms were found in Norway (33). In the Netherlands, Zwirs et al. found lower prevalence estimates of ADHD in children of immigrants, as compared to those of the native Dutch (34). In the USA, several population-based studies found lower risks of receiving an ADHD diagnosis in ethnic minority groups, including Hispanic Americans (35–41). However, a study specifically focusing on the Public Mental Health System of New York State found higher prevalences of ADHD in Hispanics and non-Hispanic Blacks than in Whites (42). Importantly, it must be noted that in the US, many studies on ethnic minority groups did not distinguish between immigrants and non-immigrants (e.g., in the case of Blacks or Hispanics), and few provide detailed information on individuals' country of origin (22).

Also when considering intellectual and learning disabilities, divergent results were observed. In Australia, Abdullahi et al. found higher prevalence rates of cerebral palsy with ID in children of mothers born in upper-middle-income countries only. They did not find evidence of increased risks of ID without cerebral palsy or cerebral palsy without ID in children of immigrant mothers (8). In two other Australian studies, mothers born abroad had no significant differences in their risk of having a child with severe ID, and when considering mild to moderate ID, foreign-born women tended to have lower risks (43, 44). A British study found lower prevalence rates of intellectual disabilities in children of immigrants, except those of Bangladeshi descent, who had higher odds of profound multiple learning difficulties, of Pakistani descent who had higher odds of profound multiple and severe learning difficulties, as well as Travelers of Irish heritage, who had significantly higher odds of moderate and severe learning difficulties (45). In France, the prevalence of severe ID was higher in areas with a high proportion of immigrants (46). In Finland, Lehti et al. found that children of immigrant parents had higher odds of developmental disorders related to speech and language, academic skills and coordination (6). Similarly, a German study found that children with foreign nationalities had higher odds of delays in gross motor skills, fine and grapho-motor coordination, grammar, memory and concentration, perseverance, abstraction, visual perception and arithmetic (47). In the US, two studies found no differences in the odds of learning disabilities in children of Latinos and Whites (37, 48), and a third study found lower odds of developmental delay, learning disabilities, and speech problems in children of immigrants (49). A Taiwanese study found lower risks of developmental delays in children of immigrants (50).

Inequalities in neurodevelopmental health may already be present at a very young age, long before children are typically diagnosed, though currently few studies on very young infants are available. In a population-based birth cohort in France, Schmengler et al. found that immigrant mothers more frequently observe early signs of neurodevelopmental problems in their 2-year-old children, as measured by a standardized parental-report screening instrument [Modified Checklist for Autism in Toddlers (M-CHAT)], compared to non-immigrant French mothers (51). In a recent Italian study on pre-term infants, children of migrant mothers from countries with a low Human Development Index (HDI) showed increased risks of general neurodevelopmental impairment at 24 months, as determined by the Revised Griffiths Mental Development Scale 0–2 years, particularly if they were exclusively fed with formula milk (52).

The timing and circumstances of migration seem to play an important role in neurodevelopmental inequalities in immigrant populations. For example, Magnusson et al. (20) reported that recent migration was particularly associated with risks of low-functioning ASD in the offspring, and children of mothers

TABLE 1 | Studies on ASD in children of immigrants [based on Augereau et al. (14)].

References	Population	Sample	Main results
Gillberg et al. (13)	Epidemiological study based on a Swedish population registry comparing migrant parents' frequency between the autism and control group	35 children with autism (20 living in urban area and 15 living in rural area) compared to 42,886 age-matched children in the general population from the same region	30% of urban children with autism have migrant parents 0% of rural children with autism have migrant parents No significant difference regarding migrant parents' frequency between urban ASD and typically developing control groups Significantly lower frequency of migrant parents in rural ASD group, as compared to typically developing controls ($p < 0.001$)
Gillberg et al. (15)	Study of autism prevalence in children born in Sweden from mothers born in Uganda	3 children with autism born in Sweden from mothers born in Uganda	Autism prevalence in children born in Sweden from mothers born in Uganda (15%) compared to the prevalence of autism in the general population (0.08%)
Gillberg et al. (16)	Epidemiological study based on a Swedish population registry comparing migrant parents' frequency between an autism and a control group	55 children with autism for 78,106 children in general population	27% of children with autism compared to 26.2% of children in general population have migrant parents (non-significant)
Lauritsen et al. (17)	Epidemiological study based on a Danish population registry comparing migrant parents' frequency between an autism and a control group	818 children with autism born in Denmark from a cohort of 943,664 children in general population	Relative risk of ASD evaluated at 1.42 when mothers were born outside European continent compared to mother born in Denmark
Keen et al. (18)	Retrospective study based on English clinical registry comparing ASD prevalence according to mother's native country and her ethnic group	428 children with autism	Relative risk of autism higher for children whose mothers were born in: - the Caribbean (RR in Lambeth = 10.01, 95% CI: 5.53–18.1; RR in Wandsworth = 8.89, 95% CI: 5.08–15.5); - Asia (RR in Lambeth = 3.97, 95% CI: 2.01–7.84; RR in Wandsworth = 2.08, 95% CI: 1.33–3.25); - Africa (RR in Lambeth = 7.92, 95% CI: 5.39–11.6; RR in Wandsworth = 3.27, 95% CI: 2.36–4.53).
Barnevik-Olsson et al. (19)	Epidemiological study based on a Swedish population registry focusing on autism prevalence in the Somali background population compared to the non-Somali population	- 250 children with autism (232 non-Somali and 18 Somali) - 113,391 children in general population (111,555 non-Somali and 1,836 Somali)	Autism prevalence was higher in Somali background children compared to non-Somali background children ($p < 0.001$)
Magnusson et al. (20)	Case-control study based on register-based cohort (*Stockholm Youth Cohort*) comparing migrant parents' frequency between an autism and a control group	3,918 children with autism (2,269 high-functioning and 1,649 low-functioning) for 589,114 children in general population	Odds of low-functioning autism were higher in migrant population when both parents were born abroad (OR = 1.5, 95% CI: 1.3–1.7), mainly if parents came from countries with low Human Development Index (HDI)
Schieve et al. (21)	Study based on an American population registry comparing ASD prevalence in US-born non-Hispanic White children and US-born Hispanic children	US-born non-Hispanic White children (n = 37,265) and US-born Hispanic children (n = 4,690)	Significantly higher ASD prevalence (1.19%) in US-born non-Hispanic White children, compared to US-born Hispanic children with foreign-born parents (0.31%)
Becerra et al. (22)	Epidemiological study based on an American population registry comparing autism prevalence according to mother's native country and ethnicity	7,540 children with autism from a cohort of 1,626,354 children in general population matched on age	Higher relative risk of autism when mothers were born in the Philippines (RR = 1.23, 95% CI: 1.08–1.40) or in Vietnam (RR = 1.45, 95% CI: 1.24–1.70), compared to White mothers born in the USA
Abdullahi et al. (8)	Register-based study on the prevalence of ASD in children born to immigrant mothers in Australia	Western Australian population-based linked data of 764,749 singleton live births from 1980 to 2010	From 1980 to 1996, but not from 1997 to 2010, children born to mothers born in low-income countries had an increased relative risk of ASD with intellectual disability
Augereau et al. (14)	Study on parents' and grandparents' migrant status in French children with ASD and French children without ASD but with language disorders	30 prepubertal male children with ASD and 30 prepubertal male children without ASD but with language disorders (control group)	Absence of significant difference between the ASD and control groups for parental migration (migratory trip and post-migration experience), whereas HDI values of native countries were significantly lower for immigrant parents and grandparents in the ASD group, compared to the control group

who migrated during the year they gave birth had the greatest risks. However, elevated risks are also observed in children of descendants of immigrants, which may suggest transgenerational effects of migration (22, 51). Importantly, risks differ strongly by parental region of origin. For example, in the USA, children of women born in the Philippines, Vietnam, and Central/South America have increased risks of ASD, compared to children of US-born White mothers, whereas no higher risks were found in children of women born in China, Japan, Korea, or Mexico (22). In France, children of mothers born in North and French-speaking Sub-Saharan Africa scored significantly higher on the M-CHAT than children of non-immigrant French mothers. However, no differences in M-CHAT scores were found when comparing children of non-immigrant French women and those born in other EU countries (51). In the UK, higher risks of intellectual and learning disabilities were mainly found in children of immigrants from Bangladesh and Pakistan, while most other immigrant groups had lower or similar risks of intellectual and learning disabilities, as compared to the non-immigrant British (45). Lehti et al. found higher odds of childhood autism in children of mothers and fathers born in Asia, the former Soviet Union, and former Yugoslavia. No higher odds were observed in children of parents born in Western countries, North Africa and the Middle East, and Sub-Saharan Africa (53). When considering ADHD, maternal migration was only associated with higher odds for children of mothers born in the former Soviet Union and Yugoslavia, Sub-Saharan Africa, and North Africa and the Middle East, whilst no higher odds were found in children of women born in Asia, Latin America, and Western countries. Paternal migration was associated with increased odds of ADHD regardless of fathers' region of birth (5). Regarding developmental disorders of speech and language, academic skills or coordination, Lehti et al. (6) found higher odds in children of women born in the former Soviet Union and former Yugoslavia, Sub-Saharan Africa, North Africa and the Middle East, Asia, and Latin America, but not other Western countries. Paternal migration was associated with higher odds of these developmental disorders regardless of fathers' region of birth, except for men born in Latin America, where no significant differences were found with non-immigrant Finnish men (6).

DIFFERENTIAL EXPOSURE TO NEURODEVELOPMENTAL RISK FACTORS

It is not clear which risk factors underlie the association between parental immigrant status and offspring neurodevelopment. Several explanations have been suggested, which include an increased prevalence of known pre-, peri-, and post-natal risk factors for neurodevelopmental disorders (54–58). For example, immigrants frequently differ from the host population on risk factors, such as obesity (59, 60), and low socioeconomic status, as well as associated risk factors, such as inadequate housing and poor nutrition (46, 61–65). Furthermore, they may be more frequently exposed to environmental pollutants, such as dioxin, PCBs, traffic-related air pollution, or heavy metals (53, 65–69). In France, children born to immigrant women of African descent have significantly higher blood lead levels than children of non-immigrant French mothers (70). Etchevers et al. hypothesized that this may partially be related to cultural habits involving the use of contaminated goods, such as imported lead-releasing ceramic cookware, herbs, or cosmetics, such as surma or kohl (70). Immigrant mothers may face language and cultural barriers, as well as discrimination when accessing prenatal care (71), which could lead to deferred treatment of pregnancy complications, which are a known risk factor of neurodevelopmental problems (55, 56, 58, 72). Indeed, pregnancy complications seem more common in some immigrant populations (73–75), yet were unable to explain higher risks of ASD in children of immigrant mothers in Sweden (20) and more early signs of neurodevelopmental problems children of immigrant mothers in France (51). Another common, yet controversial, hypothesis is that elevations in neurodevelopmental risk might be due to increased risks of vitamin D deficiency in darker skinned populations (3). Gestational vitamin D deficiency was associated with ASD-related traits at age 6 in a recent analysis of a Dutch multiethnic cohort (76). In a Swedish registry-based study, vitamin D-deficiency in pregnancy predicted ASD with, but not without, intellectual disability (77). In another Swedish study, Somali women were more likely to be vitamin D deficient than Swedish women. However, no significant differences were observed when comparing Somali mothers with and without children with autism (78). In the Netherlands, no elevation in ASD risk was found in groups known to have high levels of vitamin D deficiency, such as the Moroccan Dutch (79). Associations between vitamin D deficiency in pregnancy and increased risks of ADHD have also been reported in studies (80, 81). Other explanations focus on psychosocial stress experienced during the premigration, migration, or post-migration periods, which may adversely impact neural development of the fetus, for example through epigenetic mechanisms (82, 83). Immigrants sometimes flee from armed conflict or severe economic insecurity in their countries of origin, and may have high levels of exposure to combat, poverty and sexual violence (84). Common post-migration psychosocial stressors include acculturative stress and discrimination (85, 86).

Some evidence suggests that risks are highest if parents originate from regions with low human development, which can be considered as a proxy of the extent of adversity in the country of origin. In Finland, children of both immigrant mothers and fathers from countries with low HDI had higher odds of ADHD, compared to children of immigrant parents from countries with very high, high, or medium HDI (5). However, no such associations were found when considering developmental disorders of speech and language, academic skills or coordination (6). In Sweden, only children of mothers from medium and low HDI countries had significantly higher risks of low-functioning ASD, whereas no differences were found between children of immigrant mothers from countries with high or very high HDI and those of non-immigrant Swedish women (20). In Australia, children with ASD born to immigrants from lower income countries were more likely to have comorbid intellectual disability, and showed more

social impairments and communication difficulties (87). In France, the HDI values of native countries were significantly lower for parents and grandparents of ASD boys compared to controls (parents and grandparents of non-ASD boys with language disorders), especially for paternal grandparents, whereas no differences were found between the two groups when considering only (grand) parental immigrant status. Furthermore, HDI levels from the paternal line (father and especially paternal grandparents) were negatively correlated with autism severity, particularly for social interaction impairments (14). The authors hypothesized that social adversity-related stress experienced during the premigration period, especially by paternal grandparents, may be a factor of vulnerability for ASD (14). Furthermore, associations between low human development and the incidence of schizophrenia (which is nowadays considered a neurodevelopmental disorder), including childhood-onset schizophrenia, have been observed in immigrant populations (88–91). Unfortunately, currently very little is known about neurodevelopmental health in countries with low human development, such as those in Sub-Saharan Africa (92, 93). Importantly, bilingualism does not seem to be a determinant of developmental problems in children with and without clinical disorders, such as ASD (94–98), nor is it able to explain associations between parental immigrant status and neurodevelopmental disorders (51, 87).

DIFFERENCES IN THE DETECTION OF NEURODEVELOPMENTAL DISORDERS, HEALTH CARE ACCESS, AND TREATMENT RETENTION

Most available literature on the neurodevelopment of immigrants' children is based on disease registries and case-control studies of children diagnosed with a clinical disorder, such as ASD. These studies consider often only one neurodevelopmental disorder (e.g., ASD), despite that comorbidity is the rule rather than the exception among neurodevelopmental disorders (99–101). Furthermore, these studies miss very young children or those that remain undiagnosed (51). This is especially problematic considering that there is some evidence of ethnic diagnostic/referral bias in clinical practice, as clinicians may incorrectly attribute symptoms of neurodevelopmental disorders to other social, behavioral or language problems (102). For example, in an experimental study, Dutch pediatricians were more likely to consider an ASD diagnosis when evaluating clinical vignettes of children of non-immigrant parents, as compared to vignettes of children of Moroccan and Turkish-origin parents (102). Diagnostic bias may be less problematic when studying ethnic inequalities in ADHD, as ADHD symptoms are featured in many behavioral questionnaires, such as the Child Behavior Checklist (CBCL) and the Strengths and Difficulties Questionnaire (SDQ) (103, 104). These instruments are routinely included in population-based cohort studies, which also include undiagnosed children. Nevertheless, in studies based on clinical samples, underdiagnosis can lead to underestimation of the true extent of neurodevelopmental inequalities in the population. In clinical practice, immigrants' children with ASD may be at higher risk of not accessing highly beneficial early interventions (105).

The extent to which late or under-diagnosis is an issue for children of immigrants is not entirely clear and may vary across settings, by type of condition, and clinical severity. In several studies from the USA, children of ethnic minorities, including African Americans and Hispanics were on average diagnosed later with ASD than children of White Americans or remained undiagnosed (106, 107), suggesting that developmental problems in children from these populations may systematically be missed. Other studies found no differences or yielded opposite associations. A Dutch population-based cohort study did not find differences in the age of ASD diagnosis between children of immigrants and non-immigrants (79), whereas Abdullahi et al. found that Australian children of immigrants were diagnosed with ASD at earlier ages than their non-immigrant peers (87). However, children of immigrants in Australia were more likely to show more severe ASD accompanied with intellectual disability (87). It is therefore possible that an earlier age at diagnosis in this group is mainly explained by a higher prevalence of comorbidities and more severe phenotypes that are easier to detect in clinical practice (87). Importantly, diagnostic bias may still be present concerning children with less severe or more ambiguous symptoms, which may, for instance, explain the underrepresentation of children of immigrants in studies of children diagnosed with high-functioning ASD (20).

Disparities may exist not only in the timing of diagnosis and initial contact to health care providers, but also concern retention in high-quality neurodevelopmental care. For example, in a US study, autistic children of Latino parents with limited English proficiency received fewer hours of therapy and had higher unmet therapy needs than non-Latino White children (108). In another US study, children with ASD from immigrant families had more than twice the risk of not having a usual source of care (9). However, a study from the UK did not find ethnic differences in ASD-related care use (109). Currently, less is known about inequalities in access to ASD-related care in other European countries, where data on ethnicity are often not routinely collected (110). Nevertheless, in France, two sociological studies conducted in districts of Paris (111) and Marseille (112) that are characterized by a high prevalence of immigrant families show increased difficulties in accessing adequate care. These difficulties occurred despite the fact that France has universal healthcare access and that the principle of justice/equity within the healthcare system is inscribed in law (113). This suggests that current efforts may still be insufficient to address disparities in access to neurodevelopmental care for immigrant families in France. When considering ADHD care, studies have consistently shown lower use of ADHD medication in children of immigrants (114). For example, a study on Dutch ADHD patients aged <19 years found that higher proportions of patients with Turkish or Moroccan backgrounds reported never having used ADHD medication compared to non-immigrant Dutch patients. Furthermore, these groups showed higher medication discontinuation rates (115). Higher rates of

medication discontinuation and treatment disengagement have also been found in ethnic minorities in the US, including Latin Americans (116, 117).

BARRIERS TO THE DETECTION OF NEURODEVELOPMENTAL DISORDERS, HEALTH CARE ACCESS, AND TREATMENT RETENTION

Potential barriers to diagnosis, access to and retention in specialized care reported in studies conducted among immigrants are similar across disorders and include both structural/socioeconomic factors (e.g., financial barriers, underinsurance, problems with transportation, fragmented services, and language barriers) and social/cultural factors (e.g., lack of social support, stigma and discrimination, insufficient understanding of the host country's health system, low health literacy, and differences in values and expectations between health service providers and parents) (9, 10, 116, 118–126).

This mismatch in expectations may often stem from the fact that cultures differ substantially in their perceptions of the etiology and symptoms of neurodevelopmental disorders, on their prognosis, as well as on the effectiveness and acceptability of treatments. For example, studies suggest that the likelihood that parents attribute certain behaviors to a medical condition differs across ethnic groups (125). Some behaviors typical of ASD, such as avoiding eye contact, may be perceived as a way of expressing respect toward authority figures rather than a sign of atypical development in some Asian cultures (11, 118, 125). Likewise, the extent to which children engage in imaginative play differs across cultural groups (125). For example, Korean-Americans may be less likely to engage their children in pretend play, as compared to White Americans (127). Hence, the absence of imaginative play may be a more salient warning sign in some cultures than others (125). Such cultural differences in norms of optimal child development and parenting may influence whether and when early warning signs are recognized and reported (125). In a US study, Latina mothers were less likely to report developmental concerns and ASD symptoms than White mothers of children potentially with ASD, although children of Latino mothers who met the diagnostic criteria for ASD tended to have more severe ASD symptoms (128). Somali parents in Britain correctly identified clinical vignettes of children with autism, but had difficulties recognizing other developmental problems, which they misclassified sometimes as ASD-like symptoms (129). Indian parents may be first concerned by social difficulties, whereas US parents notice first delays in language and general development (11). Ethnic differences in reporting were also found when considering ADHD, suggesting that immigrant parents may be less likely to identify symptoms of ADHD in their children. In the Netherlands, Moroccan, Turkish, and Surinamese parents showed lower sensitivity in correctly identifying ADHD in their children than non-immigrant Dutch parents. At the same time, Moroccan and Turkish parents showed higher specificity in detecting ADHD than the non-immigrant Dutch (130). In line with this, in a Danish study, immigrant parents reported fewer symptoms of ADHD than non-immigrant parents in their children, whilst no such differences were found when considering teacher report (31). This could be because cultures may differ in the extent to which they view behavioral problems typical of ADHD as a medical issue rather than a social or spiritual problem (42, 131). A US study found that traditional gender roles and cultural values of familism were related to sociological and spiritual beliefs about the etiology of ADHD amongst Latinos (131).

Furthermore, immigrant parents may have less support from friends and family networks, and may even face stigma from their own community, which can lead to social isolation of these parents (121). For example, in some cultures, having a child with ASD is perceived as a divine punishment or a consequence of evil spirits or witchcraft (118). Perhaps more frequently, communities are not aware of the challenges of raising a child with a long-term neurodevelopmental disability, such as ASD. In a study within the Somali community in Britain, many families were unfamiliar with the term "autism" when their child was diagnosed, as ASD is not recognized in Somalia and no corresponding word exists in Somali language (132). As a result of lack of ASD awareness, parents sometimes received conflicting messages from their community and medical professionals. For example, parents were often told by members of their extended family to disregard the diagnosis of ASD, as their child may grow out of the symptoms on his or her own (132). This led frequently to a confusion between ASD as a long-term diagnosis and temporary delays in development (132). Furthermore, some parents tended to delay accessing care and to hide their children from their community for fear of negative responses to the child's disruptive behavior (132). Also immigrant parents of children with ADHD may face difficulties finding social support from within their communities. For example, a qualitative study on US Latinos reported that families often blamed parents for problematic child behavior rather than viewing ADHD as a mental health condition. As a result, some parents had a tendency to conceal their child's condition to their extended family (133). Preferences concerning treatment modalities differ also across cultural groups. For example, Latino and African American parents tend to rate behavioral treatments for ADHD more favorably than White Americans, whilst being more reluctant to accept the use of psychotropic drugs on their children (134).

SCREENING MAY ADDRESS INEQUITIES IN ACCESS TO EARLY INTERVENTIONS FOR ASD

The use of standardized screening instruments in unselected populations may help address potential referral bias and improve early detection of neurodevelopmental disorders in children of immigrants and other underserved communities, although further research focusing on multiethnic contexts is necessary. Early detection is especially important in the case of ASD, as intervention in early childhood is a critical determinant of long-term clinical prognosis (12). Some initial results on the multiethnic use of standardized screeners in

toddlers (e.g., M-CHAT) seem promising, though studies also have found differences in the performance of questionnaires across language and cultural groups. In a US study, children of ethnic minorities showed inflated screen-positive rates on the M-CHAT-R questionnaire. However, this effect disappeared when additionally considering the follow-up interview M-CHAT-R/F or subsequent clinical evaluations (135). No significant differences in the positive predictive values (PPVs) were found across ethnic groups for the M-CHAT-R with follow up (135), which was similar to the findings reported in another US study by Herlihy et al. (136). Furthermore, for children who screened at-risk for developmental delay based on the M-CHAT, Herlihy et al. found only small differences in age at evaluation between White and ethnic minority children, which suggests that standardized screening instruments may address inequalities in age at diagnosis (137). Seif-Eldin et al. found that the test performance of the M-CHAT in nine Arab-speaking countries was similar to Western contexts (138). However, some results from other studies were less encouraging, which highlights the importance of additional research on the cross-cultural validity and adaptation of neurodevelopmental screening instruments. For example, in the USA, Scarpa et al. reported poor internal consistency of the M-CHAT in children of mothers with ethnic minority background (139), and Guthrie et al. observed higher PPVs and specificity in children of Whites, as compared to those from other ethnic groups (140). Many other commonly-used ASD screeners have been assessed in terms of their cross-cultural validity, such as the Autism Spectrum Quotient (AQ)-Child questionnaire, the Social Responsiveness Scale (SRS), and the Social Communication Questionnaire (SCQ). Overall, studies support the cross-cultural use of these scales, though similar to the M-CHAT, some differences were found across settings. Carruthers et al. compared the predictive ability of individual items of the Autism Spectrum Quotient (AQ)-Child questionnaire across samples in Japan, India, and the UK. Whereas they detected cultural differences on some of the items, they found considerable cross-cultural overlap in the items that predicted most strongly ASD diagnosis (141). Wang et al. found similar sensitivity, specificity, and total scores in the Mandarin-adaptation of the social responsiveness scale (SRS) in Taiwan, compared to Western contexts (142). Similarly, Bölte et al. found satisfactory-to-good reliability and validity regarding the German adaptation of the SRS (143). However, whereas the raw SRS scores in controls in Taiwan and Germany were similar, these scores were lower than in controls from the US (142). Adequate performance of the SRS has also been found in South Korea (144) and for the Farsi adaptation of the scale in Iran (145). Studies that support the cross-cultural use of the Social Communication Questionnaire (SCQ) have been conducted in Taiwan (146), Greece (147), Germany (148), the Gulf countries (149), and Turkey (150). Detailed reviews on cultural adaptations of autism screening instruments have been performed by Soto et al. (151) and Al Maskari et al. (152).

Whereas current adaptations of screening instruments are often limited to language translations, the accuracy of these instruments could potentially be improved by making further adjustments to incorporate the norms, values, and beliefs of the adapted culture (152). For example, in Iran, Samadi and McConkey found that a screening instrument developed specifically for the Iranian context outperformed language adaptations of the M-CHAT (153). Additional difficulties relate to poor implementation of screening in clinical practice. For example, Rea et al. reported low screening completion rates and referral practices that were inconsistent with screening results in a retrospective medical chart review across three American pediatric clinics (154).

CONCLUSION

Autism spectrum disorder (ASD) has by far been the most studied neurodevelopmental disorder in terms of its relation with parental migration. It is also the disorder for which inequalities in risk have been most consistently found (1–4). Less is known about other neurodevelopmental disorders, on which studies have found heterogeneous results, with some studies reporting higher and others lower risks in children of immigrants (5–8). Importantly, there is a profound lack of research on children of immigrants living in non-Western host countries. Differences in the exposure to environmental risk factors may contribute to inequalities in neurodevelopmental health between children of immigrants and non-immigrants. Although the contribution of these risk factors to the etiology of neurodevelopmental disorders is thought to be substantial (up to 40–50% in case of ASD), research in this area is still in its infancy (155). Common limitations of existing studies include, for example, lack of prospective longitudinal designs, small sample sizes, lack of direct measurement of environmental exposures, and difficulties ascertaining the timing of exposures (155). Particularly little is known about environmental risk factors in developing countries, which may differ substantially in their prevalence and distribution from high-income countries (92). Nevertheless, some environmental risk factors (e.g., ambient air pollution, certain pregnancy complications) have been repeatedly associated with neurodevelopmental disorders in studies (55, 155). Yet, the contributions of individual risk factors tend to be small and findings are not specific enough to derive interventions to prevent neurodevelopmental disorders that go beyond the provision timely diagnosis and adequate medical care (55). There is also a scarcity of studies on the experiences of immigrant families with children with neurodevelopmental disorders in the healthcare system, and, to our knowledge, there is currently no systematic review available summarizing what is currently known about these families' interactions with providers or the utility of existing interventions. Nevertheless, the limited available evidence suggests that many barriers to detection, health care access, and treatment retention are similar across neurodevelopmental disorders and include structural/socioeconomic factors, such as underinsurance and service fragmentation, as well as social/cultural factors, such as lack of social support from the same-ethnic community and insufficient availability of culturally appropriate care (9, 10, 116, 118–126).

Several recommendations can be made to improve diagnosis and access to neurodevelopmental healthcare for children of immigrants. When addressing disparities in the age of diagnosis, it is important to be aware that different cultural groups may not be equally likely to raise concerns about their child's development. Likewise, clinicians may misattribute certain symptoms to language difficulties or cultural differences (102). The implementation of standardized screening instruments in routine medical practice may be helpful as they prompt clinicians to look specifically for early signs of ASD and other neurodevelopmental disorders that otherwise may be missed (102). Screening at day-care centers, where many young children spend a significant amount of time, may also help address barriers in access to early detection and intervention (156). Childcare professionals could be trained to identify signs of neurodevelopmental problems and ASD as early as possible and inform parents in a culturally sensitive manner. Once children are diagnosed, clinicians should be encouraged to use culturally sensitive procedures (translation services, cultural mediators) to make treatment more accessible and enhance adherence among immigrant families (11, 157, 158). In settings where sufficient resources are not available, home-based early interventions may be helpful, which are implemented by caregivers in close collaboration with healthcare professionals (159).

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HS and MM conceived and developed an initial draft manuscript in consultation with DC and ST, who regularly provided extensive feedback. All authors contributed to the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Gut Microbial Profile Is Associated With the Severity of Social Impairment and IQ Performance in Children With Autism Spectrum Disorder

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Background and Objective: Autism spectrum disorder (ASD) refers to a heterogeneous set of neurodevelopmental disorders with diverse symptom severity and comorbidities. Although alterations in gut microbiota have been reported in individuals with ASD, it remains unclear whether certain microbial pattern is linked to specific symptom or comorbidity in ASD. We aimed to investigate the associations between gut microbiota and the severity of social impairment and cognitive functioning in children with ASD.

Methods: A total of 261 age-matched children, including 138 children diagnosed with ASD, 63 with developmental delay or intellectual disability (DD/ID), and 60 typically developing (TD) children, were enrolled from the Shanghai Xinhua Registry. The children with ASD were further classified into two subgroups: 76 children diagnosed with ASD and developmental disorder (ASD+DD) and 62 with ASD only (ASD-only). The gut microbiome of all children was profiled and evaluated by 16S ribosomal RNA sequencing.

Results: The gut microbial analyses demonstrated an altered microbial community structure in children with ASD. The alpha diversity indices of the ASD+DD and ASD-only subgroups were significantly lower than the DD/ID or TD groups. At the genus level, we observed a decrease in the relative abundance of *Prevotella*. Simultaneously, *Bacteroides* and *Faecalibacterium* were significantly increased in ASD compared with DD/ID and TD participants. There was a clear correlation between alpha diversity and the Childhood Autism Rating Scale (CARS) total score for all participants, and this correlation was independent of IQ performance. Similar correlations with the CARS total score were observed for genera *Bacteroides*, *Faecalibacterium*, and *Oscillospira*. However, there was no single genus significantly associated with IQ in all participants.

Conclusions: Specific alterations in bacterial taxonomic composition and associations with the severity of social impairment and IQ performance were observed in children

with ASD or ASD subgroups, when compared with DD/ID or TD groups. These results illustrate that gut microbiota may serve as a promising biomarker for ASD symptoms. Nevertheless, further investigations are warranted.

Keywords: autism, gut microbiota, 16S rRNA, developmental delay/intellectual disability, intelligence quotient

INTRODUCTION

Autism spectrum disorders (ASDs) are a set of neurodevelopmental behavioral disorders associated with impaired social communication and restricted, repetitive behaviors in early childhood (1). A significant increase in the overall prevalence of ASD has been observed, which is now estimated at 1 in 54 in the United States (2).

In addition to atypical behavioral characteristics, such as impaired social cognition, social perception, and executive dysfunction, children diagnosed with ASD often experience other comorbidities (3), most commonly, developmental delay or intellectual disability (DD/ID). Approximately 1–3% of the global population has intellectual disabilities with below-average general intellectual function and limitations in adaptive functioning (intelligence quotient <70) according to World Health Organization statistics. In contrast, ∼70% of individuals diagnosed with ASD have some form of DD/ID (4). It is widely accepted that the clinical manifestations of ASD are highly heterogeneous in terms of symptom severity and cognitive function, ranging from non-verbal children with severe intellectual impairments to high-functioning individuals with no developmental delay and normal or superior IQ (5–7). Emerging clinical and epidemiological studies have shown remarkable differences in clinical manifestations, prognosis, and support requirements for individuals diagnosed with ASD and developmental disorder (ASD+DD) vs. ASD only. Compared to the children with ASD-only, those diagnosed with ASD+DD often exhibit obvious clinical features, including low maternal serotonin levels, low paternal IQ, and increased challenging behaviors (8, 9). Moreover, the children with ASD+DD diagnosis and relatively low IQ scores often exhibit higher levels of stereotypical behavior, self-harming, and sleep problems than individuals with ASD-only (10–12). Large-scale genome sequencing studies also suggest that the disruption rate of ASD-related genes is significantly higher in the presence of comorbid DD/ID (5, 13).

Recent studies indicate that children with ASD have an increased prevalence of gastrointestinal (GI) symptoms, including abdominal distension, constipation, and diarrhea, suggesting changes in intestinal physiology, compared to children with other developmental disorders or those who are typically developing (TD) and healthy sibling pairs (14). Further studies show that gut microbiota may play a significant role in human physiology and pathology, and the prominent GI symptoms in ASD individuals (15). The human gut microbiota is a complex population of 10^{14} microbes, including bacteria and fungi, whose genomes (microbiome) contain at least 100 times as many genes as the human genome (16). It is now well-accepted that the gut microbiota contributes to normal metabolism and immune homeostasis and could also modulate central nervous system activities and behaviors through neural, immune, and endocrine pathways (17). Evidence linking ASD and abnormalities in gut microbial function has been accumulating. Alterations in the composition of gut microbiota community structure have been reported in individuals with ASD and animal models of autism (18, 19). In human populations, the stool of children with ASD was shown with significant decreases in *Bifidobacterium*, *Prevotella*, *Blautia*, and *Dialister*, as well as significant increases in *Clostridium* compared with TD or healthy sibling pairs (20, 21). More recently, transplantation of gut microbiota from human donors with ASD or neurotypical controls into germ-free mice revealed that colonization with ASD microbiota is sufficient to induce hallmark behaviors of ASD (22). However, it remains unclear whether microbial patterns contribute to specific symptoms or comorbid medical problems in patients with ASD. In particular, there have been no studies focused on how DD/ID affects the gut microbiota of children with ASD.

Therefore, the present study aimed to investigate the gut microbiota of children with ASD in large sample size, and to clarify the association between the gut microbial spectrum and clinical manifestations (social impairment and intellectual disability) in children with ASD, by recruiting two groups of control participants, including TD and DD/ID. Furthermore, we classified children with ASD into two subgroups (ASD+DD and ASD-only), allowing us to ascertain how developmental delay or intellectual disability could influence the gut microbiota profile in a non-genetic situation and pinpoint potential microbiota-associated mechanisms and signaling pathways for related behavioral deficits in ASD.

METHODS

Ethics

The study was approved by the Ethical Committee of Shanghai Jiao Tong University School of Medicine Affiliated Xinhua Hospital and conducted following the relevant guidelines and regulations. Written informed consent was obtained from the children and their parents following the Declaration of Helsinki. We adhered to standard biosecurity and institutional safety procedures in this study.

Selection and Description of Participants

ASD subjects were enrolled from the Shanghai Xinhua ASD Registry in Shanghai, China. The subgroup of ASD-only was screened to ensure the children matched the general population. Only children with an agreed clinical diagnosis using the Diagnostic and Statistical Manual of Mental Disorders (Fifth Edition, DSM-V) were included in the ASD group ($n = 138$, 117

males and 21 females) (**Figure 1**). The diagnosis was confirmed by scores above the cut-off points of two tests: the Autism Diagnostic Observation Schedule (ADOS) and Childhood Autism Rating Scale (CARS). The ASD group was also classified according to whether the children presented with DD/ID [developmental quotient (DQ) <75 and/or intelligence quotient (IQ) <70] (23). Additionally, a group with developmental delay or intellectual disability (DD/ID) and a group of TD children were selected and matched with same-age children visiting the Outpatient Clinic of the Department of Developmental Behavioral Pediatrics and Children's Primary Healthcare at Xinhua Hospital, Shanghai, China from January to December 2020. Those who did not meet the criteria for ASD but were developmentally delayed with DQ <75 were assessed using the Gesell development scales. Those with an IQ <70 were assessed using the WISC-R or WPPSI (Wechsler Intelligence Scale for children). These children were then included in the DD/ID group (n = 63, 46 males and 17 females) (**Figure 1**). Thus, children in the DD/ID group scored below the ASD cut-off score on the ADOS and <30 on the CARS. The clinical signs and absence of illness in these children were confirmed, and all of them underwent an evaluation consisting of the ADOS, CARS, and intellectual assessment. Participants were excluded if they had undergone any antibiotic treatment for 1 month before the sample collection or presented with a diagnosis of chromosomal abnormality or other known neurological, metabolic, or genetic diagnoses.

Behavior Scale and Gastrointestinal Symptoms Scale Assessment

The CARS is made up of 15 items rated on a 7-point scale from 1 to 4. Higher scores are associated with a higher level of impairment, and scores below 30 do not indicate ASD. We categorized these items into three CARS domains: Social impairment, Negative emotionality, and Distorted sensory response (24). Social impairment is the sum of 11 subscale scores, including cars1, cars2, cars4, cars5 cars10, cars11, cars12, cars13, cars14, and cars15. Negative emotionality is the sum of 3 subscale scores, including cars3, cars6, and cars10. Distorted sensory response is the sum of 3 subscale scores, including cars7, cars8, and cars9. ADOS was used as a supplement to diagnose ASD, and comprises modules tailored to the language level and age of the individual. Four sub-items for assessment of Social interaction, Communication, Play, and Imaginative use of materials were used (25).

An evaluation of GI symptoms was completed by 247 participants (ASD:137, DD/ID:52, and TD:58). GI status was determined using the Gastrointestinal Severity Index (GSI), based on the Rome III Diagnostic Questionnaire on Pediatric Functional Gastrointestinal Disorders and other GI symptom questionnaires (26, 27). Parents/guardians completed these questionnaires and were asked to indicate the frequency of GI problems within the last 3 months, including constipation, abdominal pain, vomiting, gaseousness/bloating, and diarrhea on a Likert scale [(0) = never, (1) = rarely, (2) = sometimes, (3) = frequently, and (4) = always)]. The total GSI score was used to evaluate the severity of participants' clinical symptoms. The designation of existing GI problems was used for children rated 3 or higher on more than 1 of the 7 questions.

Sample Collection and DNA Extraction

Stool samples were collected at Xinhua Hospital of Shanghai Jiao Tong University by their parents of the study participants and kept in 1.8 ml sterile microcentrifuge tubes. After collection, the specimens were immediately frozen at −80°C until further processing. Bacterial DNA was extracted from stool samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions.

16S rRNA Sequencing Analysis

The V3–V4 region of the 16S ribosomal RNA (rRNA) gene was amplified and sequenced on the Illumina MiSeq platform and Ion PGM (Illumina, San Diego, CA, USA). The PE reads obtained first were demultiplexed using the barcodes. After quality filtering, length filtering, dereplication, and removal of model organism sequences, the final sequences were obtained. To determine the identity of bacteria in the remaining high-quality reads, sequences were analyzed using the Quantitative Insights into Microbial Ecology software package (QIIME 2, version 2018.8) and clustered into operational taxonomic units (OTUs) at 97% identity with OTU picking protocol using USEARCH. The taxonomic assignment of the OTUs was carried out using the Naive Bayes classifier (q2-feature-classifier plugin) trained on Greengenes.

Statistical Analysis

Continuous variables were presented as mean ± standard deviation, and comparisons between ASD, DD/ID, and TD were performed using the Wilcoxon rank-sum test ($p < 0.05$), Mann-Whitney U-test or ANOVA. Alpha diversity was analyzed using the core-metrics-phylogenetic for observed diversity metrics, including the evenness diversity metric, chao1 richness estimator, and Shannon's entropy.Weighted and unweighted UniFrac distances of principal coordinate analysis (PCoA) were conducted based on the Bray–Curtis dissimilarity. Between-group differences were investigated using the Kruskal–Wallis test to determine the relative abundance of bacteria at the genus level. Linear discriminant analysis with effect size (LEfSe) was used to determine the difference in microbiota genera with an effect size cut-off of 2.0 and p-value of 0.05. Correlations between microbial taxa and clinical parameters were tested using Spearman's test with adjustment for false discovery rate using the Benjamini-Hochberg procedure.

Inferred functional potential of bacterial communities was analyzed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) with Greengenes database as a reference to obtain a prediction of the Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog functional profiles (28). Group differences of KEGG pathway were illustrated using the Statistical Analysis of Taxonomic and Functional Profiles (STAMP) package (29).

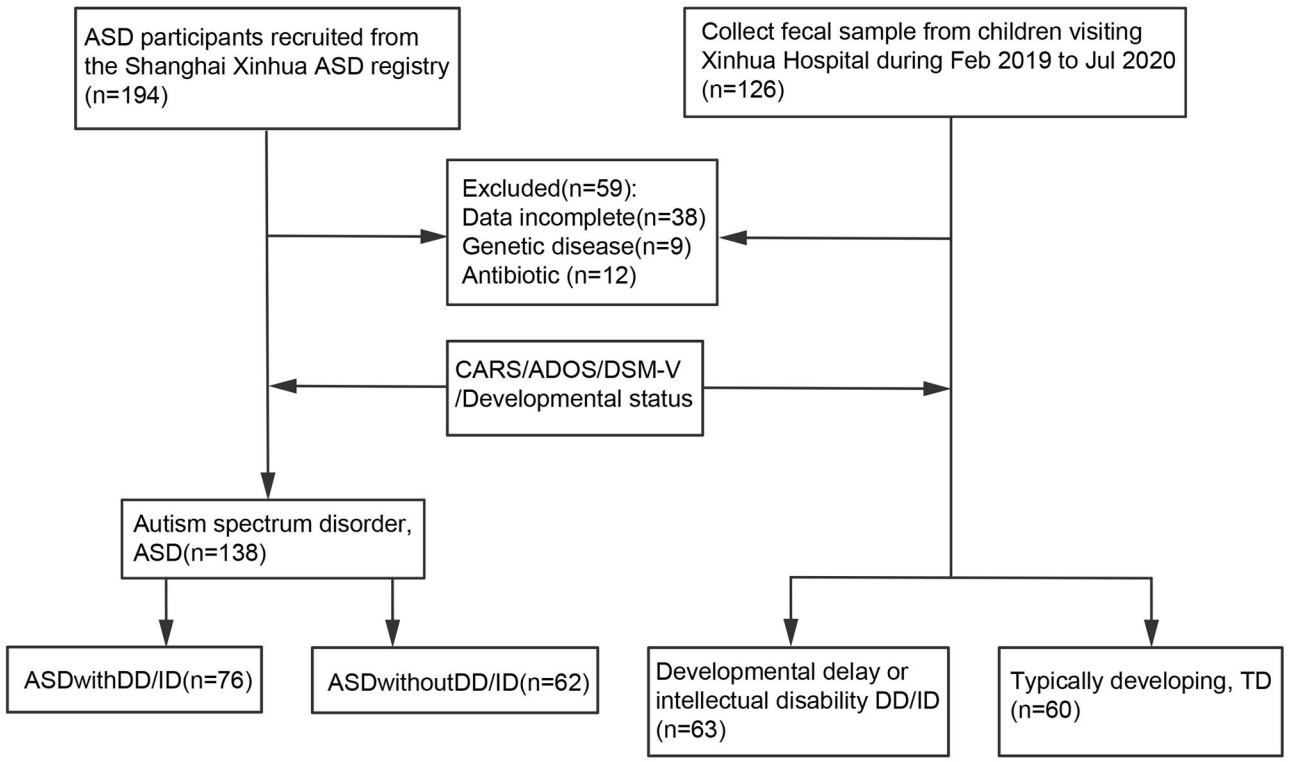

FIGURE 1 | Flowchart of the study design.

TABLE 1 | Characteristics of ASD, DD/ID, and TD.

Variables	ASD				DD/ID (*N* = 63)	TD (*N* = 60)	*p*-value (ASD vs. DD/ID)	*p*-value (ASD vs. TD)	*p*-value (DD/ID vs. TD)
	ASD+DD (*N* = 76)	ASD-only (*N* = 62)	Total (*N* = 138)	*p*-value					
Sex M/F (M %)	63/13 (82.90%)	54/8 (87.10%)	117/21 (84.78%)	n.s.	47/16 (74.60%)	27/33 (45%)	n.s.	2.25E-8	9.48E-4
Age (years)	6.32 ± 2.08	5.86 ± 1.88	6.11 ± 2.00	n.s.	6.39 ± 2.16	6.65 ± 2.22	n.s.	n.s.	n.s.
Delivery mode (Natural/C-section)	33/39	29/29	62/68	n.s.	20/19	29/18	n.s.	n.s.	n.s.
IQ/DQ	50.75 ± 8.92	88.57 ± 14.58	67.74 ± 22.24	2.10E-32	53.53 ± 12.07	105.68 ± 14.87	3.82E-8	5.90E-30	3.33E-41
CARS total	36.16 ± 4.01	32.12 ± 3.75	34.34 ± 4.37	1.28E-8	24.64 ± 2.72	17.14 ± 3.23	1.81E-45	1.63E-66	5.84E-27
Social impairment	23.20 ± 4.75	21.01 ± 2.58	22.24 ± 4.05	1.85E-3	16.86 ± 1.71	11.29 ± 2.83	4.78E-29	5.62E-48	4.73E-25
Negative emotionality	6.72 ± 1.43	6.32 ± 0.99	6.54 ± 1.26	n.s.	4.60 ± 0.72	3.59 ± 1.01	1.74E-30	1.38E-36	8.78E-9
Distorted sensory response	7.01 ± 1.59	6.31 ± 1.08	6.69 ± 1.42	3.70E-3	4.30 ± 1.05	3.21 ± 0.71	4.14E-25	5.20E-56	7.81E-10
GI problem *N*/Total (*N* %)	17/75 (22.67%)	9/62 (14.52%)	26/137 (18.98%)	n.s.	2/52 (3.85%)	4/58 (6.90%)	0.01	0.03	n.s.

ASD, autism spectrum disorder; DD/ID, developmental delay or intellectual disability; TD, typically developing; CARS, Childhood Autism Rating Scale; ADOS, Autism Diagnostic Observation Scale; GSI, Gastrointestinal Severity Index; n.s., non-significant.

RESULTS

Participant Characteristics

A total of 261 age-matched study participants were enrolled from the Shanghai Xinhua Registry (**Table 1**), including 138 ASD, 63 DD/ID, and 60 TD children. Overall, the median age of participants was 5.59 years; 191 (73.18%) were male; 111 (51.39%) had natural births, and 105 were delivered by C-section (49.63%). The ASD group was further divided into two groups: ASD with developmental disorder (ASD+DD, $n = 76$) and ASD only (ASD-only, $n = 62$) based on developmental status. As expected, these two subgroups exhibited significant differences in IQ/DQ score ($p = 2.10e\text{-}32$) and CARS total score ($p = 1.28e\text{-}8$). We also observed differences in the sex distribution of the three groups ($p < 0.01$) due to the sex specificity of ASD.

Scores on the gastrointestinal severity index (GSI) were obtained from 247 children (ASD+DD:75, ASD-only:62, DD/ID:52, and TD:58) (**Table 1**). Of the 247 participants, 32 (12.96%) were positive for GI symptoms. The children with ASD manifested GI-related problems such as constipation, gaseousness, and diarrhea, whereas the TD children did not show such problems ($p = 0.013$).

Alterations in Gut Microbial Diversity Between ASD vs. TD and DD/ID Children

To characterize the gut microbiome associated with ASD, we first compared the alpha diversity between children with ASD and the other two groups. **Figure 2** shows the distribution of Shannon index, IQ/DQ score, and CARS total score among all three groups of children. We found that the Shannon index for ASD participants decreased compared with those in the DD/ID and TD groups (**Figure 2A**). Similar results were also observed in five other measures of alpha diversity for the two ASD sub-groups when compared with those in the DD/ID and TD groups, including Chao1, observed species, ACE, Simpson, and InvSimpson indices. All six measures of alpha diversity were consistently decreased in the group of ASD-only children, while the ASD+DD group revealed significant decreases in Shannon, Simpson, and InvSimpson indices (**Supplementary Figure 1**). The above data indicate that gut microbiome in children with ASD had lower species diversity and evenness. However, no significant difference was detected in the bacterial population or beta diversity analyses between ASD+DD vs. ASD-only or DD/ID vs. TD (**Figure 2A** and **Supplementary Figure 1**).

Comparisons were also made between selected subgroups: ASD+DD vs. DD/ID ($p = 7.50e\text{-}4$) and ASD-only vs. TD ($p = 6.60e\text{-}4$, **Figures 2B,C**). Both bacterial evenness and alpha diversity indices of the ASD subgroups were significantly lower than the two control groups. These differences suggest that biodiversity might negatively correlate with ASD behaviors, regardless of developmental status. In contrast, there was little difference in any measurement of bacterial diversity within the two ASD sub-groups or within the two control groups (**Figure 2D** and **Supplementary Figure 1**), indicating a non-specific action of developmental conditions. Moreover, the PCoA analysis calculated on the Bray–Curtis dissimilarity and unweighted UniFrac distances showed no group difference (**Supplementary Figure 2**). Overall, our analyses revealed a significantly lower alpha—but not beta—diversity in the gut microbiome of children with ASD compared with DD/ID or TD children.

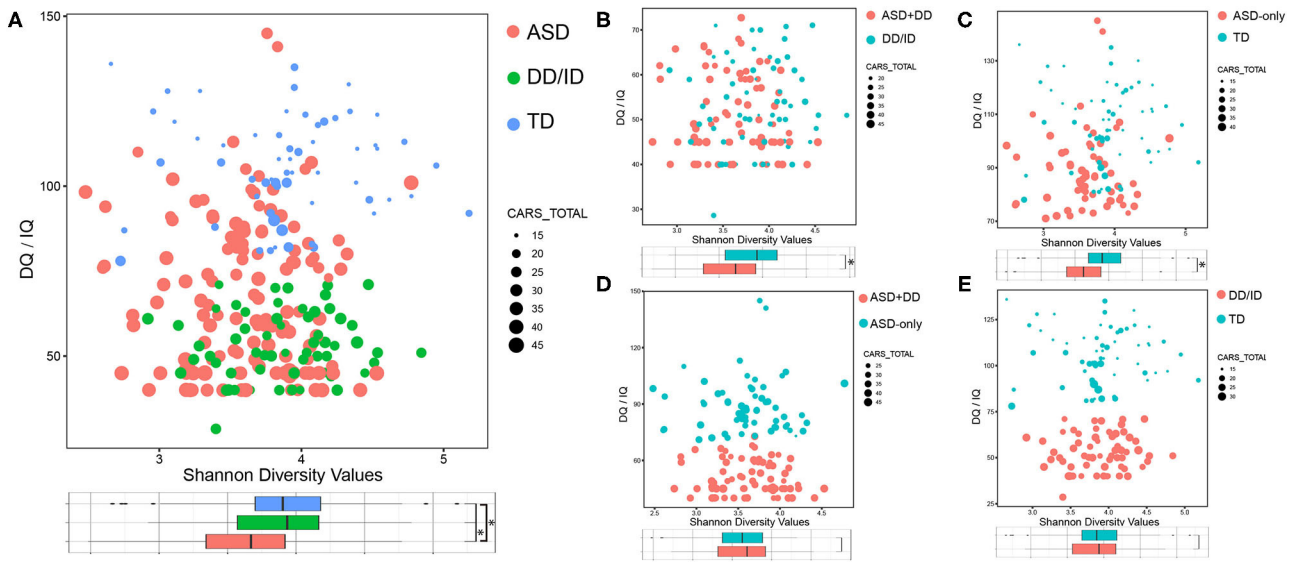

FIGURE 2 | Bacterial richness and diversity comparison according to the Shannon index depicted with IQ/DQ and CARS total scores. The size of node is proportional to the number of CARS total scores. The color of node indicates different groups. *$p < 0.05$. **(A)** ASD vs. DD/ID vs. TD. **(B)** ASD+DD vs. DD/ID. **(C)** ASD-only vs. TD. **(D)** ASD+DD vs. ASD-only. **(E)** DD/ID vs. TD. ASD, autism spectrum disorder; DD/ID, developmental delay or intellectual disability; TD, typically developing; CARS, Childhood Autism Rating Scale.

TABLE 2 | Relative abundance of bacteria in fecal microbiota of children with ASD, DD/ID, and TD.

Variables	ASD	DD/ID	TD	*p*-value (ASD vs. DD/ID)	*p*-value (ASD vs. TD)	*p*-value (DD/ID vs. TD)
Bacteroides	0.381 ± 0.166	0.306 ± 0.173	0.311 ± 0.182	0.0090	0.0153	n.s.
Faecalibacterium	0.080 ± 0.071	0.044 ± 0.040	0.059 ± 0.052	0.0001	0.0364	n.s.
Sutterella	0.032 ± 0.029	0.022 ± 0.022	0.030 ± 0.025	0.0204	n.s.	0.0206
Prevotella	0.033 ± 0.102	0.053 ± 0.122	0.055 ± 0.139	0.0043	0.0036	n.s.
Megamonas	0.014 ± 0.042	0.025 ± 0.052	0.009 ± 0.036	0.0015	n.s.	0.0178
Coprococcus	0.006 ± 0.014	0.010 ± 0.012	0.011 ± 0.013	0.0181	0.0049	n.s.
Collinsella	0.007 ± 0.030	0.007 ± 0.010	0.007 ± 0.011	0.0080	0.0246	n.s.
Desulfovibrio	0.005 ± 0.009	0.006 ± 0.008	0.007 ± 0.007	0.0069	0.0046	n.s.

Data show only the significantly ($P < 0.05$) different abundances at genus level. P-values were obtained from the Mann–Whitney U-test. ASD, autism spectrum disorder; DD/ID, developmental delay or intellectual disability; TD, typically developing; n.s., non-significant.

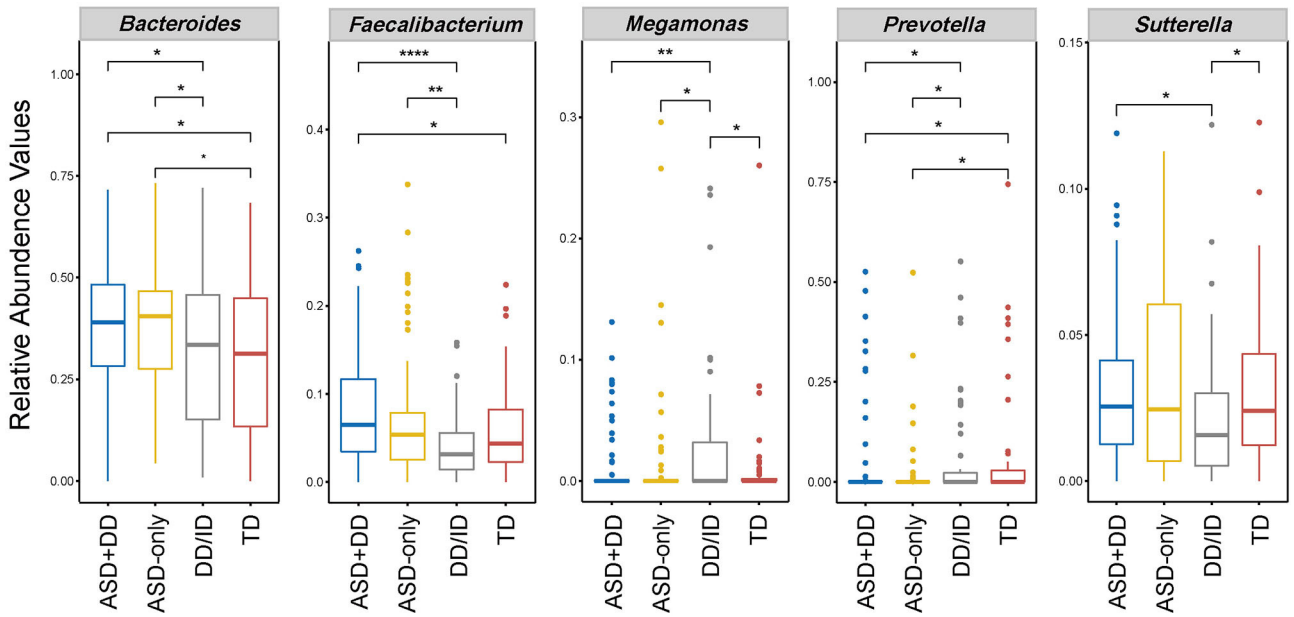

FIGURE 3 | Box plot of selected bacteria according to relative abundances of genus taxon in feces of ASD subgroups, DD/ID, and TD. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$. This figure shows only genus abundances with a value higher than 0.1%.

Differences in Microbiota Composition Between ASD, TD, and DD/ID Children

We then performed pairwise comparison to investigate the microbial compositions at genus level for all three groups (ASD, DD/ID, and TD). Data are given as median and standard deviation in **Table 2**. Significantly, in our analysis higher levels of *Bacteroides*, *Faecalibacterium*, *Sutterella*, and *Collinsella* were observed in the ASD participants. In contrast, the level of *Prevotella*, *Coprococcus*, and *Desulfovibrio* were lower than that in the DD/ID or TD groups. The results also revealed an enriched genus *Megamonas* within the DD/ID population.

Next, LEfSe analysis was used to determine significant alterations in the bacterial composition associated with ASD sub-groups, DD/ID and TD groups (**Supplementary Figure 3**). Considering the differences in relative abundance, five genera, including *Bacteroides*, *Faecalibacterium*, *Prevotella*, *Sutterella*, and *Megamonas* are shown in **Figure 3**. The abundance of *Bacteroides* and *Prevotella* ensured differences between the two ASD and non-ASD groups. An increased abundance of genus *Sutterella* was observed in ASD samples, and yet the comparison only approached significance in the context of developmental delay (ASD+DD vs. DD/ID). The abundance of *Faecalibacterium* was significantly increased in the ASD participants compared with non-ASD participants (**Table 2**). Still, no statistically significant difference was found in the context of typical development (ASD-only vs. TD). In addition, the levels of *Sutterella* and *Megamonas* varied markedly when comparison was made between the DD/ID and TD groups. However, there was no significant difference in microbiota composition between ASD sub-groups after FDR correction. Taken together, the above results confirm a microbial dysbiosis in children with ASD and, to a lesser

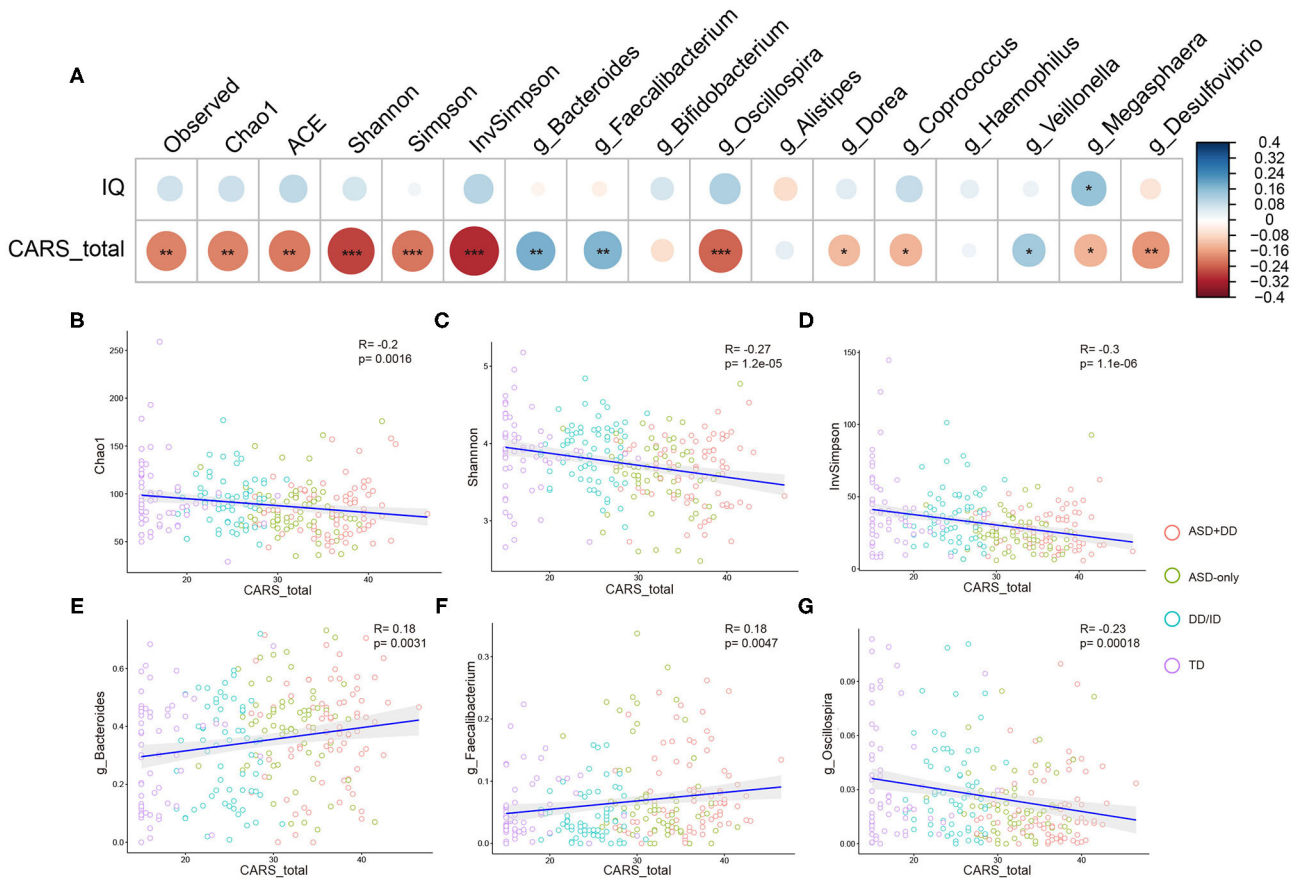

FIGURE 4 | Correlation between alpha diversity, microbiota genera, and clinical parameters in all participants. Heatmaps showing association between alpha diversity, microbiota composition, and clinical characteristics **(A)**. $^{*}p < 0.05$ and $^{**}p < 0.01$. Total CARS score is negatively correlated with Chao1 index **(B)**, Shannon index **(C)**, InvSimpson index **(D)** in all participants. Genus *Bacteroides* **(E)**, genus *Faecalibacterium* **(F)**, and genus *Oscillospira* **(G)** based on the OTU profile were associated with CARS total scores. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

extent, in the ASD subgroup in comparison with the non-ASD groups.

Correlations Between Gut Microbiota Composition and Clinical Profiles

Spearman (dichotomous variables) or Pearson (continuous variables) correlation analyses were used to explore the relationship between the gut microbiota composition and phylogenetic diversity at genus level, and the clinical profiles in ASD, DD/ID, and TD children. As the CARS total score represents the severity of ASD symptoms and the IQ score indicates intelligence, correlations of microbial profiles, CARS total score, and IQ were investigated for all participants. However, DQ and IQ were calculated using different scales, 104 ASD (ASD+DD:66, ASD-only:38), 53 DD/ID, and 56 TD participants whose IQs had been obtained were included. Indicators of bacterial biodiversity were found to strongly and significantly correlate with the CARS total score (**Figures 4A–D**). Similar correlations with CARS total score were also observed with selected genera, including *Bacteroides*, *Faecalibacterium*, and *Oscillospira* (**Figures 4A,E,F**), suggesting a broader involvement of those microbiota in ASD symptoms.

Next, we calculated potential relationships in relative abundance based on OTU counts of the microbiota at genus level and the clinical profiles across participants in each individual group. There was a significant association between the CARS total score and the abundance of *Sutterella* in the group of ASD children ($r = -0.24$, $p = 0.0046$). The result remained consistent when analyses were stratified by intellectual status, especially in the ASD-only group ($r = -0.34$, $p = 0.0072$). In addition, genus *Anaerostipes* was positively correlated with the CARS total scores in the ASD+DD group ($r = 0.35$, $p = 0.0018$, **Figure 5**).

Moreover, the correlations between microbial profile and IQ score of participants were analyzed. The abundance of *Prevotella* was positively correlated with IQ score in the DD/ID group ($r = 0.27$, $p = 0.048$) and negatively correlated ($r = -0.33$, $p = 0.014$) in the TD group. The higher abundance of *Sutterella* was associated with higher IQ scores in the ASD-only group ($r = 0.346$, $p = 0.033$) but related to lower intellectual state in the TD group ($r = -0.297$, $p = 0.026$, **Figure 5**). Taken together, these observations imply an impact of these microbiota on social ability

FIGURE 5 | Heatmaps showing correlations between microbiota genus and clinical characteristics in group ASD+DD, ASD-only, DD/ID, and TD. *$p < 0.05$ and **$p < 0.01$.

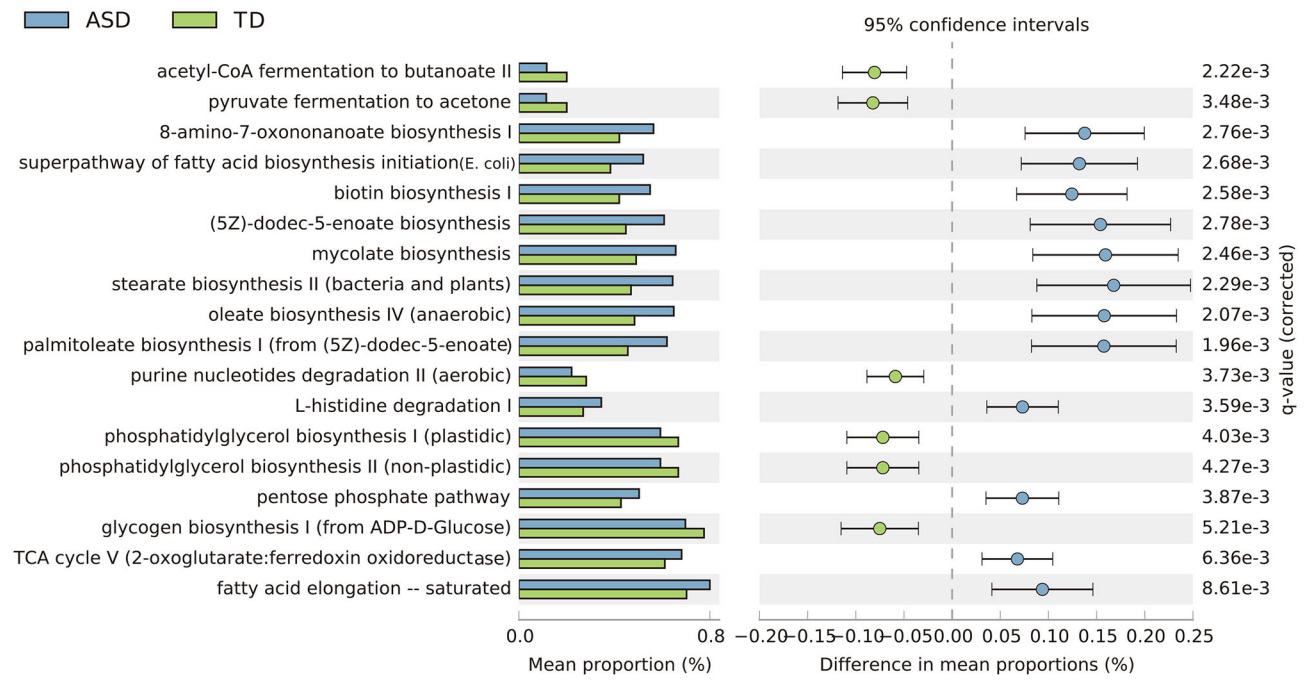

FIGURE 6 | Extended error bar plot showing 18 significantly different KEGG categories and abundance of the expression in the ASD and TD groups ($p < 0.001$, effect size >0.05. Benjamini–Hochberg FDR <0.05).

and intellectual state, for the ASD population and/or those with intellectual disability.

Predictive Microbiota Functional Profiling

The functional contributions of the bacteria in the ASD, DD/ID, and TD children were predicted based on OTUs using the KEGG microbial database. The KEGG pathways were filtered to include those with five or more alignments in at least 10% of the samples and 330 KEGG pathways were found across all samples. The effect sizes identified as significantly different ($p < 0.05$) between groups were assessed to determine discriminatory KEGG orthology.

Based on the threshold value of FDR <0.05, 18 key KEGG pathways demonstrated differential abundance between the ASD and TD groups, mainly belonging to organismal systems, cellular processes, and metabolism. KEGG pathways, including fatty acid biosynthesis, biotin biosynthesis, and L-histidine degradation, were significantly enriched in children with ASD. In contrast, several pathways—including glycogen biosynthesis, phosphatidylglycerol biosynthesis, and Nucleotide Metabolism—were relatively reduced in children with ASD and significantly enriched in TD children (**Figure 6**).

Next, the differences in representation among the microbiome in children with ASD and DD/ID were interrogated. KEGG pathways were found to vary with the same trend (**Supplementary Figure 4A**). The differences in representation between the groups of DD/ID vs. TD and groups of ASD+DD vs. ASD-only are displayed in **Supplementary Figures 4B,C**, respectively. However, no statistical significance was identified between these two pairwise comparisons after FDR correction.

DISCUSSION

Although the alterations in gut microbiota of human and animal models of ASD relative to non-ASD participants have been explored in the past decade (22, 30), human studies focusing on how particular microbial pattern links to specific ASD symptom or comorbidity remain largely lacking. In the present study, we performed bacterial taxonomy assignments using the stool samples from two age-matched control groups of DD/ID and TD participants. Associations between the microbial profile and the severity of social impairment, intellectual disability, and GI symptoms in children with ASD were systemically assessed.

The rapidly expanding body of literature on basic, translational, and clinical research on the gut microbiome suggests that the microbiota could influence brain development and have a significant impact on neurological and psychiatric diseases (31, 32). For instance, Vogt et al. characterized the bacterial taxonomic composition of stool samples from patients with Alzheimer's disease (AD). They revealed that the AD gut microbiome had decreased microbial diversity and was compositionally distinct from age- and sex-matched control individuals (33). Further analyses identified genus-wide differences in bacterial abundance, including decreased *Firmicutes* and *Bifidobacterium*, and increased *Bacteroidetes* in the gut microbiome of AD participants (34). In a longitudinal study, Carlson et al. demonstrated that the gut microbial composition of the human infant at 1 year of age could predict cognitive performance at age 2 (35). A more recent study identified a 12-genus microbial signature associated with brain structure variations and behavioral characteristics using a machine learning framework in children with ASD (36). We thus hypothesize that specific microbial pattern or signature taxa may contribute to specific dimension of ASD and other comorbidities.

The present study was a pilot exploration where we first aimed to determine the differential role of gut microbiota in social impairment and intellectual disability, as DD/ID has emerged as the most common comorbidity of ASDs. Following previous reports (37), our analysis showed a significantly lower abundance of genus *Prevotella* in children with ASD (**Figure 4**). The abundance of *Prevotella* correlated positively with the IQ score in DD/ID participants ($p = 0.048$) but correlated inversely in TD participants ($p = 0.014$). Similarly, *Prevotella* abundance has been shown to positively correlate with fluid cognition scores in chronic marijuana users but not in non-users (38). *Sutterella* abundance was also reported to have a negative association with MoCA score in patients with Sporadic Parkinson's Disease (39). Interestingly, we observed a similar negative correlation of *Sutterella* abundance and IQ score in TD children ($p = 0.026$). However, this correlation was not detected or even reversed in other subgroups of the study participants.

The cladograms generated by LEfSe indicated differences in the bacterial taxa between TD vs. DD/ID participants and ASD-only vs. ASD+DD participants (**Supplementary Figure 3**). Although it is generally believed that the genetic causes are varied and similar for DD/ID and ASDs (4), these two comparisons revealed distinct signature taxa and a unique functional enrichment of the metabolic pathways associated with DD/ID (**Supplementary Figures 3**, **4**), suggesting they may have evolved through different mechanisms of gut microbiota in DD/ID vs. in ASD with comorbid DD/ID.

We compared the alpha and beta diversities of ASD participants in relation to control participants to characterize the overall alterations in the microbiota of children with ASD and to determine the associations between the gut microbial profile and severity of social impairment in ASD. In metagenomics, alpha diversity represents the richness and diversity of the microbiome at the smallest spatial scale of analysis, in which Chaos1, ACE, Simpson, and Shannon indices are commonly used to calculate the alpha diversity (40). Previous studies lacked consistency when comparing children with ASD vs. healthy controls and some have described opposing results. For instance, several early 16S rRNA amplicon studies showed that the gut microbiome of children with ASD exhibited increases of richness (ACE and Chao1 indices), evenness (Shannon even index), and diversity (Shannon index) (20, 41) or no significant changes of Shannon index and observed species (34, 42, 43). However, relatively low microbial richness (ACE estimator) and diversity (PD and Shannon indices) have been reported in children with ASD in recent investigations (44–46). A possible reason for these inconsistent results may be due to the lack of homogeneity in terms of age, geographic area, and severity of behavioral symptoms among the ASD participants, and differences in control group selection. Altered bacterial diversity have also been described in deep metagenomic sequencing study using stool samples from ASD children, which revealed increased bacterial richness presented by Chao1 (47) and inverse Simpson diversity indices (48). Nevertheless, previous studies have also reported that the biodiversity (fisher's alpha) (49) and species richness (Chao1) (50) were significantly lower in ASD patients, suggesting different research methodologies could yield contradictory results.

In the present study, analyses of alpha diversity (Chao1, observed species, ACE, Shannon, Simpson, and InvSimpson indices) showed a minor but statistically significant decrease in the bacterial richness of children with ASD compared with non-ASD participants (**Figure 2** and **Supplementary Figure 1**). As shown in **Supplementary Figure 5**, we further examined the associations of the alpha diversities (Chao1, observed species, ACE, Shannon, Simpson, and InvSimpson indices) with the CARS total scores in participants with DD/ID (the combination of DD/ID and ASD+DD children), and then in participants without DD/ID (the combination of TD and ASD-only children). These results indicated that the association of Shannon diversity index of microbiome and the severity of social impairment is independent of IQ in children with ASD. The beta diversity refers to the total variance in the microbial community composition across varying environments. Several papers showed significant differences in beta diversity between ASD and control participants—although some did not (42, 51). Our analyses revealed no significant difference (**Supplementary Figure 2**). Correlations of microbiota were also calculated with the CARS total scores and the IQ scores. In the groups of DD/ID and ASD+DD children, genus *Bacteroides* ($r = 0.22$, $p = 0.011$) and genus *Faecalibacterium* ($r = 0.32$, $p = 9.6e\text{-}5$) were found positively associated with the CARS total scores. However, in combination of TD and ASD-only participants, genus *Streptococcus* ($r = 0.24$, $p = 0.022$) was positively correlated with the IQ scores.

Having shown that the microbiome was significantly correlated with the CARS total score in children with ASD, we further explored the associations of bacterial taxa and behavioral measurements in the three primary CARS domains representing diverse behavioral and emotional aspects in two subgroups of children with ASD (ASD+DD and ASD-only). At genus level, the abundance of *Faecalibacterium* in children with ASD only correlated negatively with the domain of Social impairment ($r = -0.33$, $p = 0.0092$). Still, it was not associated with the other two CARS domains (Negative emotionality and Distorted sensory responses). In contrast, *Sutterella* and *Prevotella* were significantly associated with all three domains in CARS (**Supplementary Figure 6**). However, these correlations were not identified in children of the ASD+DD subgroup. These analyses suggest unique and diverse correlations between the microbial pattern and behavioral and emotional features of ASD, while further investigation and validation are warranted.

Preliminary studies indicate that children with ASD frequently have GI problems and gut microbiota dysbiosis (52, 53). However, it has been reported that the microbial changes were more closely linked to the presence of ASD symptoms rather than to the severity of GI symptoms (54). Our analyses identified the genera *Bifidobacterium* ($r = 0.26$, $p = 0.0023$) and *Alistipes* ($r = 0.28$, $p = 0.00085$) had a strong and positive correlation with the severity of GI symptoms evaluated by the GSI total score of the ASD group, allowing us to speculate about the possible contributions of *Bifidobacterium* and *Alistipes* to GI symptoms. However, our current study was an initial attempt to discern the correlations in microbial changes with GI symptoms and other clinical manifestations in children with ASD. Due to limited validation of the GSI, future studies require more effective and valid questionnaires to ensure precise information.

There are some limitations to the present study. First, 16S rRNA gene amplicon sequencing has limited classification power and cannot fully resolve taxonomic profiles at the species or strain level depending on the database and classifiers used (55, 56). Thus, shotgun sequencing remains needed for comprehensive profiling of gut microbiota and to unveil the subtle differences between each subpopulation. Second, children with ASD are not a homogenous clinical population, but a group of individuals with similar behavioral characteristics and a potentially broad range of pathologies. Although associations of genera with IQ performance were identified in specific subgroups of ASD, the results warrant further validation in other independent cohort studies with larger sample sizes and stratification.

Overall, we observed alterations in bacterial taxonomic composition in children with ASD, and their associations with specific clinical manifestations. These results illustrate that gut microbiota could serve as a promising biomarker and possible therapeutic target for ASD symptoms.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI BioProject, PRJNA769228.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Shanghai Jiao Tong University School of Medicine Affiliated Xinhua Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ZC and JY designed the study. ZC, XL, YD, YL, LZ, and XD collected the fecal samples and clinical information. KS, JY, and ZC carried out the analysis, interpreted the results, and wrote the manuscript. TZ and SF analyzed the data and helped design experiments. FL and JY guided and supervised all work. 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.2021.789864/full#supplementary-material

Supplementary Figure 1 | Six measures of alpha diversity: Observed species index, Chao1 index, ACE index, Shannon index, Simpson index, and InvSimpson index in group ASD+DD, ASD-only, TD, and DD/ID. Each box plot represents the median, interquartile range, minimum, and maximum values. $*p < 0.05$.

Supplementary Figure 2 | Beta diversity analysis on fecal samples from ASD subgroups, DD/ID, and TD children. Bray-Curtis β-diversity indices of the fecal microbiota showed no distinction between four groups. ASD, autism spectrum disorder; DD/ID, developmental delay or intellectual disability; TD, typically developing.

Supplementary Figure 3 | Pairwise comparison by LEfSe analysis between groups with autism spectrum disorder (ASD), developmental delay or intellectual disability (DD/ID), and typically developing (TD) children (score >2.0 and $p < 0.05$). The cladogram of enriched taxa between ASD+DD and ASD-only **(A)**, DD/ID and TD **(B)**, ASD+DD and DD/ID **(C)**, ASD-only and TD **(D)** gut microbiome.

Supplementary Figure 4 | Extended error bar plot representing the different KEGG category abundance. **(A)** There were 42 different KEGG pathways identified in the ASD and DD/ID groups ($P < 0.001$, effect size >0.05. Benjamini–Hochberg FDR <0.05). **(B)** 9 KEGG pathways for differences among children with DD/ID and TD ($p < 0.05$). **(C)** 10 KEGG pathways were distinct within ASD subgroups ($p < 0.05$).

Supplementary Figure 5 | Correlation between the richness and diversity of bacterial and clinical characteristics in selected groups. **(A)** Correlation in participants with comorbid DD/ID (DD/ID and ASD+DD). **(B)** Correlation in participants without comorbid DD/ID (TD and ASD-only). Shannon index was negatively correlated with the total CARS score in group whether combined DD/ID **(C)** or not **(D)**. Total CARS score was negatively correlated with Simpson index in group whether combined DD/ID **(E)** or not **(F)**.

Supplementary Figure 6 | Association between bacterial relative abundances and three primary CARS domains in subgroups of children with ASD. **(A)** Heatmaps showing correlations between genus and Social impairment, Negative emotionality, and Distorted sensory responses. In the ASD-only group, the abundance of *Faecalibacterium* was negatively correlated with social impairment **(B)**, negative emotionality **(C)**, and distorted sensory responses **(D)** in the ASD-only group. The *Sutterella* genus showed significant negative correlations with social impairment **(E)**, negative emotionality **(F)**, and distorted sensory responses **(G)**.

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Prevalence and Developmental Profiles of Autism Spectrum Disorders in Children With Global Developmental Delay

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Background: Previous studies have mostly explored the comorbidities of Global developmental delay (GDD) in children with Autism Spectrum Disorders (ASD) from the perspective of ASD. The study focus on the perspective of GDD to investigate the prevalence and developmental profiles of ASD in GDD and to explore the correlation between the developmental level and symptoms of autism.

Methods: Clinical data of 521 children with GDD aged from 24 to 60 months were retrospectively analyzed. Analyses were performed first for the whole sample and then subdivided into two subgroups (GDD^+ASD^-, GDD^+ASD^+) according to whether had ASD. Symptoms of autism were evaluated by the Autism Behavior Checklist and the Childhood Autism Rating Scale. The Chinese version of the Gesell Developmental Schedules was used to evaluate the level of children's mental development.

Result: The prevalence of ASD in children with GDD was 62.3%. The total average developmental quotient (DQ) of GDD was mildly deficient and was negatively correlated with symptoms of autism ($p < 0.05$); language ability was severe and extremely severe deficient ($P < 0.05$). GDD^+ASD^- group and GDD^+ASD^+ group have some common points as well as differences in the developmental features. The language delay of children in both subgroups was the most obviously defected, thereafter followed by the item of personal social activity. In the GDD^+ASD^+ group, the DQ of gross motor skills>fine motor skills>adaptability ($p < 0.05$). There were no significant differences among the DQ of gross motor skills, fine motor skills and adaptability in GDD^+ASD^- group ($p > 0.05$). The GDD^+ASD^-group had better adaptability, fine motor skills, language ability, personal social activity than that of the GDD^+ASD^+ group, but the gross motor skills in GDD^+ASD^- group were worse ($p < 0.05$).

Conclusion: GDD children have a high proportion of comorbid ASD, and GDD children with poorer developmental levels are more likely to have ASD symptoms. Development profiles in both GDD^+ASD^- children and GDD^+ASD^+ children have common features but there are also differences. GDD^+ASD^+ group is worse than GDD^+ASD^- group in terms of the overall development level.

Keywords: global developmental delay, Autism Spectrum Disorder, developmental profiles, Gesell, comorbidity

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INTRODUCTION

Global developmental delay (GDD) is defined as a developmental disability of children under 5 years (60 months) of age, which refers to significant delays in two or more developmental domains, including gross or fine motor, speech/language, cognitive, social/personal, and activities of daily living (1). After growing up, many patients with GDD would demonstrate intellectual disability (ID) (2). It is reported in the literature that the prevalence of ID among the population receiving special education in the United States is gradually decreasing, from 8.3‰ in 2000 to 5.7‰ in 2010 (3). Autism spectrum disorder (ASD) is considered to be a neurodevelopmental disorder that can lead to severe social communication deficits, repetitive behaviors, and limited interests (4). In the past few decades, the prevalence of diagnosed ASD has increased dramatically, from 1/5000 in 1975 to 1/44 in 2021 (5), but the reason for this phenomenon remains unclear.

Both GDD/ID and ASD are neurodevelopmental disorders. These diseases overlap in their clinical manifestations and etiology. Comorbidities occur also quite often (6, 7). Previous studies have mostly explored the comorbidities of GDD/ID in children with ASD from the perspective of ASD (5, 8). Our previous studies showed that 68.3% of children with ASD under 5 years of age have combined GDD (9). Studies by the Centers for Disease Control and Prevention (CDC) in the United States showed that ∼1-third of 8-year-old children with ASD have combined ID (5, 10). Few studies have explored the comorbidities and developmental characteristics of ASD in children with GDD/ID from the perspective of GDD/ID. Some scholars believe that the increase in the prevalence of ASD in recent years may be related to the lack of understanding of ASD in the past and misdiagnosis of ASD as GDD/ID; or in case of GDD/ID combined with ASD, only GDD/ID is diagnosed, but ASD is not diagnosed (3). The treatment of ASD and GDD/ID are not all the same, current evidence supports that ASD should be primarily targeted at social communication skills complemented by management of abnormal behavior, but children with GDD may obtain greater benefit from structured daily routines, cognitive behavioral therapy and so on (11, 12). Early diagnosis and targeted treatment are of great significance to improve the prognosis (13–15). A small amount of studies have investigated ASD in the ID population, and found that the comorbidity rate of ASD in the ID population was 4.2–32.9%, and ID children with comorbid ASD had more severe intellectual disability than children with ID alone (16–18). There is no research yet to explore the developmental characteristics of Chinese children with GDD and their comorbidities with ASD. Therefore, this study examined the developmental level and autism symptoms of children with GDD admitted to our hospital from January 2018 to December 2019. By investigating the prevalence and developmental profiles of ASD in children with GDD, it aims to provide a basis for precise diagnosis and personalized treatment.

METHODS

Participants

Participants were recruited by the "Early Recognition and Intervention Program for neurodevelopmental disorders in children" project as part of the National Key Research and Development Project of China. The studies involving human participants were reviewed and approved by the Ethics Committee of the First Hospital of Jilin University. Written informed consent to participate in this study was provided by the participants' legal guardian. A total of 521 children with GDD aged 24–60 months who were treated at the Outpatient Department of Developmental & Behavior Pediatrics of the First Hospital of Jilin University from January 2018 to December 2019 were included in this study. GDD was diagnosed according to the criteria of Diagnostic and Statistical Manual of Mental Disorders V (DSM-5). The Chinese version of the Gesell Developmental Schedules (GDS) was used to evaluate the neurodevelopmental symptoms. All patients were newly diagnosed cases and received no treatment before. Children with physical or sensory disabilities, epilepsy, and genetic defects or inherited metabolic diseases, such as Fragile X Syndrome, Rett Syndrome, Angelman Syndrome, Prader-Willi Syndrome, tuberous sclerosis were excluded. According to whether the child had ASD, all the 521 participants were divided into two subgroups: GDD without ASD ($GDD^{+}ASD^{-}$, $n = 196$), GDD with ASD ($GDD^{+}ASD^{+}$, $n = 325$).

Measurements

Neurodevelopment Assessment

The Chinese version of GDS was used to assess the neurodevelopmental outcomes. It is a classic psychometrical scale widely used in China to evaluate the development of children aged from 16 days to 6 years old. This instrument covers the assessment of the following five domains: gross motor skills, fine motor skills, adaptability, language, and personal social activity. GDS generates individual developmental quotient (DQ) scores for each domain. The total average quotient was calculated as the average of five DQs. Our analysis classified them as follows: normal ($DQ \geq 86$), borderline ($DQ\ 76 \leq \sim \leq 85$), mild defect ($DQ\ 55 \leq \sim \leq 75$), moderate defect ($DQ\ 40 \leq \sim \leq 54$), severe and extremely severe defect ($DQ \leq 39$) (19). In our research, GDD was defined as mild, moderate, severe and extremely severe developmental delay ($DQ \leq 75$) in two or more domains of GDS.

Evaluation of Autism Symptoms

The autism symptoms in the participating children were assessed using the Autism Behavior Checklist (ABC) and the Childhood Autism Rating Scale (CARS) (20, 21). Both ABC and CARS are widely used in China for assessing the autism symptoms.

The ABC is a 57-item questionnaire to be completed by parent(s) or guardian(s). The questionnaire covering five aspects of autism symptoms: sensory, relating, body concept and object use, language, social and selfcare. Items are scored on a 4-point

scale, ranging from 0 (no problem) to 3 (severe problem). The higher the score, the more serious the problem (20, 22). The standard cut-off value was 53, and a score above 53 points indicated high probability of ASD (23).The reliability of ABC scale in the Chinese population is 0.97, validity is 1 (24). In order to control for potential differences in parental literacy, the survey was conducted through interviews with developmental pediatrician rather than asking parents or caregiver to fill out the written survey in this study.

The CARS includes 15 items for assessing ASD-related behavior. Each item was completed by the developmental pediatrician by observing subjects and interviewing parent(s) or guardian(s). A 7-point scale (1, 1.5, 2, 2.5, 3, 3.5, 4) was used for each of the 15 items and the total score was calculated by adding the 15 item scores together. Typically developing children show CARS scores below 30 (25). The higher the score, the more severe the symptoms (20). The reliability of CARS scale in the Chinese population is 0.73, validity is 0.97 (26).

ASD Diagnostic Evaluation

To confirm the diagnosis, all participants with an ABC score of more than 53, CARS score of more than 30 or suspected ASD were evaluated by the Autism Diagnostic Observation Schedule (ADOS). ADOS assessments were administered and scored by a trained developmental pediatrician who met requirements for research reliability. The Chinese version of ADOS used in this study was revised on the basis of the second edition of ADOS (ADOS-2) (27). The ADOS-2 is a semi-structured, play-based assessment tool used to evaluate the core features of ASD. It consists of four different modules, which are selected according to the expressive language level of the children. Each module are scored according to a specific diagnostic algorithm under two domains: social affect and restricted and repetive behaviors. Each assessment takes approximately 45 min to complete. There is a cut-off score for the total score of each module, implying confirmation of the diagnosis of ASD.

Procedure

During the first visit to the outpatient clinic, children with signs of GDD received an initial assessment by a developmental behavior pediatrician for ∼20 min. This includes the use of a self-guided general information questionnaire to investigate the current health status, birth history (antenatal, birth complication, gestation and birth weight), past medical, early developmental history, family history and a history of caregivers. Subsequently, the outpatient pediatrician would arrange an assessment checklist that includes ABC, CARS and GDS for these children. If ABC or/and CARS score was abnormal or if ASD was clinically suspected, the child would undergo an ADOS test to further confirm the diagnosis. On the day of the first visit or the following day, the parent(s) or guardian(s) of the participating children completed the ABC after receiving the guidance of a developmental behavior pediatrician in the evaluation room. Meanwhile, the pediatrician completed the CARS by observing the subjects and interviewing parent(s) or guardian(s). If the child's emotional state was good, a trained and qualified developmental behavior pediatrician would also complete the GDS on the same day. A complete GDS assessment took ∼1 h to complete. During the process, the assessment was stopped when the child experienced obvious emotional reaction, a new appointment could be made, but the assessment might be completed within 1 week. The ADOS assessment was usually completed within 45 min and usually completed within 1 week of the first visit.

Statistical Methods

SPSS Statistics, version 22.0 (IBM Corp., NY, United States) was used to analyse the whole data. The normality of the data was tested by using the Kolmogorov–Smirnov test. Continuous data were given as means ± SDs or median (P25, P75), whereas categorical data were given as number and percentages.

Chi-squared tests and non-parametric Mann–Whitney *U*-test were used to compare the demographics and clinical data differences between two subgroups. Using an approach similar to that described by Jeste et al. (28), repeated-measures analysis of variance (ANOVA) was used to analyse the comparison of five domains DQ in GDS for all participants, within subgroups and between subgroups. A Bonferroni correction was used for *post hoc* analysis to reduce the possibility for Type I errors due to multiple comparisons. Non-parametric Mann–Whitney *U*-test was used to compare the total average DQ of GDS between the two subgroups. The comparisons of all participants in the five domain DQ distributions of GDS were performed by using a chi-square goodness-of-fit test. Partial correlation was used to analyze the correlation of DQ scores and symptoms of autism in children with GDD. All tests were two-sided, with $P < 0.05$ as the significance threshold.

RESULTS

Demographic and ASD Characteristics in GDD

Patient recruitment is presented in **Figure 1**. A total of 521 children participated in this study. The characteristics of the study population are shown in **Table 1**.The average age of these

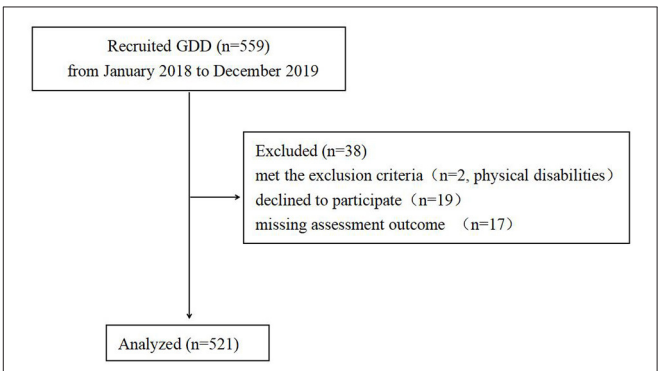

FIGURE 1 | Flow chart of participants recruitment.

TABLE 1 | The characteristics of the study population.

	GDD ($n = 521$)	GDD^+ASD^- ($n = 196$)	GDD^+ASD^+ ($n = 325$)	u/χ2	*p*
Male/female	427/94 (4.5:1)	162/34 (4.7:1)	265/60 (4.4:1)	0,103	0.749
Age at diagnosis (months)	37.0 (31.0, 43.0)	38.0 (33.0, 44.7)	35.1 (30.0, 42.8)	−1.598	0.110
Total ABC score	48.0 (36.0, 60.0)	40.0 (27.2, 51.7)	53.0 (42.0, 64.0)	8.259	<0.001
Body concept and object use	10.0 (5.0, 14.0)	8.0 (4.0, 13.0)	10.0 (7.0, 15.0)	3.513	<0.001
Sensory	7.0 (4.0, 10.0)	5.0 (2.0, 8.0)	8.0 (5.0, 11.5)	7.152	<0.001
Selfcare	11.0 (8.0, 14.0)	9.5 (6.0, 13.0)	12.0 (9.0, 15.0)	5.456	<0.001
Language	7.0 (4.0, 10.0)	5.0 (3.0, 8.7)	7.0 (5.0, 10.0)	4.718	<0.001
Relating	12.0 (7.0, 16.0)	10.0 (4.0, 13.0)	14.0 (10, 17.0)	7.062	<0.001
Total CARS score	31.0 (27.7, 34.0)	28.0 (26.0, 31.0)	32.0 (29.5, 35.5)	9.559	<0.001

GDD, Global developmental delay; ASD, Autism Spectrum Disorder; ABC, the Autism Behavior Checklist; CARS, the Childhood Autism Rating Scale; Chi-squared tests and non-parametric Mann–Whitney U-test were used to compare the demographics between GDD^+ASD^- group and GDD^+ASD^+group; $p < 0.05$ indicated statistical significance.

TABLE 2 | GDS scores of all the participants in different groups.

	GDD ($n = 521$)		GDD^+ASD^- ($n = 196$)		GDD^+ASD^+ ($n = 325$)		*p*
	Delay[a] *n* (%)	DQ (Mean ± SD)	Delay[a] *n* (%)	DQ (Mean ± SD)	Delay[a] *n* (%)	DQ(Mean ± SD)	
Adaptability	432 (82.9)	59.17 ± 17.41	139 (70.9)	64.32 ± 18.82	293 (90.2)	56.06 ± 15.73	<0.001
Gross motor skills	351 (67.4)	68.32 ± 13.70	131 (66.8)	66.17 ± 14.38	220 (67.7)	69.61 ± 13.13	0.005
Fine motor skills	387 (74.3)	63.79 ± 18.06	136 (69.4)	66.46 ± 18.13	251 (77.2)	62.18 ± 17.85	0.009
Language	511 (98.1)	42.26 ± 15.56	190 (96.9)	45.42 ± 15.28	321 (98.8)	40.36 ± 15.44	<0.001
Personal social activity	495 (95.0)	53.62 ± 13.23	182 (92.9)	56.64 ± 13.50	313 (96.3)	51.80 ± 12.75	<0.001
Total average quotient	488 (93.7)	57.43 ± 12.45	180 (91.8)	59.80 ± 12.86	308 (94.8)	56.00 ± 11.99	<0.001

GDD, Global developmental delay; ASD, Autism Spectrum Disorder; DQ, developmental quotient; GDS, the Gesell Developmental Schedules; [a]total average quotient or a subscale quotient ≤75; The repeated-measures ANOVA, Bonferroni's multiple comparison test and Non-parametric Mann–Whitney U-test were used to compare the differents between the GDD^+ASD^- group and GDD^+ASD^+group, $p < 0.05$ indicated statistical significance.

children was 37.71 ± 8.58 months (range, 24–60 months). The study consisted of 427 boys and 94 girls (male-female sex ratio of 4.5:1). The proportion of ASD in GDD was 62.3% (325/521). There were 196 children in the GDD^+ASD^- group (mean age = 38.27 ± 8.40 months, 162 Male/34 Female), and 325 children in the GDD^+ASD^+group (mean age =37.37 ± 8.68 months, 265 Male/60 Female). Both groups were matched by actual age and gender ratio ($p > 0.05$).

Developmental Features of GDD

A repeated-measures ANOVA showed a significant difference within the five subscale quotients of the GDS ($F = 311.726$, $P < 0.001$,$\eta^2 = 0.707$), indicating the existence of an unbalanced development of GDD. Language delay was the most common defect (98.1%), followed by personal social skills (95.0%), adaptability (82.9%), fine motor (74.3%), gross motor (67.4%) (see also **Table 2**). The chi-square goodness-of-fit test showed that the language DQ was mainly dominated by the distribution of severe and extremely severe defects ($\chi 2 = 236.321$, $P < 0.001$). The DQ of the remaining four regions and the total average DQ were mainly mild defects ($\chi 2_{\text{adaptability}} = 110.823$, $p < 0.001$; $\chi 2_{\text{grossmotor}} = 335.192$, $p < 0.001$; $\chi 2_{\text{finemotor}} = 115.123$, $p < 0.001$; $\chi 2_{\text{personalsocialskills}} = 211.322$, $p < 0.001$; $\chi 2_{\text{totalaverageDQ}} = 303.944$, $p < 0.001$) (see **Figure 2**).

The Association Between DQ and Autistic Symptoms in Children With GDD

After controlling gender and age, partial correlation analysis showed that the total scores of the ABC and the CARS were negatively correlated with GDS adaptability DQ, fine motor DQ, language DQ, personal social skills DQ, and total average DQ. The total scores of CARS was negatively correlated with gross motor DQ, but there was no correlation between the total scores of the ABC and gross motor DQ (see **Table 3**).

Comparison of the Developmental Features of the Two Subgroups

According to the results of the repeated-measures ANOVA, there was a significant interaction between DQ in the five sub-regions of GDS and the diagnosis ($F = 22.305$, $P < 0.001$,$\eta^2 = 0.147$), indicating that GDD^+ASD^- group and GDD^+ASD^+ group might have different development quotient profiles. After Bonferroni's multiple comparison test, it is found that the distribution trends of the DQ of gross motor, fine motor and adaptability were different, although the two subgroups were both dominated by language delay, followed by personal social backwardness. The GDD^+ASD^+ group showed a pattern of gross motor DQ > fine motor DQ > adaptability DQ ($P <$

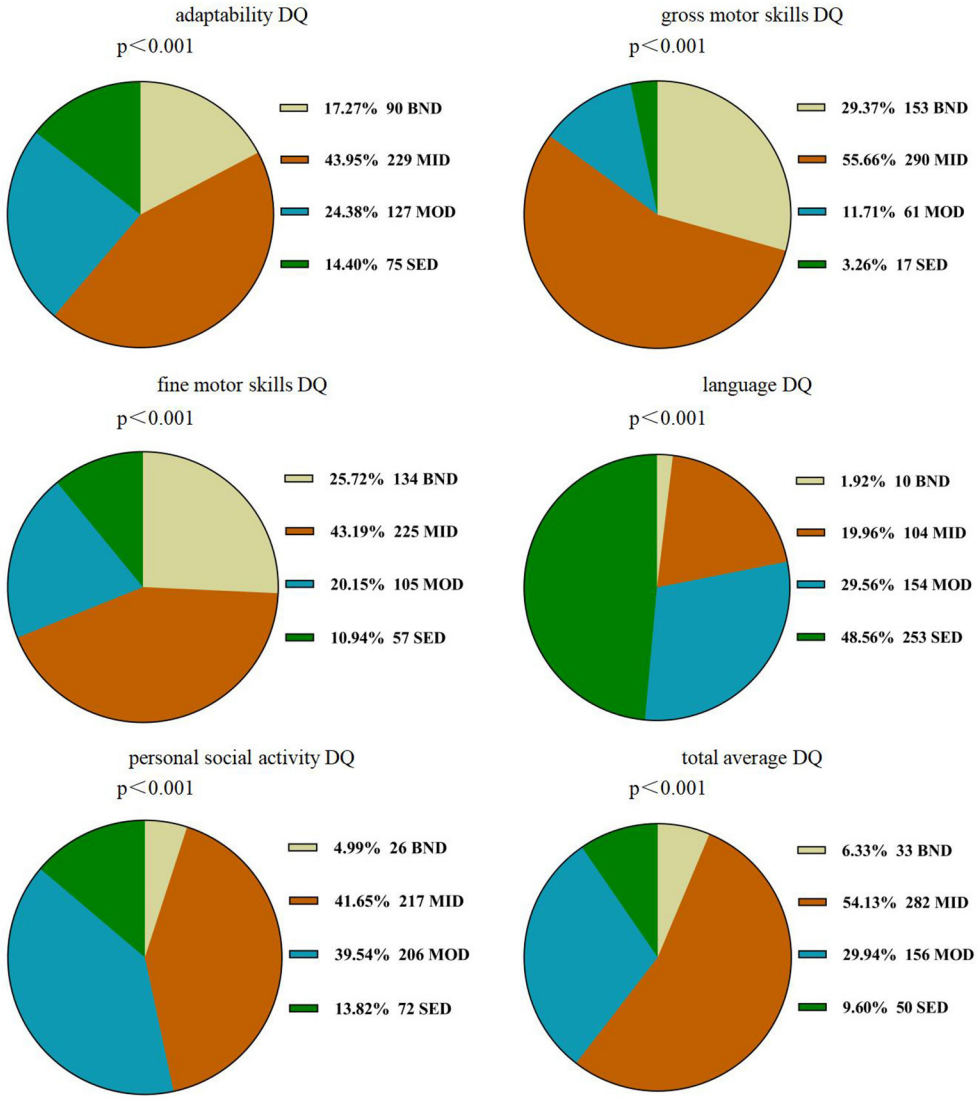

FIGURE 2 | Distribution of DQ scores of GDS in children with GDD. GDD, Global developmental delay; DQ, developmental quotient; GDS, the Gesell Developmental Schedules; BND, borderline-normal defect; MID, mild defect; MOD, moderate defect; SED, severe and extremely severe defect.

0.001); there was no statistical difference among the DQ of gross motor, fine motor and adaptability in the $GDD^{+}ASD^{-}$group (see **Figure 3**).

Description of the Developmental Features of Two Subgroups

According to the results of the repeated-measures ANOVA, Bonferroni's multiple comparison test and Non-parametric Mann–Whitney *U*-test, it is found that the $GDD^{+}ASD^{+}$ group scored lower in the DQ of adaptability, fine motor, language, personal social skills and total average DQ compared to the $GDD^{+}ASD^{-}$ group ($p_{adaptability} < 0.001$, $p_{finemotor} = 0.009$, $p_{language} < 0.001$, $p_{personalsocialskills} < 0.001$, $p_{totalaverageDQ} < 0.001$), and gross motor DQ was higher than $GDD^{+}ASD^{-}$ group ($p = 0.005$) (see **Table 2**).

DISCUSSION

In this study, we analyzed the developmental characteristics of 521 children with GDD and comorbid autism symptoms aged from 24 to 60 months. The development profile of GDD children with or without ASD was described and compared. The relationship between developmental level of children with GDD and the symptoms of autism was also discussed.

The results obtained are summarized as follows: 1. The comorbidity rate of ASD in children with GDD at 24–60 months was 62.3%. 2. Developmental imbalances was shown in the overall GDD children. The DQ of adaptability, gross motor, fine motor, personal social skills and total average DQ were dominated by mild defects, while language DQ was dominated by severe and extremely severe defects. 3. GDD

TABLE 3 | The association between developmental quotient of GDS and symptoms of autism in children with GDD.

	Total ABC score	Total CARS score
Adaptability	$r_s = -0.176$, $p < 0.001$	$r_s = -0.276$, $p < 0.001$
Gross motor skills	$r_s = -0.022$, $p = 0.619$	$r_s = -0.120$, $p = 0.006$
Fine motor skills	$r_s = -0.145$, $p = 0.001$	$r_s = -0.276$, $p < 0.001$
Language	$r_s = -0.151$, $p = 0.001$	$r_s = -0.366$, $p < 0.001$
Personal social activity	$r_s = -0.275$, $p < 0.001$	$r_s = -0.427$, $p < 0.001$
Total average quotient	$r_s = -0.193$, $p < 0.001$	$r_s = -0.383$, $p < 0.001$

GDD, Global developmental delay; GDS, the Gesell Developmental Schedules; ABC, the Autism Behavior Checklist; CARS, the Childhood Autism Rating Scale; rs was partial correlation coefficients; When P < 0.05, the correlation parameters can take negative values indicating negative correlation and positive values for positive correlations.

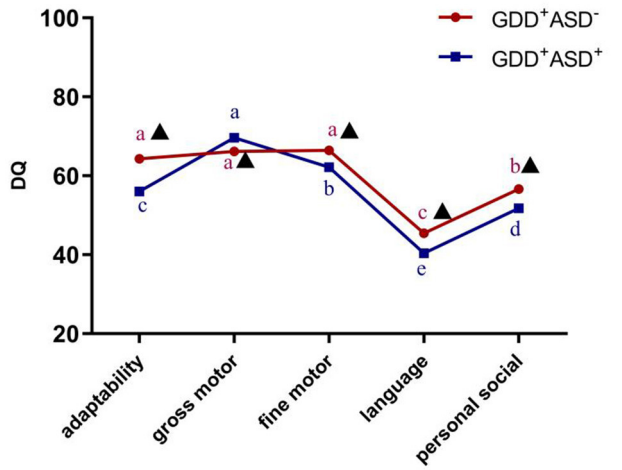

FIGURE 3 | Development profile of the Gesell Developmental Schedules in GDD^+ ASD^- group and GDD^+ ASD^+ group. GDD, Global developmental delay; ASD, Autism spectrum disorder; DQ, developmental quotient; Mean values marked with different letter (a, b, c, d or e) indicate DQ scores in the five sub-domains of Gesell Developmental Schedules, and those with the same letter exhibit no statistically significant difference while different letters represent with statistical difference (comparison within the group); ▲Indicates that there is a significant difference between GDD^+ASD^- and GDD^+ASD^+ group (comparison between groups); $p < 0.05$ indicated statistical significance.

Children with poor developmental levels were more likely to have symptoms of autism. 4. The GDD^+ASD^- group and GDD^+ASD^+ group had some similarities in the developmental features, and there were also some differences. Language delay was the most obvious defect of both groups of children, followed by personal social activity; In the GDD^+ASD^+ group, the DQ of gross motor>fine motor>adaptability. There were no significant differences between gross motor DQ, fine motor DQ and adaptability DQ in the GDD^+ASD^- group. 5. The GDD^+ASD^- group had better adaptability, fine motor skills, language ability, personal social activity than GDD^+ASD^+ group, but their gross motor skills were worse.

Prevalence of ASD in Children With GDD

Our study is the first report on the comorbidity of ASD among children with GDD in China. We found that the comorbidity rate of ASD in children with GDD aged 24–60 months was 62.3%, which is significantly higher than the 1% prevalence of ASD in the Chinese general population (29), confirming that GDD is a "high risk" sample of ASD. In addition, previous studies have also found that the proportion of GDD in children with ASD was as high as 68.3% (9), indicating that GDD and ASD have a high co-morbidity rate. Several studies have shown that the increase in the prevalence of ASD was accompanied by a decrease in the prevalence of ID (3), suggesting that the diagnosis of ASD in children with ID might be related to the increase in the prevalence of ASD. Although there is no research on the correlation between the prevalence of GDD and the prevalence of ASD, GDD is closely related to ID. The confirming diagnosis of comorbid ASD in children with GDD might also be one of the reasons for the rapid increase in the prevalence of ASD. Previous studies have found that the proportion of ASD in ID was about 4.2–32.9% (5, 16–18), which is much lower than the 62.3% ASD comorbidity rate in GDD found in this study. This phenomenon, on the one hand, may be related to the changes in the clinical phenotype of neurodevelopmental disorders with age and maturity of the central nervous system (30). On the other hand, it also indicates that the diagnosis of ASD may be insufficient. Some scholars have found that in the past, about 50% of children with ID comorbid ASD did not get a clear ASD diagnosis (31, 32). It is emphasized that clinicians need to pay more attention to and strengthen the early recognition and diagnosis of ASD comorbidities in GDD/ID. The targeted treatment should also be given to improve the prognosis.

Developmental Features of GDD

Concerning the development profile in children with GDD, we found that the total average DQ of GDD was mainly mildly defect, which is similar to the results of previous studies (33, 34). Comparing the DQ scores on the five sub-scale of GDS, it was found that children with GDD had developmental imbalance, which was different from the previous conclusion that GDD was considered to be an overall delay in development rather than developmental imbalance or developmental deviation (35). Our study also showed that language delay was the most common phenomenon among children with GDD. This is similar to the results of Liao et al. (33). The adaptability DQ, gross motor DQ, fine motor DQ and personal social skills DQ scores of GDS were mainly slightly impaired, and the language ability was severe and extremely severe defected. The language ability has predictive value for the language IQ of children with GDD (34). This reminds us that in order to optimize the developmental outcomes of the children with GDD, special attention should be paid to the training programs of their language ability.

The Association Between DQ and Autistic Symptoms in Children With GDD

There are few studies on the relationship between autism symptoms and developmental quotient in children with GDD. In our study, we found that adaptability DQ, fine motor DQ,

language DQ, personal social skills DQ and total average DQ were all negatively correlated with the symptoms of autism in children with GDD. The total scores of CARS was negatively correlated with gross motor DQ, but there was no correlation between the total scores of the ABC and gross motor DQ. Our results suggested that the lower the overall developmental level, the more severe autistic symptoms in children with GDD. This is comparable to the previous findings that ID with ASD is more severe than ID alone (16). In addition, several studies have also shown that even with intensive interventions, children with ASD and comorbid cognitive delays seemed to make only limited developmental progress over time (36, 37).This again emphasizes that it is important to pay attention to the comorbidity of ASD and GDD in the early stages of development.

Developmental Features of GDD^+ASD^- Group and GDD^+ASD^+ Group

Regarding the development profile of children in the GDD^+ASD^- and GDD^+ASD^+ subgroups, it was found that the developmental characteristics of the two subgroups had some common points, but there were also some differences. Language delay was the most obvious defect of the both groups, followed by personal social activity. In the GDD^+ASD^+ group, the gross motor DQ score was better than fine motor DQ score, fine motor DQ score was subsequently better than adaptability DQ score. There was no significant difference in gross motor, fine motor and adaptability DQ scores in the GDD^+ASD^-group. Few studies on the developmental features of GDD^+ASD^- and GDD^+ASD^+ are available for comparison. In 2005, Indian scholars conducted a comparative study on 64 children under 4 years of age with ASD and developmental delays. They found significant differences in the developmental profiles of the two groups. Compared with the developmental delay group, the significantly lower social skills and superior motor ability in the ASD group explained these differences. No common features were found in the developmental profiles of the two groups (38). As the etiology and symptoms of GDD and ASD often overlap, it is sometimes difficult to distinguish between the two conditions in the clinical practice (6, 7). Although there are a variety of screening tools available and some specific clinical symptoms are helpful for the differential diagnosis of GDD and ASD, there are still certain limitations (7, 39, 40). Research by Miller et al. found that children with a developmental level of <12 months, even if the standardized ASD diagnostic tool ADOS was used for assessment, 18.8% of children with GDD were still overestimated as ASD (7). Furthermore, the inaccurate diagnosis directly affects the follow-up rehabilitation treatment. Our study showed that children in both GDD^+ASD^- group and GDD^+ASD^+ group had similarities in their developmental profiles, but there were also clear differences, which could be helpful in the differential diagnosis of the two conditions.

Comparing development quotient of GDD^+ASD^- group and GDD^+ASD^+ group, we found that the developmental level of children with GDD^+ASD^+ was more impaired than children with GDD^+ASD^-. The adaptability, fine motor, language and personal social skills DQ scores of GDD^+ASD^+ children were lower than GDD^+ASD^- children, but gross motor DQ score of GDD^+ASD^+ children was better than GDD^+ASD^- children. A study conducted by the CDC also found that children with ASD and ID had a higher proportion of developmental problems before the age of 3 compared with children with ASD alone (36). However, children with ASD and comorbid cognitive delays appeared to make limited developmental progress over time, and even under intensive intervention, they showed serious deficits in adaptive functioning, social skills, and disruptive behaviors (41, 42). These findings again indicate that special attention should be paid to the comorbidities of GDD and ASD in the early stages of development, and targeted rehabilitation should be carried out in a timely manner according to their development characteristics to improve the prognosis.

Strengths and Limitations

This study is the first report on the comorbidity of ASD among children with GDD in China. This first report of the comorbidity rate of ASD in GDD, summarized the similarities and differences between GDD^+ASD^- and GDD^+ASD^+ children's developmental profiles, and found that the level of development was related to the symptoms of autism in GDD. Our research also has some limitations. This is a single-center cross-sectional study, and the available results cannot represent the overall situation and dynamic changes of Chinese children with GDD. Our study only compared the developmental characteristics of children with GDD alone and GDD children with ASD, but did not include children with ASD alone. Therefore, in order to achieve the early differential diagnosis and precise intervention for children with GDD/ASD, a multi-center and longitudinal follow-up study should be conducted for the entire population of ASD and GDD.

CONCLUSION

In conclusion, we found that the comorbidity rate of ASD in children with GDD was 62.3%; GDD children with poor developmental levels were more likely to have ASD symptoms. The GDD^+ASD^- group and GDD^+ASD^+ group had not only similarities, but also differences in the developmental features; The overall development level of GDD^+ASD^+ children was worse than that of GDD^+ASD^- children. These results will be helpful to discover more effective methods to identify ASD comorbidities in children with GDD.

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 the Ethics Committee of the First Hospital of

Jilin University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LS, F-YJ, and Z-DX participated in the design and definition of this study. F-YJ provided assistance for data acquisition and literature search. LS and T-TW performed the statistical analysis and drafted the manuscript. LS, F-YJ, and Z-DX edited the manuscript. All authors contributed to the article and approved the submitted version.

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The Sensory Abnormality Mediated Partially the Efficacy of Repetitive Transcranial Magnetic Stimulation on Treating Comorbid Sleep Disorder in Autism Spectrum Disorder Children

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Sleep disorder emerges as a common comorbidity in children with autism spectrum disorder (ASD), and the interaction between the core symptoms of ASD and its sleep disorder remains unclear. Repetitive transcranial magnetic stimulation (rTMS) was used on the bilateral dorsolateral prefrontal cortex (DLPFC) to investigate the efficacy of rTMS on the core symptoms of ASD and comorbid sleep problems as well as the mediation role of the ASD symptoms between rTMS intervention and sleep improvement. A total of 41 Chinese children with ASD and who met the criteria in the fifth edition of the American Diagnostic and Statistical Manual of Mental Disorders were recruited, and 39 of them (mean age: 9.0 ± 4.4 years old; the male–female ratio was 3.9: 1) completed the study with the stimulating protocol of high frequency on the left DLPFC and low frequency on the right DLPFC. They were all assessed three times (before, at 4 weeks after, and at 8 weeks after the stimulation) by the Children's Sleep Habits Questionnaire (CSHQ), Strengths and Difficulties Questionnaire (SDQ), Childhood Autism Rating Scale, Repetitive Behavior Questionnaire-2, and Short Sensory Profile (SSP). The repeated-measures ANOVA showed that the main effect of "intervention time" of CSHQ ($F = 25.103$, $P < 0.001$), SSP ($F = 6.345$, $P = 0.003$), and SDQ ($F = 9.975$, $P < 0.001$) was statistically significant. By Bayesian mediation analysis, we only found that the total score of SSP mediated the treating efficacy of rTMS on CSHQ ($\alpha\beta = 5.11 \pm 1.51$, 95% CI: 2.50–8.41). The percentage of mediation effect in total effect was 37.94%. Our results indicated the treating efficacy of rTMS modulation on bilateral DLPFC for both autistic symptoms and sleep disturbances. The sensory abnormality of ASD mediated the improvement of rTMS on sleep problems of ASD.

Keywords: autism spectrum disorder (ASD), repetitive transcranial magnetic stimulation (rTMS), dorsolateral prefrontal cortex (DLPFC), sleep disorders, sensory problems, Bayesian mediation analysis

INTRODUCTION

Autism spectrum disorder (ASD) is a severe neurodevelopmental disorder characterized by social–communicative atypicality and a restrictive and rigid repertoire of behaviors (1). Over the past 30 years, the prevalence of ASD has dramatically increased. In the United States, around one out of 59 children have been diagnosed with ASD (2). Cumulative evidence has shown that as many as 80% of children with ASD suffered from a variety of sleep problems (3), such as insomnia (4), daytime sleepiness, sleep-disordered breathing, and parasomnias (5), which is much higher than that in typically developing children (6–8). The core symptoms of ASD and their comorbid sleep disturbances interact severely with each other. The predisposition of neurophysiology and neurochemistry possibly makes autistic patients susceptible to chronic sleep–wake disorder (9)—for example, some genes (such as PER1, PER2, and NPAS2) contribute to the sleep–wake regulation processes and ASD (9). At the same time, the symptoms of ASD, such as intolerance to change and persistence in the same pattern, also make autistic children more intolerant of the sleeping environment and have more difficulty in falling asleep (10, 11). On the other hand, the sleep problems exacerbate the severity of the core ASD symptoms, such as repetitive behaviors, social, and communication difficulties (12, 13), and other maladaptive behaviors (14, 15). Exploring the interaction between them can not only help us to understand the etiology of ASD but also provide more information on effective intervention.

Recent work suggests that repetitive transcranial magnetic stimulation (rTMS) has been a promising treatment intervention for both the core symptoms of ASD (such as repetitive and stereotyped behaviors and social behavior) and sleep disturbances (16–20). However, there was no suitable stimulating protocol for both at the same time. Our previous research compared the effects of different rTMS stimulation protocols on the bilateral dorsolateral prefrontal cortex (DLPFC) for ASD. We found that high-frequency left and low-frequency right rTMS can effectively control the core symptoms of autism, especially on the improvement of the anxiety of ASD children (21). Since anxiety is closely related to sleep disorders in children and adolescents (22), some studies have confirmed that ASD symptoms and anxiety uniquely contributed to the presence and frequency of sleep problems (23). Thus, the rTMS protocol of high-frequency left and low-frequency right is supposed to work for both the core autistic symptoms and sleep disturbances.

In the current study, rTMS was applied to the left and right DLPFC with different frequencies (high-frequency left and low-frequency right) to explore the relationship between sleep profiles and core symptoms in ASD at the same time. We hypothesized that both the core symptoms and the sleep problems would be ameliorated by rTMS in ASD, and the improvement of the core symptoms would mediate the promotion of the sleeping situations of ASD children, by which we can offer (a) the verification of an rTMS protocol for both the core symptoms and the comorbid sleep problems and (b) understanding of the role of the core symptoms in the comorbid sleep problems of ASD.

METHODS

Participants

We released the recruitment information through the outpatient clinic of Tianjin Anding Hospital (the psychiatric hospital) and by online platform from October 2018 to October 2020 to recruit ASD children. The inclusion criteria were as follows: (1) it meets the diagnostic criteria of ASD in the fifth edition of the American Diagnostic and Statistical Manual of Mental Disorders, (2) age of 2–18 years old, (3) no medication during the rTMS intervention, (4) right-handed, and (5) total score of Children's Sleep Habits Questionnaire (CSHQ) at baseline $\geq$41. The exclusion criteria were as follows (21, 24): (1) contraindications to rTMS, such as metal or electronic instruments near the coil stimulation site; participants with a history of epilepsy (excluding epilepsy according to their electroencephalogram and medical record); participants with a history of brain trauma, brain tumors, and other diseases; participants with severe or recent heart disease or other major physical illnesses; (2) diagnosis of other mental illnesses (e.g., attention-deficit hyperactivity disorder, schizophrenia, and depression); and (3) other neurodevelopmental disorder, genetic metabolic disease, or severe neurological disease. The study was conducted under the Code of Ethics of the World Medical Association (Declaration of Helsinki). The study also complied with all relevant national regulations and institutional policies and had been approved by the Medical Ethics Committee of Tianjin Medical University. The participants and their parents (or legal guardians) obtained all information about the research, including the purpose, requirements, responsibilities, compensation, risks, benefits, and alternatives. All questions were answered before asking for their consent signature.

TMS Procedure

A trained electrophysiologist delivered rTMS stimulation over the cortical area, controlling the contralateral first dorsal interosseous (FDI) using a magnetic field stimulator (CCY-1, Yiruide Medical Corporation, Wuhan, China) to detect the resting motor threshold (MT). The MT was determined for each hemisphere in all individuals by gradually increasing the output of the machine by 5% until a 5-mV deflection or a visible twitch in the FDI muscle was identified in two out of three trials (24). Electromyographic responses were monitored continuously from the hand contralateral to the stimulated hemisphere using the MEP module in the magnetic stimulator (Yiruide Medical Corporation, Wuhan, China). The subjects were familiarized with the laboratory and the procedure before the first transcranial magnetic stimulation session.

In this study, rTMS was selected to stimulate the left DLPFC with a high frequency (10 Hz) and the right DLPFC with a low frequency (1 Hz) based on the evidence-based basis proposed by the European Union of Neurological Societies (25), and the electrode positioning cap was used for accurate positioning. The specific parameters are as follows: the stimulation frequency of the right dorsolateral prefrontal lobe is 1 Hz, the stimulation time is 32 s, the stimulation number is 32, the intermittent time is 1 s, the repetition number is 28, and the stimulation

intensity is 25% MT, whereas the stimulation frequency of the left dorsolateral prefrontal lobe is 10 Hz, the stimulation time is 3.2 s, the stimulation number is 32, the intermittent time is 10 s, the repetition number is 45, and the stimulation intensity is 25% MT. The intervention time of rTMS was five times/week, and the course of intervention was every 4 weeks. Each child had two courses of intervention, for a total of 8 weeks.

Assessment of the Symptoms of ASD

We used the CSHQ to evaluate the sleep quality of all participants in the current study. The caregivers were required to fill in the CSHQ according to the sleep status of the participants in a typical week in the past month (26). The participants are regarded as having poor sleep quality, with a total score of CSHQ >41. Meanwhile, the Repetitive Behavior Questionnaire (RBQ-2), the Short Sensory Profile (SSP), and the Strengths and Difficulties Questionnaire (SDQ) were used to evaluate the severity of the symptoms of repetitive behaviors, abnormal responses to sensory stimuli, and social impairments in ASD children, respectively. The Peabody Picture Vocabulary Test (PPVT) was also used to evaluate the non-verbal intelligence of autistic children. All the participants were assessed before, after one intervention course, and after two intervention courses.

Statistical Data Analysis

Statistical analyses were performed on the subject-averaged behavioral data. The primary analysis model was repeated-measures ANOVA (RM-ANOVA), with the following dependent variables: scores of CSHQ, RBQ-2, SSP, and SDQ. Repeated within-group factor was time (T_0 = before rTMS intervention, T_1 = after one course of rTMS intervention, and T_2 = after two courses of rTMS intervention). According to our previous research (25), the sample size was estimated by PASS15.05.

For the mediation analysis, a model of Bayesian mediation analysis was created, with time as the independent variable (time = 0, 1, and 2 as "before rTMS intervention", "after one course of rTMS intervention," and "after two courses of rTMS intervention," respectively), the scores of RBQ-2, SSP, and SDQ as the mediator, respectively, and the score of CSHQ as the dependent variable by using the procedure of MCMC of SAS 9.4 (27).

RESULTS

A total of 41 ASD children were recruited, of whom two withdrew during the intervention, and 39 ASD children who completed all the intervention and assessment items were finally included in the study. All the participants were right-handed. The average age of the subjects was 9.0 ± 4.4 years old, the male–female ratio was 3.9:1, and the median PPVT was 40.00 (Q1 = 0.00, Q3 = 75.50). Data on age, IQ, severity of autism symptoms, family income, and maternal education level of ASD children are shown in **Table 1**.

TABLE 1 | Demographic characteristics of autism spectrum disorder children ($n = 39$).

Variables	*N* (%)
Gender	
Male	31 (79.5%)
Female	8 (20.5%)
Age	
2.0–5.9	9 (23.1%)
6.0–11.9	21 (53.8%)
12.0–18.0	9 (32.1%)
Score of PPVT	
<70	26 (66.7%)
≥70	13 (33.3%)
Score of CARS	
<36	22 (56.4%)
≥36	17 (43.6%)
Monthly household income (RMB: Yuan)	
<5,000	6 (15.4%)
5,000–7,999	10 (25.6%)
8,000–9,999	6 (15.4%)
≥10,000	17 (43.6%)
Maternal education	
College degree or above	27 (69.2%)
High school, technical secondary school, or vocational school	7 (17.9%)
Junior high school and below	5 (12.8%)

PPVT, Peabody Picture Vocabulary Test; CARS, Childhood Autism Rating Scale.

The Results of CSHQ, RBQ-2, SSP, and SDQ After Intervention

For CSHQ, RM-ANOVA revealed that the main effect of "time" was significant ($F = 25.103$, $P < 0.001$): for the total score, the mean was 53.23 ± 6.14, 48.64 ± 5.95, and 46.97 ± 6.18, respectively; and the changes between different time points were all statistically significant (see **Table 2**). For each subscale, there was a statistical decrease after one or two courses of intervention than before, except for sleep-disordered breathing and sleep-onset delay. For details, there was a significant reduction in sleep duration at the second (T_0 vs. T_1) and third (T_0 vs. T_2) assessments. Similarly, there was a significant reduction in bedtime resistance, sleep anxiety, and daytime sleepiness after one intervention course of rTMS (T_0 vs. T_1), except parasomnias and night wakings, for which a significant reduction was observed only after two intervention courses (see **Table 2**). However, there was no reduction at all in sleep-disordered breathing and sleep-onset delay.

For RBQ-2, the main effect of "time" was not significant either in the total score ($F = 2.425$, $P = 0.093$) or in the subscales. For SSP, the main effect of "time" was significant ($F = 6.345$, $P = 0.003$), which indicated that the total score of SSP reduced significantly after rTMS intervention. For the score of the SSP subscales, there was a significant reduction in tactile sensitivity and auditory filtering, but no such significant changes were observed for the rest of the subscales (see **Table 3**). For SDQ, the main effect of "time" of the total score was also statistically significant ($F = 9.975$, $P < 0.001$), and the substantial improvement emerged at the second (T_0 vs. T_1) and the third (T_0 vs. T_2) assessment, but with no difference between the

TABLE 2 | Comparison of CSHQ before and after intervention in children with autism spectrum disorder ($n = 39$).

Items	Time[a]	Mean difference	Standard error	*P*
Total score	T0–T1	4.59	0.85	0.000
	T0–T2	6.26	0.98	0.000
	T1–T2	1.67	0.56	0.005
Bedtime resistance	T0–T1	1.18	0.25	0.000
	T0–T2	1.33	0.35	0.001
	T1–T2	0.15	0.25	0.547
Sleep anxiety	T0–T1	0.64	0.20	0.003
	T0–T2	0.85	0.24	0.001
	T1–T2	0.21	0.14	0.146
Sleep duration	T0–T1	0.67	0.20	0.002
	T0–T2	1.26	0.29	0.000
	T1–T2	0.59	0.25	0.021
Sleep-disordered breathing	T0–T1	0.10	0.08	0.210
	T0–T2	0.13	0.07	0.058
	T1–T2	0.03	0.05	0.570
Parasomnias	T0–T1	0.33	0.19	0.085
	T0–T2	0.54	0.16	0.002
	T1–T2	0.21	0.12	0.088
Daytime sleepiness	T0–T1	1.23	0.50	0.019
	T0–T2	1.59	0.52	0.004
	T1–T2	0.36	0.44	0.421
Night wakings	T0–T1	0.33	0.17	0.057
	T0–T2	0.39	0.18	0.034
	T1–T2	0.05	0.08	0.534
Sleep-onset delay	T0–T1	0.10	0.08	0.210
	T0–T2	0.15	0.12	0.205
	T1–T2	0.05	0.10	0.599

T0, before the intervention; T1, after one intervention course; T2, after two intervention courses.
CSHQ, Children's Sleep Habits Questionnaire.
[a] *Time refers to before, after one intervention course, or after two intervention courses.*

TABLE 3 | Comparison of SSP and SDQ before and after intervention in children with autism spectrum disorder ($n = 39$).

Subscales	Time[a]	Mean difference	Standard error	*P*
SSP				
Tactile sensitivity	T0–T1	1.51	0.61	0.017
	T0–T2	1.82	0.57	0.003
	T1–T2	0.31	0.47	0.520
Auditory filtering	T0–T1	1.28	0.63	0.048
	T0–T2	2.18	0.77	0.007
	T1–T2	0.90	0.58	0.130
Total score	T0–T1	4.88	2.31	0.042
	T0–T2	6.52	2.31	0.017
	T1–T2	1.64	2.31	0.825
SDQ				
Emotional problems	T0–T1	0.73	0.47	0.323
	T0–T2	1.30	0.47	0.018
	T1–T2	0.58	0.47	0.523
Conduct problems	T0–T1	0.72	0.38	0.159
	T0–T2	0.93	0.38	0.045
	T1–T2	0.20	0.38	0.934
Prosocial	T0–T1	1.00	0.54	0.192
	T0–T2	1.48	0.54	0.023
	T1–T2	0.48	0.54	0.767
Total score	T0–T1	2.65	1.08	0.047
	T0–T2	4.83	1.08	<0.001
	T1–T2	2.18	1.08	0.446

T0, before the intervention; T1, after one intervention course; T2, after two intervention courses.
SSP, Short Sensory Profile; SDQ, Strengths and Difficulties Questionnaire.
[a] *Time refers to before, after one intervention course, or after two intervention courses.*

second and the third assessment (T_1 vs. T_2). For the subscales of SDQ, there was a significant reduction at the third assessment (T_0 vs. T_2), except for the hyperactivity and inattention as well as peers/peered problems subscales, which had no change throughout the intervention (see **Table 3**).

The Result of the Bayesian Mediation Analysis

First, we performed a Bayesian mediation analysis with the total score of SSP mediating the relationship between rTMS intervention and the total score of CSHQ. The trace plots indicated a good mixing for the parameters, and the chains that mix well tend to converge sooner (see **Figure 1**), as well as the kernel density plots of the posterior distribution for the given parameter (see **Figure 1**). The posterior mean of the mediated effect of rTMS intervention through the total score of SSP on change in the total score of CSHQ was $\alpha\beta = 5.11 \pm 1.51$, with a 95% central credibility interval of 2.50–8.41. Given that 0 was not between the two credibility limits, the mediated effect of SSP was statistically significant. A further calculation ($\alpha\beta$ / c $\times$ 100%) reflected that the mediation effect was 37.94%, which means that 37.94% of the total effect between rTMS intervention and the score of CSHQ was mediated by the score of SSP (see **Table 4**). Our results indicated that rTMS intervention could promote the sleep of autistic children through improving the sensory abnormalities in addition to the direct reduction of the sleep problems. However, neither the total score of SDQ nor of RBQ-2 was the mediator due to the poor convergence of the Markov chain or the containment of 0 in the 95% central credibility interval, which meant that the mediated effect is not statistically significant.

DISCUSSION

As mentioned in the "Introduction," the high prevalence of sleep problems in children with ASD is more likely to be attributed to the pathogenesis of ASD, such as brain wave organizational and maturational differences, arousal and sensory dysregulation, circadian-relevant genes, and abnormal melatonin production (28, 29). However, there is no intervention especially for the sleep problems of autistic individuals besides the conventional measures aimed at the behavioral insomnia of children caused

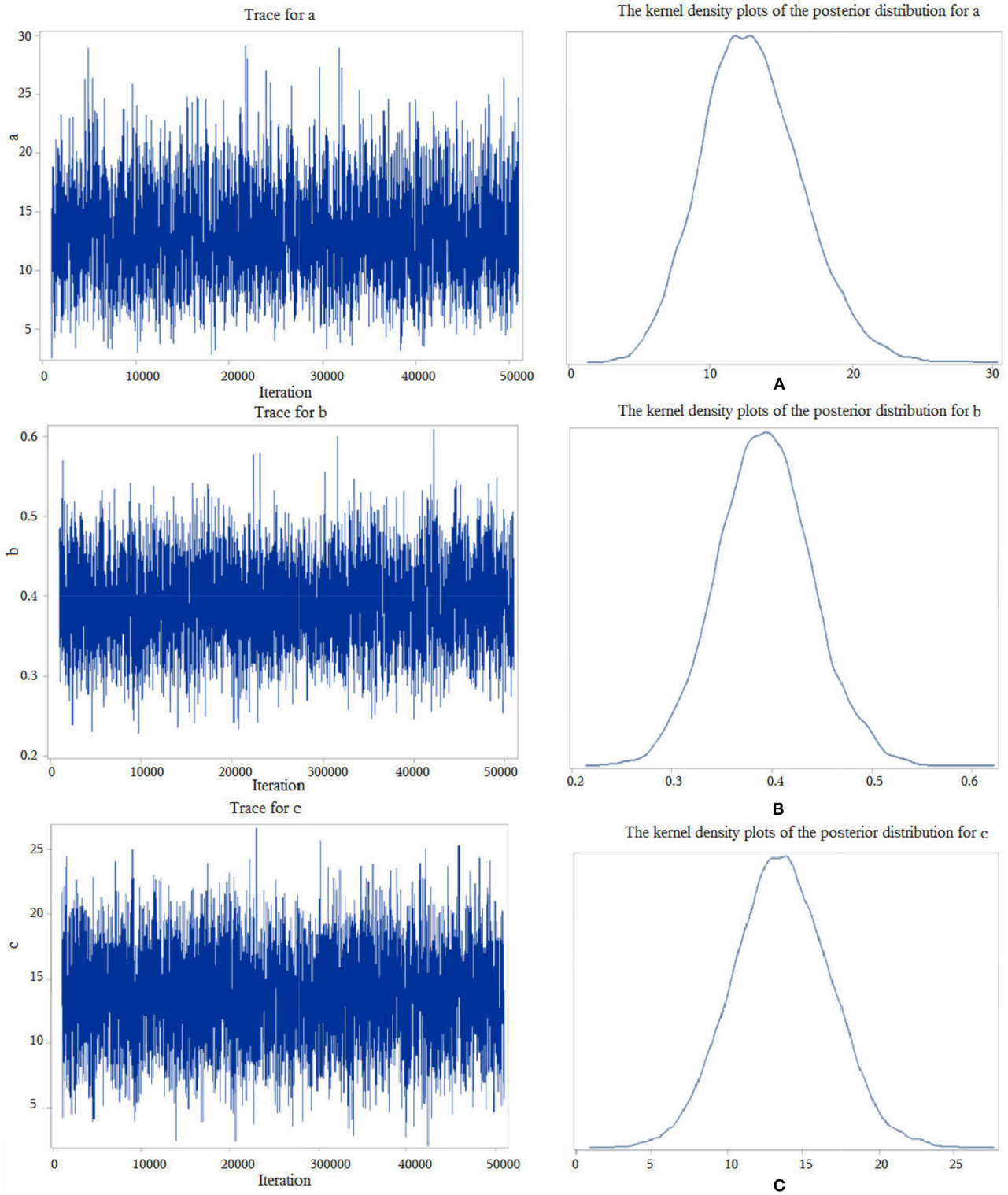

FIGURE 1 | Trace plots and kernel density plots of the posterior distribution for the parameters. "c" represents the total effect of the independent variable X on the dependent variable Y, "b" measures the relation between the mediator M and the dependent variable Y, adjusted for the independent variable X, and "a" measures the relation between X and M.

TABLE 4 | Parameter summary of Bayesian mediation analysis with SSP as mediator.

Parameter	*N*	Mean	Standard deviation	95% CI of highest posterior density	
				Lower	Upper
α	10,000	13.01	3.50	6.41	19.96
β	10,000	0.39	0.05	0.30	0.48
c	10,000	13.63	3.18	7.20	19.57
H	10,000	−0.19	0.04	−0.27	−0.12
αβ	10,000	5.11	1.51	2.50	8.41

"c" represents the total effect of independent variable X on the dependent variable Y, "αβ" represents the effect of X on Y, adjusted for the effect of the mediator M, "β" measures the relation between the mediator M and the dependent variable Y, adjusted for the independent variable X, and "α" measures the relation between X and M.
SSP, Short Sensory Profile.

by external factors, such as sleep education, sleep environment improvement, behavioral intervention, and drug treatment (such as exogenous melatonin) (28, 30). Therefore, the intervention on the sleep problems in autistic individuals should consider the treatment of the core symptoms and sleep problems (31). The current study examined the efficacy of the rTMS protocol (high frequency on the left + low frequency on the right) on bilateral DLPFC for both the core symptoms (such as social impairments, repetitive behaviors, and sensory abnormalities) and sleep problems in ASD youth. Our results revealed that both the core symptoms (such as tactile and auditory abnormalities, emotional, conduct, and social problems) and sleep problems improve significantly after two courses of intervention of rTMS. Our study provides new ideas for the intervention of sleep problems in ASD.

Previous studies have shown the efficacy of rTMS on either ASD symptoms, such as repetitive and stereotyped behaviors and social behavior (18, 32), or chronic primary insomnia, such as reducing sleep latency and increasing total sleep time and rapid eye movement latency (33, 34). However, the research on the treatment of the sleep problems of ASD individuals is far from enough. To our knowledge, the rTMS protocol for ASD comorbid sleep disturbances has not been reported yet. One of the reasons is that we lack the proper protocols for ASD symptoms and sleep problems—for example, most of the studies of rTMS for ASD symptoms chose the DLPFC for stimulating the location, but with varied protocols (35): for example, the high-frequency (10 Hz) stimulation on the left DLPFC only (36), the low-frequency rTMS on the left DLPFC (37), or the low-frequency stimulation on bilateral DLPFC (38, 39). Similarly, the rTMS protocols on sleep problems are also full of inconsistencies—for example, in terms of insomnia, most studies used the protocol of low frequency (1 Hz) on the right DLPFC (40); some researchers also chose the non-DLPFC targets to treat patients with sleep problems (41). In the current study, the high-frequency rTMS on the left DLPFC and the low-frequency rTMS on the right DLPFC worked for both ASD symptoms and sleep-related problems. The partial sharing pathogenesis of both the core symptoms and comorbid sleep problems of ASD offered the possibility of intervention of rTMS for both with the same protocol—for example, the cortical excitation/inhibition imbalances and the abnormal excitatory–inhibitory ratio in ASD patients (42, 43). The high-frequency rTMS stimulation of the left DLPFC could cause a long-term potentiation (LTP) of synaptic transmission in the stimulation area (44), and LTP could spread to the cortex and the subcortical neural network (45, 46), which led to the enhancement of excitability of the mirror neuron system in ASD patients, to improve the understanding of the social environment in ASD patients and enhance the ability of imitation (47). Thus, it finally improved the core symptoms of ASD patients. The high-frequency rTMS stimulation of the left DLPFC could also improve the sleep problems of ASD individuals by improving their arousal dysregulation and the dysregulation of the autonomic nervous system (28, 48, 49). In addition, the low-frequency rTMS of the right DLPFC could improve the core symptoms of ASD individuals by activating the inhibitory GABAergic double-bouquet interneurons to improve the excitation/inhibition balance of the prefrontal cortex in patients with ASD (50). In addition, the low-frequency rTMS on the right DLPFC could improve sleep by increasing the serum brain-derived neurotrophic factor and γ aminobutyric acid concentration and reducing motor-evoked potentials (18).

Similarly, the sharing abnormality of the gamma frequency oscillations probably interpreted the mediation effect of the sensory problems in ASD youth between the rTMS intervention and improvement of sleep. It is believed that the cortical gamma-band activity participates in a variety of mental activities, including sensory perception (51); at the same time, the gamma oscillation is the representation of the rhythm of wakefulness and sleep (52). The improvement of gamma oscillation seems to be the potential mechanism of the sensory profile of ASD children in the amelioration of their sleep disturbances by rTMS. The present findings offer a further understanding of the pathogenesis and therapeutic prospect of sleep problems in autistic patients.

There are some limitations in our study; we failed to access the sleep problems of autistic children with more objective measurements, such as by polysomnography (PSG), due to the severe discomfort caused by PSG measurement and the low tolerance of the autistic subjects. In addition, the sample size in the current research is relatively small, although the sample size calculated based on our previous study is enough. We did not try other rTMS protocols (different stimulation locations) due to the limitation of time and research funds. Future research on neuroimaging, such as functional magnetic resonance imaging or functional near-infrared spectroscopy, might extend the explanations of the abnormality of the DLPFC of ASD youth comorbid with sleep disturbances.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because their containing information in the data probably compromise the privacy of research participants. Requests to access the datasets should be directed to Xin Zhang, zhangxin@tmu.edu.cn.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

XZ and LG obtained funding, had full access to all the data in the study, and take responsibility for the integrity of the data and the accuracy of the data analysis. XZ, LG, and LT contributed to the conception and design of the study. XZ, LG, YH, Z-yQ, X-rS, and CW contributed to the acquisition, analysis, or interpretation of data. LG performed the statistical analysis. LG, LT, and XZ wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Effectiveness of Online-Delivered Project ImPACT for Children With ASD and Their Parents: A Pilot Study During the COVID-19 Pandemic

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Objective: During the COVID-19 pandemic, face-to-face intervention services for families of children with autism spectrum disorder (ASD) were limited. This study aimed to evaluate the effectiveness of an 8-week, online-delivered Project ImPACT program for children with ASD and their parents in China during the COVID-19 pandemic.

Methods: A pilot non-randomized study with a waitlist control group was conducted in 68 children with ASD and their parents in the Department of Developmental and Behavioral Pediatrics between April 15, 2020 and March 19, 2021. Participants were allocated to either the intervention (IG) or the waitlist group (WLG) according to their order of recruitment. Parents in the IG immediately received 8 weeks of the online-delivered Project ImPACT program, and the WLG received the same program with a delay when the IG had completed all sessions. Participants in both groups received treatment as usual during the research period.

Results: The online-delivered Project ImPACT program significantly improved the parent-reported social communication skills of children with ASD. Furthermore, parent's involvement in the training program produced a collateral reduction in parenting stress and an increase in perceived competence in the parental role. Parents rated the program acceptable in terms of curriculum schedule, session content, homework assignments, and therapist feedback.

Conclusions: The 8-week, online-delivered Project ImPACT program is a feasible and effective social skill training program for families of children with ASD in China during the COVID-19 pandemic. Due to the methodological limitations, randomized controlled studies with larger sample sizes are suggested to provide more solid evidence.

Keywords: autism spectrum disorder, parent-mediated intervention, e-health, social communication, parenting stress

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INTRODUCTION

Autism spectrum disorder (ASD) is a lifelong neurodevelopmental disability that is characterized by impairments in social communication and interaction as well as the presence of restricted interests and stereotyped behaviors (1). Currently, the prevalence of ASD is estimated to be 18.5 per 1,000 among children aged 8 years (2). Deficits in social communication and interaction are the most commonly reported red flags and significant core symptoms of ASD (1). These core impairments not only disrupt children's ability to develop and maintain social relationships but also impact their long-term outcomes (3, 4). Moreover, the difficulties and challenges that accompany the child's social impairment can lead to considerable parenting stress and caregiving burden, which further disrupts parental psychological wellbeing and quality of life (5). Therefore, it is crucial for early intervention services to target social skill training for children with ASD.

Best practice recommends at least 25 h per week of comprehensive interventions for children with ASD (6), but many families fail to meet this standard due to the shortage of quality services and well-trained professionals (7, 8) in China, especially some rural region. Parent-mediated intervention (PMI) is a cost-effective model to fill the existing gap. It is widely believed that PMIs can enhance many aspects of children's social development, such as social communication, adaptive behaviors, cognition, and language (9, 10). Furthermore, the involvement of parents contributes to the increase in parental self-efficacy and reduction in parenting stress (11, 12). Traditional PMIs are delivered on a face-to-face basis and parents have to travel a long distance to clinical centers, which is restricted by limited medical resources, considerable time consumption, and geographical barriers (13). Furthermore, these restrictions have become even more pressing during the COVID-19 pandemic because face-to-face intervention services for children with ASD were interrupted in most areas of China. Telehealth is a promising model of service delivery, which allows medical care services to be delivered remotely and efficiently (14). There are many advantages to remote interventions, including low cost, high reach of the target population, diverse teaching resources, and standardized instructions with high fidelity (13). Existing evidence suggests that telehealth intervention programs are highly acceptable, comparable to face-to-face interventions, and effective for the outcomes of both the implementer and the child (15). Telehealth has been successfully implemented in the care of many other chronic diseases such as dementia care (16) and psychotherapy (17). In the early assessment, diagnosis and intervention for children with ASD, telemedicine also showed satisfactory effects (18, 19).

Among the various PMI programs, Project Improve Parents as Communication Teacher (ImPACT) is an evidence-based, parent-mediated, social skill training program developed by Brooke Ingersoll and Anna Dvortcsak (20, 21). It is designed for young children with ASD and helps parents to encourage the development of children's social communication skills (e.g., social engagement, communication, imitation, and play) during daily activities and routines (20, 21). The initial feasibility and efficacy of the Project ImPACT program have been examined in previous studies (22–26). Specifically, improvements in the spontaneous use of language targets and social communication skills were observed in children with ASD (22, 24). In addition, parents exhibited a decrease in parenting stress (23), an increase in parental self-efficacy, and a positive perception toward their children (26). The feasibility, acceptability, and usability of the program were highly favorable among the target population (23).

Despite promising evidence for the effectiveness of the Project ImPACT program, most studies were conducted in developed countries. The small sample sizes and lack of positive control groups limit the generalizability of the conclusions. Furthermore, it remains to be studied whether the Project ImPACT program delivered remotely also positively affects autistic children and their parents. To enrich early intervention services and address new challenges of the global health crisis, we adapted the Project ImPACT program and delivered it remotely. This pilot waitlist control study was a preliminary examination of the effectiveness of an 8-week, online-delivered Project ImPACT program for families of children with ASD in China during the COVID-19 Pandemic. We hypothesized that the online-delivered Project ImPACT program will reduce the severity of social impairment for children with ASD, improve parental self-efficacy, and reduce parenting stress. Therefore, the primary aim of this study was to identify the effect of the online-delivered Project ImPACT program on children's social communication skills, and the secondary aim was to explore whether the online-delivered Project ImPACT program increases parental self-efficacy and reduces parenting stress.

METHODS

Study Design and Setting

A pilot non-randomized study with a waitlist control group was conducted in Shanghai, China. Participants were recruited in the Department of Developmental and Behavioral Pediatrics in Xinhua Hospital between April 15, 2020 and March 19, 2021. The study was reviewed and approved by the Institutional Review Board of Xinhua Hospital (No: XHEC-C-2019-076). Informed consent was obtained from all parents after they were told about the study procedures and agreed to participate. Participation was entirely voluntary, and all participants had the right to withdraw at any time.

Participants

The present study recruited parent-child dyads. Participants were eligible if (a) children had a diagnosis of ASD according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V); (b) children were aged 2–6 years; (c) parents were aged 20–50 years; (d) parents had the internet access to online sessions and surveys; and (e) parents were living with the diagnosed children during the intervention period. Participants were excluded if (a) children were diagnosed with other mental disorders, physical diseases, or serious genetic abnormalities (e.g., traumatic brain injury and epilepsy requiring additional medical treatments); (b) parents were diagnosed with other mental diseases that may affect their participation (e.g.,

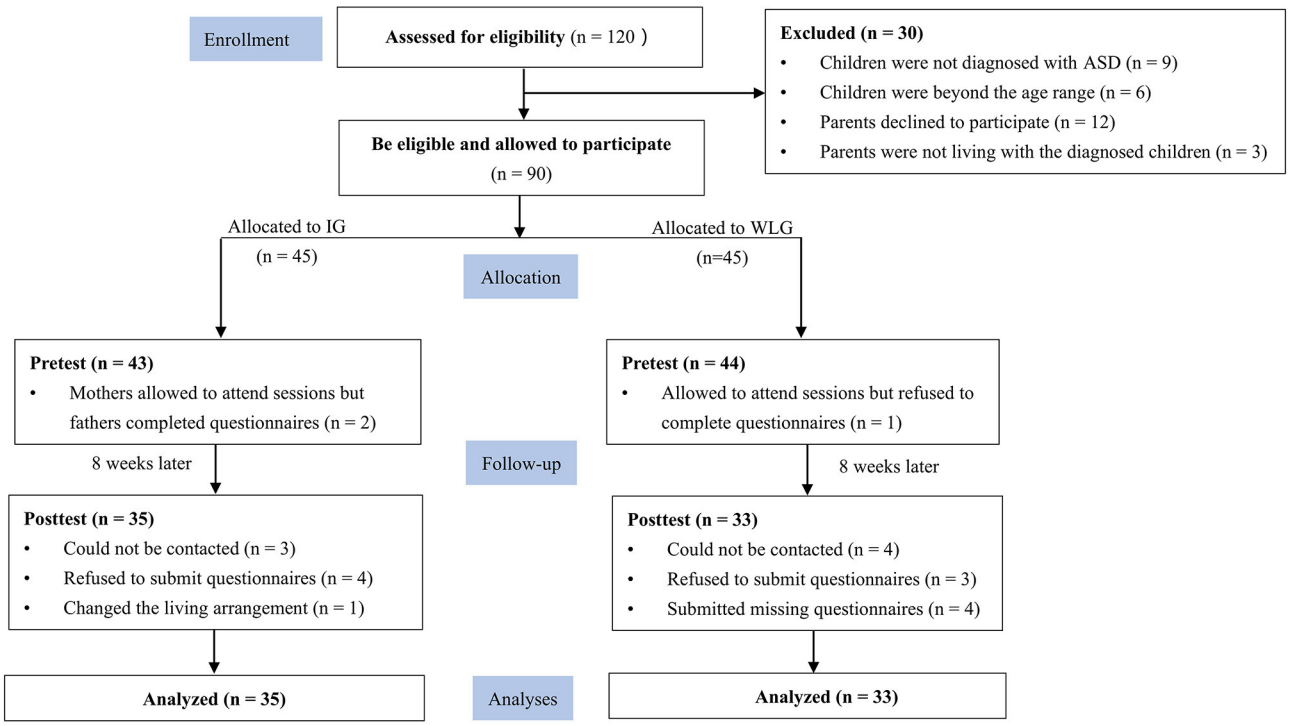

FIGURE 1 | CONSORT diagram of the study flow. CONSORT, Consolidated Standards of Reporting Trials; ASD, autism spectrum disorder; IG, intervention group; WLG, waitlist group.

depression and dyslexia); (c) parents were non-biological parents; and (d) parents had more than one child diagnosed with ASD. The participant flowchart is presented in **Figure 1**.

An independent-samples *t*-test in the PASS 15.0 Software (NCSS Corp, Utah) was used to calculate the sample size. Based on a previous study (23), we assumed that the primary outcome (Social Responsiveness Scale, SRS) at baseline was comparable between the intervention and control groups. The posttest mean (M) score on the SRS was 67.5 for the intervention group and 77.78 for the control group, with an equal standard deviation (SD) of 14, power of 0.8, an alpha of 0.05, two-tailed, and a medium effect size (Cohen's $d = 0.5$). Thus, a total of 62 participants (31 per group) were needed to obtain statistical significance. We estimated an attrition rate of 20%, and finally recruited 90 participants.

Procedures

Children's ASD diagnoses were made by developmental and behavioral pediatricians according to the DSM-V. Before being accepted into the study, ASD diagnoses were further verified by psychologists using the Childhood Autism Rating Scale (CARS) (27) and the Autism Diagnosis and Observation Scale (ADOS) (28). Recruitment leaflets that contained a detailed description of our research protocol were distributed to parents whose children had a confirmed ASD and who had registered for Project ImPACT ($n = 120$). The eligible participants who agreed to participate were recruited ($n = 90$). According to the order of enrolment, eligible parent-child dyads were allocated to either the intervention (IG; $n = 45$) or waitlist group (WLG; $n = 45$). Parents in the IG immediately received the online-delivered Project ImPACT program, and the WLG received the same program with a delay when the IG had completed all the sessions. Participants in both groups received treatment as usual during the research period. Links to online surveys were sent to all parents shortly before the beginning of the first session (T1) and 8 weeks later (T2). Those who did not complete the pretest questionnaires were not invited to submit the posttest questionnaires. The research assistant conducted the T1 and T2 assessments, and each lasted ∼40 min. Clinicians, psychologists, and therapists were blinded to treatment allocation.

Intervention Program

Given the long waitlist for early intervention services in China and the restrictions on face-to-face intervention services during the COVID-19 pandemic, some cultural adaptations to the Project ImPACT program were made to fit with the domestic medical practice. First, the original 12 weeks of sessions with 1 h per week were adapted into 8 weeks of 1.5-h sessions. Second, all sessions were delivered remotely in real-time. Third, group coaching was adopted (usually 8–10 parents per group) and WeChat group was created for on-line answer between sessions. Parents in the IG immediately received the online-delivered Project ImPACT program, with one session per week and 8 sessions in total. Each session was delivered through Tencent

Meeting Application (Tencent Corp, Shanghai) and lasted ∼1.5 h. To maintain therapist fidelity across different groups, all sessions were delivered by a certified psychological therapist from our research team, who received intensive training from the intervention developer through remote training. Session delivery was supervised by a senior administrator regularly. Parents in each group were managed in separate WeChat groups, in which an administrator, therapist, quality control supervisor, and research assistant were allocated. The training manual (Chinese version) was mailed to parents 1 week before the first session began. During the intervention period, parents in the WLG did not receive the Project ImPACT program until parents in the IG had finished all of the sessions. Parents and children in both groups received treatment as usual, which included various intervention services available in the communities.

The first session primarily involved goal setting and arrangement of the home environment. The second to eighth sessions began with a review of the content from the previous session, followed by an examination and evaluation of homework, didactic instruction of new strategies, a summary of the session, homework assignments, and finally ended with a question and answer (Q&A) section. The detailed contents of the 8-week Project ImPACT sessions are outlined in **Supplementary Table S1**. Before each session, the research assistant reminded parents to preview the corresponding chapters, and a live link to the cloud conference room was generated using the Tencent Meeting Application. Link was sent to the WeChat groups, and parents could attend the sessions by clicking the link. Between sessions, parents were allowed to ask course-related questions and upload video clips of parent-child interactions in the WeChat groups. The therapist answered their questions, watched the home videos to detect any areas for improvement and provided individualized feedback to parents on a daily basis.

Measures

Demographic Questionnaire

General data on child and parent characteristics were collected before treatment allocation. Child characteristics included age, gender, only child status, language level, diagnosis subtype, and additional intervention services received. Parent characteristics included parental role, age, marital status, educational level, employment status and residence.

Social Responsiveness Scale

The Social Responsiveness Scale (SRS) is a parent-reported questionnaire that measures the severity of children's social deficits associated with ASD in natural social situations (29). It consists of 65 items that are divided into five dimensions: social awareness, social cognition, social communication, social motivation, and autistic mannerisms. Each item is rated on a 4-point Likert scale, and a higher score indicates greater severity of social impairment. The Cronbach's α coefficient for the SRS total scale was 0.94 and the subscales ranged from 0.55 to 0.85 at pretest. The corresponding Cronbach's α coefficients at posttest were 0.93 for the total scale and 0.56–0.87 for the subscales.

Autism Treatment Evaluation Checklist

The Autism Treatment Evaluation Checklist (ATEC) is a self-reported scale designed to assess the change in autism severity in intervention studies (30). It is composed of four subscales: language/speech/communication, sociability, sensory/cognitive awareness, and health/physical/ behavior. In this study, we adopted the first two subscales only, which mainly reflect the social function of children. Each item of the sociability subscale is rated on a 3-point Likert scale, with total scores ranging from 0 to 40. The items of the language/speech/communication subscale are reverse-scored, with higher scores indicating greater severity of autistic symptoms. In this study, the Cronbach's α coefficients at pretest were 0.95 for the language subscale and 0.90 for the sociability subscale. The corresponding Cronbach's α coefficients at posttest were 0.94 and 0.91.

Parenting Stress Index-Short Form

The Parenting Stress Index-Short Form (PSI-SF) is a 36-item self-reported questionnaire that measures stress related to parenting roles (31). It consists of three subscales: parental distress, parent-child dysfunctional interaction, and difficult child. Each subscale contains 12 items rated on a 5-point Likert scale. The PSI-SF yields a total score ranging from 36 to 180, with a higher score indicating a greater level of parenting stress. A score above 90 indicates a clinical level of stress, and a score of 90 or below represents a low level of stress. The Cronbach's α coefficients for the PSI-SF in this study indicated excellent internal consistency on both pretest and posttest measures (Pretest Cronbach's α coefficient was 0.94 and posttest Cronbach's α coefficient was 0.89).

Parental Self-Efficacy of Competence Scale

The Parental Self-efficacy of Competence Scale (PSOC) was developed by Gibaud-Wallston (32), which has been used widely in ASD-related research to assess parents' belief and confidence in fostering their child's development. It is a 17-item scale that consists of two dimensions: eight items in the efficacy subscale and nine items in the satisfaction subscale. Each item is rated on a 6-point Likert scale and items in the satisfaction subscale are reverse-scored. A higher score represents higher parental self-efficacy. A previous study validated the psychometric properties of the PSOC in Chinese mothers, which demonstrated good internal consistency (33). In this study, the Cronbach's α coefficients were acceptable at pretest (Efficacy: 0.72; Satisfaction: 0.73) and posttest (Efficacy: 0.75; Satisfaction: 0.76).

Data Analysis

The SPSS 24.0 software (IBM Corp; Armonk) was used for data analyses. The normality of the continuous variables was assessed using the Shapiro-Wilk test. Continuous variables that were normally distributed were expressed as means and standard deviations ($M \pm$ SD), and if not normally distributed, as medians and interquartile ranges (IQR). Categorical variables were presented as frequencies (N) and percentages (%). Independent-samples t-tests, chi-square tests, and Mann-Whitney U-tests were performed to compare the demographic characteristics between groups. Paired-samples t-tests were used to compare

TABLE 1 | Comparison of child and parent outcomes within groups.

Variables	IG ($n = 35$)		t_1	WLG ($n = 33$)		t_2
	Pretest	Posttest		Pretest	Posttest	
SRS						
Social awareness	11.54 ± 3.09	11.17 ± 2.42	0.83	11.85 ± 2.60	12.12 ± 3.24	−0.51
Social cognition	18.11 ± 4.05	16.69 ± 3.46	2.61*	17.39 ± 4.59	18.61 ± 4.92	−1.62
Social communication	33.11 ± 8.35	29.66 ± 8.50	3.10**	33.03 ± 8.65	32.42 ± 9.33	0.48
Social motivation	15.17 ± 4.88	13.49 ± 3.81	3.28**	14.94 ± 4.94	14.15 ± 4.47	1.10
Autistic mannerisms	14.49 ± 7.03	12.74 ± 5.19	1.99	12.91 ± 6.80	13.85 ± 6.18	−0.96
Total scores	92.43 ± 22.85	83.74 ± 18.3	3.56**	90.12 ± 23.63	91.15 ± 24.74	−0.30
ATEC						
Language	13.06 ± 8.12	11.63 ± 7.59	3.43**	13.23 ± 6.70	12.70 ± 7.32	0.68
Sociability	18.03 ± 7.68	13.63 ± 6.82	5.04**	19.41 ± 6.73	16.85 ± 7.46	2.20*
Total scores	31.09 ± 12.37	25.26 ± 12.16	5.36**	32.64 ± 11.18	29.55 ± 11.92	2.20*
PSI-SF						
PD	35.77 ± 8.18	33.17 ± 7.39	2.54*	34.64 ± 11.02	32.97 ± 10.34	1.12
PCDI	31.49 ± 9.10	28.03 ± 6.45	3.50**	28.85 ± 6.55	28.82 ± 7.22	0.04
DC	35.80 ± 8.54	32.31 ± 6.18	3.41**	35.91 ± 7.48	36.18 ± 8.32	−0.28
Total scores	103.06 ± 22.21	93.51 ± 16.84	4.25**	99.39 ± 19.34	97.97 ± 21.09	0.60
PSOC						
Satisfaction	31.00 ± 6.05	30.54 ± 6.02	0.56	32.73 ± 8.04	31.79 ± 7.13	0.68
Efficacy	27.66 ± 6.30	30.49 ± 4.06	−2.51*	29.39 ± 5.76	29.85 ± 5.47	−0.49
Total scores	58.66 ± 9.78	61.03 ± 7.45	−1.93	62.12 ± 7.47	61.64 ± 5.25	0.33

IG, intervention group; WLG, waitlist group; SRS, social responsiveness scale; ATEC, autism treatment evaluation checklist; PSI-SF, parenting stress index-short form; PD, parental distress; PCDI, parent-child dysfunctional interaction; DC, difficult child; PSOC, parental self-efficacy of competence scale; t_1, within group comparison for the IG; t_2, within group comparison for the WLG.

*$P < 0.05$.

**$P < 0.01$.

the differences between the pretest and posttest outcomes Group differences in outcome measures following the intervention program were explored using one-way analyses of covariance (ANCOVA), and η^2 was calculated to evaluate the effect size of these difference ($\eta^2 \geq 0.01$: small effect, $\eta^2 \geq 0.06$: medium effect, $\eta^2 \geq 0.14$: large effect) (34).

RESULTS

Sample Characteristics and Group Differences at Baseline

Among the initial 120 parent-child dyads assessed for eligibility, 90 eligible dyads were recruited, and 68 were included in the final analyses, which resulted in a retention rate of 75.6%. Of the 22 parent-child dyads excluded from the final analyses, seven parents could not be contacted, eight refused to submit questionnaires, six submitted unqualified questionnaires, and one changed their living arrangement. The mean age of the 68 children was 3.67 years (SD = 0.99). Seventy percent of the children were from one-child families, and the majority (82.4%) were boys. Nearly one-third of children (27.9%) were non-verbal, and exactly half spoke single words or phrases. The subtypes of diagnosis were autism for 65 children (95.6%) and pervasive developmental disorder not otherwise specified (PDD-NOS) for three children (4.4%). The mean CARS score for the total sample was 36.20 (SD = 3.11). Of these, 39 children (57.4%) had a mild-to-moderate level of symptoms, and the remaining 29 (42.6%) had a severe level of symptoms (i.e., a total score > 36 and a rating of ≥ 3 on at least five items). In regard to additional intervention services, 19.1% of children received no other services, and those remaining received either family (26.5%) or institutional interventions (54.4%). The 68 participating parents consisted of 56 mothers (82.4%) and 12 fathers (17.6%), of whom 66 (97.1%) were married, with a mean age of 34.12 years (SD = 4.71). The majority had a college degree or above (77.94%) and 70.59% of them were employed. Sixty-two (91.2%) parents lived in urban areas. There were no significant group differences in any of these child and parent characteristics between groups (**Supplementary Table S2**). Sample characteristics were also compared for the completers and non-completers, and no significant difference was detected (**Supplementary Tables S3**, **S4**).

Changes in Child and Parent Outcomes Within Groups

Following 8 weeks of intervention, there was a significant improvement in children's social communication skills [$t_{(34)} = 3.56$, $P = 0.001$], language [$t_{(34)} = 3.43$, $P = 0.002$], sociability

TABLE 2 | Analyses of covariance for child and parent variables.

Source	SS	df	MS	*F*	*P*	η2
Child's social impairment						
T1 SRS	15,678.01	1	15,678.01	66.64	<0.001	0.51
Group	1,353.91	1	1,353.91	5.76	**0.019**	0.08
Child's sociability						
T1 ATEC_sociability	493.29	1	493.29	18.15	<0.001	0.22
T1 SRS	168.62	1	168.62	6.21	0.015	0.09
Group	125.88	1	125.88	4.63	**0.035**	0.07
Parenting stress						
T1 PSI-SF	14,346.83	1	14,346.83	97.91	<0.001	0.60
Group	836.09	1	836.09	5.71	**0.020**	0.08
Parents' perceived competence in the parental role						
T1 PSOC_efficacy	262.10	1	262.10	13.93	<0.001	0.18
Group	95.30	1	95.30	5.06	**0.028**	0.07

SS, sum of squares; MS, mean square; SRS, social responsiveness scale; ATEC, autism treatment evaluation checklist; PSI-SF, parenting stress index-short form; PSOC, parental self-efficacy of competence scale; η2 ≥ 0.01, small effect; η2 ≥ 0.06, medium effect; η2 ≥ 0.14, large effect. P values in bold indicate a statistically significant difference (P < 0.05).

[$t_{(34)} = 5.04$, $P < 0.001$], parents' perceived competence in the parental role [$t_{(34)} = -2.51$, $P = 0.017$], and parenting stress [$t_{(34)} = 4.25$, $P < 0.001$], but the change in the parental satisfaction domain of the PSOC was not significant [$t_{(34)} = 0.56$, $P = 0.58$]. In contrast, children and parents in the WLG did not show significant improvements during the intervention period in terms of children's social communication skills [$t_{(32)} = -0.30$, $P = 0.76$], language [$t_{(32)} = 0.68$, $P = 0.50$], parenting stress [$t_{(32)} = 0.60$, $P = 0.55$] or parental self-efficacy [$t_{(32)} = 0.33$, $P = 0.74$], except for the sociability domain of the ATEC [$t_{(32)} = 2.20$, $P = 0.04$]. Results of the comparisons within groups are provided in the **Supplementary Material** (**Table 1**).

Differences in Child and Parent Outcomes Between Groups

Results of the ANCOVA showed that the baseline SRS score had a significant effect on children's social impairment at posttest [$F_{(1,65)} = 66.64$, $P < 0.001$, partial $\eta^2 = 0.51$], and there was also a significant effect of treatment allocation on children's social communication skills [$F_{(1,65)} = 5.76$, $P = 0.019$, partial $\eta^2 = 0.08$], where children in the IG showed greater improvement than those in the WLG (**Table 2**). Adjusted mean SRS scores at posttest in the IG and WLG were 83.0 and 91.94, respectively. When we examined the effect of intervention on the SRS subscales, we found a significant effect of treatment allocation on social cognition [$F_{(1,65)} = 8.09$, $P = 0.006$, partial $\eta^2 = 0.11$].

There was no significant effect of group allocation on children's language [$F_{(1,64)} = 1.02$, $P = 0.315$, partial $\eta^2 = 0.02$], which was mainly predicted by the language level at baseline [$F_{(1,64)} = 224.51$, $P < 0.001$, partial $\eta^2 = 0.78$]. For sociability, results showed that the baseline level of sociability [$F_{(1,64)} = 18.15$, $P < 0.001$, partial $\eta^2 = 0.22$] and SRS score [$F_{(1,64)} = 6.21$, $P = 0.015$, partial $\eta^2 = 0.09$] had a significant effect on posttest sociability. Furthermore, there was a significant effect of group allocation on post-intervention sociability [$F_{(1,64)} = 4.63$, $P = 0.035$, partial $\eta^2 = 0.07$], with children in the IG showing greater improvement than those in the WLG (**Table 2**). The adjusted sociability score of the IG was 13.85 after the intervention compared with 16.61 of the WLG.

The results of the ANCOVA demonstrated that parenting stress after the intervention was mainly predicted by the stress level at baseline [$F_{(1,65)} = 97.91$, $P < 0.001$, partial $\eta^2 = 0.60$]. Moreover, the effect of treatment allocation on parenting stress was also significant [$F_{(1,65)} = 5.71$, $P = 0.02$, partial $\eta^2 = 0.08$], where parents in the IG showed a lower level of stress after the intervention (**Table 2**). The adjusted PSI-SF score was 99.30 in the WLG, whereas that of the IG was 92.26 after the intervention. When we examined the effect of intervention on the subscales, we found a significant effect on the parent-child dysfunction interaction [$F_{(1,65)} = 4.67$, $P = 0.03$, partial $\eta^2 = 0.07$] and difficult child [$F_{(1,65)} = 9.30$, $P = 0.003$, partial $\eta^2 = 0.13$]. It should be noted that the proportion of parents with clinically significant stress in the IG decreased from 71.4 to 60%, whereas in the WLG, there was an increase from 69.7 to 72.7% during the research period.

We did not find a significant group difference in parents' satisfaction with the parenting role after the intervention [$F_{(1,65)} = 0.07$, $P = 0.79$, partial $\eta^2 = 0.001$], which was mainly predicted by the satisfaction level at baseline [$F_{(1,65)} = 27.59$, $P < 0.001$, partial $\eta^2 = 0.30$]. The perceived competence in the parental role at baseline had a significant effect on the posttest competence level [$F_{(1,64)} = 13.93$, $P < 0.001$, partial $\eta^2 = 0.18$]. In addition, there was a significant effect of group allocation on posttest competence [$F_{(1,64)} = 5.06$, $P = 0.028$, partial $\eta^2 = 0.07$], where parents in the IG showed greater improvement than those did in the WLG (**Table 2**).

Feasibility of the Online-Delivered Project ImPACT Program

Among the parents in the IG, 87.5% evaluated the schedule of sessions to be reasonably arranged, and 98.2% considered

the contents well prepared and precisely targeted. Three-quarters of parents reported that could deal with the homework between sessions and believed that the homework facilitated their mastery of strategies and techniques. Furthermore, 98.2% of parents reported that they received instant feedback from the therapist and that the feedback helped them solve problems.

DISCUSSION

This pilot waitlist control study evaluated the effectiveness of an 8-week, online-delivered Project ImPACT program for families of children with ASD in China during the COVID-19 pandemic. Findings from our study showed positive changes in child and parent outcomes following the intervention program. Consistent with our hypothesis, the online-delivered Project ImPACT program significantly improved child's social communication skills compared with that of the WLG, especially in the domains of social cognition. Moreover, this was further supported by the analysis of the sociability subscale of the ATEC. For parents, there was a significant reduction in parenting stress among those in the IG compared with their counterparts in the WLG, and the reduction was especially marked in the domains of parent-child dysfunctional interaction and difficult child. Furthermore, we found an obvious reduction in the proportion of parents with clinically significant stress in the IG. In contrast, the proportion of parents with clinically significant stress in the WLG showed a slight increase. Moreover, parents in the IG showed a significant improvement in perceived competence in the parental role, whereas that of the parents in the WLG remained relatively stable. There were no significant improvements in children's language or parents' satisfaction with the parenting role after the intervention. Parents rated the program highly in terms of curriculum schedule, session content, homework assignments, and therapist feedback. Taken together, these results provide preliminary evidence for the effectiveness of the online-delivered Project ImPACT program for families of children with ASD in China during the COVID-19 pandemic.

The pervasive impairment of social communication is one of the core symptoms for children with ASD, and the online-delivered Project ImPACT program specifically targets the critical domains of children's social development. As noted in a previous study (24), children who receive 12 weeks of the Project ImPACT program demonstrate significantly greater gains in social communication skills compared with those in the community. Although the intervention program in the current study was adapted to 8 sessions and delivered remotely, our results corresponded to those of the previous study. There are several potential reasons for the positive changes. First, *via* the Project ImPACT online training, parents consciously adjusted their communication strategies to interact with children more efficiently, and they tended to be more sensitive and responsive to their child's social cues, which increased the opportunities for meaningful parent-child interactions. Parents who act synchronously with their children's focus and intentions are more likely to enhance their children's social communication skills (35). Second, the improvement in children's social communication skills may be attributed to the active involvement of parents. Parents are usually the family members who spend the most time with children, and this is particularly true during the COVID-19 pandemic. Coaching parents to be the mediators of social skill interventions for children allows the training to be integrated into daily life and routine activities, which maximizes the exposure of children to a favorable communicative environment (36). Finally, all parents in our study were managed using WeChat groups, which provided a platform for reinforced learning and opportunities for booster training between sessions. Parents were permitted to ask questions and upload video clips of parent-child interactions to the groups, and the therapist offered feedback on an individual basis. The support and supervision from professionals may motivate parents to constantly engage in practicing social interactions with their children.

We also observed a significant reduction in parenting stress and an increase in perceived competence in the parental role following the intervention. It is well established that parents of children with ASD experience an elevated level of stress compared with those of typically developing children and children with other developmental disabilities (37, 38). Moreover, these parents seldom obtain emotional reciprocity with their children during daily interactions; thus, they are susceptible to emotional frustration and low parenting self-efficacy (39). As an enabling process, the Project ImPACT program equipped parents with strategies and techniques for social communication, and parents were empowered to flexibly and effectively interact with their children during daily activities (22, 24, 26), which increased parents' confidence in parental tasks and reduced the pressure on parental roles. The Project ImPACT program delivered online encouraged parents to utilize various resources and maximize multimedia functions (e.g., interactive learning, group discussion, video telephone, and online Q&A). Additionally, parents could share disease-related information, parenting experience, and emotional distress in the WeChat group. The cultivation of a peer support network among parents may facilitate the improvement of parental psychological wellbeing and increase their confidence in the parental role (40).

Although we detected a significant effect of the online-delivered Project ImPACT program on children's social communication skills and parenting stress, it is worth noting that the level of social impairment at baseline was also a significant predictor of social communication skills after the 8 weeks of intervention. Similarly, baseline parenting stress also positively predicted post-intervention stress levels. Following the intervention program, children's language and parents' satisfaction with the parenting role did not show a significant difference in either group. Two implications are informed for future course optimization: (1) professionals should pay special attention to children with initially severe social impairment or a low language level, who may benefit more from customized sessions with a longer duration and booster training and (2) some parent support programs aimed at stress

management and psychological adjustment can be integrated into current sessions.

Parent feedback showed an optimistic attitude toward the online-delivered and group-based Project ImpACT program. Families of children with ASD in our outpatient department come from across the country, and more than half of our study cohort (62.38%) are living outside of Shanghai. Parents of children with ASD tend to be young and thus are receptive to new delivery models of medical services. In addition, there is a long waitlist for early intervention program. All these characteristics laid the foundation for cultural adaptation of Project ImPACT program. We introduced Project ImPACT and delivered remotely in a group format. The Project ImPACT program delivered online allowed for maximum inclusion of families who have limited access to on-site training programs. In fact, distance training for parents of children with ASD has increased in recent years, particularly during the COVID-19 pandemic. Parent training delivered remotely has the potentials to enhance parental knowledge, increase parent intervention fidelity, and improve the social behaviors and communication skills of children with ASD (19). The use of telehealth programs to train parents of children with ASD in evidence-based intervention techniques is a promising area of research in future studies. In addition, we adopted group coaching due to the long waitlist and limited early intervention services. It is a highly recommended practice in resource-limited areas, because sessions delivered in a group format is not only cost and time effective, but also facilitate the building support network among parents.

LIMITATIONS

Although the online-delivered Project ImPACT program show preliminary effectiveness for children with ASD and their parents, parents were allowed to access usual care in the community for ethical consideration, which makes it necessary to be cautious of the conclusion. Meanwhile, there are several limitations to this study. First, a lack of randomization in treatment allocation and the relatively large rate of attrition weakened the methodological quality of the study. Second, the intervention period lasted 8 weeks, and no long-term follow-up data were collected; therefore, we could not evaluate maintenance effects. For future studies, randomized controlled studies with larger sample sizes and longer follow-up periods are recommended to provide more solid conclusions for the program. Third, the outcome measures in this study were parent-reported. To avoid reporter bias, more reliable measures (e.g., video coding, clinical interviews, and physical assessments) were recommended in future studies. Finally, we did not collect information on parents' implementation of techniques at home (e.g., fidelity, facilitators, and barriers) and the details of parental usage of online Q&A (e.g., video unloading and therapist feedback). Thus, qualitative studies may offer a deeper insight into parental perceptions of the program and the process of changes from a different perspective.

CONCLUSIONS

The 8-week, online-delivered Project ImPACT program is an effective intervention program for families of children with ASD in China during the COVID-19 pandemic. It has the potential to improve children's social impairment, alleviate parenting stress, and increase parents' perceived competence in the parental role. Given the scarcity of early intervention services for children with ASD in low-resource areas, the Project ImPACT program delivered online may be a promising program to promote intervention accessibility and improve health outcomes for children with ASD and their parents.

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 Institutional Review Board of Xinhua Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FēL was responsible for the design of research, collection and analyses of data, and drafting of the manuscript. DW was responsible for the delivery of all sessions and online answer in the WeChat group. FR, LS, MXue, and JY were involved in the organization and arrangement of sessions. FeL and LJ provided guidance on the design of the study and supervised the implementation of the research. MXu was in charge of the quality control of session delivery and coordinated between our research team and the Project ImPACT developers. YT, LZha, XL, and MT were involved in participant recruitment, data preparation, statistical analyses, and manuscript revision. LZho was responsible for the language quality 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/fpsyt.2022.806149/full#supplementary-material

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Empathy, Theory of Mind, and Prosocial Behaviors in Autistic Children

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Background: Previous research has suggested that children with autism spectrum disorder (ASD) display fewer prosocial behaviors, and the role of empathy or Theory of Mind (ToM) in prosocial behaviors of autistic children remains unclear.

Methods: Data were obtained from an ongoing longitudinal study in Guangzhou, China. A total of 96 autistic children and 167 typically developing (TD) children were enrolled. Prosocial behaviors were assessed using a subscale of the Strength and Difficulties Questionnaire and Dictator Game (DG) paradigm with stickers as incentives. Empathic traits and ToM ability were measured using the children's Empathy Quotient and the Chinese version of ToM toolkit. Generalized linear models were used to assess the differences of prosocial behaviors and empathic traits, ToM ability between the two groups and the associations between empathic traits, ToM ability and prosocial behaviors in autistic children.

Results: Compared with TD children, autistic children exhibited worse ToM ability and performed less pro-socially in the DG paradigm, while there were no differences regarding empathic traits. In autistic children, empathic traits especially affective empathy, were positively associated with parent-reported prosocial behaviors [$\beta = 0.17$, 95% confidence interval (CI): 0.07–0.27; $\beta = 0.47$, 95%CI: 0.33–0.60]. ToM ability was associated with DG paradigm ($\beta = 1.03$, 95%CI: 0.16–1.89).

Conclusion: Autistic children showed less pro-sociality and ToM ability than TD children. In autistic children, empathic trait was associated with parent-reported prosocial behaviors while their ToM ability was associated with prosocial behaviors in experimental condition. Our findings indicated that better ToM ability and empathic trait might promote prosocial behaviors in autistic children.

Keywords: autism spectrum disorder, prosocial behavior, empathy, Theory of Mind, Dictator Game

INTRODUCTION

Prosocial behaviors, which refer to actions that one engages in to benefit others, like helping, sharing, and comforting, is often believed to be the basis of human relationships (1). Children with autism spectrum disorder (ASD) might display fewer prosocial behaviors compared with typically developing (TD) children which are likely due to the social-communication deficits associated with an ASD diagnosis (2). For example, school-aged autistic children presented fewer prosocial behaviors in daily life than typically developing (TD) children when using parent- or teacher-reported rating scales (e.g., the Strength and Difficulties Questionnaire) (3, 4). However, in experimental settings, several studies have found that preschool autistic children do show helping behaviors (5, 6). In resource allocation tasks [e.g., Dictator Game (DG) and Ultimatum Game (UG)], autistic children showed a higher preference for equality than self-interest compared to TD children (7–10); but they tended to accept unfair offers (7, 10). Different measures might identify different situation of prosocial behaviors, resource allocation tasks such as DG is believed to be powerful to illuminate individuals' social interactions because it could examine the extent to which individuals attain their own goals while simultaneously displaying altruistic behavior toward unrelated individuals. However, directly measure of prosocial behavior in specific experimental environment may be lack of ecological validity (11). Therefore, it is particularly important to evaluate prosocial behaviors from different dimensions. In China, the study of prosocial behaviors in autistic children is limited and more studies are needed.

Empathy and Theory of Mind (ToM) are generally considered to be the major determinants of prosocial behaviors (12, 13). Empathy has been described as the ability to infer and share the emotional experiences with another (14), and consists of two components (15): the ability to understand the emotional state of others and distinguish another's feelings from one's own (i.e., cognitive empathy), and the ability to vicariously experience of the emotional experiences of others and respond emotionally in an appropriate way (i.e., affective empathy) (15). ToM, on the other hand, has been referred to as the attribution of mental states, such as emotion, desires, intentions and beliefs to others (16, 17). Given the theoretical similarities, cognitive empathy refers to a complex cognitive capacity, largely overlapping with ToM ability (12, 15). However, increasing studies have argued that ToM ability differs from empathy because ToM does not denote a sharing of another person's affective states, but rather a cognitive understanding of another person's mental states (18) (**Supplementary Table S1** in **Appendix A**). ToM deficits have been considered as one of the major features in autistic children (19, 20). Studies have also shown impaired cognitive empathy but intact affective empathy in autistic children (21–24). Specifically, previous studies also indicated that there might be different roles of empathy or ToM ability on prosocial behaviors in children. Numerous researches have shown that prosocial behaviors, such as helping, sharing and comforting, are associated with preschool- and school-aged children's disposition to empathize with others (25–30). A meta-analysis of 6,432 children (2–12 years) revealed that children with advanced ToM abilities were more likely to show prosocial behaviors (r = 0.19), especially cooperating behaviors (r = 0.24) (13). When studying prosocial behaviors in relation to empathy and ToM simultaneously, Abrams et al. (31) found that empathy was positively associated with prosocial behaviors, but the association between ToM and prosocial behaviors was only shown in the non-competitive situation. Longobardi et al. (32) found that both empathic concern and ToM had direct positive effects on prosocial behaviors in primary school children. However, few studies focus on the associations between empathy, ToM and prosocial behaviors in autistic children with inconsistent results. For instance, a study of 20 school-aged autistic children and 20 language-age matched counterparts found that affective empathy was strongly related to peer interaction and prosocial behaviors (helping and sharing) in school-aged autistic children (33), while other studies found no associations between empathic responses and prosocial behaviors in preschool-aged and school-aged autistic children (34, 35). These earlier studies of autistic children with relatively small sample sizes only consider the role of empathy and ToM ability in prosocial behaviors separately, and to our knowledge, no studies have considered the role of empathy and ToM ability in prosocial behaviors simultaneously or defined them distinctly. In this study, we measured empathy and ToM simultaneously and evaluated prosocial behaviors via both parent-reported rating scales and experiments in autistic children and TD children in mainland China. We aimed to investigate the role of empathy or ToM ability in prosocial behaviors between these two groups.

METHODS

Participants

We used the baseline data obtained from an ongoing study "the Guangzhou Longitudinal Study of Autistic Children" examining the developmental trajectories of 6- to 12-year-olds autistic children in Guangzhou, China. The participants were recruited between April, 2017 and February, 2020 from the Research Center of Children and Adolescent Psychological and Behavioral Development. Participants were included if they had a historical diagnosis of ASD which was confirmed by a combination of the Childhood Autism Rating Scale (CARS) and an expert clinician. Two professional child psychiatrists (Jin Jing and Xiu-Hong Li) further confirmed their diagnosis using Diagnostic and Statistical Manual of Mental Disorders, Fifth Revision (DSM-5) criteria. We also recruited a group of TD children at the same time, and the additional inclusion criteria for both groups were as follows: (1) chronological age between 6 years 0 months and 12 years 0 months; (2) voluntarily participation of the children's parents; (3) absence of dyslexia, attention deficit and hyperactivity disorder, emotional disorder and other disorders those would interfere with social ability; and absence of seizures, head trauma, cerebral palsy, or other movement disorders that would interfere with study assessments; and (4) absence of known genetic or chromosomal abnormalities or severe visual or hearing impairment. Only one child per family was recruited to ensure the independence of observations. If two or more

children from one family were in the eligible age, we included the firstborn child (36). A total of $n = 209$ autistic children and $n = 170$ TD children were included in this longitudinal cohort. In this study, we selected a subsample of $n = 96$ autistic children (83 boys and 13 girls) and $n = 167$ TD children (97 boys and 70 girls) with complete questionnaire data, who were able to understand the instructions and able to complete all the behavioral assessments (detail of the inclusion flowchart was shown in **Supplementary Figure S1** in **Appendix A** and comparison of demographic characteristics of included and excluded children was shown in **Supplementary Table S2** in **Appendix A**).

MEASURES

Assessment of Prosocial Behaviors, Empathic Traits and Theory of Mind

Children's prosocial behaviors were evaluated via parent-reported rating scales and face-to-face experiments. The rating scale we used was the subscale of prosocial behavior in the Chinese version of the Strengths and Difficulties Questionnaire (SDQ). The subscale has five items rated on a 3-point Likert scale (0 = not true, 1 = somewhat true, 2 = certainly true). The total score of the five items ranges from 0 to 10 (higher scores represent more prosocial behaviors). The Chinese version of SDQ has been validated with a Cronbach's α of 0.73 for total score and 0.65 for subscale of prosocial behaviors (37).

Children's prosocial behaviors were also assessed using the Dictator Game (DG) paradigm (38), which has been validated in TD children aged 3–11 years in our previous study (39). In the DG paradigm, the dictator is given a windfall resource to allocate between him/herself and the virtual recipient who has no right to reject the offer. We arranged three settings to exclude factors that may affect children's decisions: (1) to avoid the influence of social distance, we set up a virtual anonymous character with the same gender and age as the participant (40); (2) to eliminate repeated interactions that might affect participant's willingness to share (i.e., in the repeated interaction settings, individuls may change his/her willingness to share by looking forward to the next round of feedback from the partner), we set up a one-shot interaction (40, 41); (3) to decrease the reputation effect caused by bystanders, we left the child alone in the room when he/she made decisions (42). We used stickers as allocated resources and prosocial behavior was measured based on the number of stickers that children shared and the decision to share or not. We also asked the children about their preference regarding the stickers by scores from 0 to 10.

The children's Empathy Questionnaire (EQ-C) was used to assess empathic traits. It has 27 items (13 reverse-scored items) rated on a 3-point scale: 2 = strongly agree, 1 = agree, 0 = disagree/strongly disagree (43). The Taiwan version was revised to 20 items (8 reverse-scored items) with three subscales of affective empathy, cognitive empathy and disrupt behaviors (44). We calculated the total score for all items and subscale scores for affective and cognitive empathy, and higher scores denoted stronger traits. The EQ-C and subscales have been validated with a Cronbach's α of 0.84, 0.69, 0.79, and 0.74 (44) (The detail of Taiwan version of EQ-C is shown in **Supplementary Table S3** in **Appendix A**).

We used a set of ToM tasks which were adapted for use in China and have previously shown a Cronbach's α of 0.75 (45). Three subtasks were included: emotion attribution task, unexpected content task (46), and Sally-Anne task (47). In each subtask, children were marked with a pass when they gave the correct answer. We defined passing the ToM tasks when the children gave correct answers for all the three subtasks.

The detailed materials of the DG paradigm and the ToM tasks are provided in **Appendix B** of the **Supplementary Material**.

Assessment of Covariates

Demographic information about children's age, gender, and being intervened formerly and/or currently, maternal age and education level, and per capita monthly household income was obtained via questionnaires.

Intelligence quotient (IQ) was assessed via the Chinese version of Wechsler Intelligence Scale for children, fourth edition (WISC-IV), which is suitable for children aged 6 years to 16 years 11 months. WISC-IV provides a full scale intelligence quotient (FSIQ) based on the sum of scores from the 10 core subtests, as well as four index scores: Verbal Comprehension Index, Perceptual Reasoning Index, Working Memory Index, and Processing Speed Index (48).

Severity of ASD symptoms was assessed via the Chinese Social Response Scale (SRS) which is a 65-item questionnaire used for children between 4 to 18 years. Each item is scored on a Likert scale ranging from 1 (not true) to 4 (almost always true). Total score was calculated ranging from 0 to 195, and higher scores indicated severe ASD symptoms. Cronbach's α coefficient for total scale wase 0.90 (49).

Considering that altruistic sharing behaviors might be somewhat constrained by the child's ability to inhibit control (50), we also assessed executive function by the Chinese version of Behavior Rating Scale of Executive Function (BRIEF) for children aged 6 to 18 years. The BRIEF is parent reported with 86 items using responses as follow: Never, Sometimes, or Often, coded as 1, 2, or 3, respectively. Higher score indicated greater perceived impairment of executive function, the subscales and total score were calculated and standardized into Z-score with the Cronbach α coefficient ranged from 0.74 to 0.96 (51).

Procedure

Children underwent face-to-face measures performed by trained psychometrists, graduate students, or research assistants at the research center. Information on symptom severity, daily activities, executive functioning skills, and social cognitive abilities was obtained through in-person interviews with primary caregivers or validated tools/questionnaires. All the parents of the participants provided written consent. The study was approved by the Ethical Review Committee for Biomedical Research, Sun Yat-sen University (2015-No.29).

Statistical Analyses

We calculated means and standard deviations for continuous variables and percentages for categorical variables. We compared the basic information between autistic children and TD children using chi-square tests and *t*-tests. We analyzed the correlations between all variables used in this study by using Pearson's correlation coefficient for two continuous variables, Cramer's V for two binary variables and Point biserial correlation coefficient for continuous variable and binary variable (see **Supplementary Tables S4, S5** in **Appendix A** of supplement).

Generalized linear models were used to compare the differences of prosocial behaviors, empathic traits and ToM ability between the two groups, and to investigate the associations of empathic traits and ToM ability separately with prosocial behaviors in autistic children. We fitted crude models without any adjustments. We fitted adjusted models by adjusting for covariates including child's age, FSIQ, SRS total score, BRIEF total score, maternal age which were selected based on the correlation analyses. In order to eliminate the influence of sharing value due to the difference in sticker preference, we also adjusted for the degree of sticker preference when analyzing DG paradigm. Both empathic traits and ToM ability were entered into the same adjusted model to see whether they were independent of each other.

We conducted all statistical analyses with R 4.0.3 statistical software (R Core Team 2019). The results were presented as coefficient estimates (β) or odd ratios (OR) with a 95% confidence interval (CI). We considered a two-sided *P*-value < 0.05 as statistically significant.

RESULTS

The characteristics of the children were shown in **Table 1**. The mean age of the included autistic children was 7.4 ± 1.5 years and 86.5% of them were boy. Most of the autistic children were being intervened formerly and/or currently (72.9%). Compared with TD children, autistic children had lower FSIQ and scored higher on the SRS and BRIEF ($t = -11.03$, 18.99, and 9.50, all $P < 0.01$). Autistic children's mothers had low education levels with low household income compared with their counterparts ($\chi^2 = 9.71$ and 38.14, all $P < 0.01$). There were no differences between groups in child's age ($t = 1.53$, $P = 0.11$) or maternal age $t = 0.57$, $P = 0.56$).

As shown in **Figure 1** (detailed data shown in **Supplementary Table S6** in **Appendix A**), after adjusting for potential covariates, there were no significant difference in EQ-C total score and the subscale score of affective empathy and cognitive empathy between autistic children and TD children (10.8 ± 4.5 vs. 19.4 ± 6.2, 3.4 ± 2.6 vs. 6.5 ± 3.0, 2.4 ± 1.9 vs. 5.5 ± 2.7; $\beta = -1.96$, 95%CI: −4.05∼0.12, $P = 0.06$; $\beta = -0.52$, 95%CI: −0.27∼-0.58, $P = 0.40$; $\beta = -0.12$, 95%CI: −1.06∼0.82, $P = 0.80$). However, autistic children showed a lower passing rate in ToM toolkit compared to TD children (25.0% vs. 88.0%, $OR = 0.10$, 95%CI: 0.03–0.34, $P<0.01$). There was no significant difference between groups in the score of prosocial behaviors in SDQ (4.9 ± 2.0 vs. 6.5 ± 2.1, $\beta = 0.50$, 95%CI: −0.36∼1.36, $P = 0.25$). In DG paradigm, autistic children shared less stickers (1.7 ± 1.6 vs. 1.8 ± 1.4, $\beta = -0.78$, 95%CI: −1.42∼-0.13, $P = 0.02$) with a lower proportion of sharing (63.5% vs. 79.6%, $OR = 0.29$, 95%CI: 0.09–0.88, $P = 0.03$) than those in TD children, and the frequencies were shown in **Supplementary Table S7** in **Appendix A.**

Table 2 showed the associations between empathic traits, ToM ability and prosocial behaviors assessed by the SDQ subscale in autistic children. In the adjusted model, autistic children who had higher EQ-C total score and higher affective empathy score scored higher in the prosocial behavior subscale ($\beta = 0.17$, 95%CI: 0.07–0.27; $\beta = 0.47$, 95%CI: 0.33–0.60; all $P < 0.001$).

Table 3 showed the associations of empathic traits, ToM ability and prosocial behaviors assessed using the DG paradigm in autistic children. In the adjusted model, children who passed ToM toolkit shared more stickers compared to those who did not pass the tasks ($\beta = 1.03$, 95%CI: 0.16–1.89, $P = 0.02$). We did not observe significant associations of EQ-C total score and subscale score of affective or cognitive empathy with the number of shared stickers in autistic children.

The results were similar when both empathic traits and ToM ability were entered into the same adjusted model (**Supplementary Table S8**).

DISCUSSION

When using parent-reported measurements, we did not find differences in prosocial behaviors between autistic children and TD children; and empathic traits, especially affective empathy, was associated with prosocial behaviors in autistic children. However, we found poorer prosocial behaviors in autistic children than those in TD children when using experimental measurements, which were found to be associated with ToM ability.

Inconsistent results were previously obtained in three observational studies [one in Japan (3), two in the UK (4, 52)], indicating that parent-reported prosocial behaviors in 6–12 year-old autistic children were poorer than TD children. These earlier studies did not consider several important confounders (i.e., socio-economic and cognitive factors), while our study employed a large sample size with comprehensive information on these potential confounders. However, the inconsistency might have also resulted from cultural differences, such as culturally-specific norms and different socialization processes, thus affecting Chinese parents' evaluation of their children's prosocial behaviors (53). Unlike parent-reported measurements, we found that autistic children performed less pro-socially in experimental conditions. Similarly, several studies conducted in Western countries with small sample sizes found that autistic children can share things in resource allocation tasks (6, 10), but showed a higher preference for equality than self-interest compared to TD children (7, 54). In our study, prosocial behaviors measured

TABLE 1 | Demographic and Clinical characteristics of autistic children and TD children.

	ASD (*N* = 96) *N* (%)/Mean (SD)	TD (*N* = 167) *N* (%)/Mean (SD)	*P*-value[a]
Age	7.4 (1.5)	7.1 (1.2)	0.11
Gender			**<0.01**
Boy	83 (86.5)	97 (58.1)	
Girl	13 (13.5)	70 (41.9)	
FSIQ	90.1 (18.3)	113.3 (12.6)	**<0.01**
CARS total score	30.9 (3.4)	-	-
SRS total score	89.4 (19.8)	43.7 (16.8)	**<0.01**
BRIEF total score	64.9 (8.7)	54.2 (9.0)	**<0.01**
Being intervened formerly and/or currently			-
Yes	70 (72.9)	-	
No	26 (27.1)	-	
Maternal age	29.9 (3.8)	29.7 (3.4)	0.56
Maternal education level			**<0.01**
Low (primary, secondary, high school)	44 (45.8)	45 (26.9)	
High (university and above)	52 (54.2)	122 (73.1)	
Per capita monthly household income			**<0.01**
Low (<¥8,000)	57 (59.4)	36 (21.6)	
High (≥¥8,000)	39 (40.6)	131 (78.4)	

ASD, Autism spectrum disorder; TD, Typically developing; SD, Standard deviation; FSIQ, Full-Scale Intelligence Quotient; CARS, Childhood autism rating scale; SRS, Social Response Scale; BRIEF, Behavior Rating Scale of Executive Function.

[a]*The t-tests was used for the comparison of continuous variable while chi-square tests was used for the comparison of categorical variable.*

The bold values meant the P -value was statistically significant.

by the DG paradigm is a form of strong reciprocity, which is a behavior that people may increase the fitness of unrelated individuals at a cost to themselves. This kind of prosocial behaviors is crucial for humans to establish cooperation (55, 56). In this paradigm, advanced social cognitive functions interact with others and an understanding of social norms are required when participants display prosocial behavior toward unrelated individuals (11, 55). Our results indicated that experimental measurements might be able to capture behavioral characteristics of prosocial behaviors in autistic children, and future studies should confirm our findings by using both parent-reported and experimental measurements.

When studying ToM ability and empathic traits simultaneously, we found that autistic children exhibited worse ToM ability but similar empathic traits than TD children. Consistent with previous studies, we confirmed that the ToM ability of autistic children was significantly lower than that of TD children (20, 57–59). However, autistic children had similar empathic trait compared to TD children, which was inconsistent with previous studies (43, 44, 60). When we further considered different types of empathy, we did not find differences in both affective and cognitive empathy. The results of affective empathy were consistent with most previous studies, indicating an intact affective empathy in school-aged autistic children (21, 23, 34, 61). However, parent-reported cognitive empathy in autistic children was not significantly lower than those in TD children, which was not in line with some of the previous findings (23, 61). This inconsistency might have resulted from the differences between self-reported questionnaires and behavioral assessments (21–23, 62). Moreover, most of the behavioral measurements of cognitive empathy were bundled together or conflated with ToM ability, resulting in the difficulties to distinguish cognitive empathy and ToM ability in these studies (21, 22). Fletcher-Watson and Bird (24) proposed that empathy can be broken down into four component stages: (1) noticing another's feeling; (2) correctly interpreting another's feelings; (3) feeling empathy; (4) responding in line with social norms. Although the subscale of cognitive empathy can reflect the second component of empathy, some of the items in this subscale might overlap with other components (i.e., item of 8 and 17 listed in the **Supplementary Table S3** of **Appendix A**), limiting the power of this subscale. Therefore, more studies are needed to consider different components of empathy when studying the behavioral characteristics of autistic children.

In the DG paradigm, we found that ToM ability played an important role in the prosocial behaviors of autistic children. However, our previous study found that ToM ability did not contribute to prosocial behaviors of the DG paradigm in Chinese TD children, while other complex cognitive functions (i.e., inhibition control) may play a role in deciding resource allocation (39). In China, routinely enforced parental instruction that children should share things might eliminate the need for social insight to act as a trigger for sharing behavior for TD children in experimental conditions (13). Regrading autistic children, the development of social cognition is affected by the social motivation (63). Social Motivation Model suggested

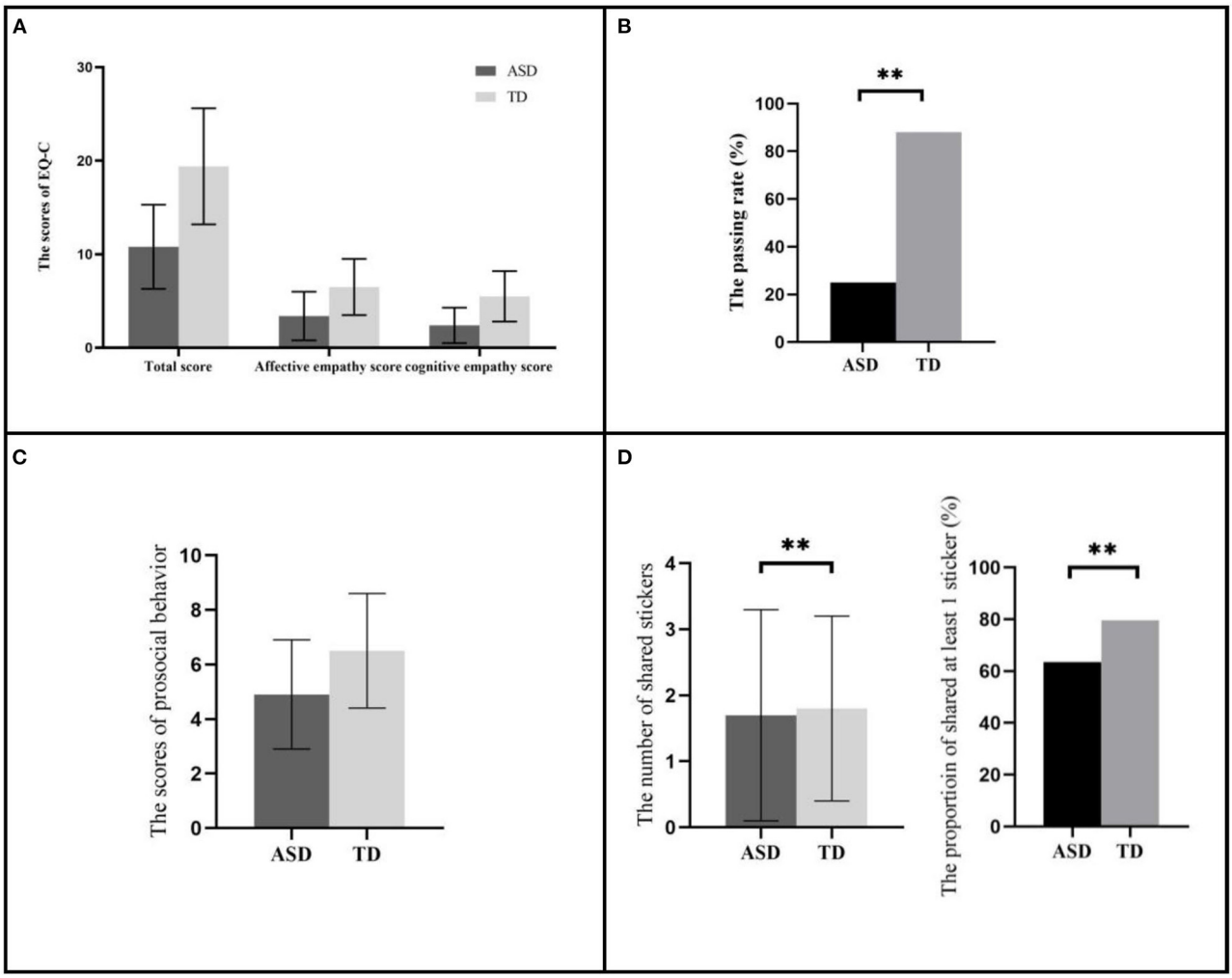

FIGURE 1 | The comparison of empathic traits, ToM ability and prosocial behaviors between autistic children and TD children. **(A)** The comparison of EQ-C scores between the two groups. **(B)** The comparison of passing rate of the ToM toolkit between the two groups. **(C)** The comparison of prosocial behaviors score between the two groups. **(D)** The comparison of the number of shared stickers and the proportion of shared at least 1 sticker in DG paradigm between the two groups. All the comparison were adjusted for child's age, child's sex, FSIQ, SRS total score, BRIEF total score, maternal age, maternal education level and per capita monthly household income. The comparison of prosocial behavior in DG paradigm was further adjusted for degree of stickers preference. ASD, Autism spectrum disorder; TD, Typically developing; EQ-C, Children's version of Empathy quotient; ToM, Theory of mind. $^{**}P < 0.01$.

that early-onset impairments in social attention ultimately deprive the child of adequate social learning experiences, causing the imbalance in attending to social and non-social stimuli and subsequently disrupting social cognitive development (63). The resource allocation task in the context of game theory (i.e., DG paradigm) is developed based on the assumption that individuals can predict other people's actions when they understand others' motivations, preferences, and beliefs (18). Consequently, better ToM ability might contribute to prosocial behaviors of DG paradigm in autistic children. Our results might have clinical insights for behavioral interventions targeting social skills in real life since prosocial behaviors are of importance to social interaction, cooperation and adaptation (64).

In this study, we only observed a positive association between empathic traits and parent-reported prosocial behaviors in autistic children, and the associations were more pronounced when considering affective empathy. Autistic children might understand the emotions of others and respond appropriately since they have intact affective empathy (21, 34, 61). According to the Intense World Theory, autistic children might have hyper-perception and hyper-emotionality caused by hyper-functioning of local neural microcircuits (65). The vicariously emotional experience with others in autistic children might be intact or amplified, which could promote them to perform more prosocial behavior. Meanwhile, parents of ASD children tended to believe that their children behaved more pro-socially when they had higher affective empathic traits in Western countries

TABLE 2 | Associations between empathic traits, ToM ability and parent-reported prosocial behaviors in autistic children.

	The score of the prosocial behavior subscale ($N = 96$)			
	Crude model		Adjusted model[a]	
	Estimates (95%CI)	*P*-value	Estimates (95%CI)	*P*-value
EQ-C				
Total score	**0.21 (0.13, 0.29)**	**<0.001**	**0.17 (0.07, 0.27)**	**<0.001**
Affective empathy score	**0.52 (0.40, 0.64)**	**<0.001**	**0.47 (0.33, 0.60)**	**<0.001**
Cognitive empathy score	**0.27 (0.06, 0.48)**	**0.01**	0.06 (−0.18, 0.29)	0.65
ToM				
Not pass	1 (Reference)		1 (Reference)	
Pass	0.50 (-0.44, 1.44)	0.30	0.27 (−0.76, 1.30)	0.61

EQ-C, Children's version of Empathy quotient; ToM, Theory of mind; CI, confidence interval.
[a]Generalized linear models were used to investigate the associations of empathic traits and ToM ability with prosocial behaviors in autistic children. Crude model was fitted without any adjustment. Adjusted model was fitted with adjusting for child's age, FSIQ, SRS total score, BRIEF total score and maternal age.
The bold values meant the P -value was statistically significant.

TABLE 3 | Associations between empathic traits, ToM ability and DG paradigm in autistic children.

	The number of shared stickers ($N = 96$)			
	Crude model		Adjusted model[a]	
	Estimates (95%CI)	*P*-value	Estimates (95%CI)	*P*-value
EQ-C				
Total score	0.00 (−0.07, 0.07)	1.00	0.02 (−0.07, 0.11)	0.65
Affective empathy score	0.02 (−0.10, 0.15)	0.71	0.00 (−0.15, 0.15)	0.97
Cognitive empathy score	0.02 (−0.14, 0.20)	0.79	0.19 (−0.02, 0.39)	0.08
ToM				
Not pass	1 (Reference)		1 (Reference)	
Pass	**0.97 (0.24, 1.71)**	**0.01**	**1.03 (0.16, 1.89)**	**0.02**

EQ-C, Children's version of Empathy quotient; ToM, Theory of mind; CI, confidence interval.
[a]Generalized linear models were used to investigate the associations of empathic traits and ToM ability with prosocial behaviors in autistic children. Crude model was fitted without any adjustment. Adjusted model was fitted with adjusting for child's age, FSIQ, SRS total score, BRIEF total score, maternal age, and degree of sticker preference.
The bold values meant the P -value was statistically significant.

(35). However, the results should be interpreted cautiously because empathy traits and prosocial behaviors in daily life were parent-reported, indicating the potential measurement error (34). Parents might report higher levels of empathic traits or prosocial behaviors due to exaggerated parental perceptions (66). One previous study in the Netherlands showed that the prosocial responses to peer distress were similar in autistic children and TD children in a computer task, indicating that the association between affective empathy and prosocial behaviors was less pronounced in experimental conditions (34), which was consistent with our findings. Since this is the first study to reveal the different role of empathic traits and ToM ability on prosocial behaviors in autistic children, more studies are needed to confirm our findings.

This study has several limitations. First, we used simple ToM toolkit instead of advanced ToM tasks, which might introduce a ceiling effect in TD children. However, our previous study has confirmed that most autistic children may have struggled with the advanced ToM tasks (20, 58, 59). Further research is needed to develop appropriate paradigms to compare ToM ability between autistic children and TD children. Second, we were not able to measure empathic traits and ToM ability by parent-report and experiment condition, which might introduce potential confounding of our findings. Third, we only performed one single task of the DG paradigm, and therefore it might be subject to measurement errors. Fourth, the behavioral characteristics of prosocial behaviors might be different when offering different resources (e.g., food, toys, attachment objects) (67). We only used stickers in this study as allocation resources although we have investigated the degree of sticker preference, and if the children disliked the stickers, we might not have been able to elicit prosocial behaviors. We found weak correlation between the prosocial behaviors measured by parent-report and by experiments ($r = -0.10$) which indicated that children might behave differently in their natural social environment than in experimental conditions. Future research is needed to study children's sharing behaviors of different resources in their natural environment. Fifth, we did not match on age and intelligence between the two groups despite of the use of statistical control in this study.

CONCLUSIONS

Our study found that autistic children showed less prosociality and ToM ability than TD children. In autistic children, empathic trait was associated with parent-reported prosocial behaviors while their ToM ability was associated with prosocial behaviors in experimental condition. Our findings indicated that better ToM ability and empathic trait might promote prosocial behaviors in autistic children.

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 Ethical Review Committee for Biomedical Research, Sun Yat-sen University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JJ, X-HL, and J-HG: conceptualization, supervision, project administration, and funding acquisition. XW: methodology, formal analysis, and writing-original draft preparation. BA and L-ZL: study design and manuscript revise. XW, NP, QC, J-JC, S-YL, and M-XD: data collection. L-ZL: data curation. XW, BA, NP, QC, J-JC, S-YL, M-XD, J-HG, X-HL, and JJ: writing–review and editing. 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.2022.844578/full#supplementary-material

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SYSTEMATIC REVIEW
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Efficacy and Safety of Stem Cell Therapy in Children With Autism Spectrum Disorders: A Systematic Review and Meta-Analysis

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Aim: There is insufficient evidence regarding the efficacy and safety of stem cell therapy for autism spectrum disorders. We performed the first meta-analysis of stem cell therapy for autism spectrum disorders in children to provide evidence for clinical rehabilitation.

Methods: The data source includes PubMed/Medline, Web of Science, EMBASE, Cochrane Library and China Academic Journal, from inception to 24th JULY 2021. After sifting through the literature, the Cochrane tool was applied to assess the risk of bias. Finally, we extracted data from these studies and calculated pooled efficacy and safety.

Results: 5 studies that met the inclusion criteria were included in current analysis. Meta-analysis was performed using rehabilitation therapy as the reference standard. Data showed that the Childhood Autism Rating Scale score of stem cell group was striking lower than the control group (WMD: −5.96; 95%CI [−8.87, −3.06]; $p < 0.0001$). The Clinical Global Impression score consolidated effect size RR = 1.01, 95%CI [0.87, 1.18], Z = 0.14 ($p = 0.89$), the effective rate for The Clinical Global Impression was 62% and 60% in the stem cell group and the control group, respectively. The occurrence events of adverse reactions in each group (RR = 1.55; 95%CI = 0.60 to 3.98; $p = 0.36$), there was no significant difference in the incidence of adverse reactions between the stem cell group and the control group.

Conclusions: The results of this meta-analysis suggested that stem cell therapy for children with autism might be safe and effective. However, the evidence was compromised by the limitations in current study size, lacking standardized injection routes and doses of stem cells, as well as shortages in diagnostic tools and long period follow-up studies. Hence, it calls for more studies to systematically confirm the efficacy and safety of stem cell therapy for children with autism spectrum disorders.

Keywords: autism spectrum disorders (ASD), stem cell therapy, meta-analysis, efficacy, safety

INTRODUCTION

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders characterized by social deficits, communication inabilities and stereotypic behaviors (1). The incidence is estimated to be 1–2% of all children, according to the U.S. Centers for Disease Control and Prevention (2). Despite its increasing prevalence (3, 4), the etiology of ASD is not fully understood yet, which can be interpreted as including both genetic and environmental factors (5). The processes of inflammation and immune activation may act to modify the risk of ASD gene expression or destruct process of typical neural development in ASD (6). ASD patients are a heterogeneous group with different symptom characteristics (7), thus there is no definitive treatment for ASD patients (8).

Given the potential effects of sustained immune disorders and inflammation in ASD and known paracrine (9), homing (10, 11), immunomodulatory (12) and multi-directional differentiation capacity (13, 14) of stem cells, they are receiving attention as a potential therapeutic approach. Growing numbers of research reports have confirmed the efficacy and safety of stem cell therapy with different methods including autologous bone marrow mononuclear cells (15–17), fetal stem cells (18), human cord blood mononuclear cells (19) and umbilical cord mesenchymal stem cells (20) in patients with autism. However, the great mass of these case reports or case series studies are limited to a few geographical regions, thus fail to provide sufficient guidelines for clinical decisions. Moreover, these studies have several shortcomings, such as small sample size, non-standard control groups, non-standard assessment scale and short-term follow-up. Most importantly, when we began this study, there was no systematic evidence-based medical review demonstrating the efficacy and safety of stem cell therapy for ASD.

Collectively, we aim to rigorously screen and extract all preclinical trial data on stem cell therapy for autism, objectively evaluate and summarize evidence concerning the effectiveness of stem cell therapy for autism symptoms through systematic review and meta-analysis.

METHODS

The study design was developed by the steering group, followed by the standard Cochrane Neonatal Review Group methods and PRISMA reporting guidelines. We have already submitted a registration application at Prospero (CRD42021285384, https://www.crd.york.ac.uk/prospero/).

Population

Children and adolescents (age 0–18 years) were diagnosed with autism, regardless of region, gender, or race.

Intervention

Multiple kinds of stem cells interventions on children with autism were investigated within the current systematic review and meta-analysis, with no limitations on injection times, administration route and dose. Studies of stem cells in combination with other treatments, such as antipsychotic drugs, are also being considered.

Comparisons

Rehabilitation therapy includes sensory integration therapy, auditory training, behavioral intervention, occupational therapy, speech therapy and music therapy.

Outcomes

The main indicators are the scores of Clinical Global Impression (CGI) and Childhood Autism Rating Scale (CARS) or any other evaluation tools suitable for ASD in the corresponding studies.

Study Types

Randomized controlled trials (RCTs) and controlled clinical trials (CCTs) were included in this study, both paralleled or crossover. For trials that had a crossover design, we included all the data before and after the crossover.

Data Sources

The following English and Chinese databases were searched from their inception to 24th JULY 2021: PubMed/Medline, Web of Science, EMBASE, Cochrane library, China Academic Journal (through China National Knowledge Infrastructure [CNKI], [WanfangData], [Cqvip], [SinoMed]) and Clinicaltrials.gov. A detailed illustration of search strategies is available in Supporting information 1 (S1. Search Strategy). No date restrictions or language restrictions are used for retrieval. Finally, references were tracked and included in the study to ensure that no RCTs and CCTs were missed by the search strategy.

Study Selection

All prospective controlled clinical studies of stem cell therapy on autism patients were included, as were trials in which stem cells were part of a complex intervention. We excluded qualitative studies, uncontrolled trials, case studies and case series, as well as trials that developed between different cell types, and studies that failed to provide detailed results.

Data Extraction and Quality and Validity Assessment

Two independent reviewers evaluated the retrieved studies for inclusion and assessed the methodological quality of included studies. Elements extracted included study characteristics (author, country, publication year and experimental design), participant characteristics (sex, age range and diagnostic criteria), intervention details (types of cells, dose ranges, administration and frequency), outcome measurement, follow-up time and adverse reactions. The risk of bias was assessed using the Review Manager 5.3. The disagreements were thrashed out by the two reviewers.

Data Analysis

Data entry and analysis were performed using Review Manager 5.3 software. The data required for meta-analysis were obtained by direct input or indirect calculation based on the original data (The data of CARS can be obtained directly, and the effective improvement number of CGI needs to be calculated based on

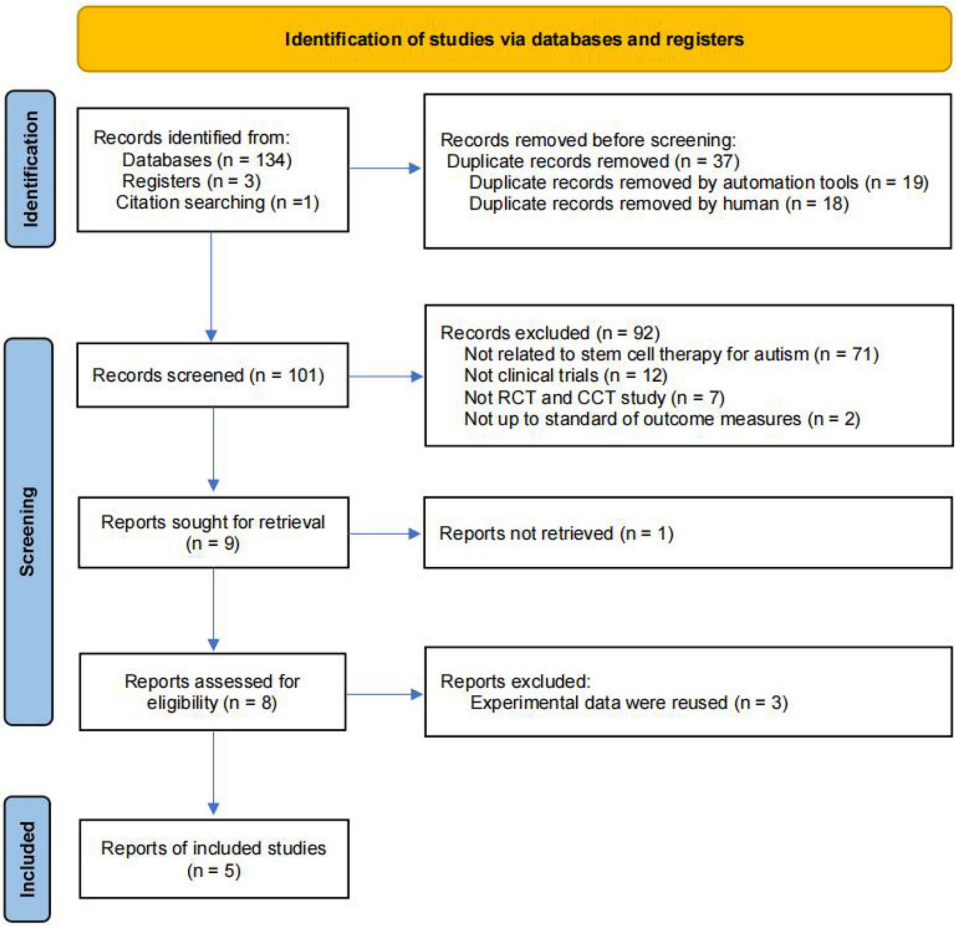

FIGURE 1 | The inclusion flow chart of the literature was retrieved.

the total efficiency provided in the original text multiplied by the number of each group, like Dawson *et al.*'s study). When studies of multiple intervention groups are compared, the "shared" control group is split equally in each comparison (21) and the weighted average difference (WMD) and risk ratio (RR) were used to compare continuous variables (CGI and CARS) and dichotomous variables (Adverse events) respectively. All results obtained were reported with 95% confidence intervals (CI). Heterogeneity among studies was determined by Q test and I^2 statistics (I^2 equals or exceeds 50%, $p < 0.05$ is considered to have greater heterogeneity). The random effect model or mixed effect model was selected according to the size of heterogeneity. With high heterogeneity, sensitivity analysis or subgroup analysis was used to detect the source of heterogeneity; if the source of heterogeneity cannot be found, a descriptive analysis was conducted.

RESULTS

Results of the Search

A flowchart describing the selection of eligible trials is presented in **Figure 1**. A total of 137 articles from 9 databases were retrieved: Web of Science ($n = 15$) databases, PubMed/MEDLINE ($n = 12$), Cochrane ($n = 7$), Embase ($n = 36$), CNKI ($n = 11$), Wan fang Data ($n = 30$), Cqvip ($n = 19$), Sino Med ($n = 4$), Clinicaltrials.gov ($n = 3$). We also included 1 latest research reports through citation searching to ensure that the retrieved literature includes all the studies in the published meta-analysis (26). Finally, 5 studies were included within our meta-analysis.

Characteristics of the Studies

The characteristics of the included studies are listed in **Table 1**. Diagnosis criteria were performed mainly through the Diagnostic and Statistical Manual of Mental Disorders-4 (DSM-4) (60.0%) or Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) (40.0%) criteria. The 5 studies comprised 3 RCTs and 2 CCTs, sample sizes ranged from 36 to 180. A total of 325 subjects were included in the systematic review and meta-analysis, 319 of whom were analyzed for safety, including 265 males and 54 females. However, in Dawson's study (27), two pilot participants and 2 participants were found to be ineligible [bipolar disorder ($n = 1$), the primary caregiver does not speak English ($n = 1$)] after randomization, thus were excluded from the efficacy

TABLE 1 | Summary of clinical studies of stem cells therapy for autism spectrum disorders.

Study	Country	Design	Sample size		Age, y	Diagnostic criteria	Therapy			Control		Follow up	Main outcome measures	Adverse reaction
			(aggregate/ analyzed)	(male/ female)			Intervention	Administration	Sample size	Control intervention	Sample size			
Sharifzadeh et al. (22)	Iran	RCT	36/32	27/5	5-15	DSM-5	BMMSC, first,0.5-1 × 10^8 cells per 2.0 mL.s,0.3-0.5 × 10^8 per 2.0 mL.	Intrathecally injection.	14	Rehabilitation therapy and risperidone	18	6 and 12 M	CARS, GARS-II, CGI	In general, injection-related side effects of 12 months were not observed in any of the patients.
Liu (23)	China	CCT	42/42	35/7	3–12	DSM-4	Liu 2010 a: MNCs, cells per serving was 2 × 10^6/kg.	Intravenous infusion once, lumbar puncture subcobweb space injection 3 times.	14	Rehabilitation therapy	14	4W,4M	CARS, CGI	There were 12 cases of low fever, 4 cases of headache, 3 cases of lumbago, 4 cases of fatigue, and 2 cases of nausea and vomiting, without serious adverse reactions.
							Liu 2010 b:MNCs and MSCs. Cells per serving was 2 × 10^6/kg.	Intravenous infusion once, lumbar puncture subcobweb space injection 3 times.	14					
Dawson et al. (24)	USA	RCT	180/176	143/37	2–7	DSM-5	CB, the number of therapeutic cells≥2.5 × 10^7 cells/kg.	Peripheral intravenous infusion.	119	The placebo (TC199 + 1% DMSO) and behavioral intervention	61	24W	VABS-3, PDDBI, CGI-S, CGI-I, and EOWPVT-4	3 in the placebo arm, 1 in the autologous CB cohort,and 2 in the allogenic CB cohort. None of these events was related to the study product.
Lv et al. (19)	China	CCT	37/36	35/1	3–12	DSM-4	Lv 2013a: CBMNC and UCMSCs,cells per serving was 2 × 10^6/kg.	Once intravenous and three times intrathecal infusions.	9	Rehabilitation therapy	13	4W, 8W, 16W, 24W	CARS,ABC,CGI	No significant safety issues related to the treatment and no observed severe adverse effects.

(Continued)

TABLE 1 | Continued

Study	Country	Design	Sample size (aggregate/ analyzed) (male/female)		Age, y	Diagnostic criteria	Therapy			Control		Follow up	Main outcome measures	Adverse reaction
							Intervention	Administration	Sample size	Control intervention	Sample size			
							Lv 2013b: CBMNC cells per serving was 2×10^6/kg.	Once intravenous infusion and three times intrathecal injections.	14					
Chez et al. (25)	USA	RCT	30/29	25/4	2–6	DSM-4	AUCB, at least 10×10^6/kg, 50 ml.	Peripheral intravenous infusion.	29	Infusion of 0.9% saline placebo	29	12W, 24W, 37W, 49W	EOWPVT-4,ROWPVT-4,Stanford Binet,CGI	No adverse events required treatment. There were no observed allergic reactions or serious adverse events associated with the administration of AUCB.

RCT, randomized controlled trial; CCT, controlled clinical trail; DSM,Diagnostic and Statistical Manual of Mental Disorders; BMMSC, bone marrow mesenchymal stem cell; MNCs, mononuclear cells; MSCs, mesenchymal stem cells; CB, cord blood; UCMSCs, umbilical cord mesenchymal stem cells; AUCB,autologous umbilical cord blood; DMSO, Dimethyl sulfoxide; CARS, childhood autism rating scale; CGI, Clinical Global Impression; VABS, Vineland Adaptive Behavior Scales; PDDBI, The PDD Behavior Inventory; EOWPVT, Expressive One-Word Picture Vocabulary Test; ABC, Autism Behavior Checklist; ROWPVT, receptive one word picture vocabulary test; M, month; W, week.

analysis. Collectively, only 315 subjects were included in the efficacy analysis.

Methodological Quality

The **Figures 2**, **3** showed the assessment results of bias risk and methodological suitability of the included studies. Studie by Dawson *et al* was considered high quality and low risk of bias. Lv *et al.* 's study was high risk in the field of random sequence generation and have "Some concerns" in multiple domains that substantially lowers confidence in the result. The other three studies should be considered "Some concerns" according to Cochrane's book in their one or two domains.

Meta-Analysis

Five eligible articles were meta-analyzed using a random effects model, with CARS (**Figure 4**) and CGI (**Figure 5**) as primary and secondary indicators to evaluate the effectiveness of stem cell therapy for autism, and adverse reactions as safety indicator (**Figure 6**).

As can be seen from the forest diagram in **Figure 4**, heterogeneity test $p = 0.14$; $I^2 = 42\%$, indicating moderate heterogeneity. Data showed that the CARS score of the stem cell group was significantly lower than the control group [WMD: −5.96; 95% CI (−8.87, −3.06); $p < 0.0001$] (**Figure 4**). A higher score of CARS refers to severe disease. Overall, our results showed that the stem cell group had better efficacy in ASD treatment than the control group. In addition, sensitivity analysis was conducted to eliminate outliers in another intervention group (Lv 2013b) of Lv *et al.*'s study and the results were found to be stable (WMD: −7.08; 95%CI [−9.46, −4.70]; $p < 0.0001$; heterogeneity test $p = 0.53$; $I^2 = 0\%$).

We found that the forest plot of CARS had moderate heterogeneity. According to the results of the methodological quality assessment, we concluded that the research quality of Lv *et al.*'s study was low. Afterward, we conducted a subgroup analysis based on the methodological quality, and found that the combined results of CARS in the high-quality group were stable [WMD: −6.37; 95%CI (−9.11, −3.63); $p < 0.00001$, heterogeneity test $p = 0.55$; $I^2 = 0$] (**Figure 7**). The results of Lv *et al.* 's study showed high heterogeneity and instability in the low-quality group [WMD:−4.83; 95% CI (−13.78, 4.12); $p = 0.29$; heterogeneity test $p = 0.02$; $I^2 = 82\%$] (**Figure 7**). Therefore, Lv *et al.*'s study might be the source of heterogeneity. The results in **Figure 5** show no significant difference in CGI. Consolidated effect size RR = 1.01, 95%CI [0.87, 1.18], Z = 0.14 ($p = 0.89$), the effective rate for CGI was 62 and 60% in the stem cell group and the control group, respectively. Heterogeneity test $I^2 = 0\%$, $p = 0.72$, indicating no heterogeneity.

The forest plot in **Figure 6** reflects the occurrence events of adverse reactions in each group [RR = 1.55; 95%CI = (0.60, 3.98); $p = 0.36$; **Figure 6**], there was no significant difference in the incidence of adverse reactions between the stem cell group and the control group, with heterogeneity test $p = 0.28$; $I^2 = 22\%$. Based on the forest map, we have visual outliers. The sensitivity analysis found that the study of Dawson *et al* was an outlier

and the cause of heterogeneity. Whereas, after Dawson *et al*'s study was excluded, the results were more stable [RR = 4.70; 95%CI = (0.90, 24.63); $p = 0.07$; Heterogeneity test $p = 0.95$; $I^2 = 0\%$].

DISCUSSION

Stem cells are defined as tissue units of biological systems which is responsible for the regeneration and development of organs and tissues. Stem cells are capable of self-renew and differentiate into multiple cell line ages, therefore, these cells can also be units that evolve through natural selection (28, 29). Hematopoietic stem cells were primarily discovered and used for the treatment of blood-system failure induced by nuclear radiation (30). In recent years, the clinical results show that stem cells have shown promising effects in a variety of chronic and difficult diseases, such as spinal cord injury (31–34), graft-vs. -host disease (GVHD) (35, 36), diabetes and complications (37–39), stroke (40, 41), *etc*. As expected, more and more researchers are attempting to determine the efficacy of stem cell therapy for ASD.

Martínez (26) published the first systematic review and meta-analysis of stem cell therapy for autism in September 2021, but they included uncontrolled studies in the analysis to compensate for the insufficient number of studies. Especially in the studies within the control group, they only extracted data from the intervention group, which might reduce the clinical guiding significance of the conclusion that stem cell therapy significantly improves scales in patients with ASD. Our study demonstrates that stem cell therapy for children with autism appears to be safe and effective. CARS - the primary outcome measurement confirmed the efficacy, whereas, the secondary outcome measurement—CGI, showed no difference between stem cells and control treatment. Furthermore, Bieleninik, Ł (42) found total costs of ASD including health services costs and societal costs, were estimated to be around 2834 EUR in 2 months by analysis with 5 European countries and 4 non-European countries. However, the median total charges and costs for stem-cell transplant hospitalization were $270,198 and $92,717 from 2002 to 2015 (43). Given the persistence

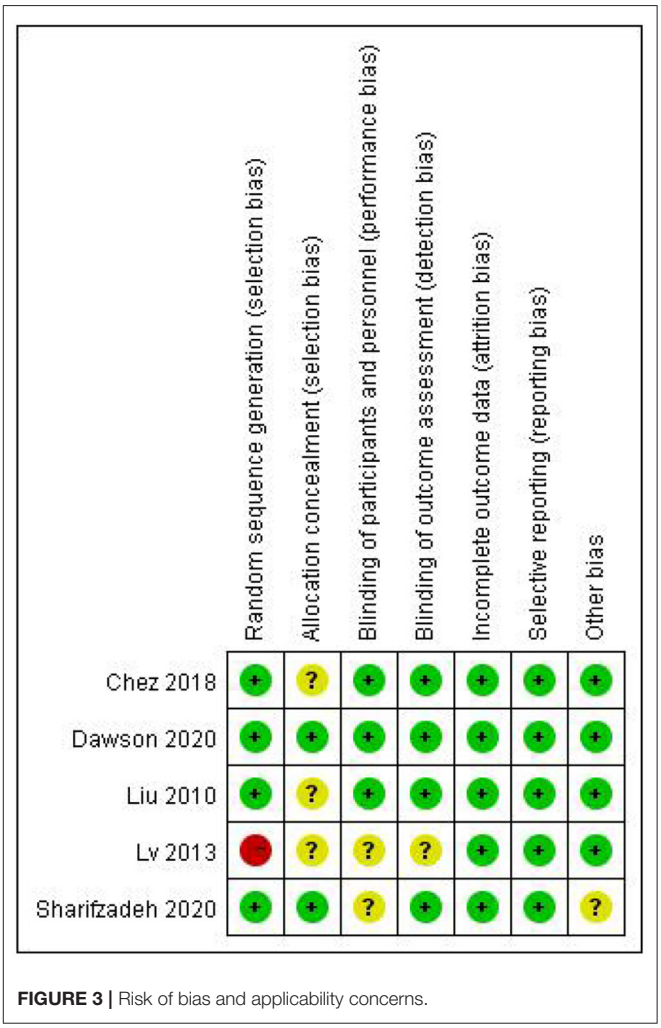

FIGURE 3 | Risk of bias and applicability concerns.

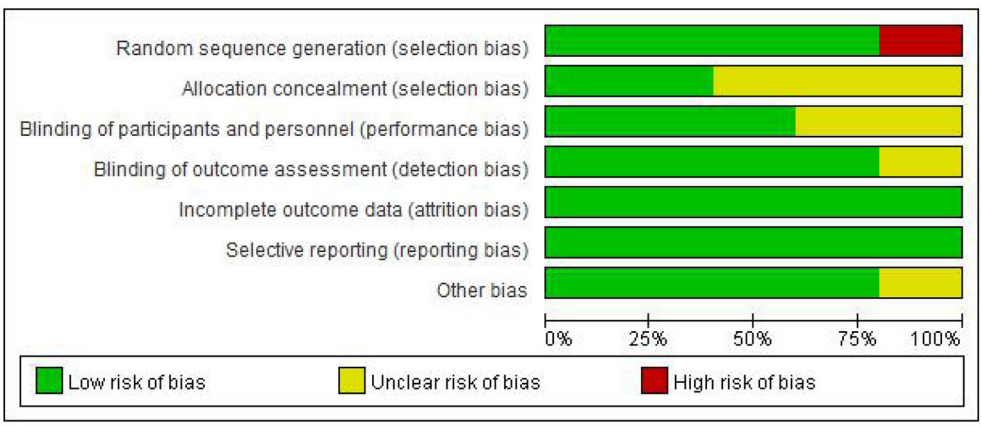

FIGURE 2 | Risk of bias and applicability concerns.

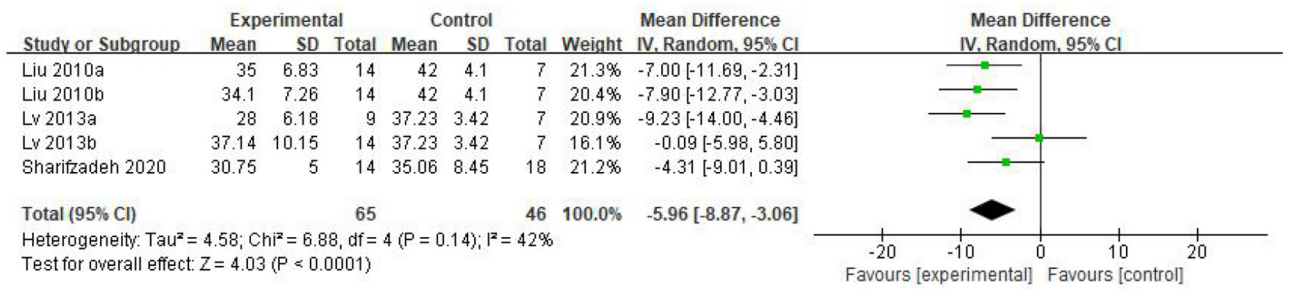

FIGURE 4 | CARS forest plot included in the study.

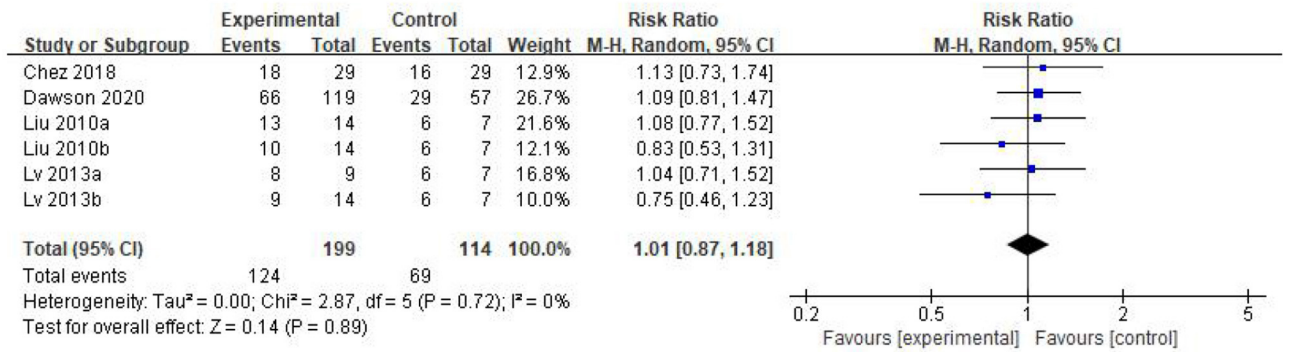

FIGURE 5 | CGI forest plot included in the study.

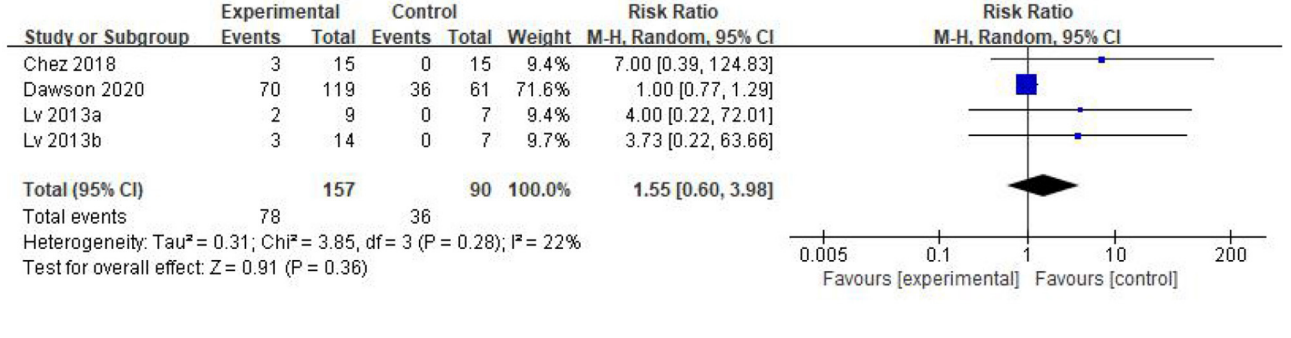

FIGURE 6 | Forest plot of adverse events.

of autism, the hospitalization cost also increased dramatically. Therefore, it is extremely important to make the expensive stem cell therapy to gain greatly therapeutic effect. Currently, stem cell treatments for autism is mostly considered a new mean of clinical trials and have just been conducted in only a few places. It is urgent to form the standardized treatment methods and improve the curative effect before that they are popularized in clinical practice.

We can glimpse from the included studies where future autism stem cell therapy needs to be standardized. Firstly, we noted that the doses of cell injections in the included studies were varied. In Sharifzadeh's study (22), subjects received a total of 0.5-1 $\times$ 10^8 cells in the first injection and 0.3-0.5 $\times$ 10^8 cells in the second injection. In other studies (19, 24, 25, 44), subjects received injections ranging from 2 $\times$ 10^6/kg to 2.5 $\times$ 10^7/kg cells at a time. Different doses of cell injection may account for the inconsistent efficacy. Owing to these limited studies, we could not analyze the influence of dosage on the efficacy and safety of stem cell injections. Secondly, there are two ways of cell injection: intravenous injection and intrathecal injection. Intravenous infusion of cells may limit the homing effect, cells could be trapped in organs such as lungs, heart, liver or kidney

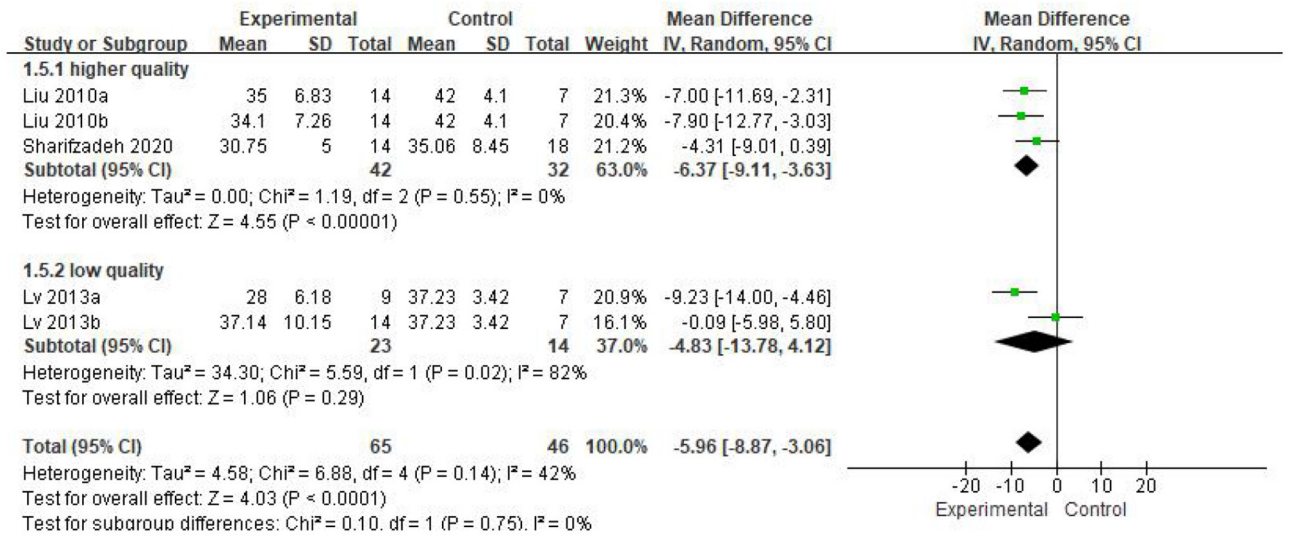

FIGURE 7 | CARS Forest plot of subgroup analysis.

and get blocked by the blood-brain barrier, which might reduce the therapeutic effect on ASD (45, 46). Furthermore, only two studies were followed up for 12 months, such a short period is not conducive to observing progress in the improvement of core symptoms of autism. Previous studies have suggested improvements observed after 12-month and 18-month follow-up, particularly in the Clinical Global Impression Scale (17, 27, 46). The CGI rating scale has been widely used in psychiatry to evaluate the degree of symptom and therapeutic efficacy, and the Improvement (CGI-I) scale is used to assess the extent a patient has improved or worsened following an intervention, but they are non-ASD specific (47), which might explain why there was no significant difference in the CGI scores between the two groups. Additionally, ASD is a complex neuropsychiatric disorder with substantial phenotypic and genetic heterogeneity (48), reducing the impact of heterogeneity on treatment and evaluation studies is quite important. Moreover, there are multiple sources of stem cells, and the therapeutic effects of stem cells from different sources may vary. Lv (19) and Liu (44) suggest that compared with the control group, the effect of cord blood mononuclear cells (CBMNC) transplantation was significant, however, the combination of CBMNC and umbilical cord mesenchymal stem cells (UCMSCs) had a greater therapeutic effect than CBMNC alone. The small sample size of included studies is also a problem that cannot be ignored. As mentioned above, there are many studies in the field of stem cell therapy for autism that have directly or indirectly demonstrated its effectiveness. However, they did not meet the criteria for inclusion in our analysis and were not meta-analyzed, but their results were equally important.

Overall, the major limitations of the included studies were small sample size, non-specific outcome measures, treatment regimens were not uniform, and lacking adequate follow-up.

CONCLUSIONS

In conclusion, the use of stem cells to treat children with autism may be effective and safe, but we believe that the current evidence is in-sufficient, the conclusions are based on studies that do not have a uniform treatment protocol. Besides, due to the high cost of stem cell therapy and the lack of widespread clinical application, guardians of children with autism need to be discreetly about enrolling in clinical trials of stem cell treatments for autism. It is urgent to establish a standardized treatment protocol through a large number of trials, such as the most suitable stem cell type, administration method and dose need to be screened; the post-treatment evaluation of stem cell therapy need to be improved. These may lead to the discovery of stem cell therapy for autism and its pathogenesis, thus further improving the therapeutic effect. We expect stem cell therapy to be used in the clinical treatment of autism and have significant therapeutic effects, but it is still a lot of work to be done before this can happen.

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.

AUTHOR CONTRIBUTIONS

JQ: conceptualization, writing-reviewing and editing, data extraction, and assessing the risk of bias. ZL: conceptualization, writing-reviewing and editing, data extraction, and assessing the risk of bias. JY: writing-original draft, study selection, research retrieval, and statistical analysis. MZ: study selection, research retrieval, and statistical analysis. LL: study selection,

and data extraction. ZZ: writing-reviewing and data extraction. ZH: assessing the quality of studies. LZ: article revision and grammar revision. YL: writing-original draft and statistical analysis. 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/fped.2022.897398/full#supplementary-material

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A Probable Way Vitamin D Affects Autism Spectrum Disorder: The Nitric Oxide Signaling Pathway

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Vitamin D (VD) deficiency during pregnancy and early brain development is an important environmental risk factor for autism spectrum disorder (ASD). Its specific mechanism of action is still unclear. However, one study on the correlation between metabolomics and VD levels in children with ASD has found that the whole-blood arginine (Arg) levels of children with ASD are significantly negatively correlated with serum VD levels, suggesting that the effect of VD on ASD may be related to the signaling pathway involving Arg. Arg is a precursor of nitric oxide (NO), and changes in its levels most directly affect NO levels and signal transduction pathways. NO, a biologically active free radical, is both a neurotransmitter and a neuromodulator in the central nervous system and is related to the pathogeneses of various neurological diseases. The NO signaling pathway is not only affected by VD levels but also closely related to ASD through a series of mechanisms, such as neurotransmitter imbalance, immune disorders, and oxidative stress. Therefore, the effect of VD on ASD may be achieved via regulation of the NO signaling pathway. The current review discusses the relationship among VD, NO, and ASD as suggested by a large body of evidence in the literature in an effort to provide clues for researchers on the pathogenesis of ASD and the mechanism of VD's impact on ASD.

Keywords: autism spectrum disorder, nitric oxide, vitamin D, nitric oxide synthase, arginine

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INTRODUCTION

Autism spectrum disorder (ASD) refers to a group of neurodevelopmental disorders that start in early childhood. These disorders, whose main symptoms are social communication impairment and repetitive stereotyped behaviors and interests, have a high prevalence and high disability rate. ASD is one of the fastest-growing severe diseases in the world (1). In 2016, the World Health Organization estimated that 1 child among every 160 has ASD worldwide. In 2018, the US Centers for Disease Control and Prevention reported that the prevalence of ASD in 8-year-old children was as high as 1/59 (2); in 2020, the prevalence reached 1/54 and still maintained a large male-to-female ratio (4.3:1) (3). The onset of the disease occurs during childhood, and the disease often persists throughout life. Its etiology and pathogenesis are still unclear, so it remains one of the most challenging global health problems. It is generally believed that ASD is the result of the combined effects of genetic factors and environmental factors (4, 5).

Nutrition is an environmental factor that interacts with genes from the beginning of the embryonic period and has major impacts on organisms. Vitamin D (VD) is an important nutrient associated with ASD. In 2008, Cannell's epidemiological survey found that the levels of VD in the American population decreased year over year, while the prevalence of ASD increased year over year during the same period; in addition, the prevalence was high in areas with heavy air pollution, cities, and high-altitude areas where VD absorption is reduced. This evidence, combined with evidence of the effects of VD on brain development, led to the hypothesis that VD deficiency may be the etiological factor leading to ASD (6). Since then, research on the correlation between VD and ASD has received extensive attention in academic circles. Subsequent studies have shown that maternal VD deficiency during pregnancy and early brain development is an important environmental risk factor for the development of ASD. However, its specific mechanism of action remains unclear. In a study on the correlation between metabolomics and VD levels in children with ASD, our research team found that the levels of whole-blood arginine (Arg) in children with ASD are significantly negatively correlated with the levels of serum VD, suggesting that the effect of VD on ASD may be related to signaling pathways involving Arg (7). Arg is the precursor of nitric oxide (NO), which most directly affects the NO signal transduction pathway. NO, a biologically active free radical, is both a neurotransmitter and a neuromodulator in the central nervous system and is related to the pathogeneses of various neurological diseases (8). According to both domestic and international studies, the NO signaling pathway is not only affected by VD levels but also closely related to ASD through a series of mechanisms, such as neurotransmitter imbalance, immune disorders, and oxidative stress. Therefore, the effect of VD on ASD may be achieved via regulation of the NO signaling pathway. The current review discusses the relationship among VD, NO and ASD as suggested by a large body of evidence in the literature in an effort to provide clues for researchers on the pathogenesis of ASD and the mechanism of VD's impact on ASD.

VD AND ASD

VD is a fat-soluble vitamin and is the only chemical substance in our body that is obtained from sunlight. It is also known as the "sunshine vitamin." VD is obtained in very small amounts from a normal diet, but it is primarily synthesized endogenously in the skin after irradiation by ultraviolet light. After being synthesized, VD binds to plasma VD-binding protein and is transported to the liver, where it is catalyzed by 25-hydroxylase to generate 25(OH)D; then, 25(OH)D is further hydroxylated by 1α-hydroxylase in the kidneys to generate the biologically active compound $1,25(OH)_2D$. 1, $25(OH)_2D$ can also activate a variety of target genes through the VD receptor (VDR) to affect the activities of most organs in the body, including the brain. 25(OH)D is the main form of VD in the blood circulation and is used as an indicator of the nutritional VD status.

It was previously believed that the main role of VD was to regulate calcium and phosphorus metabolism and affect bone growth and development. In recent years, however, studies have shown that changes in VD levels can increase the risks of breast cancer, diabetes, dyslipidemia, cardiovascular disease, etc. (9–11). With the discovery that large amounts of 1α hydroxylase (the key enzyme in 25(OH)D synthesis) and VDR are widely present in the brain, it has been gradually recognized that VD plays important roles in brain activity (12). For example, VD plays a very important role in brain development (13, 14). VD deficiency in early life can lead to abnormal brain development and can impair learning, memory, cognition and other functions (15, 16). VD deficiency is also closely related to depression, seasonal psychosis, schizophrenia, Alzheimer's disease and other diseases (17–21). These findings suggest that this "sunshine vitamin" is a valuable sun-derived resource for humans and that its deficiency at different stages of human development and under different genetic backgrounds may have significant impacts on human health.

Studies Have Shown That Low VD Levels Are Closely Related to ASD

In 2010, Meguid et al. reported that the serum 25(OH)D levels of Egyptian children with ASD were lower than those of normal control children (22). Subsequently, decreased VD levels in children and adolescents with ASD have been reported around the world (23–28) and found to be correlated with the clinical symptoms of ASD (29–34). Maternal vitamin D levels during pregnancy were inversely associated with the risk of autism in children (35–37). Lower first trimester maternal serum levels of 25(OH)D were associated with an increased risk of developing autism in offspring (38). In a study of 4,229 mother-child pairs, Vinkhuyzen et al. found that scores of autism-like behavior (measured by the Social Responsiveness Scale) in 6-year-olds were significantly greater among children whose mothers had VD deficiency in the second and third trimesters (39). Multiple meta-analyses on the association between ASD and VD levels have shown that 25(OH)D levels in children with ASD are significantly lower than those in healthy controls (40), and insufficient VD levels during pregnancy are a risk factor for ASD in offspring (41).

High-Dose VD Can Improve the Core Symptoms of ASD

Jia et al. administered VD to children with ASD and found that high-dose supplementation with VD could improve the core symptoms of the disorder (32, 42–44). In an international open-label prospective study, mothers who had a child with ASD were given 5,000 IU of daily VD supplementation when they became pregnant again, and the mothers continued to give the newborns 1,000 IU of daily VD supplementation after delivery until the age of 3 years. The prevalence (5%) was significantly lower than the reported comorbidity rate of ASD siblings (20%) (45). In contrast, Aizza and Kerley et al. found that VD was ineffective in improving the clinical symptoms of ASD (46), but the therapeutic dose of VD used in those two studies (2,000 IU/day) was much lower than that of Jia et al. (150,000 IU, muscle, once/month for 3 consecutive months), and the serum 25(OH)D levels of the children with ASD at the end of the

study were <40 ng/ml. Another study showed that under the condition of supplementing the same dose of VD (2,000 IU/day), the level of VD in children with ASD was significantly lower than that in children with asthma (47). These findings have been suggested that children with ASD require higher doses of vitamin D supplementation and that increasing the levels of 25(OH)D in the serum of children with ASD to above 40 ng/ml can exert a therapeutic effect (48). The latest randomized controlled trials and meta-analyses of VD in the treatment of ASD have also shown that VD can improve the core symptoms of ASD (49, 50).

Animal Model Studies Suggest That VD Deficiency Is Associated With ASD

Valproic acid (VPA), a deacetylase inhibitor, is an anticonvulsant and mood-stabilizing drug. Prenatal exposure to VPA can induce ASD behavior in male offspring, which is one of the methods for constructing ASD animal models (51). Studies have shown that the serum 25(OH)D levels of VPA-induced ASD animal models are significantly lower than those of normal controls. Compared with controls, pups of VD-deficient rats (VDD rats) fed a diet deprived of VD exhibit altered ultrasonic vocalizations and stereotyped repetitive behaviors, as well as impaired social interactions during adolescence (52). Early VD supplementation in infant rats with VPA-induced ASD significantly improves related developmental and behavioral issues (53).

In conclusion, VD deficiency is closely related to the pathogenesis of ASD, and supplementation with high-dose VD can improve the core symptoms of ASD. However, the mechanism of interaction between VD and ASD is still unclear.

THE NO SIGNALING PATHWAY AND ASD

NO is a highly active intracellular and intercellular messenger molecule that is widely distributed in various tissues, especially nervous tissue. NO has physiological functions such as immune regulation, neurotransmission, blood pressure regulation and platelet aggregation inhibition, and changes in its concentrations are closely related to physiological functions (54). In the central nervous system, NO is both a neurotransmitter and a neuromodulator. At low concentrations, NO plays physiological roles in the functions of nerve cells and vascular cells, while at high concentrations, NO can be toxic and lead to cell death, which is related to the pathogeneses of various neurological diseases, including stroke and neurodegenerative diseases (55).

NO is generated from Arg in a reaction catalyzed by NO synthase (NOS). There are two types of NOS: native and inducible. Native NOS is inherent in cells and is divided into neuronal NOS (nNOS) and endothelial NOS (eNOS). Under normal physiological conditions, NO is produced in appropriate amounts through the action of native NOS, thereby exhibiting important physiological functions. nNOS is the major subtype of NOS in the central nervous system. The N-methyl-D-aspartate receptor (NMDAR) at the postsynaptic membrane is activated by glutamate (Glu), which in turn causes Ca^{2+} influx, activates nNOS, catalyzes Arg reactions, and generates citrulline and NO. NO acts on adjacent neurons through diffusion, thereby inducing long-term potentiation (LTP) related to learning and memory and exerting its biological activity (56). eNOS enables endothelial cells to continuously release NO so that vascular smooth muscle is in a state of relaxation, which plays an important role in the regulation of cerebral blood flow. iNOS is mainly distributed in glial cells (including microglia and astrocytes) in the brain. Unlike native NOS (nNOS or eNOS), inducible NOS (iNOS) does not exert biological activity under normal physiological conditions; rather, it is generated and activated only under pathological conditions (such as inflammation, cancer, trauma, etc.) to further generate large amounts of NO.

There are three main pathways downstream of NO. First, NO can activate soluble guanylate cyclase to generate cGMP, which leads to the transcriptional activation of different genes through the cGMP-PKG-CREB pathway. Second, NO can directly or indirectly lead to S-nitrosation (SNO) of many proteins and receptors, thereby changing the activity of receptors, the interactions between proteins and the localization of proteins, etc., resulting in changes in related signaling pathways (57–60). Dysregulation of NO and SNO signaling is involved in the progression of many neurodevelopmental, neurobehavioral and neurodegenerative diseases. In addition, elevated NO levels increase nitrosative stress, nitrite formation, and tyrosine nitration of proteins and may ultimately induce cell death. NO reacts with superoxide anion radicals (O^{2-}) to generate peroxynitrite, which ultimately damages DNA, fats and proteins during oxidative stress (61, 62) (**Figure 1**).

Although the pathogenesis of ASD is unclear, neurotransmitters, oxidative stress, and immune and nutritional factors are believed to play important roles in the pathogenesis of ASD. These factors are closely related to the NO signaling pathway. Multiple studies have shown that the levels of NO signaling pathway members are altered in children with ASD. These findings suggest that NO may play an important role in the pathogenesis of ASD.

Children With ASD Have Abnormal Levels of Molecules in the NO Signaling Pathway

Both clinical studies and meta-analyses have found that serum and red blood cells (RBC) NO or nitrite a (metabolite of NO) levels in children with ASD are higher than those in normal children (63–68). Abnormal transport of L-Arg, the raw material of NO, is also related to ASD. Metabolomics studies have found that children with ASD have increased plasma Arg levels and decreased renal Arg clearance (69). Arg is transferred into neurons via cationic amino acid transporter 3 (CAT-3), and genetic testing of children with ASD has identified missense mutations in the *CAT-3* gene (70).

In Addition, There Is Some Indirect Evidence That ASD Is Closely Related to NO

ASD patients have abnormal levels of neurotransmitters, and abnormal Glu metabolism is considered to be one of the causes of ASD (71). Glu can activate NMDARs, thereby activating nNOS and resulting in NO production. Changes in Glu levels can

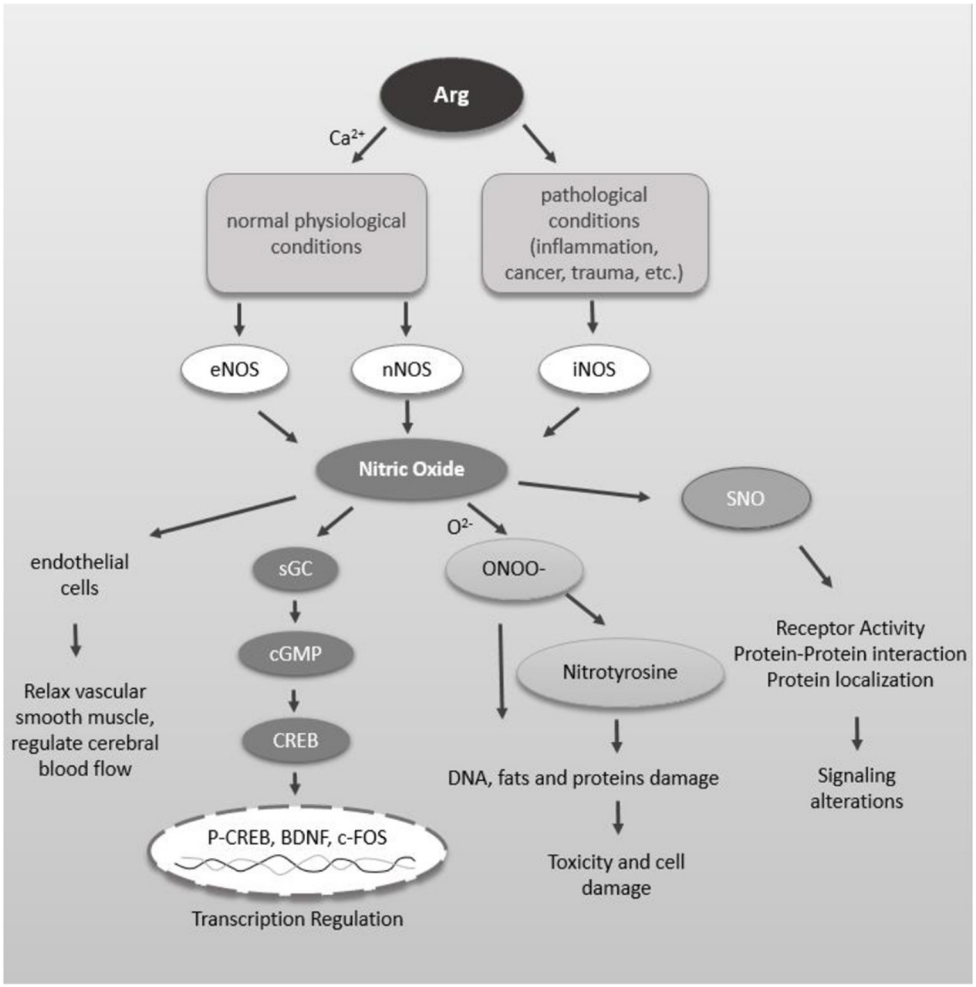

FIGURE 1 | Schematic diagram of NO function in the brain. NO can relax vascular smooth muscle, thereby regulating cerebral blood flow. Arg is catalyzed by different NOS to generate NO under physiological and pathological conditions. NO activates soluble guanylate cyclase to produce cGMP which interacts with many intracellular proteins such as PKG. PKG leads to CREB phosphorylation which leads into transcriptional activation of different genes. NO, directly and indirectly, leads to S-nitrosylation (SNO) of many proteins and receptors. SNO of proteins can alter the receptor activity, protein-protein interaction and protein localization leading to alteration in signaling. Increased level of NO increases proxynitrite formation, tyrosine nitration of proteins, which ultimately may lead into cell death.

lead to changes in NO levels in the nervous system. Immune disturbance mechanisms are also recognized to be involved in the pathogenesis of ASD. Lipopolysaccharide (LPS), a complex of lipids and polysaccharides, is the main component of the cell walls of gram-negative bacteria. LPS injection in pregnant rats can cause ASD symptoms in offspring and is thus currently a commonly used method to induce an immune ASD model in rats (72). However, LPS can induce the expression of iNOS in glial cells (73), thereby causing the production of large amounts of NO. In addition, there is an imbalance between oxidation and antioxidant capacity in children with ASD, and oxidative stress can increase the production of NO by neurons, which has a synergistic effect with the neuronal damage caused by oxygen free radicals (74). Furthermore, the ratio of males to females in ASD is ∼4–5:1; this sex difference in the incidence of ASD has long been a popular research topic. Notably, NO is closely related to the hypothalamic-gonadal axis, and the activity of nNOS in neurons is also strictly regulated by estrogen (75). Therefore, NO is closely related not only to ASD but also to the intersection of the abovementioned pathogeneses of ASD and is very likely to be a direct target for the treatment of ASD induced by various causes.

VD AND NO

$1,25(OH)_2D$ is closely related to NO production, and $1,25(OH)_2D$ and NO can modulate each other's concentrations. VD is a steroid hormone that is mainly derived from the conversion of 7-dehydrocholesterol in the skin under the action of ultraviolet rays. After two-step hydroxylation, the active product $1,25(OH)_2D$ is formed, which binds to VDR. VDR forms a heterodimer with retinoic acid receptor (RXR), which

specifically recognizes the VD response element (VDRE) and regulates the transcription of target genes. The VD signaling pathway can regulate NO and NOS levels. In a study on VDR-knockout mice, the loss of VDR resulted in decreased levels of native NOS and increased expression of arginase 2, which catalyzes the hydrolysis of Arg. Arginase 2 further competed with NOS for Arg to hydrolyze it into ornithine (Orn) and urea (UR), resulting in reduced Arg bioavailability and reduced NO levels (76). *In vitro* studies have found that $1,25(OH)_2D$ can inhibit LPS-stimulated changes in iNOS mRNA levels in macrophages and significantly reduce NO production (77). $1,25(OH)_2D$ also regulates NO production and iNOS expression in macrophages, endothelial cells, osteoblasts, microglia, macrophages, and astrocytes (78, 79). Population studies have revealed significant negative correlations between serum NO and VD levels in normal adolescents (80). In addition, NO can inversely regulate $1,25(OH)_2D$ levels. CYP27B1 is a 1α hydroxylase gene whose expression is bidirectionally regulated by NO levels. The transcription of CYP27B1 can be upregulated at low concentrations of NO but inhibited by high concentrations of NO, thereby further affecting the levels of $1,25(OH)_2D$ (81). Therefore, VD levels are closely related to NO levels, and the two influence each other.

CONCLUSIONS

VD deficiency during pregnancy and early brain development is an important environmental risk factor for ASD, and high-dose VD can improve the core symptoms of ASD. The serum VD levels in children with ASD are significantly negatively correlated with the levels of the raw material for NO synthesis, Arg. NO, one of the most important signaling molecules in the brain, is involved in the occurrence of various brain-related diseases and is an important pathological molecule in ASD, Its role in ASD is mediated by a series of mechanisms, such as neurotransmitter imbalance, immune disorders, and oxidative stress. VD can regulate the levels of molecules in the NO signaling pathway. This review hypothesizes a potential mechanism in which abnormal levels of NO during the critical period of brain development caused by various factors (including abnormalities in neurotransmitters, immune disorders, etc.) are important factors in the pathogenesis of ASD, and VD affects the pathogenesis and severity of ASD by regulating NO levels. If this hypothesis is verified, monitoring NO levels during pregnancy and early brain development may become an important strategy for ASD risk prediction, and NO levels may guide the timing and dosing of VD supplementation. Researches on this pathway could contribute to detect more objective criteria for the diagnosis of ASD. The combination of VD and NO- related issues might indicate possible subtype of ASD. Eventually this may lead to more effective prevention and treatment of ASD.

AUTHOR CONTRIBUTIONS

BW, LX, and HD contributed to conception of the review and wrote sections of the manuscript. BW, HL, and XY participated in the literature search. BW wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Different Eye Tracking Patterns in Autism Spectrum Disorder in Toddler and Preschool Children

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Background: Children with autism spectrum disorder (ASD) have been observed to be associated with fixation abnormality as measured eye tracking, but the dynamics behind fixation patterns across age remain unclear.

Materials and Methods: In this study, we investigated gaze patterns between toddlers and preschoolers with and without ASD while they viewed video clips and still images (i.e., mouth-moving face, biological motion, mouthing face vs. moving object, still face picture vs. objects, and moving toys).

Results: We found that the fixation time percentage of children with ASD showed significant decrease compared with that of TD children in almost all areas of interest (AOI) except for moving toy (helicopter). We also observed a diagnostic group (ASD vs. TD) and chronological age (Toddlers vs. preschooler) interaction for the eye AOI during the mouth-moving video clip. Support vector machine analysis showed that the classifier could discriminate ASD from TD in toddlers with an accuracy of 80% and could discriminate ASD from TD in preschoolers with an accuracy of 71%.

Conclusion: Our results suggest that toddlers and preschoolers may be associated with both common and distinct fixation patterns. A combination of eye tracking and machine learning methods has the potential to shed light on the development of new early screening/diagnosis methods for ASD.

Keywords: autism, eye tracking, percentage of fixation time, machine learning, toddler, preschool, support vector machine

INTRODUCTION

Autism spectrum disorder (ASD) is a common neurodevelopmental disorder. Literature suggests that early intervention can significantly impact the prognosis of individuals with ASD and reduce cost of care (1–4). Thus, early detection plays an important role in the treatment of ASD (5, 6).

In recent years, eye tracking (ET), a non-invasive and convenient measurement tool, has drawn the attention of investigators (7–10). The rationale behind applying eye tracking in ASD research is that ASD is associated with different attention patterns from typical development (11–14). Thus, measuring eye movements and gaze patterns using eye tracking technology may help us understand the aberrant behavioral associated with individuals with ASD, and distinguish individuals with ASD from typically developing (TD) individuals.

Faces convey rich personal, emotional and social information starting soon after birth. During even a brief encounter, individuals can automatically attend to and quickly perceive the complex information present in a face, recognizing emotional state and social context, and often remembering the individual face later (15). Brain imaging studies have suggested that eye contact can evoke activity in brain areas in the social brain network, while developmental studies have shown evidence for preferential orienting toward, and processing of faces with direct gaze from early in life (16). Accumulating evidences suggest ASD is associated with an atypical pattern of eye contact behavior (17); thus it is generally agreed that autism involves deficits in face processing. Nevertheless, the precise nature of these deficits and the relationships between anomalous face processing and atypical socio-emotional function in ASD remain unclear (15).

Similarly, biological motion (BM) also conveys information that allows for the identification of affective states and intentions (18, 19). It represents the ability of individuals to detect, label and interpret human movement and to associate certain emotional states with these movements. Thus, biological motion reflects a certain level of social perception (18). Moreover, neurotypically developing individuals have been shown to be able to readily extract socially relevant information from sparse visual displays. Specifically, point-light displays (PLDs), which portray biological motion with points located only on major joints, are readily recognized as depicting differing actions by neurotypically developing individuals, which requires integration across these discrete points of light in order to disambiguate the form (20).

Thus, videos or photographs of human faces and biological motion are the most commonly used in eye tracking paradigms (12, 21–31). With the aid of these paradigms, researchers have identified unique gaze patterns in children with ASD. In a recent meta-analysis, which included 122 independent studies and 1,155 comparisons, Frazier and colleague (21) found a reliable pattern of gaze abnormalities in individuals with ASD, suggesting that individuals with autism have difficulty selecting socially relevant vs. irrelevant information for attention. This problem persists across ages and worsens during perception of human interactions. In addition, the researchers found that although the gaze abnormalities were present across a wide array of stimuli and AOIs, adding perceptual complexity to stimuli or increasing cognitive load during attention-heavy tasks does not increase gaze abnormalities in autism (21).

Nevertheless, few studies have investigated the eye tracking differences across different chronological age groups. Brain imaging studies found that the brain functional and structural alterations associated with ASD may differ across different age groups (5, 6), which indicates that pathophysiology associated with ASD may vary across different ages. In an early study (32), investigators examined temporo-spatial gaze patterns in children (age range 2.8–9) and adults with and without ASD while they viewed video clips. They found that children and adults were separated on the plane, showing a clear effect of age on gaze behaviors. They also found that typically developed infants preferred to watch the mouth rather than the eyes during speech, a preference that reverses later in development. These results highlight the importance of taking the effect of age into account when addressing the gaze behaviors that are characteristic of ASD.

To the best of our knowledge, no study has been carried out to investigate/compare eye tracking patterns between toddlers (ages 1–3) and preschoolers (ages 3–5) with and without ASD. We are particularly interested in these two chronological age groups because despite a clinical diagnosis of ASD, a proportion of the children identified with possible autism before age 3 do not meet criteria for autism at later follow-up (33). Furthermore, although accurate diagnosis of autism spectrum disorder at earlier than 18 months is feasible and overall is more stable than other diagnostic categories (34); earlier diagnosis is associated with many factors such as greater symptom severity, high socioeconomic status, and greater parental concern about initial symptoms (35). Many children are not diagnosed with ASD until age 3 or older despite displaying symptoms much earlier (33, 36–38). Also, 3 years old is cutoff between toddler and preschooler, which represents different developmental milestone.[1] This calls for intensive investigation of the difference between the toddler and preschooler.

Elucidating the eye tracking pattern differences between the toddler and preschooler will significantly enhance our understanding of the dynamic characteristics of eye tracking patterns. Thus, the primary aim of this study is to investigate the eye tracking pattern differences between ASD and TD children across toddler and preschool stages using simple paradigms consisting of short videos clips and pictures (**Figure 1**).

Recently, investigators have tried to use different methods to distinguish ASD from TD in addition to traditional statistical methods, such as the logistic regression model (13). Machine learning techniques, including the support vector machine (SVM), have been applied to identify biomarkers for ASD (39–41). As a subset of artificial intelligence in the field of computer science, machine learning is a procedure that trains the computer algorithm to analyze a set of observed data and statistically learn the latent patterns without being explicitly programmed. One characteristic of SVM is that it does not require any prior assumptions about the underlying relationship between variables (42). In the past, SVM has been used to distinguish between individuals with ASD and TD using multiple measurements such as behavior (43, 44), brain structure (45, 46), activity (47), connectivity (48), and eye tracking (11, 49), and has achieved encouraging results. Thus, this study also aims to explore the eye tracking features that can be used to distinguish ASD from TD across different age groups using SVM.

[1] https://www.cdc.gov/ncbddd/childdevelopment/positiveparenting/

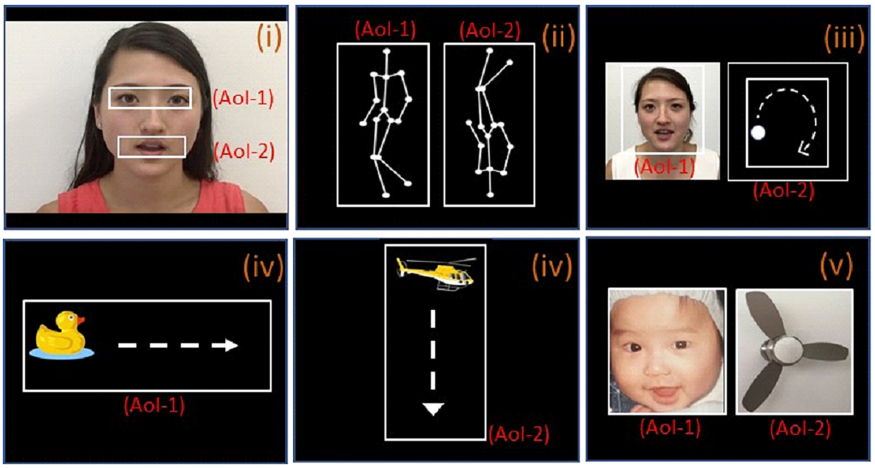

FIGURE 1 | The paradigms used for eye-tracking with area of interest (AOI). **(i)** Female face with moving mouth; AOI-1 and AOI-2 include the eye and mouth regions, respectively. **(ii)** Biological motion; AOI-1 and AOI-2 are point-light display figure of a person walking upright and in the other side, inverted images (upside down), respectively. **(iii)** People and geometry (same size); AOI-1 and AOI-2 are same-sized images of human face with moving mouth and geometry (moving white dot) respectively. **(iv-1)**, a clipart duck moving horizontally from the left side of the screen to the right side, the moving pathway is AOI-1, **(iv-2)**, a clipart helicopter moving vertically from the top of the screen to the bottom, moving pathways is the AOI-2; **(v)**, baby and object (same size); AOI-1 and AOI-2 are same-sized images of baby face and fan respectively.

MATERIALS AND METHODS

Participants

Participants in the ASD group were recruited from the Child Mental Health and Rehabilitation Center of the Shenzhen Maternity & Child Healthcare Hospital in China. Similar to our previous study, inclusion criteria were: (1) aged 1.5–3 or 3–5 years old and (2) Childhood Autism Rating Scale (CARS) score greater than 30 (for those 2 years and older), in line with the ADOS standard. All diagnoses were made by experienced physicians based on the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5).

TD children were recruited as controls from (1) a kindergarten in the same city or (2) routine annual check-ups in an outpatient ward of the Department of Child Psychiatry and Rehabilitation, Shenzhen Maternity and Child Healthcare Hospital. Specifically, we used the Ages and Stages Questionnaires (Chinese Version) (ASQ-C) (50), Chinese children warning signs checklist (51) and Developmental Screening Parent Questionnaire for Children Aged 0–6 (52) to assess TD children's communication/language, physical ability, social skills, problem-solving skills, and intelligence. All screenings were performed by a study physician by onsite assessments and communicating with the parents or guardians. Only children that passed the screening, with no history of psychiatric or neurological disorders were included in the study. All participants (ASD and TD) had normal or corrected vision.

A portion of preschool data using paradigm (video clip) data had been used in a previous study to explore whether different parts of body (eye, mouth, nose, hair, and body) could help distinguish ASD from TD (53). However, this study used more comprehensive paradigms and the findings presented in this study have never been published before. The study protocol was approved by the Ethics Committee of the Shenzhen Maternity and Child Healthcare Hospital, Shenzhen, China, and all parents provided and signed informed consent. Informed consent was obtained for publication of identifying information/images in an online open-access publication in the Materials and Methods section.

Experimental Procedure

Similar to our previous study (53), the SMI RED250 portable eye tracking system was used in data collection. Screen resolution was set to 1,024 × 768 pixels with a sampling frequency of 250 Hz and spatial resolution of 0.03 degrees. Children were seated in front of a 22-inch widescreen LCD monitor in a dark and soundproof room. The center of their vision was aligned with the center of the monitor, with an eye-to-monitor distance of 65 cm. Before the short video presentation, eye position correction was performed by having the subjects fixate on a dynamic pink rabbit (five-point calibration). Those who failed to follow the dynamic pink rabbit fixation did not continue in the experiment. After five-point calibration, silent video clips were presented. The stimulus described below (**Figure 1**) was presented. Gaze patterns were recorded on a BeGaze data analysis software system. To assess the quality of the data, before the data analysis, the eye tracking instrument Gaze Replay (overall path playback) was used to exclude the subjects whose fixation point deviates from the screen to reduce systematic errors. Only the data of the participants who cooperated, completed the whole experiment and passed the quality check were included in the data analysis.

Stimuli

The stimuli consisted of several simple video clips and pictures prepared by co-authors (JP, JK, XJK, copy right obtained to use

for study and publication). Please see **Figure 1** for details of stimuli and AOIs.

Paradigm i [Video 1 (10 s)]: This video consisted of a woman sitting and mouthing the alphabet without sound. The eyes, which play an important role in social communication and emotional expression, constituted one area of interest (AOI) and the mouth, which represents functional responses related to (early) stages of normative language development, constituted another (11). A similar paradigm (with different background) had been used in a previous study (11).

Paradigm ii [Videos 2a and b (5 s each)]: A point-light display figure of a person walking upright was shown on one side of the screen. On the other side, the same figure was shown rotated 180 degrees, with the person appearing to walk upside down. Each figure was determined as an AOI. A similar paradigm has been used in previous studies (7, 11). Scenario 2b was identical to video 2a, but with the positions of the figures switched (left vs. right side of the screen). The fixation time percentage in 2a and 2b was averaged during data analysis. Preferential attention to biological motion is a fundamental mechanism facilitating adaptive interaction with other living beings. Previous studies have found that children with ASD fail to orient toward point-light displays of biological motion (12, 28).

Paradigm iii [Videos 3a and b (5 s)]: A video of a white dot moving along a circular path (dynamic moving dot) was displayed on one side of the screen, while a video of a woman mouthing the alphabet was shown on the other side (dynamic character). A similar paradigm has been used in a previous study (11). Each video was an AOI with the same size. Video 3b was identical to Scenario 3a, but with the positions of the videos switched. The fixation time percentage in 3a and 3b was averaged during data analysis. This paradigm aims to test if the child favors a moving dot or a woman's face with a moving mouth to study children's social interest and social cognition.

Paradigm iv [Videos 4a and b (10 s)]: A clipart duck was shown moving horizontally from the left side of the screen to the right side (5 s, iv-1). This was followed by a clipart helicopter moving vertically from the top of the screen to the bottom (5 s, iv-2). The two moving pathways were the AOIs. The videos aim to test the ability of keeping attention on a moving animal and object.

Paradigm v [Video 5 (5 s)]: An image of an electronic fan was presented next to an image of an infant's face. The aim of the task is to assess whether the child favors looking at the face or the object (fan) to study children's social interest and social cognition.

Data Analysis

BeGaze software was used for data pre-processing. The software applied filtering, interpolation and smoothing to preprocess the data, including the missing data. The fixation threshold settings were set to duration > 100 ms and max displacement <1°of visual angle. Similar to previous studies (11, 54), we used the percentage fixation times allocated to each AOI. The fixation time percentage on each AOI was automatically calculated (time allocated to an AOI/duration of stimulus presentation) by BeGaze and exported to excel file for statistical analysis.

Data analyses were processed with R-studio version 3.5.2. Sociodemographic data between the ASD and TD groups were analyzed using two independent sample *t*-tests and $\chi 2$. AOI fixation time percentage analysis was performed with ANCOVA (for each AOI separately). Fix effects include diagnostic group (ASD vs. TD), age group (toddler vs. preschool) and their interaction. Covariate includes age and gender. For each AOI analysis, a test for equality of variance (Levene's) was applied for Assumption Checks. For AOIs with $p < 0.05$ for the Levene's test (assumption was not met), weighted least squares (WLS) was applied. *Post-hoc* analysis (Tukey correction) was applied to compare the differences between the preschool ASD vs. the preschool TD, and toddler ASD vs. the toddler TD (except for AOIs of eye and helicopter).

For AOIs of eye and helicopter, the age group by diagnosis group interaction was significant in the ANCOVAs, and therefore the contrasts between ASD and TD and Toddler and Preschool could not be tested using *post-hoc t*-tests. We fit linear regression models for these AOIs, including age-group, diagnosis group and their interaction, and adjusting for age and gender, and calculated estimates and standard errors for the contrasts of interest, using the regression coefficient estimates and estimated variance-covariance matrix of these coefficients from the regression model results. We adjusted for multiple comparisons using the multivariate-t method in the "multcomp" library in "R" (55, 56).

Discriminant Analysis

To explore the feasibility of using eye-tracking for the diagnosis of ASD, we applied machine learning with a support vector machine (SVM) to discriminate children with ASD from TD children, using the percentage of fixation time on each AOI as data for this classification, as in our previous publications (48, 53). Since the two cohorts of children (toddler and preschool) exhibited different characteristics in fixation time percentage on different AOIs (see Results for details), we performed the analyses for toddlers and preschool children separately.

Performance of the procedure was assessed by the percentage correctly classified for ASD subjects (sensitivity), and TD subjects (specificity). The accuracy of the procedure was defined to be the percentage of correct classifications combining the two groups. All analyses were based on leave-one-out cross validation by dividing *N* participants ($N = 95$ for toddler; $N = 74$ for preschool) into N-1 training samples and 1 test sample. The same procedure was repeated *N* times to make sure that each participant was used as a test sample once. We then predicted the group assignment for each subject with a separate classifier, determined using only the data from the other subjects, omitting the one predicted. Finally, we determined the performance measures (sensitivity, specificity, accuracy) using these predictions, along with the true group assignments.

RESULTS

For the toddler cohort, 55 ASD children and 40 age-matched TD children were included in data analysis. For the preschool cohort, 37 ASD children and 41 age-matched TD children were included in data analysis. There were no significant differences in age or gender between ASD and TD in the two age groups (**Table 1**).

Participants' Fixation Time Percentage at AOIs

ANCOVA showed a significant main effect of diagnostic group (ASD vs. TD) on all AOIs except the moving helicopter, and a significant main effect of age group (toddler vs. preschool) only on the dynamic moving dot. Interestingly, we found significant group and age interaction on fixation time percentage for the eye and moving helicopter AOIs (**Table 2**).

Because of the significant interactions in the ANCOVAs for eye and helicopter AOIs, we tested the significance of contrast of interest using linear regression, as discussed in the Materials and Methods section. Analysis on the eye AOI showed that p = 0.93 for toddler (ASD vs. TD), p = 0.006 for preschool (ASD vs. TD), p = 0.98 for ASD (toddler vs. preschool), p = 0.68 for TD (toddler vs. preschool). For the moving helicopter AOI, p = 0.65 for toddler (ASD vs. TD), p = 0.31 for preschool (ASD vs. TD), p = 0.37 for ASD (toddler vs. preschool), p = 1.0 for TD (toddler vs. preschool).

As an exploratory analysis, we all applied *post-hoc* analysis on the ASD and TD differences across different age groups on all AOIs, and calculated the effect size of each comparison, the results are presented in **Table 3**.

Preferential attention to biological motion is a fundamental mechanism facilitating adaptive interaction with other living beings. Thus, we also investigated biological motion across different age groups. The within-group analysis (paired t-test) in four groups (respectively) between the walking upright AOI and walking upside down AOI showed that only preschool TD preferentially attend to biological motion (walking upright) (p = 0.01), and there is no significant difference in all other groups (preschool ASD, toddler TD and toddler ASD, p = 0.84, 0.59, 0.69, respectively).

TABLE 1 | Demographic and clinical traits for all participants (mean ± SD).

Characteristics	Toddler cohort			Preschool cohort		
	ASD (n = 55)	TD (n = 40)	P-value	ASD (n = 37)	TD (n = 41)	P-value
Age (years)	2.4 (0.5)	2.2 (0.6)	0.27	4.6(0.5)	4.8(0.3)	0.11
Gender† (boys/girls)	50/5	33/7	0.22	33/4	33/8	0.29
CARS score	32.3 (1.5)	–	–	33.0 (2.1)	–	–

†*The p-value was obtained by χ^2 test; other p-values were obtained by a two independent samples t-test. CARS, childhood autism rating scale. Of the 55 toddlers, there were eight children who were younger than 2 years old (age ranging from 1.6 to 1.8 years old). The CARS scores were only calculated on children 2 years or older in the Toddler group.*

TABLE 2 | Fixation time percentage of the areas of interest (AOIs).

AOIs	Toddler cohort		Preschool cohort		Effect of group (ASD vs. TD)	Effect of age (Toddler vs. Preschool)	Effect of interaction
	ASD	TD	ASD	TD			
Mouth†	9.1 (13.6)	23.1 (20.3)	11.4 (13.2)	18.0 (16.8)	$F_{(1,167)}$ = 15.40, $p < 0.001$	$F_{(1,167)}$ = 0.20, p = 0.65	$F_{(1,167)}$ = 2.21, p = 0.14
Eye#	15.4 (17.1)	17.6 (20.1)	15.1 (17.5)	29.2 (20.3)	$F_{(1,167)}$ = 7.56, p = 0.007	$F_{(1,167)}$ = 0.19, p = 0.67	$F_{(1,167)}$ = 3.99, p = 0.048
Walking on hands	19.4 (18.1)	35.5 (21.3)	22.1 (19.3)	36.2 (16.9)	$F_{(1,167)}$ = 26.41, $p < 0.001$	$F_{(1,167)}$ = 2.01, p = 0.16	$F_{(1,167)}$ = 0.36, p = 0.55
Walking upright	20.7 (17.3)	33.3 (20.0)	21.5 (16.5)	28.0 (14.9)	$F_{(1,167)}$ = 11.94, $p < 0.001$	$F_{(1,167)}$ = 1.14, p = 0.29	$F_{(1,167)}$ = 1.56, p = 0.21
Dynamic character†	18.9 (16.1)	32.3 (18.6)	19.8 (18.1)	25.2 (12.1)	$F_{(1,167)}$ = 12.75, $p < 0.001$	$F_{(1,167)}$ = 0.63, p = 0.42	$F_{(1,167)}$ = 2.61, p = 0.11
Dynamic moving dot	20.1 (16.1)	39.4 (20.4)	25.7 (18.6)	37.5 (17.8)	$F_{(1,167)}$ = 32.79, $p < 0.001$	$F_{(1,167)}$ = 8.79, p = 0.003	$F_{(1,167)}$ = 3.62, p = 0.06
Helicopter#	20.6 (15.4)	16.5 (12.5)	19.8 (14.3)	25.5 (15.0)	$F_{(1,167)}$ = 0.21, p = 0.644	$F_{(1,167)}$ = 0.65, p = 0.42	$F_{(1,167)}$ = 3.79, p = 0.05
Duck†	20.0 (17.0)	22.2 (13.9)	19.1 (15.0)	27.6 (13.4)	$F_{(1,167)}$ = 5.65, p = 0.019	$F_{(1,167)}$ = 3.59, p = 0.06	$F_{(1,167)}$ = 1.03, p = 0.31
Baby face	20.0 (18.8)	36.9 (21.6)	21.6 (17.7)	32.1 (16.2)	$F_{(1,167)}$ = 22.75, $p < 0.001$	$F_{(1,167)}$ = 2.31, p = 0.13	$F_{(1,167)}$ = 1.72, p = 0.19
Electric fan	16.7 (17.0)	19.8 (14.3)	16.9 (16.9)	29.3 (17.0)	$F_{(1,167)}$ = 8.80, $p <= 0.003$	$F_{(1,167)}$ = 0.67, p = 0.41	$F_{(1,167)}$ = 2.56, p = 0.11

Data were presented in mean (SD); †Indicates for AOI with $p < 0.05$ for the Levene's test (assumption of the equal variance was not met), weighted least squares (WLS) model was applied. #Indicates significant interaction between the diagnosis group and age group. See Results section for results from contrasts of interest using linear regression.

TABLE 3 | Fixation time percentage differences (AOIs).

AOIs	Toddler (ASD vs. TD)		Preschool (ASD vs. TD)	
	P-value	Effect size	*P*-value	Effect size
Mouth	$p < 0.001$	0.81	$p = 0.25$	0.44
Eye[#]	$p = 0.93$	0.12	$p = 0.006$	0.74
Walking upside down	$p < 0.001$	0.81	$p = 0.01$	0.78
Walking upright	$p = 0.005$	0.67	$p = 0.4$	0.41
Dynamic character	$p < 0.001$	0.77	$p = 0.54$	0.35
Dynamic moving dot	$p < 0.001$	1.05	$p = 0.03$	0.65
Helicopter[#]	$p = 0.65$	0.23	$p = 0.31$	0.37
Duck	$p = 0.86$	0.14	$p = 0.08$	0.60
Baby face	$p = 0.001$	0.83	$p = 0.07$	0.62
Electric fan	$p = 0.80$	0.20	$p = 0.01$	0.73

P-value derived from the post-hoc analysis (Tukey correction) between ASD and TD across different developmental stages. Effect size is measured by Cohen's d.
[#]Indicates comparisons of interest using linear regression, as described in Materials and Methods section.

To explore the association between gaze fixation time percentage and clinical outcome (CARS), we applied a multiple regression analysis including age and gender as covariates for all ASD children. We found no significant association between fixation time percentage and CARS (*p*-values range from 0.13 to 0.99).

Discriminant Analysis

Support vector machine analysis showed that the classifier could discriminate ASD from TD in toddlers with an accuracy of 80%, a sensitivity of 80%, and a specificity of 82%; and could discriminate ASD from TD in preschoolers with an accuracy of 71%, a sensitivity of 81%, and a specificity of 61%.

Single AOIs, including the moving mouth, walking upright, moving dot, and baby face provided higher accuracies in classification for toddlers. The moving duck, moving helicopter, electric fan picture, and eyes were ranked as the least discriminative features and did not show significant classification accuracies (below the red dashed line in **Figure 2A**) for ASD toddlers. In contrast, the eyes, moving duck, infant face, and electric fan were the four most important AOIs for discriminating ASD preschoolers from TD preschoolers, and the dynamic character and moving helicopter were ranked as the least discriminative features (**Figure 2B**) for ASD preschoolers.

DISCUSSION

In this study, we investigated fixation time patterns between ASD and TD children in toddler and preschool stages. We found that ASD is associated with significant fixation time percentage reduction in most of the AOIs as indicated by the main effect of groups (ASD vs. TD), but only one significant difference between the toddler and preschool groups as indicated by main effect of age group. Interestingly, we also found that toddler and preschool-aged children with ASD are associated with different fixation patterns during the video of the moving mouth (video 1). The fixation time percentage on eye AOI between the ASD and TD children in the toddler is similar, but in the preschool-aged children with ASD, the fixation time percentage on eye ROI is significantly reduced compared to TD (**Table 3**). The support vector machine analysis showed that the classifier could discriminate ASD from TD with an accuracy of 80%, a sensitivity of 80%, and specificity of 82% in toddlers, and an accuracy of 71%, sensitivity of 81%, and specificity of 61% in preschoolers.

The paradigm 1 is a video clip of a female moving mouth with eye and mouth areas as AOIs. In an earlier study on fifteen male adolescents and young adults with autism and 15 matched control, Klin et al. (7) found that individuals with autism are associated with reduced salience of eyes and increased salience of mouths, bodies, and objects. Fixation times on mouths and objects but not on eyes are strong predictors of degree of social competence. In a following study, Dalton et al. (22) conducted two separate fMRI studies (one with 14 male adolescents with ASD, another with 15 male adolescents with ASD) using eye tracking while measuring functional brain activity during facial discrimination tasks. In both studies, the authors found that activation in the fusiform gyrus and amygdala was strongly and positively correlated with the time spent fixating the eyes in the ASD group, suggesting a heightened emotional response associated with gaze fixation in autism. Following studies provide a more complicated view on eye tracking patterns in ASD. For instance, in a review article on this topic (57), investigators showed that although individuals with ASD often give less preferential attention to social objects and events (faces, people, and social actions), the excessive mouth and diminished eye gaze hypothesis of ASD (58) is not generally supported. Different age groups may be associated with different patterns.

We found significant group and age group interaction on fixation time percentage for the eye AOI. Specifically, we found that for the eye AOI, toddler ASD, and TD is similar, but preschool TD is associated greater fixation time than ASD. For the AOI of the mouth, preschool ASD and TD is similar, but toddler TD is associated with significantly greater fixation time. One potential interpretation is that perhaps toddler TD children tend to take more use of visual mouth information than children with ASD who often have language delays (57). Thus, the toddler TD children pay more attention to the mouth, which in turn reduces attention to the eyes (11). As age increases, the language (sound) itself becomes more meaningful for preschool children. This may explain why preschoolers pay preferential attention to the eyes and have similar attention to the mouth.

In the paradigm ii, we investigated biological motion across different age groups. Preferential attention to biological motion is a fundamental mechanism facilitating adaptive interaction with other living beings. An early study found that 2-year-olds with autism fail to orient themselves toward point-light displays of biological motion (7). In a following study, investigators found that children with autism aged 3–7 years old (28) failed to show preferential attention to biological motion, but age-matched TD do. In our study, we found that only preschool TD preferentially attend to biological motion (walking upright AOI compared to walking upside down AOI). This finding is consistent with the previous study (28), in which investigators

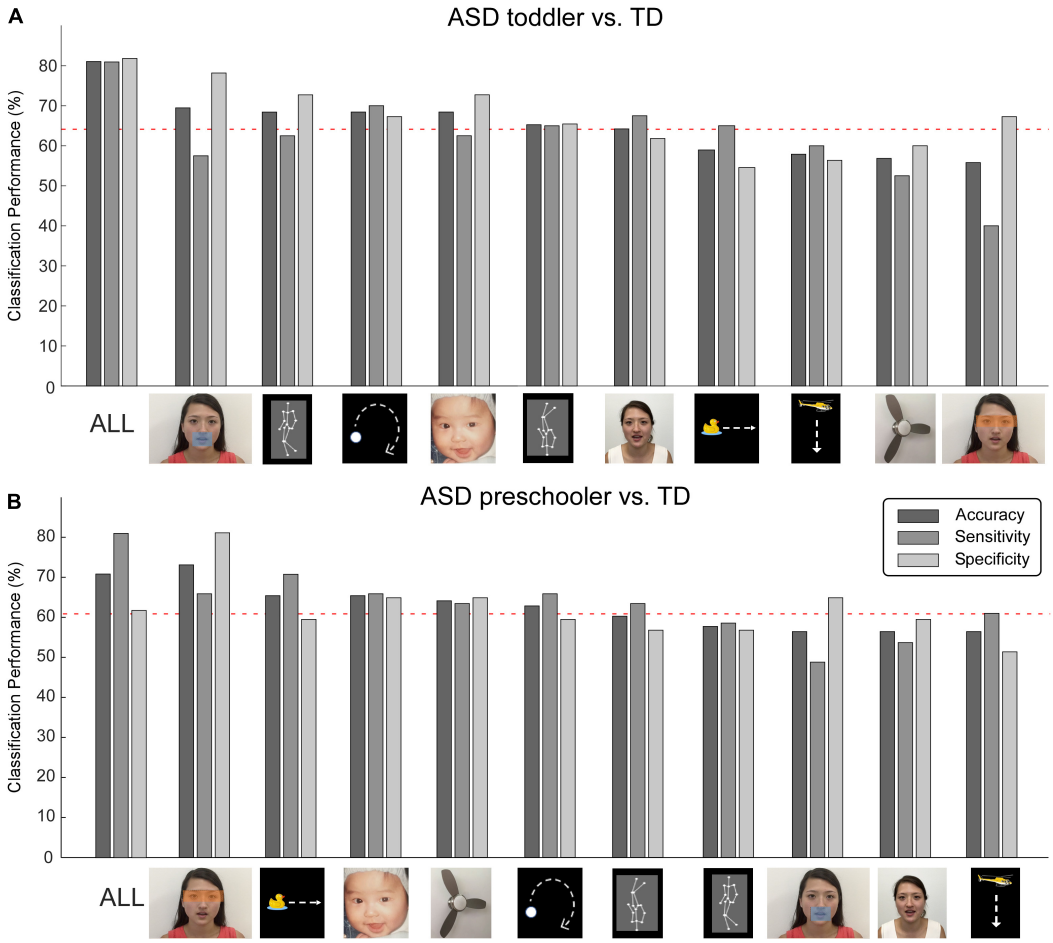

FIGURE 2 | Machine learning accuracies for discriminating autism spectrum disorder (ASD) and typically developing (TD). Classification accuracy, sensitivity, and specificity for all AOIs and 10 single AOIs, for toddlers **(A)** and preschoolers **(B)**, respectively. Red dashed lines represent the 95% confidence interval obtained from non-parametric permutation testing.

found that children with ASD between 3 and 7 years old (28), but not age-matched TD, failed to show preferential attention to biological motion. Our result is only partly consistent with the earlier study on 2-year-old children (7) in which the authors found toddler ASD failed to show preferential attention to biological motion. However, we found that toddler TD also failed to show preferential attention to biological motion, which is not consistent with findings from the previous study (7). It is worth noting that the age range for our study is between 1.5 and 3, while the previous study focused only on 2-year-olds, so we cannot exclude the possibility that the discrepancy is due to age range difference. Further studies are needed to better understand this inconsistency.

In paradigm iii, we compared a white dot moving along a circular path (dynamic moving dot) on one side of the screen and a woman (dynamic character) mouthing the alphabet on the other side. We found a significant main effect on diagnostic group and age (with preschoolers showing greater fixation percentage) on the AOI of the dynamic moving dot in the paradigm. *Post-hoc* analysis indicates there is no significant difference when we analyze the ASD and TD separately in different stages; nevertheless, the effect size is much larger in Toddler group compared to preschool group (1.05 vs. 0.65, **Table 3**).

Paradigm iv is a video clip of a clipart duck moving horizontally from the left side of the screen to the right side, followed by a clipart helicopter moving vertically from the top of the screen to the bottom. We use this to test attention to moving objects. We found that there is no significant diagnostic group main effect on the helicopter AOI, but a significant main diagnostic group effect on the moving duck AOI. We also found a significant interaction between the group and age on the helicopter AOI, but there is no significant difference detected on *post-hoc* analysis (**Table 3**). These findings suggest that for some objects such as the moving helicopter, the preferential attention between the ASD and TD is similar in toddler and preschool children with ASD and age-matched TD children.

In paradigm v, we compared an image of an electronic fan and a baby's face. The results showed that TD is associated with high fixation time percentage as compared to ASD for both AOIs. Further analysis showed that toddler (but not preschool)

ASD showed less fixation time percentage to a baby's face (person related picture). This suggest that only toddler ASD is associated with less preferential attention to social objects and events (faces). We are not sure if this is due to relatively larger sample size in the toddler groups. Further studies are needed to validate this finding.

In this study, we also employed support vector machines (SVM) on fixation time percentage of pre-defined AOIs to discriminate ASD from TD. SVM is a supervised learning model and binary classifier that is used to perform classification analysis. In a study conducted (49) on children from 4 to 11 years old, Liu et al. (49) applied a machine learning method to identify the participants with ASD based on eye tracking data during a facial recognition task, and found they discriminated ASD from TD children with 86.2% specificity and 93.1% sensitivity. Fujioka et al. (11) explored visual fixation time on objects in movies in male adolescents and adults with ASD and found fixation time on objects with large effect sizes for group differences could be used to distinguish ASD from TD participants (sensitivity = 81.0%, specificity = 80.0%). More recently, we (53) conducted an experiment with ASD and TD preschoolers (4–6 year-old) using a moving mouth paradigm (paradigm i) and found that fixation time for the moving mouth and upper body (shoulder) could successfully distinguish ASD from TD (accuracy = 82.8%, sensitivity = 79.3%, specificity = 86.2%).

We found that the classifier could discriminate ASD from TD in toddlers with an accuracy of 80%, a sensitivity of 80%, and a specificity of 82%, and it could discriminate ASD from TD in preschoolers with an accuracy of 71%, a sensitivity of 81%, and a specificity of 61%. Although the paradigm applied in this study produced discriminative accuracy comparable to previous studies, our findings are unique in two respects. First, our study population was between 1 and 3 years old (in the toddler stage), which is much younger than the previous studies mentioned above that applied SVM to distinguish ASD from TD. Toddler years are a crucial period for ASD diagnosis and intervention (33, 36, 37), and the ability to distinguish ASD from TD during toddler years using the machine learning method demonstrates the potential of applying eye tracking for early screening.

Also, a recent meta-analysis (59) on accuracy of Machine Learning Algorithms for the Diagnosis of ASD using Brain Magnetic Resonance Imaging tools showed a sensitivity of 0.83 (95% CI 0.76–0.89), a specificity of 0.84 (95% CI 0.74–0.91) using structural MRI, and a sensitivity of 0.69 (95% CI 0.62–0.75), specificity of 0.66 (95% CI 0.61–0.70) using functional MRI. Our findings based on the eye tracking are comparable with results obtained from sophisticated brain imaging tools. Our results are also comparable with that of studies using traditional statistical methods such as logistical regression analysis. Frazier and colleagues have tried to build an autism symptom index based on eye tracking data collected while the children viewed a 5-min video that included 44 dynamic stimuli from seven distinct paradigms. They found that the autism risk index had high accuracy for ASD diagnosis (area under the curve [AUC] = 0.86), whereas the autism symptom index was significantly associated with ADOS-2 total severity scores (13).

Additionally, we found that the roles of different paradigms in discriminating ASD from TD differ significantly. For instance, the moving duck, helicopter, electronic fan, and eyes (accompanied by moving mouth) cannot be used to distinguish ASD from TD toddlers. Yet, it can be used to distinguish ASD from TD preschoolers. Elucidating the role of different paradigms in ASD classification across different stages may shed light on developing paradigms for earlier ASD detection, particularly for toddlers between 1.5 and 3 years old or even younger.

There are several limitations to this study. First, the ASD diagnoses were made by licensed pediatric psychiatrists based on the DSM-V and CARS (for those 2 years or older), but not confirmed by the ADOS/ADI-R due to limited access to these diagnostic tools in China. Although the TD were screened by licensed pediatrician for psychiatric or neurological disorders and have normal communication/language, physical ability, social skills, problem-solving skills, and intelligence, we did not assess the CARS in TD Children. Second, the sample size was relatively small, and we did not include independent data sets to validate our findings. Thirdly, we did not include developmental assessment or use developmental age in this study. As ASD display significant heterogeneity, predominant ASD studies used chronological age instead of developmental age. Also, in practice, a child transition from Toddler to preschooler is typically based on the chronological age without developmental testing. Thus, we believe that our study of using chronological age is in consistent with previous ASD studies. Future studies may also need to consider adding a developmental test in research on this topic. Forth, we decided not to report positive predictive value (PPV) and negative predictive value (NPV) in this study as it is well-known that PPV and NPV change as prevalence changes. Since the prevalence of autism is very low (1–2%), one can expect a low PPV and high NPV. For example, using the above toddler values of 80% sensitivity and 82% specificity, and assuming 1% prevalence in the population as does the reviewer, the PPV is approximately 4.2%. On the other hand, using these same values, NPV is greater than 99%. Readers should be able to calculate PPV and NPV based on the sensitivity, specificity, and prevalence or *a priori* probability. This decision of not including PPV and NPV is consistent with previous ASD studies on this topic (11, 48, 49). Finally, although we believe that the technology we present is promising and could ultimately lead to a significant improvement in the screening/diagnosis of ASD, this is still an early stage of paradigm development and data analysis for the application of eye tracking in ASD research, the identifying of the most sensitive features to distinguish ASD from TD, and continuous input on this topic is needed for the improvement of clinical implementation of this valuable tool for ASD screening and diagnosis.

In particular, we compared typical cases of ASD with a group of TD with no clear ASD symptoms. Children with a history of psychiatric or neurological disorders were excluded from the TD sample, which may have produced spectrum bias (60). Thus, the ability of the eye tracking to discriminate between the ASD and non-ASD as shown in our study may be overestimated in medical practice. Future studies should include a broad spectrum of the ASD and non-ASD participants to further validate our findings.

In summary, we investigated the fixation time of toddler and preschool children with ASD using different paradigms. Results indicated that toddler and preschool ASD showed both common and unique eye tracking patterns. These findings may shed light on our understanding of the development (age) of ASD and facilitate the development of new screening methods for early ASD detection.

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 Ethics Committee of the Shenzhen Maternity and Child Healthcare Hospital, Shenzhen, China. 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) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

X-JK, ZW, GWa, JK, and ZF: experiment design. BS and GWa: data collection. YT, MC, SY, YH, and JK: data analysis. JK, JP, GWi, GWa, MC, and X-JK: manuscript preparation. All authors contributed to the article and approved the submitted version.

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Functional Brain Networks in Preschool Children With Autism Spectrum Disorders

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Objective: The present study aims to investigate the functional brain network characteristics of preschool children with autism spectrum disorder (ASD) through functional connectivity (FC) calculations using resting-state functional MRI (rs-fMRI) and graph theory analysis to better understand the pathogenesis of ASD and provide imaging evidence for the early assessment of this condition.

Methods: A prospective study of preschool children including 32 with ASD (ASD group) and 22 healthy controls (HC)group was conducted in which all subjects underwent rs-fMRI scans, and then the differences in FC between the two groups was calculated, followed by graph-theoretic analysis to obtain the FC properties of the network.

Results: In the calculation of FC, compared with the children in the HC group, significant increases or decreases in subnetwork connectivity was found in the ASD group. There were 25 groups of subnetworks with enhanced FC, of which the medial prefrontal and posterior cingulate gyrus and angular gyrus were all important components of the default mode network (DMN). There were 11 groups of subnetworks with weakened FC, including the hippocampus, parahippocampal gyrus, superior frontal gyrus, inferior temporal gyrus, precuneus, amygdala, and perirhinal cortex, with the hippocampus and parahippocampal gyrus predominating. In the network properties determined by graph theory, the clustering coefficient and local efficiency of the functional network was increased in the ASD group; specifically, compared with those in the HC group, nodes in the left subinsular frontal gyrus and the right middle temporal gyrus had increased efficiency, and nodes in the left perisylvian cortex, the left lingual gyrus, and the right hippocampus had decreased efficiency.

Conclusion: Alterations in functional brain networks are evident in preschool children with ASD and can be detected with sleep rs-fMRI, which is important for understanding the pathogenesis of ASD and assessing this condition early.

Keywords: preschool children, ASD, rs-fMRI, graph theory, functional connectivity

Abbreviations: ASD, autism spectrum disorder; HC, healthy control; rs-fMRI, resting state magnetic resonance imaging; BOLD, blood oxygen level-dependent; ADOS, autism diagnostic observation schedule; CARS, childhood autism rating scale; 3D-SPGR, sagittal three-dimensional SPGR sequencing; FD, framewise displacement; ROI, regions of interest; FC, functional connectivity; DMN, default mode network; AAL, Anatomical Automatic Labeling; NBS, Network-based Statistic.

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INTRODUCTION

Patients with autism spectrum disorder (ASD) are characterized by impaired social interactions and communication skills and typically demonstrate restricted interests and/or repetitive behaviors (1). The prevalence of ASD is increasing yearly, with the CDC reporting a prevalence of 1.7% (2, 3) in children aged 8 years in 2014 versus 1.85% in 2020 (1). The causes of ASD are complex, and it is now recognized that interactions between genetic and environmental factors may lead to early brain changes that result in the development of ASD (4). The diagnosis of autism in children is currently based on typical clinical presentations and an assessment of clinical scales, but some objective indicators and markers are still lacking.

With advances in imaging technology, resting-state functional magnetic resonance imaging (rs-fMRI), based on blood oxygen level-dependent (BOLD) imaging, has been widely used to study functional brain networks in patients with ASD. The form that functional brain connectivity takes in ASD is controversial, with differing views on whether it is under- or overconnected (5), some studies proposing that both types of connectivity abnormalities coexist in the form of reduced long-distance connectivity and increased short-distance connectivity (6), while other studies present evidence to the contrary (7). Uddin et al. reviewed several papers and found that the above inconsistencies may be correlated with patient age, i.e., functional brain connectivity is reduced in adolescents and adults with ASD but elevated in children with ASD (8).

Resting state magnetic resonance imaging imaging generally requires the subject to be awake, so it is often performed on adolescents or adults with ASD who are more compliant than children (9), for whom rs-fMRI is thus difficult to perform. Some researchers (10) have compared functional brain connectivity between awake and sedated states and found that although certain brain regions showed reduced activity after sedation, functional connectivity (FC) was still more significant, suggesting that stable spontaneous brain activity remains under sedation.

In light of these studies, we performed rs-fMRI imaging on children with ASD aged 2–6 years and explored their functional brain network characteristics using FC and graph theory-based brain network analysis methods in the hope of providing an imaging basis for the early assessment of ASD.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Research Ethics Committee of Children's Hospital of Chongqing Medical University [IRB number: (2018) Ethical Review (Research) No. (82)]. All parents of the subjects gave written informed consent after they had been informed of the possible risks and benefits of the research and were assured of the security and privacy concerning the children's medical records.

Participants

All participants were recruited at the child health care department of Children's Hospital of Chongqing Medical University, a tertiary hospital and National Children's Medical Center in China, from July 2014 to July 2016. ASD was rigorously diagnosed according to the DSM-5 criteria (11) and a Childhood Autism Rating Scale (CARS) score of more than 30 and confirmed by the Autism Diagnostic Observation Schedule (ADOS-2). ASD patients were included if they were 2–6 years old and were first-time patients who had not undergone appropriate rehabilitation. Exclusion criteria included other neurological disorders and genetic conditions, such as ASD with fragile X chromosome and Rett's syndrome. The healthy controls (HC) group was composed of healthy children aged 2–6 years. HC group children were volunteers openly recruited from July 2014 to July 2016 who underwent a general physical examination, MRI routine sequence scan, and the Gesell Developmental Scale (GDS) test. Inclusion criteria included no significant abnormalities in growth and development on the general physical examination, no significant organic diseases or delayed myelination on the MRI routine sequence scan, and GDS score of > 75 in all categories.

MRI Data Acquisition

Each subject's MR data were obtained by a 3 Tesla MR scanner (Achieva, Philips, Netherlands) with an 8-channel head coil. Since most of the children under the age of 6 needed to be sedated, all subjects were to undergo sleep deprivation for a certain period of time (usually 6–8 h). Oral 10% chloral hydrate 0.3 ml/kg was administered with a maximum dose $\leq$ 15 ml. After the subject had fallen asleep, the level of consciousness was tested, and if there was no response to mild painful stimuli, the subject was then placed flat on the examination bed (in the supine position). During the examination, the subject was removed from the study if he or she awoke and was unable to continue with the examination.

Each subject underwent a routine MRI scan first, followed by sagittal three-dimensional SPGR sequencing (3D-SPGR) and rs-fMRI if no significant intracranial lesions were found.

The parameters of the routine MRI sequences were as follows: axial T1WI: inversion recovery sequence (IR), repetition time (TR) = 2,000 ms, and echo time (TE) = 20 ms; axial T2WI: turbo field echo sequence (TFE), TR = 3,500 ms, and TE = 80 ms; T2FLAIR sequence: TR = 8,000 ms, and TE = 125 ms. All axial scanning fields of view (FOVs): 230 mm $\times$ 191 mm $\times$ 143 mm, slice thickness, 5 mm, interval, 1 mm, and a total of 20 layers.

The parameters of the 3D-SPGR sequence were as follows: TR = 7.7 ms; TE = 3.8 ms; flip angle, 8°; FOV, 256 $\times$ 256; voxel size, 1 mm $\times$ 1 mm; matrix: 250 $\times$ 250; slice thickness, 1 mm; and total time: 155 s.

The parameters of the rs-fMRI were as follows: TR = 2,000 ms, TE = 35 ms, FOV: 192 $\times$ 192; matrix: 64 $\times$ 64; flip angle, 90°; slice thickness, 4 mm; gap, 0.5 mm; and total time: 8 min 6 s, with a total of 240 time points acquired.

MR Image Processing and Analysis

Preprocessing of the rs-fMRI data was performed in the SPM12 (SPM Neurology, London, United Kingdom) toolkit. In this study, three patients with HC and one with ASD did not meet the requirements and were excluded. We also calculated the FD (framewise displacement) for each subject,

and a two-sample *t*-test revealed no significant differences in FD values between the two groups (ASD mean = 0.175, HC mean = 0.169, $p = 0.83$). The corrected images were normalized to the EPI template, and voxels were resampled to 3 mm × 3 mm × 3 mm.

The normalized images were further processed, including spatial smoothing, detrend, filtering, and regression of 24 head movement parameters, the cerebrospinal fluid mean signal and the white matter mean signal. The Anatomical Automatic Labeling (AAL) template cortex and 90 subcortical brain regions were used as regions of interest (ROIs). **A**natomical Automatic Labeling (AAL) is a software package and digital atlas of the human brain. It is typically used in functional neuroimaging-based research to obtain neuroanatomical labels for the locations in 3-dimensional space where the measurements of some aspect of brain function were captured. In other words, it projects the divisions in the brain atlas onto brain-shaped volumes of functional data (12). The time series signal of each ROI was extracted, and the Pearson correlation coefficient between the ROIs was analyzed to obtain the correlation matrix for each subject. The Fisher Z-transformation was applied to the correlation matrix to obtain the Z matrix. The values of the Z matrix satisfied a normal distribution and were used for statistical analysis. The average connection matrix for the two groups is shown in **Figure 1**.

For the ASD and HC groups, a one-sample *t*-test was used to detect those edges where the FC value was significantly not equal to zero. A statistical significance threshold of $p < 0.05$ was assumed, and edges above the threshold formed a connected mask. Comparisons of FC between the two groups were made within this mask. It should be noted that only the positively connected edges were considered for this study value, as the negatively connected edges were not well interpreted in clinical practice.

Network-based Statistic (NBS) was used to test for differences between FC groups. The processing steps for NBS are as follows: Briefly, (1) first test the difference in FC between ASD and control on each edge to obtain the statistical value of each edge; (2) take a statistical threshold of $|T| = 3.26$ (corresponding to $P = 0.001$), remove edges below the threshold and retain those above the threshold; (3) estimate the connectivity components composed of the retained edges, each of which can be considered as a connectivity sub-network; (4) estimating the significance level of each connected component using a permutation test (5,000 times). Specifically, for each permutation, all subjects were randomly assigned to the ASD and HC groups, and the group differences of each edge were recalculated to obtain the statistical value of each edge, and then the size of the maximum connected component (number of edges) was calculated by retaining the edges above the threshold. After 5000 permutations, the permutation distribution of the maximum connected component was obtained, and the significance level of each true connected component was determined based on this distribution; (5) $P < 0.05$ for the connected component was considered significant.

Calculation of Network Properties for Functional Connectivity

Each subject's functional network properties were calculated according to its FC matrix. The specific process is shown in **Figure 2**.

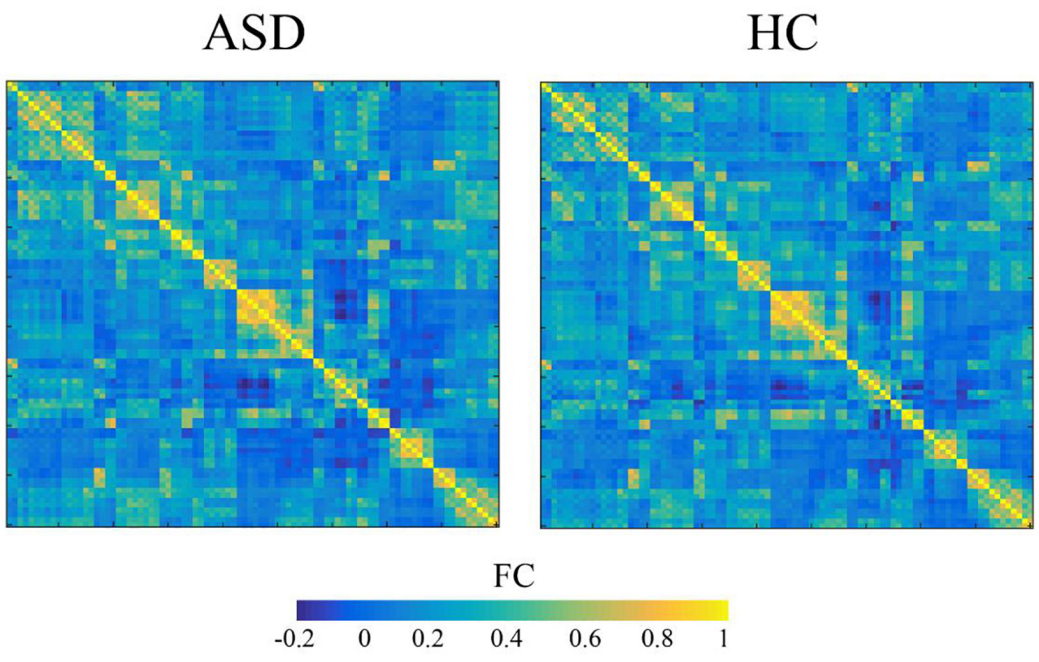

FIGURE 1 | Average functional connection matrix of the ASD group and HC group.

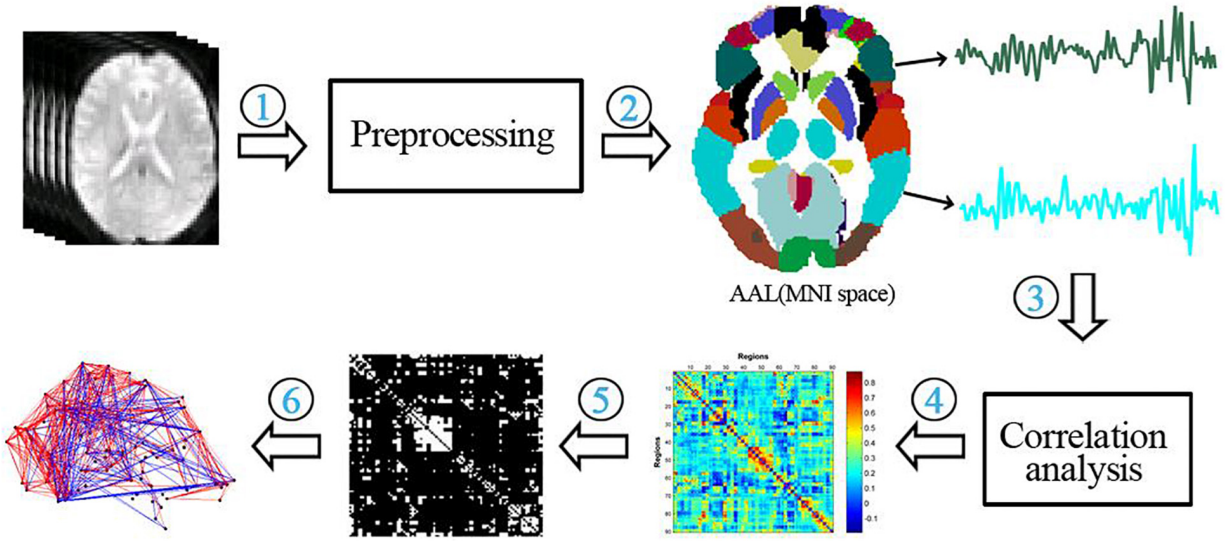

FIGURE 2 | Flow chart of the functional network analysis. (1) preprocessing of functional data; (2) extraction of temporal signals from 90 brain regions of the AAL template; (3) correlation analysis of temporal signals between brain regions; (4) construction of a functional connectivity matrix; (5) thresholding of the functional connectivity matrix; (6) calculation of network properties.

Global properties of the brain networks were calculated, including the clustering coefficient (C), local efficiency (Elocal), shortest path length (L), global efficiency (Eglobal) (L), normalized clustering coefficient (C), normalized shortest path length (L), global efficiency (Eglobal), normalized clustering coefficient (L), normalized shortest path length (L), and small-worldness (S).

Statistical Analysis

Statistical analysis was performed by using Statistical Package for Social Sciences (IBM-SPSS) software. For each network attribute, we calculated the area under the curve (AUC). A two-sample *t*-test was used to detect differences in network attributes between the ASD and HC groups. For the network global attributes, $P < 0.05$ was considered significant for differences. For the node attributes of the network, the significance threshold range was set at $P < 1/N$ (where N is the number of nodes, 90).

RESULTS

Demographic Data

The demographic data of the participants are shown in **Table 1**. Among participants in the ASD group, the chronological age and CARS score were 3.03 ± 0.99 years and 33.47 ± 1.67, respectively. Six of the ASD patients (19%) were female, and 26 (81%) were male. A total of 22 healthy controls were included in the HC group. Among them, 5 individuals (23%) were female, 17 individuals (77%) were male, and the chronological age was 2.95 ± 0.90 years.

TABLE 1 | Demographic characteristics of the participants.

Variables	ASD (*n* = 32)	HC (*n* = 22)	*P*-value
Age (mean ± SD)	3.03 ± 0.99	2.95 ± 0.90	0.82[a]
Sex (male/female)	26/6	17/5	0.71[b]
CARS score (mean ± SD)	33.47 ± 1.67		

[a]*Two-sample t-test,* [b]*Chi-square test.*

Analysis of Functional Connectivity

After the calculation of FC, significant increases or decreases in subnetwork connectivity was found, as shown in **Tables 2**, **3** and **Figures 3**, **4**.

Analysis of Brain Network FCs

Calculating the brain network FC properties through graph theory revealed that the FC network of ASD had small-world properties. It is well known that Watts and Strogatz (13) combined the concept of path length (the minimum number of edges required to establish a connection between nodes) with the concept of clustering coefficients between nodes (i.e., the probability that nodes j and k that are directly connected to node I are also directly connected to each other) to create the concept of small-world properties. This means that the brain networks under study between regular and random networks have both higher clustering properties similar to regular networks and shorter shortest path lengths similar to random networks (14). The stronger the "small world" property of the network. The clustering coefficient (C) and local efficiency (Elocal) were both increased in the ASD group relative to the HC group, as shown in **Figure 5**.

After calculating the nodal efficiency, it was found that the nodes that had increased efficiency in the ASD group were the inferior frontal gyrus, opercular part (left IFGoperc), and middle

TABLE 2 | Subnetworks with increased FC in the ASD group relative to the HC group (p = 0.003).

ROI i	ROI j	Mean FC in ASD	Mean FC in HC	*T*-value
Frontal_Inf_Oper_L	Frontal_Sup_Orb_L	0.34	0.16	3.84
Temporal_Mid_R	Frontal_Mid_L	0.26	0.08	3.27
Frontal_Inf_Tri_R	Frontal_Inf_Oper_L	0.58	0.39	3.32
Cingulum_Post_L	Frontal_Inf_Oper_L	0.21	−0.02	3.78
Cingulum_Post_R	Frontal_Inf_Oper_L	0.14	−0.05	3.30
Temporal_Mid_R	Frontal_Inf_Oper_L	0.22	0.02	3.65
Temporal_Mid_R	Frontal_Inf_Tri_L	0.28	0.09	3.42
Frontal_Sup_Medial_L	Frontal_Inf_Orb_L	0.61	0.41	3.53
Frontal_Sup_Medial_R	Frontal_Inf_Orb_L	0.48	0.29	3.36
Frontal_Med_Orb_L	Frontal_Inf_Orb_L	0.57	0.35	3.45
SupraMarginal_L	Frontal_Sup_Medial_R	0.16	−0.03	3.90
Temporal_Inf_L	Frontal_Med_Orb_L	0.43	0.20	3.78
Temporal_Inf_L	Frontal_Med_Orb_R	0.33	0.14	3.35
SupraMarginal_L	Rectus_L	0.11	−0.06	3.58
Parietal_Inf_R	Cingulum_Post_L	0.11	−0.12	3.86
SupraMarginal_L	Cingulum_Post_L	0.16	−0.03	3.42
Temporal_Mid_L	Cingulum_Post_L	0.5	0.27	3.80
Temporal_Inf_L	Cingulum_Post_L	0.51	0.21	4.51
Temporal_Inf_R	Cingulum_Post_L	0.45	0.24	3.27
Parietal_Sup_R	Cingulum_Post_R	0.11	−0.06	3.51
Temporal_Inf_L	Cingulum_Post_R	0.37	0.13	3.85
Temporal_Inf_R	Cingulum_Post_R	0.41	0.22	3.60
Temporal_Mid_L	Angular_L	0.58	0.32	3.47
Temporal_Inf_L	Angular_L	0.66	0.40	3.31
Temporal_Inf_L	Temporal_Mid_R	0.58	0.37	3.40

TABLE 3 | Subnetworks with decreased FC in the ASD group relative to the HC group (p = 0.003).

ROI i	ROI j	Mean FC in ASD	Mean FC in HC	*T*-value
ParaHippocampal_R	Frontal_Sup_L	0.05	0.24	−3.37
ParaHippocampal_R	Frontal_Sup_Medial_L	0.15	0.31	−3.29
ParaHippocampal_R	Rectus_L	0.18	0.36	−3.71
Precuneus_R	Hippocampus_L	0.06	0.25	−3.69
Temporal_Inf_R	Hippocampus_L	0.09	0.27	−3.81
Precuneus_L	Hippocampus_R	0.05	0.21	−3.26
Precuneus_R	Hippocampus_R	0.06	0.24	−3.53
Temporal_Inf_R	ParaHippocampal_L	0.09	0.3	−3.55
Temporal_Inf_R	ParaHippocampal_R	0.17	0.41	−4.27
Temporal_Inf_R	Amygdala_R	0.11	0.26	−3.41
Precuneus_R	Calcarine_L	0.36	0.54	−3.49

temporal gyrus (right MTG), and the nodes that had decreased efficiency in the HC group were the left lingual gyrus (left LING), calcarine fissure and surrounding cortex (left CAL), and right parahippocampal gyrus (right pHIP), as shown in **Figure 6**.

DISCUSSION

To date, the etiology of ASD remains uncertain. In the past, it had been suggested that ASD is primarily an abnormality in the structure of a particular brain region. More studies have found widespread cortical underconnectivity, local overconnectivity, and mixed results suggesting disrupted brain connectivity as a potential neural signature of autism (15, 16). Resting-state FC has enabled the identification of the primary networks of the brain, and the default mode network (DMN) has been implicated in social-cognitive deficits in ASD (17). Some studies have found that abnormal FC extensively exists within some networks in children with ASD (18), while others have also concluded that children with ASD have decreased FC in a number of regions in the action observation network (19). A diffusion tensor imaging (DTI) study found increased topological network efficiency in preschoolers with autism (20). In a review of numerous studies, Padmanabhan et al. found that the DMN is in a state of increased connectivity during childhood and shows decreasing connectivity with age (21), and Harikumar et al. concluded that the DMN in ASD shows a mixed pattern of strong and weak FC patterns in the DMN. One reason for the mixed trend in ASD could be that different brain regions of the social cognitive network of children with ASD are at different levels of development, such as underdevelopment of specific brain regions of the social cognitive network and reduced connectivity in social cognition-related areas, while other behavioral and connectivity patterns show typical development (17).

The present study found that the brain regions of the DMN were in a state of increased connectivity in preschool children with ASD, which may be related to their symptoms of social impairment. The brain regions with increased FC in the ASD group included the superior frontal gyrus, middle frontal gyrus, inferior frontal gyrus, superior temporal gyrus, middle temporal gyrus, inferior temporal gyrus, posterior cingulate gyrus, superior limbic gyrus, and angular gyrus, of which the medial prefrontal, posterior cingulate, and angular gyrus are all important components of the DMN, an important brain system that processes information about the "self" and the "outside world" and is strongly activated in the absence of external stimuli, while the attention network is activated once external stimuli are perceived, after which the DMN is suppressed.

In this study, in addition to brain areas belonging to the DMN, there was also increased FC in the temporal lobe, including the superior temporal gyrus, middle temporal gyrus and inferior temporal gyrus, areas belonging to the auditory network and responsible for speech perception. A behavioral experiment found that people with early ASD have impairments in speech recognition (22). In addition, people with ASD have difficulty recognizing and learning unfamiliar sounds but are indistinguishable from the general population in recognizing well-known sounds (23). This phenomenon may be related to abnormal brain connections in speech perception (24). The abnormalities in FC in the temporal lobe found in this study may suggest that preschool children with ASD also have abnormalities in speech perception.

The current study also found that FC was decreased in some brain regions of children with ASD, including the hippocampus, parahippocampal gyrus, superior frontal gyrus, inferior temporal gyrus, precuneus, amygdala, and perirhinal cortex of the talar fissure, with the hippocampus and parahippocampal gyrus predominating. The hippocampus and amygdala are key

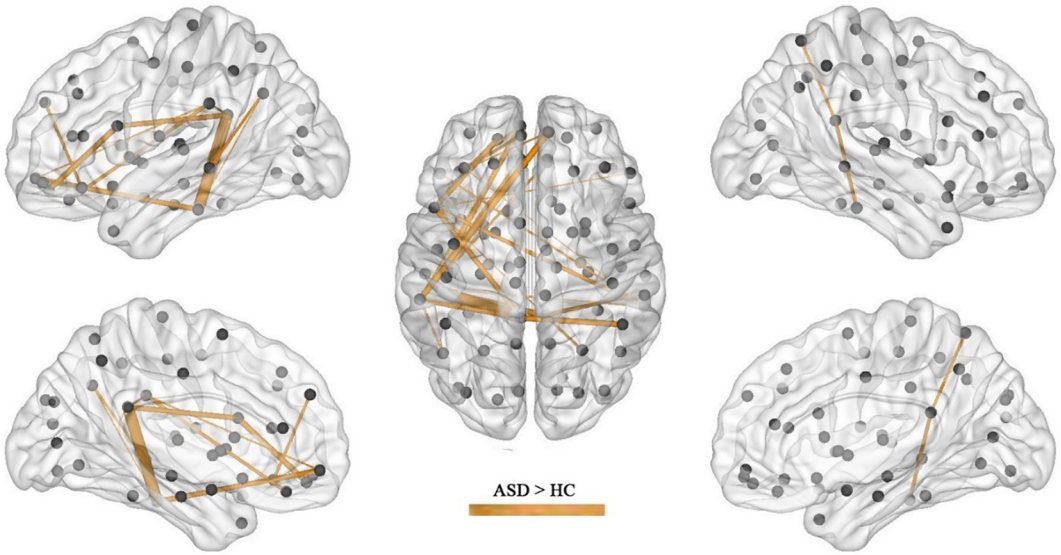

FIGURE 3 | Subnetworks with increased FC in ASD (p = 0.003).

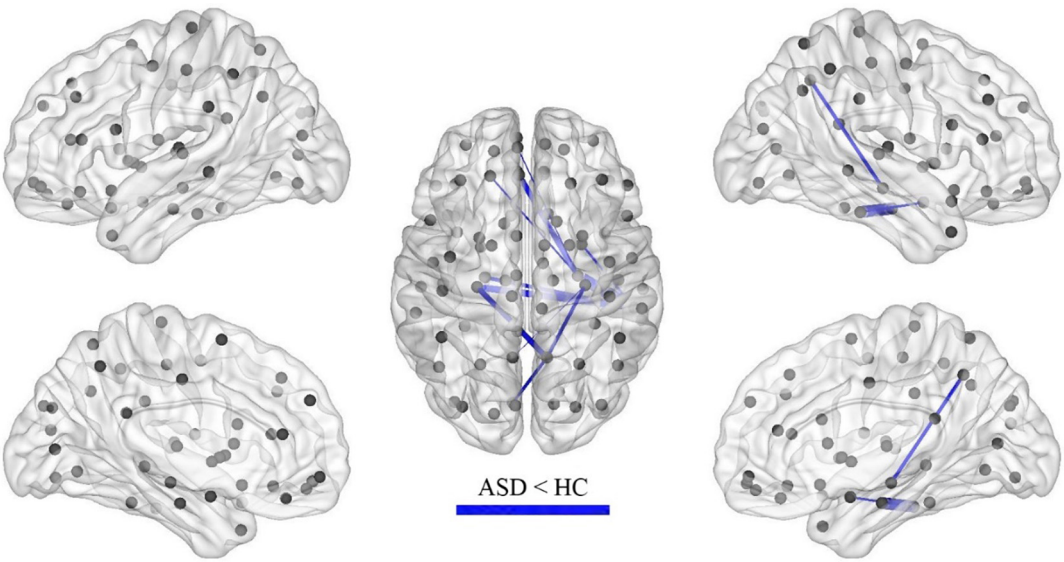

FIGURE 4 | Subnetworks with decreased FC in ASD (p = 0.003).

components of the nervous system for emotion regulation and perception regulation, which have been suggested to be associated with the development of ASD in previous studies (25). The decreased connectivity of this brain region found in this study may be associated with emotional alterations in ASD. The perisylvian cortex is part of the emergent network and the present study suggests that FC of the emergent network is reduced in preschool children with ASD, consistent with an enhanced DMN.

Lateralization of brain function or structure can occur in the developing brain. The centers of left-sided brain lateralization include the DMN (posterior cingulate cortex, medial prefrontal cortex, temporal junction) and language areas (e.g., Broca's and Wernicke's areas), whereas the centers of right-sided lateralization include the attentional control network (e.g., medial parietal sulcus, anterior insula) (26). Abnormal lateralization of the structure and function of the brain is often associated with neuropsychiatric disorders such as ASD (27, 28). In our study, we also found a tendency for left-sided lateralization in regions of hyperconnected brain networks in children with ASD, which may be related to a tendency for right-sided lateralization of reduced brain network connectivity.

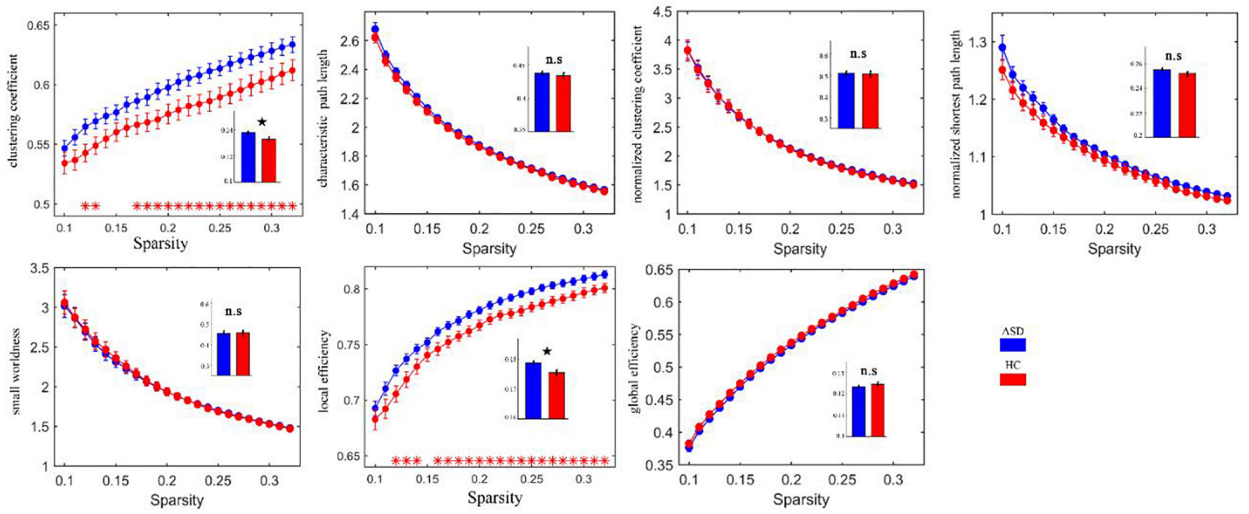

FIGURE 5 | Clustering coefficients and local efficacy of the abnormally elevated network FC in ASD. Red * indicates that the network attributes of the two groups are significantly different under this threshold ($p < 0.05$). The histogram represents the area under the curves of the two groups. The black star indicates that there are significant differences in the AUC values of the two groups' network attributes. ns, no significance.

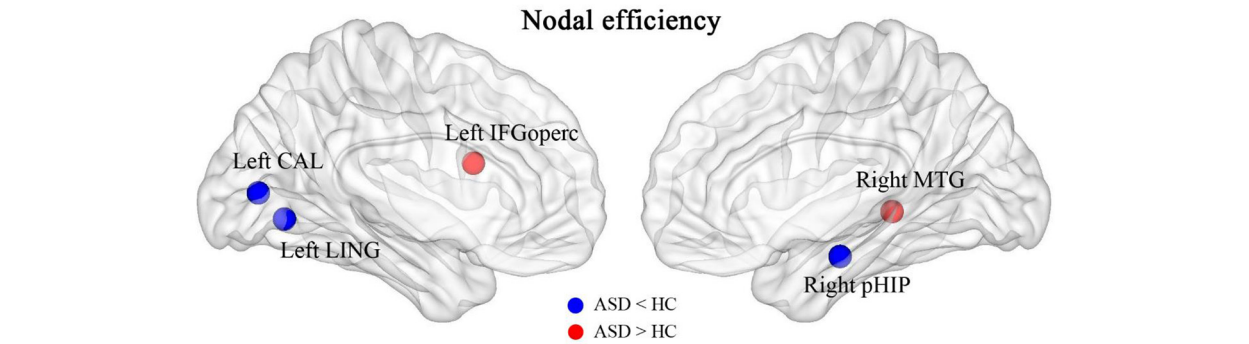

FIGURE 6 | Brain regions with abnormally altered nodal efficiency in ASD. The red brain regions had significantly increased nodal efficiency in ASD. The blue brain regions had significantly decreased nodal efficiency in ASD.

We also found that brain networks in both the ASD and HC groups had small-world properties. The clustering coefficient (C) and local efficiency (Elocal) were both greater in the ASD group than in the HC group, suggesting an increase in both direct and indirect connectivity of nodes in the functionally connected brain network but not in the efficiency of the global network, which is partially consistent with the theory proposed by Belmonte et al. that network performance in ASD patients is characterized by an increase in short-range connectivity and a decrease in long-range connectivity (6). In the ASD group, the nodal efficiency of the FC was enhanced in the left subinsular frontal gyrus and the right middle temporal gyrus and reduced in the left lingual gyrus, the left perirhinal cortex of the talar fissure and the right parahippocampal gyrus relative to the HC group. It is suggested that these brain regions may play a central nodal role in school-aged patients with ASD, but this role should be explored in subsequent studies.

This study still has some limitations and due to the poor completion of natural sleep MRI in younger children, chloral hydrate sedation was chosen to assist in the completion of MRI. A recent study on brain activity in rats pointed out that brain activity in rats sedated with chloral hydrate has a rhythm similar to natural sleep, but of different duration (29). Therefore, our results on FC in the preschool children are not a true rs-MRI and more preparation will be needed to achieve a true resting state MRI, such as more sleep deprivation, late night examination time, more attempts, etc.

CONCLUSION

Alterations in functional brain networks are evident in preschool children with ASD and can be detected with sleep rs-fMRI, which is important for understanding the pathogenesis of ASD and assessing this condition early.

DATA AVAILABILITY STATEMENT

The original contributions presented in this 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 Research Ethics Committee of Children's Hospital of Chongqing Medical University [IRB number: (2018) Ethical Review (Research) No. (82)]. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YZ: designing the topic, collecting data, and writing the manuscript. BQ: MR parameter setting, post-processing, and statistical analysis. LW: scanning and image quality assessment of MR. JC and TL: quality control of the experimental process and the analysis and discussion of the results. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Current View on the Paradox of Pain in Autism Spectrum Disorders

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder, which affects 1 in 44 children and may cause severe disabilities. Besides socio-communicational difficulties and repetitive behaviors, ASD also presents as atypical sensorimotor function and pain reactivity. While chronic pain is a frequent co-morbidity in autism, pain management in this population is often insufficient because of difficulties in pain evaluation, worsening their prognosis and perhaps driving higher mortality rates. Previous observations have tended to oversimplify the experience of pain in autism as being insensitive to painful stimuli. Various findings in the past 15 years have challenged and complicated this dogma. However, a relatively small number of studies investigates the physiological correlates of pain reactivity in ASD. We explore the possibility that atypical pain perception in people with ASD is mediated by alterations in pain perception, transmission, expression and modulation, and through interactions between these processes. These complex interactions may account for the great variability and sometimes contradictory findings from the studies. A growing body of evidence is challenging the idea of alterations in pain processing in ASD due to a single factor, and calls for an integrative view. We propose a model of the pain cycle that includes the interplay between the molecular and neurophysiological pathways of pain processing and it conscious appraisal that may interfere with pain reactivity and coping in autism. The role of social factors in pain-induced response is also discussed. Pain assessment in clinical care is mostly based on subjective rather than objective measures. This review clarifies the strong need for a consistent methodology, and describes innovative tools to cope with the heterogeneity of pain expression in ASD, enabling individualized assessment. Multiple measures, including self-reporting, informant reporting, clinician-assessed, and purely physiological metrics may provide more consistent results. An integrative view on the regulation of the pain cycle offers a more robust framework to characterize the experience of pain in autism.

Keywords: pain, autism, perception, reaction, evaluation, expression

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INTRODUCTION

> "*On the one hand, some people with autism can tolerate extreme heat, cold or pressure and seem relatively insensitive to pain. On the other hand, they may experience intense pain from idiosyncratic sources but struggle to communicate it.*" Citation from Spectrum Autism Research News (1).

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder, affecting around one birth out of 150 worldwide (2). According to new report from Centers for Disease Control and Prevention, the incidence is as high as 1 in 44 children, leading to a prevalence estimated at 2.3% (3), a male-to-female ratio around 3:1 (4). ASD significantly decreases the person's educational, social and employment opportunities. Among other comorbidities, pain is frequent, sometimes undiagnosed or diagnosed with delay in children with ASD (5–8). Children with autism are about twice as likely as their typical peers to experience chronic or recurrent pain (9). Various co-morbidities in ASD, such as epilepsy (10, 11), joint hypermobility-related disorders (12), gastrointestinal disorders (13), anxiety (14), and sleep problems (15) may be additive sources of pain. Individuals with ASD face significant inequities in healthcare, despite their high rate of medical comorbidities. They tend to have poorer health outcomes (16) and higher mortality rates (10, 17, 18) than their neurotypical peers. Aside from other core symptoms, such as socio-communicational difficulties, repetitive activities and/or interests and atypical sensory modulation, some individuals may present high levels of Self-Injury Behaviors (SIB; 19) which may be related to pain reactivity in ASD (20, 21).

The paradox of pain perception in ASD has attracted the attention of researchers for many years. Atypical pain perception, expression and difficulties in pain assessment in people with ASD have been described (22–27). On one hand, some studies report a decrease or absence of pain reactivity in daily life mostly based on reports from self or others and clinical observations (22, 28). On the other hand, various results show equal or greater responsiveness to painful stimuli in experimental conditions (29–31). Atypical pain sensations, like allodynia (extreme sensitivity to usual non-painful tactile stimulation causing intensive pain), paradoxical heat sensation (gentle cooling perceived as hot or burning), and hypoesthesia (decreased pain sensitivity) are also reported for ASD (32). Various hypotheses attempted to explain observed alterations. However, none of these hypothesis by themselves were able to reconcile these apparently contradictory findings.

The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with, or *resembling* that associated with, actual or potential tissue damage" (33). On one hand, pain is a universal "alert sign," considered to be an instinct to prevent chronic and repetitive injuries. On the other hand, pain is a subjective, personal, and multifaceted construction that may be modulated by various biological, psychological, and social (cultural and contextual) factors. It may be provoked by physical, emotional, or social triggers (e.g., evoked by the observation of the suffering of others; 34). Pain regulation is essential for successful adaptation to life events, development of social interactions skills and empathy (35, 36) and has been implicated in social difficulties in autism (37).

The physiological mechanisms and both peripheral and central pathways of pain processing are well documented. Following activation of specific nociceptors, pain signals are transmitted *via* the peripheral nervous system (PNS) to the central nervous system (CNS), composed of the spinal cord and the brain. The CNS is responsible for processing, integrating, and interpreting the information sent from the PNS, ultimately elaborating into a complex sensory experience (**Supplementary Table 1**). The subcortical and cortical centers in the brain coordinate all the motor responses produced to diminish or avoid painful input. Both thresholds and intensity of pain perception may be modulated by internal mechanisms. Endogenous pain modulation includes the "***endogenous analgesia"*** which refers to the pain-inhibiting pathway originating in the brainstem and terminating in the spinal cord. Another mechanism, "***descending pain modulation system,"*** consists of a large network in the brain which regulates pain sensory input to the CNS and behavioral reactivity. The internal mechanisms can change the meaning of pain based in previous exposure and pain expectancy, its influence on our emotional state, and its relevance to our survival. However, these processes may be affected by mood, neurological disorders, environmental and genetic factors (36, 38, 39).

Whatever the medical condition or the noxious input, individuals can use a large number of cognitive or behavioral strategies for coping with pain. These reactions include cognitive self-instruction; visual imagery and distraction; body relaxation training; seeking for social support or, alternatively, withdrawing from social contact; worrying or even catastrophizing; each of which can either decrease or increase pain sensation (40). Therefore, pain perception and pain responses form dynamic feedback loops (41–43). Interruptions in this "pain cycle" (**Figure 1**) may cause long-term plasticity in pain perception and regulation mechanisms which can be responsible for increased or decreased pain sensitivity, or even allodynia (44).

When one considers the perspectives of pain research in ASD, one may ask what would be the origins of altered pain sensitivity and reactivity in ASD? In other words, ***where*** and ***when*** did the circle of pain start to go wrong, and for ***whom***? Another important question, **what** should be done to overcome that issue?

To address this, we will review in the second section, the recent literature on pain processing and regulation in individuals with ASD, from initial stages to brain response in the "*pain matrix*." We will propose that altered cognitive and emotional control of pain may also contribute to the altered pain response in ASD, in line with the chronic pain disorders hypothesis (42).

In the third section, pain responses in individuals with ASD and clinical methods for pain assessment (self-reports and reports by others, physiological measures, body responses) will be synthesized, highlighting the need for objective and multimodal approaches. The significance of an

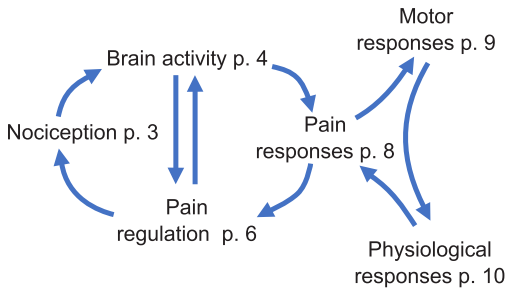

FIGURE 1 | The simplified view of the cycle of pain (for illustrative purposes) with the numbers of corresponding pages of the paper.

individualized approach for pain assessment and management will be highlighted.

Thereafter, the altered pathophysiological pathways and brain circuits involved in the emergence of ASD will be reviewed and discussed with regard to their possible involvement in the pain regulation. We will conclude by proposing an integrative view of the pain cycle, which, along with existing mechanisms of pain alterations in ASD, defines key targets for further pain research in ASD.

Introduction Highlights. *Individuals with ASD demonstrate a wide range of alterations in pain reactivity. However, the question of pain perception in autism is understudied. Processing of pain includes several stages with feed-back and feed-forward interactions, forming "a pain modulation circle." Reactivity to and coping with pain in autism may be altered because of various interruptions of this cycle; these can be configured differently in each individual with ASD.*

NEUROPHYSIOLOGICAL PROCESSING OF PAIN IN AUTISM SPECTRUM DISORDER

Initial Stages of Pain Signaling: From Nociceptors to the Central Axis

While pain perception arises from the combination of different somatosensory modalities (45), processing of a painful stimulus starts from nociceptors (**Supplementary Table 1**), which are specialized for perception of painful stimuli of different modalities, detecting potential thermal, mechanical, and chemical tissue damage. The signal is transmitted to the CNS *via* more rapid, myelinated Aδ (A-delta) fiber axons responsible for acute pain transmission and slow, non-myelinated C-fibers (small fibers). The combination of these signals creates two phases of pain perception; fast and intense, followed by diffuse and dull pain (36). In individuals with ASD, atypical small fiber density was recently described (46, 47). These alterations were associated with autistic symptoms, and may also contribute to hypoesthesia (already explained above) and allodynia in autism (47). However, increased pain and touch sensitivity were observed specifically in areas innervated by C-fibers, providing a candidate mechanism for atypical avoiding behaviors or hypersensitivity in ASD. Another hypothesis could be a pathological activation of silent nociceptors, which are normally unresponsive to noxious stimuli, but become responsive to mechanical stimulation (30).

The initial stages of noxious signaling are usually characterized by the so-called nociceptive "thresholds," i.e., the lowest intensity at which noxious stimuli are perceived to be painful. The thresholds may be different depending on whether noxious stimuli are thermal, mechanical, or due to compression. Studies in individuals with ASD have reported contradictory findings on pain thresholds depending of the type of stimuli (24, 25, 29, 31, 32, 48). No difference between neurotypical subjects and individuals with ASD was reported for electrocutaneous pain (48) and thermal pain (14, 49) thresholds. Lower thresholds (i.e., higher sensitivity) were observed for pressure-induced pain (29, 30) and heat-induced pain (31). By contrast, subjects with ASD displayed hyposensitivity to pinprick-induced mechanical pain (32).

In addition to these inconsistent results, individuals with ASD may display hypersensitivity to stimuli usually considered painless. These responses may be accompanied by absence of reactivity to potentially hazardous and noxious stimuli (1). Such *stimulus overselectivity,* when an individual responds only to a limited category/amount of incoming sensory information, was not confirmed on group level, however, (50). Furthermore, sensory processing patterns are known to change with time, depending on the context in which they are estimated (51). Both hyper- and hyposensitivity to the same type of stimuli may exist in the same person with ASD, challenging the suggestion that only the PNS is implicated in the observed alterations (52). In addition, the results of such psychophysical evaluation of pain detection and discrimination may be confounded by other factors like attentional resources, levels of anxiety or task performance capacities which may be different in autism.

At the level of the spinal cord, complex interactions between excitatory and inhibitory interneurons have been shown to modulate pain signal transduction. According to the "gate control" theory (53), concurrent activation of large sensory afferents from the skin (Aβ-fibers) could suppress transmission in small unmyelinated C-fiber afferents and therefore block pain perception. As a consequence, tactile stimulation of a painful area may relieve pain (54).

The relation between self-stimulation and self-injuring behavior and response to pain in autism has been discussed (20). A study measured SIBs and pain signs in non-verbal individuals with ASD (55) revealed increased behavioral signs of pain in adults with chronic self-injury. That suggests that the SIBs might be a coping strategy to manage chronic pain. Children with ASD showed a significantly reduced pain sensitivity and increased tactile sensitivity after somatosensory- directed therapeutic manipulations (with touch, proprioception, vibration, and stereognosis; 56); this effect was not find in the control group. The authors suggested that repetitive somatosensory distraction, by an increase of affective-motivational input affects pain processing and alleviates pain sensation, in line with findings in various chronic pain conditions (57–59). Although the effects of these

therapeutic manipulations may be ascribed to central pain processing and top-down regulation, normalization and integrity of peripheral sensory perception likely also contributes. However, the causal relations between SIB and altered pain perception in ASD is far from understood.

Initial stages of neurophysiological processing of pain in ASD highlights. The evidence for alterations in pain transduction at the level of peripheral receptors in ASD is limited to reports of decreased nociceptor density. That question needs to be more robustly corroborated, given that pain thresholds in individuals with ASD and the control groups weren't different in most studies. However, peripheral nerve alterations may partly explain sensory dysfunctions like *allodynia* – painful atypical response to touch, and *hypoesthesia* – hyposensitivity to injurious stimuli, but also *paradoxical heat sensation* documented in the battery of sensory tests in autism.

Atypical Brain Activity for Pain Processing in Autism Spectrum Disorder

The Pain Matrix: First Level of Response "The Nociceptive Matrix" (Thalamus, Somatosensory Cortex)

The Thalamus

The thalamus, a regulatory hub for sensory inputs from different modalities, is involved in pain processing (60). It can be reorganized following chronic pain, altering maps of noxious and innocuous stimulation and leading to the perception of innocuous stimuli as nociceptive (61). In individuals with ASD, both structural and functional deviations in the thalamus have been found (62–66; **Figure 2**). The available studies on the resting state or anatomical connectivity yielded heterogeneous results. Both hypo-(64, 67) and hyperconnectivity (65, 68) between thalamus and cortical areas were demonstrated in ASD. During an aversive sensory stimulation, thalamocortical connectivity was attenuated, while connectivity between thalamus and subcortical areas (putamen, hippocampus, and amygdala) was, in contrast, enhanced in individuals with ASD when compared to controls (69). This hyper-connectivity in response to aversive stimuli could indicate a lack of cortical inhibition. Additionally, connectivity between thalamus and amygdala is thought to play a role in directing attention to emotionally salient information. This suggests that modified thalamus connectivity may be responsible for over-attribution of "pain" salience to benign tactile stimuli (allodynia).

In a recent study investigating different stages of pain-induced brain responses, Failla et al. (62) showed that processing of noxious stimuli in the brain differs between subjects with ASD and neurotypicals, with regard to the duration and type of pain. In this study, adults with ASD showed significantly less brain activation in the thalamus compared with controls during sustained pain stimulation (62). In contrast, acute brain responses to pain were similar across groups. That reorganization of thalamocortical and thalamosubcortical pain-processing pathways may be responsible for reduced pain awareness due to attenuated thalamocortical signaling. In addition, increased unspecified distress due to upregulated signaling between thalamus and sub-cortical structures noxious may result in possible perception of neutral stimuli as in ASD.

The Somatosensory Cortex

The functional and structural modifications in one of the earliest brain areas activated by noxious stimuli, the primary somatosensory cortex, S1 have been detected in subjects with ASD (73, 74) along with altered sensitivity profiles (32, 75). In addition, reduced gray matter volume in somatosensory brain areas and possible disruptions in thalamocortical white matter fiber pathways were associated with higher SIB scores in autism (76). In the above-mentioned study (62) sustained painful stimulation (heat stimuli administered to a right lateral calf), was associated with significantly less activation in the left S1 and bilateral secondary somatosensory cortices S2 in the group of participants with ASD than in their neurotypical peers. Again, brain activation during the initial stage of stimulation was not different across these groups.

The Pain Matrix: Salient Encoding of Pain Stimuli "The Salience Matrix" (The Cingulate Cortex, Prefrontral Cortex and Insula), and Their Interactions With Third Order Areas

Activation of "salience matrix" regions is not specific to painful stimulation. These circuits may be activated by any sensory stimulus that rises above the sensory threshold. Such co-activation adds the "salience" meaning of noxious stimuli and triggers attentional control of pain-evoked response (70). The coordinated response within the sensory-specific and second-order networks (**Supplementary Table 1** and **Figure 2**) is crucial to create links between pre-conscious nociception and conscious pain (36, 43).

The cingulate cortex: Besides its role in the attentional component of pain stimulus processing (**Supplementary Table 1**), the cingulate cortex plays a role in the brain response related to negative affect and goal-directed behavior, resulting in "urgency to act" (77). The alterations in cingulate cortex function have already been documented in ASD (78, 79). Decreased engagement of the anterior cingulate cortex in participants with autism was also observed in the above-mentioned study of sustained pain responses (62). In addition, during a heat-induced pain, both amplitudes and latencies of evoked potentials in the cingulate gyrus were found to be lower in adults with ASD compared to controls, while pain scores were not significantly different between groups (71). Such results suggest that the cingulate cortex seems to be less implicated in general, but may have a specific role at the latest stages of pain processing in ASD, contributing to the motor hyporesponsiveness of individuals with autism.

The insula is involved both in the initial perceptual stage and in the cognitive encoding of pain (80). Structural and functional changes in the insula, as well as alterations in connectivity, were observed in autism, as reviewed in Caria and de Falco (81), Uddin and Menon (82). Decreased insula activation in participants with autism was also observed during sustained pain (62). The insula is considered as a hub for *interoception*, i.e., representation of our own internal word, (83, 84)*; self-awareness* and *bodily self-consciousness* (85); which have generally been reported as atypical

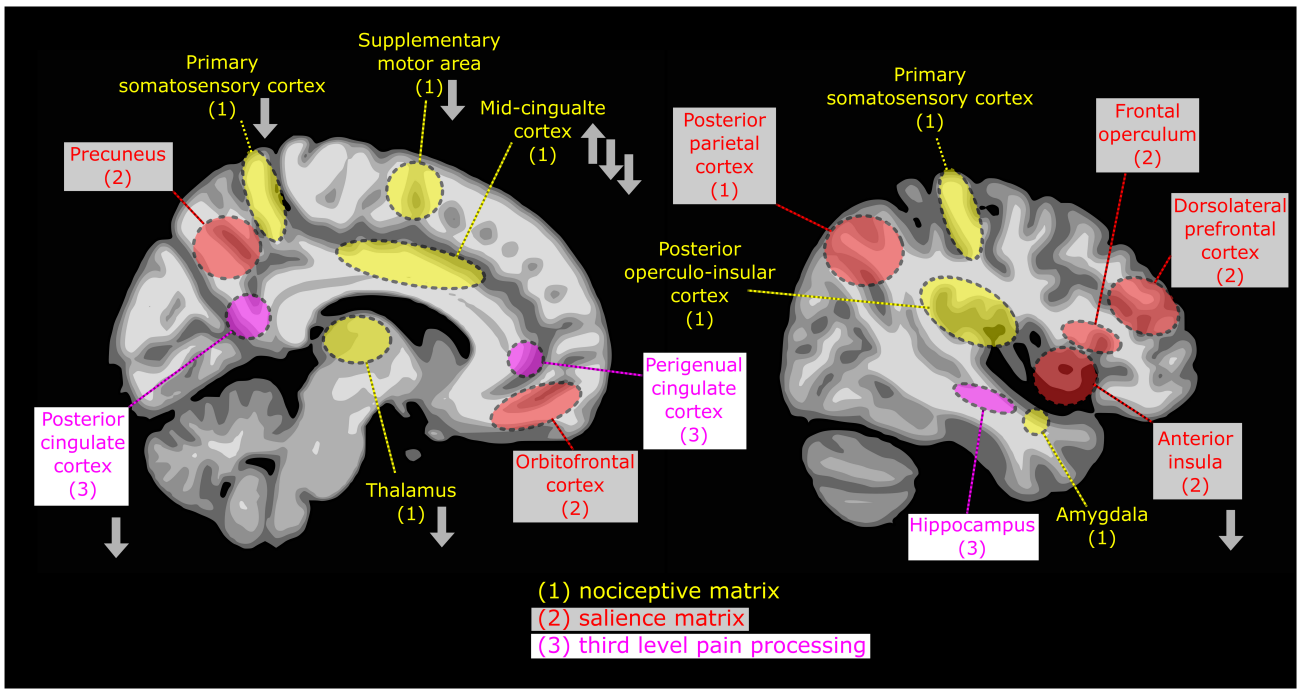

FIGURE 2 | Basic view of ascending pain processing, delineated on two brain sections, with emphasis on early, sub-conscious *(1, "nociceptive matrix"*: the posterior operculo-insular cortex, the primary sensory areas, p-mid-cingulate cortex, supplementary motor area and the amygdala) and conscious pain perception (2, "*salience matrix*": the anterior cingulate cortex, the anterior insula, posterior parietal, prefrontal and orbitofrontal cortices), and (3, "*areas of third-order brain activation*": the hippocampus and the anterior and posterior cingulate). Nociceptive cortical processing is initiated in parallel in sensory, motor and limbic areas. Some activation may last longer than voluntary motor reaction. Based on brain response dynamics and models described in Bushnell et al. (42), Garcia-Larrea and Bastuji (70). The alterations in pain-induced responses in the brain of individuals with ASD are shown with gray arrows according to Failla et al. (62), Chien et al. (71), and Gu et al. (72).

in population with ASD (86–92). Disrupted engagement of the insula during pain processing may be responsible for altered pain awareness and/or cognitive pain representation (49).

The (pre-)frontal cortex participates in top-down cognitive and anti-nociceptive short- and long-term controls over pain perception and reaction (93, 94). Less prefrontal cortex engagement may be related to reduced antinociceptive feedback (**Supplementary Table 1** and **Figure 2**). Even if pain-related prefrontal cortex activation does not seem to differ between individuals with and without ASD (62), it is negatively correlated with levels of perceived pain in participants with ASD. Another recent results reported a reduced prefrontal cortex response to painful stimulation in participants with ASD (95).

The reciprocal connections between *prefrontal cortex, hippocampus, and amygdala* are known to be implicated in different stages of pain processing (96). It has been reported that noxious stimuli modulate prefrontal-hippocampal connectivity and impact behavioral performance (97). Chronic pain causes neuroplastic changes in these regions of the brain, resulting in aversive states and memory deficits (98, 99). In ASD, structural and functional alterations have been observed in the hippocampus-prefrontal cortex-amygdala network (100–102). The interruption of maturation of these brain areas caused by chronic pain in early life has been proposed as a predisposing factor for ASD (103). Taking into account recent theories about reorganization of pain processing pathways during the transition from acute to chronic pain proposed by De Ridder et al. (104), one may speculate that such a reorganization during perinatal development could explain alterations observed in ASD. Further research on this topic may provide a more unified account of how sensory and social peculiarities impact together the perception and processing of pain in subjects with ASD (37).

Default mode network (DMN) may also be related to pain processing in autism. According to recent models (104), chronic pain results in reorganization of pain networks in the brain and also implicates the DMN (*the medial prefrontal cortex, posterior cingulate cortex, inferior parietal cortex and the precuneus*; 105–107). That leads to integration of pain into body schema and changes in its appraisal. Little is known about DMN activity during pain processing in subjects with autism, but numerous alterations of DMN activity and in its connections with other brain areas have been described in subjects with ASD (108).

By referring to the model of chronic pain, we may suggest reorganization of thalamocortical and thalamosubcortical pain-processing pathways in ASD. That plasticity could be partly responsible for reduced pain awareness due to attenuated thalamocortical signaling. Second, reduced activation of the second-order brain areas may lead to deviant pain consciousness and hypo- or delayed activation of structures implicated in goal directed behaviors and motor responses. Finally, increased

unspecified distress due to upregulated signaling between thalamus and sub-cortical structures, might lead to perception of neutral stimuli as nociceptive.

The "pain matrix" in ASD highlights. *Results of neuroimaging studies of pain processing in ASD are limited but suggest atypical brain activation patterns. Decreased activation in most of the areas of the pain matrix, starting from earliest cortical and sub cortical structures, during sustained pain stimulation in contrast to acute pain, supports the hypothesis of "feedback default" or "atypical top-down pain regulation" in ASD. Possible re-organization of pain processing pathways in early stages of development in ASD may be responsible for such alterations. Taken together, available information on central pain processing in ASD may explain the paradoxical dissociation between (near to-) intact low-level perceptive abilities in pain detection tasks and altered salience and awareness of pain and its affective components.*

Pain Perception Modulation in Autism Spectrum Disorder

The Descending Pain Control Pathways

The descending pain modulatory system involves connections between the (pre-)frontal cortex, the thalamus, the periaqueductal gray (PAG) and the *amygdala* (**Supplementary Table 1** and **Figure 2**). Among these brain structures, *the amygdala* appears as a key component of top-down regulation. The amygdala allocates attention and assigns emotional value – either positive or negative – to sensory information, thus leading to adaptive behavioral and affective responses and also contributing to emotional memory. Under short-term aversive conditions, such as stress or fear, amygdala activation induces hypoalgesia. Contrary to this, during chronic pain caused by various medical conditions, long-lasting functional plasticity of amygdala activity is related to enhanced nociceptive responses, including hyperalgesia, aversive behavioral reactions and anxiety-like states (109, 110). The amygdala has received considerable attention in ASD studies (111). For example, the amygdala in individuals with ASD show both microscopic (112) and macroscopic structural regional abnormalities. Recently, in a large sample of 1,571 participants with ASD, increased thickness in the frontal cortex and reduced subcortical amygdala volumes were reported (113) as well as connectivity atypicalities (114). In line with the so-called "weak amygdala's emotional modulation hypothesis in ASD" (115), such deviant regulation may yield atypical behavioral and social/psychological responses (116). Reduced connectivity between the prefrontal cortex and the amygdala during unpleasant stimulus processing was recently observed in children with ASD (117). That suggests that alterations in the interconnectivity of these structures may play a role in the blunted behavioral responses to pain in autism. Importantly, the amygdala is activated by pain as early as the first-order cortical areas (118). Thus if prefrontal networks are unable to exert inhibitory modulation, it may remain over-activated, producing a cascade of autonomic and behavioral reactions.

The descending pain modulation pathways can be both facilitatory as well as inhibitory. Facilitatory pathways are the ones which enhance pain perception, while inhibitory pathways suppress pain perception. Endogeneous opioids are released at synapses at multiple points in the PNS to block the ascending pain transmission signal. Moreover, during chronic pain, these pathways are plastic, resulting in pain sensitization or other perceptual alterations such as allodynia or nocebo (119). Alterations of the balance between descending controls, both excitatory and inhibitory, are involved in some dysfunctional and chronic pain states such as fibromyalgia (120–122) that has been associated with higher rate of autistic traits (123).

Of the endogenous pain modulatory mechanisms, the Diffuse Noxious Inhibitory Control (DNIC), is essential and often been described as "pain inhibits pain." That occurs when response to a painful stimulus is inhibited by another, often spatially distant, noxious stimulus. The DNIC is predominantly studied in humans using the psychophysical paradigm of Conditioned Pain Modulation (CPM; 124, 125). The most commonly investigated test stimuli are pressure pain threshold (PPT) and cold water immersion is the most frequently studied conditioning stimulus (126). In ASD, recent results suggested a preserved CPM effect. The measures of PPTs increase significantly after a cold conditioning stimulus in both adults with and without ASD (32, 127). Nevertheless, as the pain scores were very variable in individuals with ASD, with a greater range of extreme scores than in control group, results must be interpreted with caution (127).

Anticipation of Painful Stimuli

Brain activity patterns accompanying pain anticipation and processing were studied in high functioning adults with ASD and matched neurotypical individuals (72). Stronger activation in the anterior cingulate cortex was observed during the anticipatory phase in the group of participants with autism, while brain responses during painful stimulation were not different from neurotypical peers, in line with (62). The increased anticipatory brain response was paralleled by augmented behavioral sensitivity to *anticipated (expected)* pain in individuals with autism. This may seem to be contrary to previous observations of decreased response to sustained pain stimuli. This may be explained by the fact that in Gu et al. (72), contrary to Failla et al. (62) a cued paradigm was used to force participants to attend to incoming noxious stimuli and concentrate on the sensory aspects of stimulation. It should be noted that in this study, participants with ASD pre-selected a lower level of painful stimulation than healthy individuals, consistent with their hypothesized nociceptive hypersensitivity. The idea being developed here is that one may explain one of the paradoxes of pain perception in autism: expected pain perception is enhanced due to stronger attentional engagement.

Pain Resonance and Empathy

A growing body of evidence suggests that the ability to understand and feel compassion for another's pain, so called "empathy for pain" (128) is underpinned by the same neural

structures that are involved in the direct experience of pain. Observing someone experiencing pain induces brain response in the *insula, anterior cingulate cortex, the brainstem and the cerebellum*, but also emotional and physiological responses similar to those caused by a direct experience of pain (129–131). Empathy for pain is hypothesized to be based on the subject's own experience of pain. It may be related to vicarious pain perception, the sensation of pain on one's own body while observing the pain of others (132). Results from studies of vicarious pain perception and empathy for pain in ASD are contradictory. Some studies reported no difference from typical peers in behavioral and neuronal signatures of empathy for pain, while others suggest atypical brain activations and reactions in ASD (29, 109, 133–135).

Observation of someone's injured hand vs. non-injured hand (from a first-person perspective) induced strong and consistent activations of *cingulate, somatosensory and insular* cortices both in participants in control and ASD groups (109). These responses are accompanied by the same level of pupil dilation, an estimator of physiological arousal. Similarly, no group difference was found in the activation of the *insula* during a task based on painful stimulation applied to both the other's hand and one's own (48). Similar results were obtained from the observation of facial expressions of others experiencing painful stimuli (134, 135); both neurotypical subjects and participants with ASD exhibited activation in regions involved in pain processing (*the insula, somatosensory cortex, supplementary motor area, periaqueductal gray, and prefrontal cortex*), in affective reaction (*orbito-frontal cortex, the amygdala*) and in face and body part processing (*fusiform face area, extrastriate body areas*). These results would suggest that typical brain processes involved in shared representations of pain or emotional contagion are intact in autism.

In contrast, other results revealed atypical brain activations and physiological and behavioral responses to vicarious pain in ASD. In the same study cited above (135), videos of someone's limbs in painful vs. non-painful situations caused less activation in the *inferior frontal gyrus, the precentral gyrus, the frontal orbital cortex and the medial prefrontal cortex* in adults with ASD compared to neurotypical controls. As these regions are known as important parts of motor resonance and empathy for pain network (136) their hyporesponsiveness may contribute to emotional resonance alterations in autism.

In the study of Gu et al. (133), subjects observe images of someone's hands or feet (from first-person perspective) in painful or non-painful situations. In that study subjects with autism were less able to discern pain in others, but demonstrated higher activation *in the anterior insula and extrastriatal body areas*, and lower activation of right *prefrontal cortex* compared to controls. In addition, participants with ASD had augmented empathetic pain-related skin conductance response. That response correlated with brain activation evoked by images, suggesting higher level of physiological arousal and sympathetic nervous system engagement, compared to control participants.

In general, empathic responses are described as non-typical in ASD (137). Although general empathy and empathy for pain are distinct, they may share common neurological and physiological mechanisms. It has been reported that in subjects with ASD and their neurotypical peers, lower empathy is correlated with greater *anterior cingulate cortex and insula* activation in a pain anticipation task (72). Consistent with this, empathy scores are negatively correlated with insula activation only in participants with ASD during vicarious pain perception (133). In addition to autonomic hyperarousal evoked by these stimuli, it is plausible that alterations in the regulation loop between central and peripheral responses during subjective and vicarious pain experiences may be related to empathy modulation in ASD. It is also possible that difficulties in recognizing and reporting on one's own (and others') emotions, referred to as alexithymia, may be more responsible for observed alterations in empathy than core autistic features themselves (48, 110). For example, it has been shown that higher levels of alexithymia is related to increased arousal and diminished habituation to negative emotional stimuli (138). However, this conjecture remains unresolved (139).

The role of embodiment, e.g., the integration of sensations from one's own bodily experience (in the present as well as in the past) in the construction of conscious perception in order to understand our own experience, and the experiences of others (140) in ASD is discussed (92). Embodied cognition is based on the same neurophysiological mechanisms which are activated during observation of stimulation applied to others as during perception by self (141–143). When participants observed someone's body parts being injured, participants with ASD had increased activation in the *somatosensory cortex* (S1/S2) and decreased activation in the *medial prefrontal cortex* comparatively to controls (29). In addition, only participants with ASD demonstrated that S1/S2 overactivation evoked by vicarious pain was associated with lower pain pressure thresholds (e.g., higher pain sensitivity) and, paradoxically, to reduced unpleasantness rating scores. These results suggest that atypical coupling between embodiment and atypical sensory processing influences perception of vicarious pain in ASD. Consistent with these findings, reduced embodiment reaction in response to emotional stimuli is demonstrated in autism (144). Interestingly, production of an empathic response to other's pain requires directed attention from individuals with ASD (145), which is attenuated when the individual is distracted.

The attentional demands required to detect others' pain may partially explain discrepancies in insular cortex responses in subjects with ASD: overactivation of the insula demonstrated in Gu et al. (133) or inhibition of its activity shown in Fan et al. (29). While in the former study the images of different type (pain/no pain) were counterbalanced and randomized, Fan et al. presented stimuli in blocks of consecutive events, creating the possibility of priming the subject to the valence (pain/no pain) of the upcoming stimulus. This latter observation may be congruent with lower insula activation for sustained pain stimulation observed in Failla et al. (62). Taking into account the integrative role of the insula in the perception of the self, these observations may demonstrate

alterations in upper-level integrative processes in ASD during vicarious pain perception.

In summary, results from the limited number of studies on brain reactivity to vicarious pain in ASD need to be replicated to clarify the patterns of activation of each area of the pain matrix, compared to typical activation. Previous discrepancies concerning somatosensory or insula activities during empathy for pain may arise from differences in experimental paradigms, as already described in similar studies of neurotypical development (146). Nonetheless, these studies identified potential specific alterations in brain activity, similar to those reported in studies of direct pain stimulation, especially in the (pre-)frontal brain areas. This suggests a possibility of shared origins between atypical responses to pain stimuli and difficulties in empathy (at least for pain) in ASD. According to embodiment theories, altered perception of pain in oneself may be related to anomalous reactions to signs of pain, and may be related to other socio-cognitive deviations in ASD, such as attenuated distinction between positive vs. negative emotions (124).

Social Touch and Down-Regulation of Pain in Autism

The social component of pain modulation must not be underestimated in the global processing of pain, particularly in ASD. The observation of someone's pain induces a need for action in observers, an urge to provide help or reassure the person in distress. One of the most common ways to respond to someone's pain, to reassure and calm, is through the so called "social," or "soft," "interpersonal," or "affective" touch, a specific type of tactile stimulation provided by others, promoting pleasant feelings, approach-related behaviors and reductions in pain (125). It has been reported that brain correlates of soft touch-induced analgesia, included modification of pain-related activation in "nociceptive" brain areas as well as secondary ("salience") and third-order pain perception areas, and in sub-cortical regions such as amygdala, hypothalamus, and PAG (125). Affective touch signals are conveyed to the brain *via* the specific variety of type C nerve fibers, so called C-tactile, CT fibers (127). CT fibers stimulation activates the insula, but not somatosensory areas S1 and S2 and underlies positive affective aspects of touch (147). However, these types of interactions are often reported as stressful by people with ASD, provoking unpleasant feelings or touch-aversion behavior (148).

Recent studies demonstrated that brain correlates of social touch are modified in ASD (149–151). These alterations in brain activation were correlated with the autism severity (150). Moreover, while activation of the insula and other socio–emotional brain regions were reduced, social touch evoked atypical hyper-reactivity in primary somatosensory cortex (151), consistent with increased tactile sensitivity in areas innervated by CT-fibers (30). This may explain the unpleasantness of social touch and interruption of the mechanism of soft touch-induced analgesia in autism.

Social touch promotes communication through oxytocin-dependent mechanisms (152). *Oxytocin*, a neuropeptide receiving increased attention because of its prosocial effects, has potential in social interactions improvement in autism (153, 154). The regulatory role of oxytocin on pain processing may be found on various levels from peripheral antinociception to high-level emotional pain, pain anticipation and pain memory (155). Some studies demonstrated that oxytocin treatment potentiated social brain connectivity and behavioral improvements in subjects with ASD (156, 157) and reduced heat pain intensity ratings and amygdala activation during painful stimulation (158, 159). This suggests the therapeutic potential of oxytocin in modulation of pain down-regulation by others in autism.

Formation of adequate top-down regulation of pain requires conscious appraisal of it emerging from coordinated co-activation of numerous brain areas. Decoupling between first and second order brain areas of the pain matrix (for example, under anesthesia) leads to nociceptive matrix-elicited physiological and hormonal responses (160, 161), but absence of down-regulation from upper brain areas (162). Such blunted reactions may have long-term consequences such as anxiety, pain sensitization, depression, or post-traumatic stress disorder (70, 163). In ASD, an increase in SIB (21, 28) or other-injurious (164) behavior after painful procedures, along with increased physiological and hormonal responses (165) could be considered consequences of such dysregulation.

To conclude, a body of evidence suggests atypical neuronal processing of pain in ASD. Although some recent results indicate possible alterations in nociceptors (46) or suggest possible excitatory/inhibitory imbalance in the spinal cord level (5), most of the findings focus on atypical brain processing. Together with evidence of hypoactivation in areas of the second and third order brain areas, more consistently in (pre-)frontal cortex, scientific findings support the hypothesis of a "feed-back defect" or "altered top-down regulation" in pain processing, in ASD (62). In addition, the crucial role of attentional engagement in resulting pain-evoked response may be proposed. In many studies these alterations correlated with participants' assessment of direct pain, linking central mechanism dysfunction with atypical expression of pain in ASD. This section emphasizes the linkages between deviations in social regulation and pain perception in ASD.

Pain perception modulation in ASD highlights. *Pain processing in the brain relies on coordinated activation of multiple brain areas. Reorganization of that response may decrease or increase levels of perceived pain. Affective and cognitive aspects of pain perception, as well as in vicarious pain experience and down-regulation of pain response by social touch seem to be modified in people with ASD. Observed alterations should be taken into account when considering the role of social reciprocity in pain regulation in autism.*

ATYPICAL PAIN REACTIONS IN AUTISM SPECTRUM DISORDER

Emotional expressions (bodily, verbal, and facial) have an important social function in communicating one's own feelings to others to receive adequate responses. Pain is a complex socio-communicative experience which includes biological, physiological and cognitive but also social determinants. Expressions of pain can be enhanced or suppressed by the

presence of others and social context, as well as by the personal history of reassurance and support, in reference to the "social communication model of pain" (166). Displays of pain command the attention of observers and provoke reflexive behaviors that facilitate down−regulation by various mechanisms of distraction, reassurance or social referencing (36). Alternatively, individuals with autism may mask or camouflage their feelings (167), which may be associated with adverse mental health outcomes. Alterations of any of the top-down modulation steps including clear pain communication (**Figure 1**) may lead to aggravation of painful experience (168).

Different levels of pain communication exist. They include more or less volitionally regulated facial and body expressions, vocalizations, withdrawal from the source of the pain and also visible physiological signs of distress, like increased pupil diameter, sweating, pale skin, increased heart and respiratory rate, mediated by specific changes in hormonal and neurochemical responses (36). It is still debated (24, 27) whether atypical patterns of pain expression in ASD are mostly attributed to alterations in pain processing and regulation itself, or in the motor response realization, as alterations in motor skills are frequent in autism (169).

Challenges of pain assessment in ASD. Historically, hypo-responsiveness to painful stimuli was included as one of the symptoms of ASD as "apparent indifference to pain" (19), also largely reported by clinicians and relatives of the patient (22, 25, 170). During evaluation of every-day pain reactivity, among several types of behaviors rated as "very often occurred" after a painful incident in people with ASD, some were similar to individuals without ASD ("seeking comfort," "crying") and some were atypical ("being difficult to distract," "jumping around," "agitated," and "fidgety"; 171). However, despite the higher incidence of pain-evoking accidents (172), missing, delayed or aberrant reactions to painful stimuli are often reported in daily observation of subjects with ASD.

Observer-rated pain assessment may provide a long-term profile, and thus is well-suited for chronic pain conditions. Various scales have been specifically adapted for pain assessment in populations with communication and cognitive alterations, as a function of the age and global mental functioning of the person. We can cite the short-form McGill Pain Questionnaire, SF-MPQ (49, 173); the Non-communicating Children's Pain Checklist, NCCPC-R (174, 175), the Pre-Linguistic Behavioral Pain Reactivity Scale, PL-BPRS (21, 165); the Faces, Legs, Activity, Cry and Consolability – Revised (FLACC-R) or the Faces Pain Scale-Revised and a Numeric Rating Scale (176). These scales are focused on behavioral signs, extracted from body language and facial expression, in particular on visible signs of agitation, stress and discomfort. Importantly, in ASD many signs of emotions including stress, anxiety, discomfort, but also pain are considered as "idiosyncratic." Thus these methods of pain evaluation in individuals with ASD, who are known to express atypical responses, should be applied with caution. Parents and observers must be familiar with the "common behavior" of the person, in order to catch the new "signal" and to recognize it as the response to pain (24, 26, 177).

Self-assessment of pain gives direct, but subjective information and requires familiarization with the scales. Tools that obtain quantified self-reports of pain intensity usually allow the individual to state a number or point to a face that correlates with a number, such as the Wong-Baker Faces Pain Rating Scale (178) or the Pain-O-Meter, a plastic tool with a moveable marker (179). Self-evaluation could be difficult in an acute pain situation, even more so if the person has intellectual and conceptual difficulties and cannot follow instructions for self-reporting or has limited understanding of concepts necessary for tool utilization. Ely et al. promoted an alternate *interactive* approach to pain assessment in ASD based on individualized consideration and estimation of pain assessment methods. Utilizing the help and knowledge of parents appeared to be essential in identifying the presence of pain and accurately estimating its intensity (177). More research must be completed to advance knowledge and practice for assessing the pain of the individual with ASD. Researchers developing new tools should recruit parents for assistance.

Global motor response. Based on the use of these various scales, some studies have reported a reduced behavioral reactivity to pain in children with autism, whether at home (parental assessment), in institutions or during a venipuncture (professional assessment; 165, 180, 181), but hyper-reactivity was also suggested in similar experimental conditions as well as in other clinical settings, such as during dental cleaning (182, 183). In addition to behavioral observations with traditional scales, delayed or aberrant reactions to painful stimuli are often reported in daily observations by parents and clinical reports (171). These are: lack of protective body position or withdrawal reactions (7); a wide range of deviant pain-evoked responses like: hyperactivity, paradoxical laughs, aggressiveness, stereotyped behaviors and SIB (20, 22, 165, 184); a general expression of discomfort without localization of the source of pain, as well as other variations (171).

Very few tools to capture atypical signs that are not listed in traditional scales have been tested or adapted for individuals with autism. Only one adaptative scale: the "Simplified Pain Evaluation Scale for Dyscommunicative Autism Spectrum Disorders: ESDDA" has been proposed, but it currently lacks psychometric validation (185). The development of reliable and validated assessment tools remains challenging, because of the high heterogeneity of ASD clinical features but also because pain is defined as a highly subjective experience that varies among individuals in general.

Facial expression of pain in ASD. Facial expressions of pain are a crucial component of social signaling (186–188), even in non-communicative or unconscious patients (189).

Facial emotion recognition and the ability to convey emotion through facial expression has been a topic of interest in autism research for more than three decades. In people with ASD, facial expressions are often perceived as awkward or atypical for a review, see (190). The idea that people with ASD spontaneously express less frequent and shorter emotional expressions and of lower quality (less accurate and intense) than their neurotypical peers is widely accepted (191, 192), described as "amimia"

in clinical reports (191). Many screening and diagnostic tools for ASD include the items: "range of facial expression" or "inappropriate facial expressions," as one of the early signs of ASD (e.g., the Social Communication Questionnaire, Modified Checklist for Autism in Toddlers, Childhood Autism Rating Scale, Autism Diagnostic Interview).

Studies examining pain expression in autism have generally documented mixed results that have failed to provide a consensus. For example, during a venipuncture, some authors reported more facial expressions in children with ASD than in their neurotypical peers (175) whereas no differences were identified in another sample (193). To note, the existing methodologies based on manual scoring of facial expression are strongly dependent on the abilities of the observer (194–196). To minimize the impact of subjective bias and context, several studies were designed to explore facial expressions accompanying pain through standardized conditions and tools and to compare observational rating scores with physiological markers of pain perception during routine clinical procedures, such as venipuncture (165, 175, 193, 195) or dental procedures (183).

Nader et al. (175) has analyzed facial expressions in children using the most frequently used tool in traditional research on facial emotion expressions: the Child Facial Action Coding System for children (FACS) during a venipuncture. The study demonstrated that participants with ASD had greater facial response during the needle insertion phase than the children in the control group. However, other results indicated a lack of concordance between the observer and parental scores of pain evaluations (193). In addition to this, facial reactivity was compared with the motor and physiological responses (heart rate) in children with ASD, children with developmental delay and neurotypical controls.

Only children with ASD demonstrated elevated facial and motor reactivity after the end of the venipuncture, and authors indicated that the age-dependent decrement of pain-induced facial reactions was not observed in this group (193). To avoid observer-related subjectivity, automated facial emotion recognition technologies based on FACS were developed and tested in experimental settings (197–205), however, they need to be adapted to individual features of a person with ASD. To our knowledge, currently no experimental design has been proposed to test and refine algorithmic detection of pain in individuals with ASD.

Physiological correlates of pain perception in ASD. The experience of pain evokes numerous physiological responses driven by the sympathetic and parasympathetic nervous system (206–208). The acute experience of pain causes an increase in heart and respiratory rate, skin conductance response, blood pressure, and pupil diameter, all features of sympathetic nervous system activation. Painful stimuli also provoke heart rate variability changes, mostly an increase in low frequency vs. high frequency ratio (209), muscle tension and a decrease in skin temperature see for review (210). Most of the existing results reported similar physiological responses in individuals with and without ASD, with regard to skin conductance responses (72) and heart rate (193). Only one study indicated increased heart rate in children with ASD compared to children without ASD, after a venipuncture (165). After venipuncture, the low-functioning autism participants demonstrated increased rates of injurious behavior directed toward others (164). Importantly, prediction models based on the integration of various physiological signals (electrodermal activity and cardiovascular activity) with motor response measured by accelerometry may be applied to anticipate the behavioral outcome in non-verbal patients with autism (211), and, potentially, to prevent negative affect and pain after manipulation.

Several limitations, related to the medical manipulation itself, should be mentioned. As venipuncture or dental care include not only painful stimulation, but also different manipulations of the subject's hand or mouth (a tourniquet placement, numerous touches to find the vein, disinfection of the skin, or mixed sensory stimulations – lights, odors. . .), these procedures may generate certain level of stress. Taking in mind high unpleasantness of touch or odors for some individuals with ASD and possible impact of other psychological issues (increased anxiety, for example), these stressors may obscure the response to pain. Therefore, it would be optimal to disentangle several factors of such manipulation to characterize the specific pain-induced reactions.

To conclude, more research is needed to address the broad heterogeneity of pain expression in ASD. Experimental and clinical assessment of pain expression in ASD should be addressed by a multimodal approach, combining data from self-assessed pain, as well as the assessment of pain by an observer, with an effort to adapt and validate the traditional scales. In addition, pain reactions should be assessed *via* the automated analysis of facial expressions and body movements, as well as skin temperature and conductance; vocalizations, and other physiological signals, to create a complete profile of pain-evoked response (212, 213). Moreover, during studies that exploit the analysis of pain-evoked responses in clinical settings, efforts should be made to minimize anxiety and distress due to novelty and anticipation of pain as it may undesirably magnify pain perception in individuals with ASD (168). Paradoxically, in a recent study (175), the children who were the most reactive to pain were those who were described as the least sensitive and reactive to pain by the parents. To explain these results, the authors propose that the expression of pain differs according to the type of pain (care-related acute pain, daily acute, or chronic pains). While participants with ASD would have atypical or reduced behaviors in an everyday situation, they would express themselves in an exaggerated way during a care situation (175). Thus, in the future, preference should be given to non-invasive or more naturalistic methods with minimum manipulations or novelty.

To achieve this goal, new technologies provide some innovative approaches (190, 211). The use of wearable devices allows to assess a range of physiological and behavioral reactions in individuals with ASD (214); however, to date, these studies were not aimed to evaluate pain-evoked responses. Neuro-feedback based strategies, which would

join individual neurophysiological correlates of pain experience with simultaneously recorded expressions of pain, may provide a pathway for people with ASD to express their feelings and solicit reflexive behaviors from social partners.

Atypical pain reactions in ASD highlights. *Production of an adaptive reaction to a painful event is indispensable to receive adequate social support. In individuals with ASD, reactivity to pain is often changed, leading to the absence of proper pain management and corruption of pain cycle functioning. The dissociation between central and autonomic responses to pain may be a cause of alterations of pain processing in ASD. The need for an integrative evaluation of pain responses utilizing modern real-life technologies is necessary to address the individualized needs of each patient.*

MECHANISMS AND PERSPECTIVES

Summary of Common Mechanisms, Involved in Both Autism Spectrum Disorder and Pain Regulation

Many neurochemical pathways involved in determining ASD are also known to participate to pain processing in typical development (see; 215). The pain regulation pathways are predominantly noradrenergic and serotonergic, but implicate other neuromodulators including dopamine and opioids, as reviewed in Yam et al. (35). Here we will discuss the role of some neurotransmitters in ASD in relation to the regulation of pain.

Opioids and Gut Hormones

Endorphins are important components of endogenous pain control (35, 208, 216). Peripherally, beta-endorphins produce analgesia by binding to opioid receptors. At the level of CNS, mu-opioid receptors block pain processing and activate descending pain control circuits including the amygdala and PAG (216). The "Brain Opioid Theory of Social Attachment" (BOTSA), in which social and affiliative behaviors were proposed to depend on endogenous opioid peptide levels, has been based on similarities between social attachment and drug addiction (217). Many symptoms of ASD seems to resemble behaviors induced in animals or humans by opioid administration, including: reduced socialization, repetitive stereotypies, motor hyperactivity and especially insensitivity to pain. This has led to the *neurochemical opioid hypothesis* in ASD, based on evidence from animal models and human studies and from clinical trials with opioid antagonists (naloxone and naltrexone), in the early 90s. Blood and cerebrospinal fluid studies have reported that beta-endorphin levels were altered in individuals with ASD (218–222). In children with ASD, compared to neurotypical children, increased opioid levels were found after exposure to painful manipulation (venipuncture), which were correlated with autism severity (165). However, these results failed to be replicated, that may been ascribed to measures biases (165, 219–222).

After years of disinterest, the role of opioids in ASD could be updated. In light of recent views on the opioid antagonists effect (223) and findings on modifications in particular opioid receptors in subjects with ASD (224), imbalance in the opioid system may be proposed as one of the mechanisms of pain sensitization, hyperalgesia and increasing of pain vulnerability (225, 226). It has been hypothesized that chronic pain conditions in individuals with ASD could be responsible for SIB, which would have a calming effect through opioid release (24). SIB may be related both to enhanced expressions of pain (227), and to reduced pain sensitivity (228). In neurodevelopmental disorders, SIB has been proposed to represent a nociceptive coping behavior, mediated by altered nociception, allodynia or hyperalgesia and neuroinflammatory mechanisms similar to those in chronic neuropathic pain disorders (229). In a recent review of 10 clinical trials testing opioid antagonists and specific drugs in children with ASD, the authors concluded that only in a sub-group with elevated endorphin levels, naltrexone was effective in improving SIB, hyperactivity and restlessness (223).

Endogenous opioids are implicated not only in pain downregulation, but also play a role in many high-level processes like regulation of social behavior. Thus, imbalance of opioid signaling in ASD may be implicated in altered pain perception and SIB as an internal pathophysiological mechanism of opioid tone regulation, but also may contribute to social symptoms. However, the nature of the relationship between self-harm, endogenous opioids and pain processing is not clear yet.

An alternative supposition has been formulated by Jonhson et al. (230, 231), elaborating the links between opioids and ASD. The authors proposed that autism may be related to a genetic predisposition in sub-groups of individuals, triggered by administration of exogenous opioid hormone/medication at birth or during labor that interferes with the natural fetal opioid balance. However, this hypothesis hasn't been documented in a large cohort of babies born to opioid-addicted mothers (232). To note, pre-conception opioid prescription was associated with 2.43 times more for the odds of ASD compared to mothers without prescriptions (233).

Recently, the relation between opioids and gastrointestinal disorders, which is often comorbid in autism, has been discussed (234–236). The gastrointestinal tract problems in ASD include impairments in bowel mucosa function, selective permeability, gut immune response, potentially creating a source of chronic irritation and visceral pain in these patients (237). Increased gastrointestinal tract symptoms are significantly associated with post-stress cortisol concentrations in ASD (238). Gut hormones may play an important role in such relations (239). Among them, ghrelin, a peptide hormone regulating appetitive behavior, reward, stress and anxiety response (240) is decreased in children with autism (241). Ghrelin may also be involved in pain modulation, as central ghrelin injections increased beta endorphin concentrations in PAG and reduced behavioral pain responses (242). Taken together, these observations underpin possible interactions between co-occurrence of chronic pain, SIB and gut dysfunctions, pointing toward a role of endogenous opioids and stress hormones in dysregulated pain perception in ASD.

The Gamma-Aminobutyric Acid Role

The excitatory-inhibitory misbalance in the neuronal system Is implicated in the pathophysiology of pain (243, 244). Gamma-aminobutyric acid (GABA) is known as the main inhibitory neurotransmitter in the adult brain. However, during prenatal stage, GABAergic regulation possesses excitatory role (245, 246). In typical development, GABAergic system switches from excitatory to inhibitory function. The *"GABA" hypothesis* suggests that early dysfunction of the GABAergic signaling disables normal excitatory/inhibitory switch and is responsible for ASD-related symptoms (247–250). This suggestion is in line with model of "developmental heterochrony," according to which, autism-related traits may be explained by delays or truncation of typical development (251). In support of this, various studies in ASD have reported reduced GABA concentration, specifically in visual, auditory, somatosensory areas, both in children and adults (252–254).

Recent findings demonstrated that lack of GABA transmission *via* deficiency in long-term potentiation and learning mechanisms impacts the ability of *the amygdala* to form fear memory (255) and may be responsible for autistic-like features. Improvement in ASD symptoms related to Bumetanide therapy (a drug promoting of GABA shift from excitation toward inhibition) in young children (<6 years old) was associated with normalization of GABA/Glutamate ratios specifically in the insula cortex (256). This may relate GABA dysfunction and the role of negative affect in socio-emotional learning of pain perception. Two other recent studies analyzed the relationship between GABA levels in several brain regions and multiple aspects of sensory processing alterations in ASD. The first study revealed that GABA concentration in the sensorimotor cortex of adults with ASD was lower than in neurotypical adults. In addition, GABA levels were positively correlated with self-reported tactile hypersensitivity in adults with ASD, but not in neurotypical adults (257). In line with these findings, another study in adults with ASD revealed a negative association between left ventral pre-motor cortex GABA concentration and the severity of sensory hyper-responsiveness scores on the Adolescent/Adult Sensory Profile (258). To our knowledge, no study has examined whether GABA concentrations in the brain were associated with altered pain experiences in people with ASD. Future work should evaluate GABA levels in the brain pain matrix as a significant biomarker and therapeutic target for autistic sensory processing disorder and specifically pain processing.

The Role of the Endocannabinoid System

In direct relation with GABA neurotransmission, cannabinoid receptors are present in brain and peripheral neurons, the vagus nerve, gastrointestinal lining, immune cells and skin (259). The ECS participates in brain development, synaptic plasticity, sensory processing and integration, stress regulation and neuromodulation (260). This system modulates social interactions, anxiety and social reward (261). In the context of dysregulation of pain perception, cannabinoid receptors have also been found to play a role in sensory transmission and pain integration (262). Endocannabinoid system dysregulation has been proposed as a pathophysiological mechanism of autism (263, 264). Decreased levels of endocannabinoids were found in children with ASD (265); see for systematic review (266, 267) to link various symptoms in ASD (inflammation, microglia dysfunction, and social symptoms; 268). Three recent papers published by the same team (269–271) focused on the modulation of the brain's excitatory and inhibitory systems in adults with ASD and neurotypical controls, after a single dose of 600 mg of cannabinoids. This treatment increased levels of GABA *in the dorsomedial prefrontal cortex* of controls, but decreased GABA in that region of participants with ASD. In light of these findings, new avenues of therapeutic intervention in the treatment of autism have emerged, on one hand from empirical experience of patients and members of their families, and on the other hand, leveraging genetic and metabolic research. In relation to the above-mentioned gut-brain axis dysfunction in ASD, the potential for the endocannabinoids in alleviating gastrointestinal (272) and behavioral disorders in autism (263, 273–275) along with pain management (262) should be explored. However, the precise mechanism of action of cannabinoids in ASD is far from complete. For example, the two main active components, tetrahydrocannabinol and cannabidiol, interact divergently with cannabinoid receptors. The former activates the receptors, whereas the latter seems to block them (260). However, both components were found to improve behaviors in autism (273, 276), indicating needs to future investigations.

Thus, numerous alterations of neurochemical pathways implicated in autism (**Table 1**) may provide a rich background for integrating numerous behavioral and neurophysiological alterations documented in pain perception, as well as in the development of strategies for pain treatment.

Global Sensory Processing Alterations

Sensory atypicalities are recognized as diagnostic criteria in ASD (19). 90% of individuals with ASD have atypical sensory experiences and regulation, described as both hyper- and hypo-reactivity, with altered responses to tactile or auditory stimulations (75). This topic has been the focus of intensive research over the last 10 years, including studies on pain perception, leading to the "*hypothesis of sensory dysfunction" as a* cause of altered pain sensitivity in ASD (14, 30, 32). These anomalies affect all the sensory channels of the child and the adult (see section "Initial Stages of Pain Signaling: From Nociceptors to the Central Axis") and complicate the integration and cognitive processing of stimuli, making each pain percept unique and poorly integrated in a general concept of the self.

Altered Central Pain Processing and Modified Regulation of Pain Responses

A growing body of evidence delineating alterations of central (both cortical and subcortical) and peripheral pain transduction, transmission and processing are challenging the idea of impaired pain processing due to a single factor, and calls for an integrative model. To summarize the findings regarding the central brain responses and altered pain reactivity in ASD (see section

TABLE 1 | Summary and discussion of mechanisms involved in both ASD and pain dysregulation.

Mechanism	Description	Justification	Criticism
Neurochemical modulations: *Opioid system dysregulation*	As endogenous opioids are an important component of pain downregulation, modifications of opioid signaling in ASD may cause altered pain perception	Increased levels of opioids observed in ASD after painful manipulation; opioid antagonists seems to alleviate ASD symptoms opioid receptor dysfunction found in ASD	Elevated opioid levels were not confirmed in subsequent studies
Neurochemical modulations: *Interrupted GABA switch*	Pain perception is altered by excitatory-inhibitory imbalance in the developing nervous system	Various studies have reported reduced GABA concentrations in perceptive areas in ASD	No studies demonstrate pain alleviation in ASD with GABA agonists/antagonists or any relation between GABA brain levels and pain
Neurochemical modulations: *Endocannabinoid dysregulation*	Endocannabinoid system dysregulation has been proposed as a pathophysiological mechanism of autism	ECS participates in sensory transmission and pain integration. Cannabinoids alleviate autistic behavior symptoms	No studies demonstrate relation between ECS brain levels and pain perception in ASD
Pain processing modulations: *Global sensory processing alteration*	Atypical pain perception in autism due to global alterations of sensory perception mechanisms (peripheral or central)	Sensory alterations are present in the majority of cases and are commonly recognized as diagnostic criteria in ASD; modifications in the structure of nociceptive sensory endings were reported in ASD; consistent with these findings, allodynia, paradoxical heat sensations and alterations in C-tactile soft touch perception are documented in autism	Intact, hypo-, and hypersensitivity may be observed depending on methodology. Pain-related responses in population with ASD are heterogeneous. Sensory processing patterns are known to change over time, depending on the context in which they are observed
Brain pain processing modulations: *Altered pain processing and response regulation*	*pain cycle imbalance in ASD:* reduced engagement of the pain matrix and in-adequate physiological responses disable adequate cognitive and emotional regulation of pain perception	In ASD, decreased response of the pain matrix might be a result of reorganization of pain processing pathways. Altered interoceptive abilities and self-consciousness in ASD do not allow cognitive appraisal of all aspects of pain and thus makes impossible proper down-regulation, which may lead to inadequate pain-related responses, like self-injury behavior	Descending noxious controls have been found to be normal in autism Little data is available on brain processing of pain in ASD
Modulation of both pain expression and social feed-back: *Socio-communicational issues*	Social and cognitive complications in ASD prevent communication about perceived pain and requests for help	According to theories regarding the role of cognitive and emotional control of pain, altered embodiment of emotions in ASD and atypical pain expressions in ASD may lead to atypical development of recruiting the help of others in managing pain in ASD	In spite of some alterations in brain processing. perception of vicarious pain seems to be normal in ASD

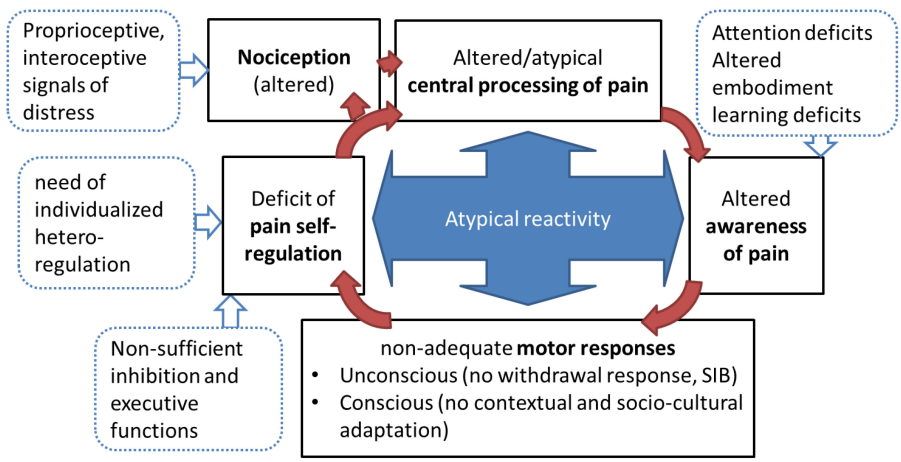

FIGURE 3 | The pain cycle in ASD with possible impacts from suggested mechanisms, modified integrated model, adapted from Dubois et al. (23), Rattaz et al. (24), and (277).

"Neurophysiological Processing of Pain in Autism Spectrum Disorder"), we propose the integrated model of the pain cycle in autism (**Figure 3**). Within this framework, evidence for altered self-awareness and interoception, and reciprocal relations between altered top-down regulation of pain processing and pain coping in autism may lead to inadequate and deviant

pain reactivity. By analogy with other chronic pain conditions, we speculate that altered pain processing in the brain of individuals within the spectrum results from reorganization of pain pathways. This suggestion is supported by recent findings from neuroimaging studies (62, 71, 95). However, more research is needed to delineate causal dependencies between these diverse processes, and to demonstrate how pre-conscious nociception relates to conscious pain perception in subjects with autism, given their self-awareness and interoceptive abilities. Although the basic processes of pain down-regulation seem to be unchanged, the capacity for conscious appraisal of pain and the communication of pain to others might be limited in ASD, especially in early life, when imbalances in physiological and hormonal responses lead to dysregulation of pain management, and probably to long-term consequences like increases in anxiety and depression (168).

Socio-Communicative Issues

Communicational model of pain highlights the social role of pain expression and intrapersonal reciprocity in down-regulation of pain (277). Observed dissociation between enhanced physiological and biological stress responses and behavioral reactions in autism (28, 165) suggest that, at least for some individuals, the neurochemical and brain pathways are preserved but pain communication is altered (8, 177, 181). Based on the various findings reported above (see section "Atypical Pain Reactions in Autism Spectrum Disorder") atypical motor response and altered reciprocity may inhibit the observer's empathy, raising a "double-empathy issue" (278) that was highlighted in recent publications (23, 24). As in most cases, people with ASD have no intellectual disability (279) and may use alternative strategies of social adaptation (280), the potential of specific cognitive-behavioral therapy (281) for pain understanding in autism should be explored.

Perspectives

Do core autism symptoms (sensorial and social) ***initially*** cause altered pain perception and expression, which are then worsened by comorbidities and atypical social modulation experiences? Or does pain processing in both neurotypical and ASD individuals share ***common vulnerabilities*** that lead to association of ASD symptoms and pain dysregulation? Would it be possible to compensate or prevent alteration of the pain modulation as early as ASD is diagnosed? General heritability of ASD is approximately 80% (282). Among the numerous genes associated with ASD, some have also recently been implicated in pain processing (283, 284). These genes are involved in various cytomolecular mechanisms controlling of C-fiber excitability thresholds, or glutamate pathways (27) and induce alteration in pain processing, in both animal and human models (5) if muted. However, findings in animal models of autism report wide divergence of pain perception and responses (27), pointing out the possible impact of epigenetic factors on relevant genetic alterations of ASD. To note, most animal models are monogenic mutation models, in contrast with human. Additional research is needed to better understand the common genetic and biomolecular pathways (283, 284), and propose innovative and preventative therapeutics.

Pain processing develops with age, starting during gestation, and matures with social experiences throughout life (285, 286). Given that ASD is considered a developmental condition, future research need to address this developmental aspect, proposing longitudinal studies, including early therapeutics and preventative strategies.

Social development interacts with pain management. Positive social experiences impact pain modulation and may alleviate pain perception. Oppositely, negative social experiences as isolation, bullying, and social rejection among others, may aggravate perceived pain. Such "psychological" pain itself produces similar brain response in pain matrix as does physical pain (287, 288) and have been associated with SIB in ASD (289). That bidirectional aspect of social interactions on pain perception and expression in ASD should be more explored.

Mechanisms and perspectives highlights. *Numerous mechanisms involved in ASD pathogenesis have been proposed to explain the alterations in pain processing. Among them, alterations in neurotransmitters and neuropeptides (opioids, endocannabinoids, GABA), in global sensory processing, in brain response, together with socio-communicational issues are thought to be associated differently in individuals with ASD contributing to both autism core symptoms and pain dysregulation. In an attempt to integrate these mechanisms, we propose our all-inclusive model of the pain cycle in autism. However, a number of open questions still remains, requiring an integrative approach to resolving the paradox of pain in ASD.*

CONCLUSION

The research of pain in ASD is complex. Recent findings in alterations of brain pathways, summarized as an integrated model of the pain cycle in autism (**Figure 3**), suggest more than nociceptor and neuronal dysfunctions and implicate altered self-awareness and interoception, which interact with top-down regulatory pathways for processing and coping with pain. Both peripheral and central deviations in pain signal processing are documented in autism. The high variability in pain-related responses in this population makes the group-based approach challenging. To date, other- and self-rated pain assessments aren't sufficient to characterize the specificity of pain-related processes in autism and to drive interventions. New technologies, already used in emotion recognition research in ASD, joined with continuous physiological activity screening, may provide more quantitative and integrative approaches to specify what is atypical in expressions of pain in people with ASD. Future directions may address the role of these various alterations at different stages of pain signaling and regulation in ASD symptomatology and may yield promising candidates for global therapy, social improvements and behavioral amelioration. The importance of adequate, objective and multiple approaches for the assessment of pain in individuals with autism is

essential not only for health outcomes but also to prevent the worsening of social disorders. A better understanding of the complexity and individuality of pain regulation may resolve the paradox of pain in autism, leading to the development of individualized pain management strategies, and with novel therapeutic approaches.

AUTHOR CONTRIBUTIONS

OB: conceptualization and writing – original draft. VB: visualization and writing – review and editing. AP, MB, and NM: writing – review and editing. J-RC: funding acquisition, supervision, and writing – review and editing. AA: funding acquisition, supervision, conceptualization, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Case report: One child with an autism spectrum disorder who had chronically elevated serum levels of CK and CK-MB

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Some patients with autism spectrum disorder (ASD) exhibit elevated serum creatine kinase levels, which are believed to be associated with mitochondrial dysfunction. Although a few articles have reported this situation in the past and the increase mostly ranges from 100 to 300 U/L, there is a paucity of previous study focusing on the serum creatine kinase MB isoenzyme. This article discusses a 5-year-old girl with ASD, whose serum creatine kinase and creatine kinase MB isoenzyme have been rising for nearly 2 years, fluctuating at 584–993 and 111–625 U/L respectively. Except for behavioral and language symptoms associated with ASD, the child appears normal in other aspects. The child's laboratory tests showed no abnormality, except that the serum levels of lactic acid was slightly higher than normal (1.89 mmol/L, normal 1.33–1.78 mmol/L). The child was prescribed with a traditional Chinese medicine during the process and the serum creatine kinase MB isoenzyme level decreased dramatically to 111 U/L after the treatment. This study firstly recorded the serum creatine kinase levels and the MB isoenzyme in patients with autism spectrum disorder for nearly 2 years, indicating that patients with ASD may experience long-term increases in serum creatine kinase and creatine kinase MB isoenzyme, and that the traditional Chinese medicine decoction Xinfukang can temporarily reduce the serum creatine kinase MB isoenzyme level in patients. Nevertheless, the effect is not sustained. Therefore, it is of great importance to conduct long-term longitudinal studies so as to elucidate the potential mechanism responsible for long-term elevation of serum creatine kinase level.

KEYWORDS

autism (ASD), creatine kinase, biomarkers, traditional Chinese medicine, case report

Background

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental disorder characterized by defective communication capacity, social impairment, stereotyped behaviors, and limited interests (1). The latest prevalence of ASD in the United States is 2.3% (2). There are at least 78 million people with ASD which makes it one of the fastest

growing diseases in the world (3). It was reported that in 2013, the prevalence of ASD was 0.7% in China (4). While ASD's pathogenesis is still unknown, recent studies have found that some ASDs may be associated with inborn errors of metabolism (5). Some metabolic disorders can only be identified by non-targeted biochemical markers (5) and serumcreatine kinase (CK) values may be one of the potential indicators to it (6).

The reversible transfer of high-energy phosphate bonds between creatine and ATP can be catalyzed by CK, also referred to as phosphocreatine kinase (CPK). It is a significant regulatory enzyme provides energy for muscle contraction (7). It exists in skeletal muscle, cardiac muscle, brain tissue and other tissues that require a large amount of energy supply (7). The creatine kinase MB isoenzyme (CK-MB) is one of the isoenzymes of CK, and the two are often combined as a laboratory indicator for clinical judgment of myocardial injury (8, 9).

However, the results of current studies regarding serum CK levels in patients with ASD are inconsistent. Some studies have found that serum CK levels in ASD patients are higher than those in healthy people. Hassan et al. (10) compared the serum CK values in 73 ASD men and those in 73 age-matched healthy men, and revealed that the serum CK value in the ASD group was greater than that of the healthy control group ($P < 0.05$) (the serum CK value was 137 ± 12.96 U/L). It is consistent with the findings of El-Ansary et al. (11). Nonetheless, this study did not take into account the gender difference of patients and the highest serum CK value in the ASD group was only 63 U/L. Other studies found that not all ASD patients exhibit elevated serum CK levels. According to Mosalem et al. (12), the serum CK value of ASD children was 72.35% higher than that of normal children, with a serum CK value of 284.277 ± 242.477 U/L. A retrospective study conducted by Polling et al. (13) found that only 47% of ASD patients had abnormally elevated serum CK values. Cohen et al. (14) found that there are no differences in serum CK levels between children with ASD and normal children ($P > 0.05$). However, none of these studies discussed the serum CK-MB.

For traditional Chinese medicine therapy of patients with ASD, the choice of medicine is mainly based on the experience of physicians for which there is no unified standards to refer. Choosing traditional Chinese medicine based on tongue manifestations, pulse manifestation and clinical symptoms can improve symptoms associated with ASD (15). In particular, it can significantly improve patients' verbal communication ability and social communication ability, whereas its effect on improving patients' stereotyped behaviors is poor (16). Traditional Chinese medicine decoction Xinfukang is used for heart failure in clinical practice and other cardiac diseases. Although there were no studies focusing on the use of traditional Chinese medicine decoction Xinfukang for ASD patients, a study including 150 children with elevated serum level of CK-MB showed that traditional Chinese medicine Xinfukang can better reduce the serum levels of CK-MB of pediatric patients, compared with the western medicine group (oral vitamin C, vitamin E, inosine tablets and coenzyme Q10 for intramuscular injection) (17). In an animal study, traditional Chinese medicine Xinfukang administered orally by gavage can decrease the serum levels of CK-MB in rats with exercise-induced myocardial injury (18).

Though the serum CK levels were reported to be elevated in ASD patients, no report mentioned that the serum CK levels exceeded 900 U/L. There is a paucity of previous study focusing on the serum CK-MB levels in ASD patients, and there has been no report on the simultaneous monitoring of serum CK and CK-MB for 2 years continuously, making this research unique in this field. The case of an ASD child with abnormally elevated serum CK and CK-MB levels for almost 2 years, fluctuating between 584 and 993 (reference range, normal 29.0–168.0 U/L) and 111–625 U/L (reference range, normal 0.0–24.0 U/L), respectively, is described in this article. Apart from ASD-related behavior and language performance, the child is symptom free. During this period, the child was treated with the Chinese herbal decoction Xinfukang. After the treatment, the serum CK-MB level decreased to 111 U/L, but then increased again.

Case presentation

A 5-year-old female Asian child with communication disorder, stereotyped behavior, and limited interest was diagnosed with ASD. The patient had no history of epilepsy or febrile seizures. The parents of the child are healthy, without any history of genetic disease in the family. The mother was healthy during her pregnancy, and the child is a first-born only child. The vaccination was administered on time and no adverse reactions were reported. She is a child with normal limb development, good nutrition, normal gross motor development, and delayed fine motor development. By the age of 2, parents observed that the child refused to communicate with others, showing a lack of eye contact, no responded to names, poor language skills, confusion or repetition of words, self-talk, rigid language, occasional strange noises, no response to teaching, not responding to commands, limited interest, pacing back and forth, irritability, and night terrors. The child's Autism Behavior Rating Scale (ABC) score was 132, while the Childhood Autism Rating Scale (CARS) score was 35. The child's doctor diagnosed her with ASD. The prescribed medications for her were Lactulose 10 ml/day, Singulair 4 mg/day, Cetirizine 2.5 mg/day, and Folic acid 5 mg three times per day. The child's serum CK value was 993 U/L and the CK-MB value was 200 U/L when he was 3 years old (May 2020), both of which were higher than normal. Besides, the results of electrocardiogram, liver and renal function tests and cardiac color ultrasound were normal. Upon multiple rechecks, serum CK and CK-MB values were higher than normal (see Figure 1). The results of electrocardiogram, cardiac color ultrasound and liver and

renal function tests were normal. Serum level of folic acid showed unremarkable, serum level of lactate acid was 1.89 mmol/l, slightly higher than normal (normal 1.33–1.78 mmol/l). We performed whole exome genetic screening, blood genetic metabolic disease amino acid and acylcarnitine, urine organic acid comprehensive analysis, troponin, myoglobin, rheumatoid factor, antinuclear antibody, C-reactive protein, antistreptolysin "o" test, myositis-specific antibodies tests (including anti-KU antibody, anti-JO-1 antibody, anti-RO-52 antibody, anti-PM-SCL75 antibody, and anti-MDA5 antibody) and other related tests. As a result of all the tests, there are no abnormalities. Nevertheless, muscle biopsy, a serum lymphocyte test, and a platelet mitochondrial test were not performed due to parental refusal. The child showed no symptoms of fever, palpitation, chest pain, suffocation, cyanosis, muscle pain, or changes in muscle tone throughout the entire process. Based on the above examinations and clinical manifestations, doctors ruled out possible causes such as myocardial damage, liver damage, myositis, juvenile idiopathic arthritis, hypokalemia, and hypothyroidism.

During the periods of treatment, western medicine seemed to has no effect on serum CK and CK-MB level of the child with ASD. Thus when the child aged 4 (March 2021), her parents asked physician to add traditional Chinese medicine to the treatment regimens and physician gradually decreased the use of other medicine and added traditional Chinese medicine decoction Xinfukang according to clinical manifestations and tongue manifestations, pulse manifestations (see Figure 2). Traditional Chinese medicine was decocted with water for 30 mins, and the decoction was administered about 150 ml each time orally. Within 1 month of oral treatment, the serum CK-MB level of the child decreased to 111 U/L, and the serum CK value decreased to 707 U/L (see Figure 1). In May 2021, the parents discontinued taking traditional Chinese medicine on their own since the child caught a cold. This child took the traditional Chinese medicine decoction Xinfukang for treatment again in July 2021, and the decoction was taken orally until the end of the monitoring. No side effects were observed during the period. Unfortunately, the serum CK and CK-MB levels of the child were not present significantly decreased.

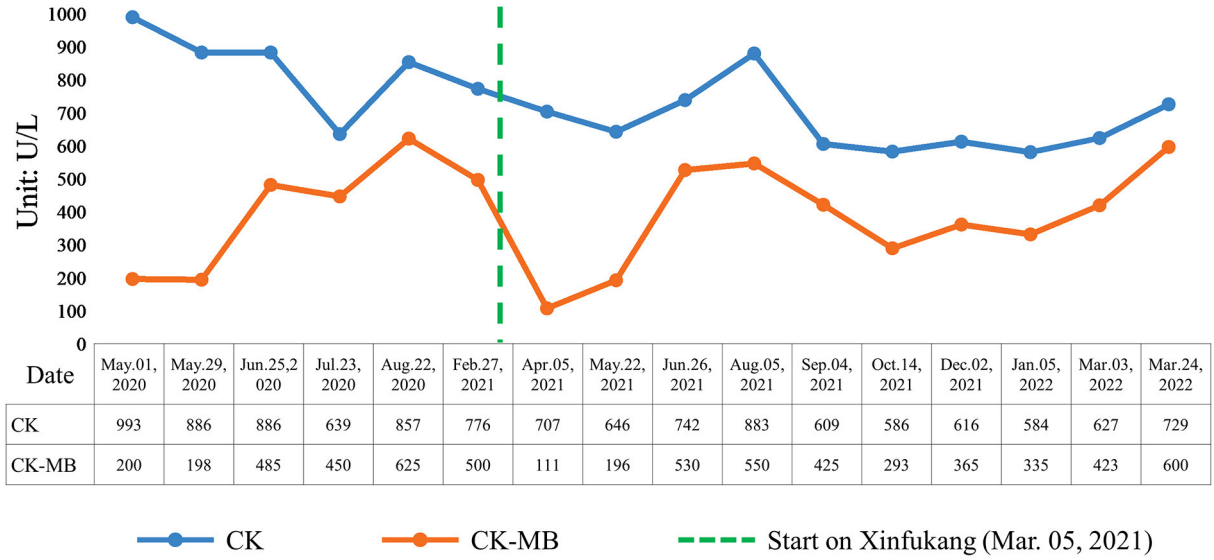
During 2 years of monitoring and treatment, the doctor observed that the child's language comprehension and expression abilities occasionally regressed, while the overall trend was improving. The number of night terrors gradually decreased and eventually disappeared. At the end of the study, the child's ABC volume represented "60 points" and the CARS volume represented "34 points." The parents of the child believe that the child is displaying enhanced social skills, with more eye contacts with others. Moreover, the child is able to express simple ideas through language or gestures, and can complete some simple command actions. It has been shown that Chinese medicine has a significant effect on improving moods and sleep in the child. The child's teacher believes that the child's attention has improved. However, neither the physician nor the child's parents thought that the child's behavior, language development, mood, sleep, and other aspects were not related to serum CK and CK-MB. For instance, the child's parents noted that when the child visited the doctor on August 22, 2020, the child showed improved comprehension and more stable mood in that month, but with relatively high serum CK and CK-MB levels were relatively high, at 993 and 625 U/L, respectively. During January 2022, the child was irritable without obvious inducements, and his comprehension abilities were poorer than before, though the serum CK levels did not fluctuate significantly in comparison to before (see Figure 1). Therefore, it appears that serum CK and CK-MB levels are not associated with the condition of children with ASD.

Discussion

The serum CK and serum CK-MB are often used in combination as laboratory indicators of myocardial injury (8, 9). In addition, the elevation of serum CK levels can also be seen in hereditary or acquired myopathies such as muscular dystrophy, metabolic myopathy, congenital myopathy, drug- or toxic-induced, inflammatory myopathy, and endocrine myopathy, etc. (19). Serum CK elevation can also be caused by non-myopathy factors, such as getting black, high fever, strenuous exercise, trauma, viral infection, malignant hyperthermia, and idiopathic hyperckemia (19, 20). Neurological diseases such as epilepsy (21), schizophrenia (22), etc. may increase serum CK levels. According to previous studies on adult psychosis, acute psychosis can cause elevated serum CK levels, whereas chronic psychosis patients present normal serum CK levels (22).

CK-MB is a hybrid dimer composed of M-type and B-type monomer subunits, which has reversible catalytic functions for creatinine and ATP phosphorylation (23). In normal serum, CK-MB constitutes less than 5% of CK (24). It is generally believed that serum CK-MB/CK $\geq$ 6% indicates myocardial injury, and that CK-MB activity can also be increased in skeletal muscle disease. However, serum CK/CK-MB is less than 5% in most cases (25). The possibility of myocardial injury in the child is ruled out based on her clinical symptoms and the outcomes of related laboratory tests despite the serum CK-MB/CK of the child described in this article being higher than 6%. Xiao et al. (24) found that patients with traumatic brain injury or brain diseases may have significantly elevated CK-MB and that in some cases, even the inversion of CK-MB and CK measurements would emerge. Although the serum CK-MB level of this child did not exceed the serum CK level, its significant increase may be related to autism.

It is unclear what causes the rise in serum CK levels in ASD. However, it has previously been explained in terms of mitochondrial dysfunction. In ASD patients, mitochondrial dysfunction is a common metabolic disorder (5). In their



Date	May.01, 2020	May.29, 2020	Jun.25,2020	Jul.23, 2020	Aug.22, 2020	Feb.27, 2021	Apr.05, 2021	May.22, 2021	Jun.26, 2021	Aug.05, 2021	Sep.04, 2021	Oct.14, 2021	Dec.02, 2021	Jan.05, 2022	Mar.03, 2022	Mar.24, 2022
CK	993	886	886	639	857	776	707	646	742	883	609	586	616	584	627	729
CK-MB	200	198	485	450	625	500	111	196	530	550	425	293	365	335	423	600

FIGURE 1
Changes of serum CK and CK-MB levels in the child.

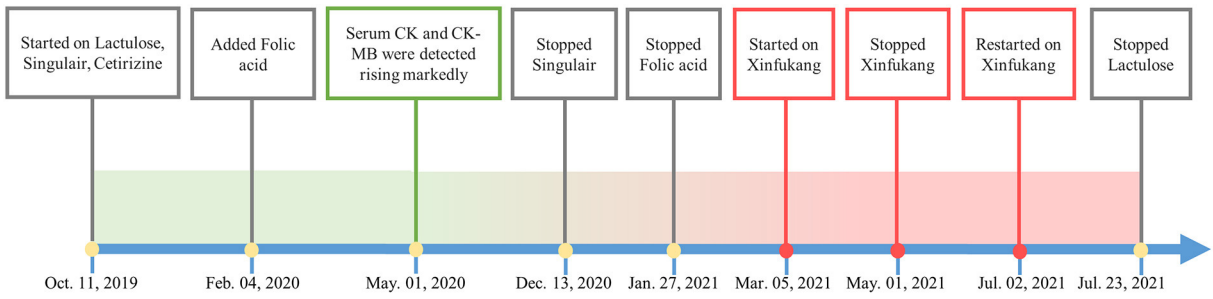


FIGURE 2
Medication history.

study, Rossignol et al. (26) argue that mitochondrial dysfunction can result in the loss of cellular integrity in specific organs, including muscle and liver, enabling the release of CK into the bloodstream, leading to increased serum levels of CK. However, not all ASD patients with mitochondrial dysfunction have elevated serum CK levels. In their study, Frye et al. (27–29) found that only a minority of ASD patients with mitochondrial disease had elevated serum CK levels. In a study of 25 patients who had ASD and mitochondrial dysfunction, it was found that only 32% had elevated serum CK levels (30). Furthermore, not all elevated serum CK levels are caused by mitochondrial dysfunction. According to a meta-analysis, 5% of ASD patients suffer from mitochondrial disease, while 47% of ASD patients have elevated serum CK, significantly higher than 5% (31). Therefore, further studies should be conducted to explore whether there are other potential mechanisms responsible for the elevation of serum CK level in patients with ASD.

Muscle biopsy is frequently used to detect mitochondrial diseases (32), as is mitochondrial examination of serum lymphocytes and plates can also be a laboratory indicator of mitochondrial disease (33). Lactate is a key biomarker for identifying mitochondrial disease (31, 34). The parents of the child described in this article refused muscle biopsy and serum lymphocyte and platelet-related mitochondrial tests. The child's serum lactate acid level was 1.89 mmol/L, slightly higher than normal. Studies have shown that children with ASD and mitochondrial disease may suffer from fatigue, gastrointestinal problems, abnormal types of neurodevelopmental degeneration, seizures, and motor retardation (35). However, these symptoms were not observed in the patient described in this article. Further, over a period of nearly 2 years, the doctor monitored the

child's serum CK level has been rising, which is inconsistent with the intermittent increase in serum CK levels in ASD patients with mitochondrial disease previously reported by Weissman et al. (30). Therefore, it cannot concluded that the increase in serum CK in this patient is linked to mitochondrial dysfunction.

Traditional Chinese medicine decoction Xinfukang can decrease the serum levels of CK-MB of rats with exercise-induced myocardial injury by decreasing the serum levels of cortisol and elevating the serum levels of testosterone which to protect myocardial cell (18). On the other hand, Xinfukang oral liquid has effects on mitochondrion; Qilin et al. (36) found that Xinfukang oral liquid can adjust mitochondrial proteomics in myocardial cells of rats with heart failure by upregulating the expression of α subunit of ATP synthetase related to energy metabolism which can improve energy metabolism disorder in myocardial cell to a certain degree and downregulate the expression of mitochondrial stress-70 protein in myocardial cell at the same time. Stress-70 protein family played an important role in preventing and treating cell damage caused by stress and repairing damaged cells. Xinfukang oral liquid can alleviate stress reaction and protect damaged myocardium by downregulating the expression of mitochondrial stress-70 protein. Physician expected to decrease serum levels of CK-MB of the child with ASD and adjust mitochondrial function to improve her clinical manifestations related to ASD by giving her Xinfukang. At the time of 1 month after the initial use of Xinfukang, serum levels of CK-MB of the child with ASD indeed decreased but increased again without subsequent improvement in spite of the continual use of Xinfukang. Luckily, however, language expression abilities and moods of the child with ASD seemed to be improved with the traditional Chinese medicine.

Patients with ASD may have elevated serum CK levels, which have previously been associated with mitochondrial dysfunction. The article first reported a child with ASD who had significantly elevated serum levels of CK and CK-MB for approximate 2 years and recorded the changes of serum CK, CK-MB in details. After the use of traditional Chinese medicine decoction Xinfukang, the decrease of serum CK-MB once appeared. However the effect was not persistent and the function mechanism remains unclear. As the case indicated, child with ASD may have the chronically elevated serum levels of CK and CK-MB and traditional Chinese medicine decoction Xinfukang can improve child's behavioral and language symptoms, moods and so on, but cannot improve the serum levels of CK and CK-MB in the long term. Due to no reports of similar cases in the past and no studies about traditional Chinese medicine decoction Xinfukang therapy for ASD, it is a huge challenge to diagnose and treat ASD. One of the limitation of this study is lacking of related evidence to support it. The next step should be the completion of a long-term longitudinal study of CK and CK-MB levels in ASD patients to clarify the potential mechanisms responsible for long-term elevation, so as to explore further the possibility that Xinfukang might be effective in treating ASD.

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 authors.

Ethics statement

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

PR and RM contributed to conception and design of the study. SZ wrote the first draft of the manuscript. QF, XZ, and MC wrote sections of the manuscript. LY and YS collected information about the patient's condition. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

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Reliability and validity of the repetitive behavior scale-revised for young Chinese children with autism spectrum disorder in Jiangxi Province

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Restricted and repetitive behaviors (RRBs) are one of the two main diagnostic features of autism spectrum disorder (ASD). To date, a growing body of research on RRB in children with ASD has recently attracted academic attention. The Repetitive Behavior Scale-Revised (RBS-R) was primarily intended for use in evaluating RRBs observed in ASD. This study recruited 381 Chinese children with ASD aged 2–4 years to measure the reliability and validity of the RBS-R. Confirmatory factor analysis (CFA) was applied to the structuring models of the four proposed structural models, indicating that a 6-factor model demonstrated good internal consistency and the best fit based on common overall fit indices. These findings suggest the utility of the Chinese version of RBS-R.

KEYWORDS

restricted and repetitive behaviors (RRBs), Repetitive Behavior Scale-Revised, autism spectrum disorder (ASD), confirmatory factor analysis (CFA), validity, reliability

Introduction

Autism spectrum disorder (ASD), defined as a neurodevelopmental disease, has two core symptoms including social communication deficits and restricted and repetitive behaviors (RRBs) and sensor stimuli behaviors (1). Genes, environment, and the interaction between genes and environment are the etiology causes of ASD (2). The Autism and Developmental Disabilities Monitoring (ADDM) network estimated the prevalence of ASD among children aged 8 years, showing 0.66% in 2002 (3), 1.46% (4) in 2012, 1.68% (5) in 2014, and 1.85% (6) in 2016, respectively. A Chinese nationwide multi-center population-based study (7) recruited 125,806 participants aged 6–12 years

to determine the prevalence of ASD, revealing that 0.7% of these children were diagnosed with ASD. This figure was higher than the 0.118% reported in a meta-analysis conducted in China (8), which included 25 studies correlated with the prevalence of ASD. As the prevalence of autism increases, there is an increasing focus on the condition around the world.

Restricted and repetitive behavior is a series of behaviors characterized by high frequency and invariant repetition; this results in the child desiring environmental sameness (9). RRBs have four subtypes of behavior in the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5). The first is the repetition of actions, speech, or use of objects, such as turning around and parroting. The second is insisting on sameness such as walking the same route. The third behavior is restricted interests, including obsessing with wheels. The last one is sensory processing abnormalities, for example, smelling people's hair (10). It is well known that the social communication of ASD has gained considerable research attention over the years; however, the field of RRBs lags far behind the former. In many infants later diagnosed with ASD, RRBs are detectable after 12 months of age, which may be some of the earliest detectable behavioral markers of ASD (11). RRBs are also present in typically developing (TD) infants, toddlers, and developmental delay (DD) children (12). Through literature review, we found that most of the measurements of RRBs were derived from some items of the Autism Diagnostic Interview-Revised (ADI-R) (13) and the Autism Diagnostic Observation Schedule (ADOS) (14) and a scale named the Repetitive Behavior Scale (Revised Edition), a 43-item informant-based rating scale, was used to assess RRBs only. This scale includes six subscales: (a) Rituals, (b) Self-injurious Behavior, (c) Stereotypic Behavior, (d) Compulsive Behavior, (e) Restricted Interests, and (d) Sameness. The RBS-R aimed to measure the presence and severity of a variety of RRBs and its items were drawn from other instruments and conceptually categorized based on the authors' clinical experience (15). Numerous investigations have been conducted on the psychometric properties of the RBS-R including in Canada (16), German (17), Italy (18), Spain (19), United States (20), and so on. RBS-R was adopted in a study (21) of 163 Chinese children aged 3–8 with ASD enrolled, indicating that a 5-factor model was more appropriate for evaluating RRBs, with a Comparative Fit Index (CFI) of 0.99 and a Goodness of Fit Index (GFI) of 0.73. Another reliability and validity study (16) of RBS-R from Canada showed that the goodness-of-fit indices of the hypothesized latent-factor models of the RBS-R failed to achieve anticipated success. Another study in the United States found that the four- and six-factor models both demonstrated adequate-to-good fit in a sample of 350 children with ASD aged 2 to 9 years (20). In conclusion, the psychometric analysis of the RBS-R was confirmed with different nations and samples.

Since the RBS-R is a widely used analytical tool for researching the RRBs of ASD, it is crucial to clarify the appropriateness of using the RBS-R in Chinese children with ASD. Currently, there are only two domestic studies on the reliability and validity of RBS-R in ASD. In our study, we aimed to increase the sample to measure the reliability and validity of the RBS-R among children with ASD aged 2–4 years in Jiangxi province of China. According to Rouquette A (22), a minimum sample size of 300 subjects is generally acceptable but should be increased when the number of factors is large. This research was also designed to determine the feasibility of employing the RBS-R as an assessment tool for the identification and diagnosis of ASD in the future.

TABLE 1 Socio-demographic and diagnostic characteristics.

Demographic and diagnostic variables	*N*	Percentage (%)
Age		
2–3	189.00	49.61
3–4	119.00	31.23
4–5	73.00	19.16
Gender		
Boy	305.00	80.05
Girl	76.00	19.95
The severity of autism spectrum disorder		
Mild	144.00	37.80
Severe	225.00	59.06
Missing	12.00	3.15
Age of father		
<30	146.00	38.32
30–40	165.00	43.31
≥40	44.00	11.55
Missing	26.00	6.82
Age of mother		
<30	206.00	54.07
30–40	134.00	35.17
≥40	17.00	4.46
Missing	24.00	6.30
Education level of father		
Junior high school diploma or below	116.00	30.45
High school diploma	160.00	41.99
Undergraduate degree	72.00	18.90
Graduate degree or above	16.00	4.20
Missing	17.00	4.46
Education level of mother		
Junior high school diploma or below	137.00	35.96
High school diploma	137.00	35.96
Undergraduate degree	78.00	20.47
Graduate degree or above	12.00	3.15
Missing	17.00	4.46

The severity of autism spectrum disorder was evaluated by the score of CARS. 30–35 was defined as mild and ≥36 was defined as severity.

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Materials and methods

Participants and procedures

We recruited children aged 2–4 years old from a hospital in Jiangxi province. Inclusion criteria were: (1) native Jiangxi province children, (2) no parent-reported visual and auditory impairment, epilepsy, cerebral palsy, trisomy 21 syndrome, or other psychiatric diseases. These children were selected from those who received the assessment of the Childhood Autism Rating Scale (CARS) and based on a score of no less than 30. Children were then diagnosed with ASD according to the DSM-5. The parents or caregivers of eligible children were given written informed consent and the demographic questionnaire, RBS-R, and Autism Behavior Checklist (ABC). The researchers verified their eligibility once they completed these questionnaires, and a second RBS-R was issued to the parents who consented to complete the questionnaire a second time one week after the first one was completed. When conducting the assessment, researchers would prompt participants to complete any incomplete questionnaires.

Measures instrument

The original Repetitive Behavior Scale (RBS) had three subscales: (a) Stereotypic Behavior, (b) Self-injurious Behavior, and (c) Compulsions which was established by Bodfish (23). RBS-R-based RBS was built by Bodfish in 2020 (15), which was intended to assess the variety of RRBs observed in individuals with ASD and the 43 items can be categorized into the six following subscales: stereotyped behavior (6 items), self-injurious behavior (8 items), compulsive behavior (8 items), ritualistic behavior (6 items), sameness behavior (11 items), and restricted behavior (4 items). All items are rated on a four-point Likert scale of severity; the higher the scores, the more severe the conditions (i.e., 0 = behavior does not occur, 1 = behavior occurs and is a mild problem, 2 = behavior occurs and is a moderate problem, 3 = behavior occurs and is a severe problem). The Chinese version was cited by Li et al. (24) and translated from English to Simplified Chinese by two native Chinese speakers who majored in developmental-behavioral pediatrics and were also fluent in English. To guarantee the consistency of the message, two professors then proofread and translated the Chinese version back into English. In our study, we added descriptions of some items by utilizing more pertinent and detailed explanations from knowledgeable doctors who evaluated numerous individuals with ASD. The RBS-R used in this study was completed by the parents/caregivers of the participants during the evaluation of CARS. The questions on this scale were given to the parents/caregivers to respond to with reference to the preceding month.

TABLE 2 Frequency of endorsement (affirmative answer) for items of the RBS-R and the spearman's correlation coefficients between items of the total score.

Item		Frequency of endorsement in subscale (%)	Mean	SD	*r*
Stereotypy subscale		96.85			0.633**
1	Body movements	12.60	0.17	0.48	0.262**
2	Head movements	21.52	0.26	0.53	0.326**
3	Finger movements	36.22	0.57	0.90	0.395**
4	Locomotion	77.17	1.38	1.04	0.361**
5	Object usage	81.10	1.48	0.98	0.308**
6	Sensory	61.15	0.94	0.92	0.441**
Self-injurious subscale		33.86			0.394**
7	Hits w/body	18.37	0.25	0.60	0.290**
8	Hits against surface	13.91	0.18	0.51	0.200**
9	Hits w/object	3.15	0.03	0.17	0.274**
10	Bites self	6.04	0.08	0.37	0.176**
11	Pulls hair/skin	2.89	0.04	0.24	0.199**
12	Rubs/scratches	1.84	0.02	0.16	0.123**
13	Inserts finger/object	1.31	0.02	0.18	0.175**
14	Picks skin	3.67	0.06	0.31	0.236**
Compulsive subscale		68.50			0.713**
15	Ordering	28.08	0.42	0.76	0.442**
16	Completeness	19.69	0.30	0.69	0.461**
17	Washing	6.56	0.09	0.35	0.236**
18	Checking	4.99	0.07	0.33	0.327**
19	Counting	5.25	0.07	0.31	0.353**
20	Hoarding	5.77	0.07	0.30	0.374**
21	Repeating	37.01	0.53	0.78	0.271**
22	Needs to touch/tap	21.26	0.28	0.60	0.329**
Ritualistic subscale		82.94			0.617**
23	Eating/mealtime	34.38	0.54	0.86	0.334**
24	Sleeping/bedtime	24.93	0.37	0.71	0.328**
25	Self-care routine	6.56	0.09	0.35	0.365**
26	Transportation routine	12.86	0.18	0.51	0.447**
27	Play/leisure routine	17.06	0.20	0.48	0.478**
28	Communication	65.62	1.07	0.98	0.175**
Sameness subscale		71.92			0.761**
29	Placement of objects	8.92	0.11	0.39	0.331**
30	No new places	14.17	0.17	0.44	0.383**
31	No interruption	38.06	0.49	0.71	0.421**
32	Walks certain way	8.92	0.12	0.41	0.412**
33	Sits certain place	4.99	0.06	0.30	0.256**
34	Appearance/behavior of others	6.82	0.11	0.45	0.257**
35	Uses certain door	2.62	0.03	0.22	0.384**
36	Videotapes	34.12	0.57	0.90	0.487**
37	Difficult transitions	28.61	0.35	0.62	0.467**
38	Insists on routine	10.50	0.13	0.43	0.271**
39	Insists on time	3.67	0.04	0.23	0.324**
Restricted subscale		77.69			0.620**
40	Preoccupation with subject	43.04	0.72	0.96	0.411**
41	Attached to object	30.18	0.50	0.85	0.366**
42	Preoccupied with part of object	38.58	0.64	0.93	0.283**
43	Preoccupation with movement	43.57	0.70	0.94	0.413**

**$P \leq 0.01$.

Data analysis

Confirmatory factor analysis (CFA) was applied to test the overall fit of the data to the scale model with 43 items.

Statistical indices used to evaluate the model fits include the model of chi-square/degrees of freedom ($\chi 2/df$), the comparative fit index (CFI), the goodness of fit index (GFI), the root mean square error of approximation (RMSEA), the standardized root means square residual (SRMR), and the Akaike information criterion (AIC). The index criteria for well-fitting models were: CFI > 0.9, GFI > 0.9, RMSEA < 0.08, and $2 < \chi 2/df < 5$ (25). The reliability of the RBS-R was determined by the test-retest reliability and internal consistency. Test-retest reliability was estimated by an intra-class coefficient (ICC), which was calculated by the correlation between the first and second completion of the scale. The recommended ICC value is ≥0.7, and a value of ≥0.6 is considered acceptable (26). Cronbach's alpha was used to determine the internal consistency of the scale. An acceptable cutoff value is not below 0.7 (27). The CFA was performed using AMOS, version 17, and SPSS, version 19 was used for other statistical analyses.

Result

Demographic characteristics

The sample of the current study consisted of 381 Chinese children with ASD, including 305 boys and 76 girls, with a mean age of 3.14 years old (range 2–4.92). The mean CARS score was 36.04 (range 30–44). The descriptive statistics for these subjects, including both demographic and diagnostic information, are shown in Table 1.

Item analysis

Table 2 provides details on the positive response indices, frequency of endorsement, and spearman's correlation coefficients between the components of the total score. The frequency of positive responses ranged between 1.31% (i.e., for item 13: Inserts finger/object) and 77.17% (i.e., for item 4: locomotion). The correlation between items of total score ranged between 0.123 and 0.487.

Confirmatory factor analysis

We hypothesized a range of three to six factors for the specific models, as shown in Figure 1.

The CFA results for all four models (i.e., Models 3, 4, 5, and 6) in Table 3, indicated that the 3-, 4-, 5-, and 6-factor models were all reasonably good fits for the data, based on the fit statistics guidelines previously described. However, considering the optimal statistical fit, we suggested the 6-factor to be the best

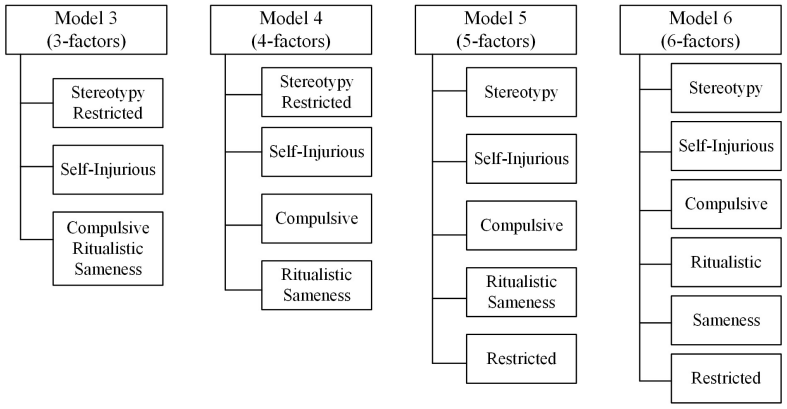

FIGURE 1
The 3–6 factors competing hypothesized models of the RBS-R factor structure.

TABLE 3 Goodness-of-fit indices for the hypothesized latent factor RBS-R models (N = 381).

	χ^2	df	χ^2/df	CFI	GFI	RMSEA	SRMR	AIC	BIC
Model 3	1539.768	849.000	**1.814**	0.703	0.840	**0.046**	**0.062**	1733.768	2116.474
Model 4	1458.895	848.000	**1.720**	0.737	0.847	**0.043**	**0.060**	1654.895	2041.546
Model 5	1437.682	845.000	**1.701**	0.745	0.849	**0.043**	**0.059**	1639.682	2038.169
Model 6	1411.453	840.000	**1.680**	0.754	0.852	**0.042**	**0.054**	1623.453	2041.667

RBS-R, behavior scale-revised; CFI, comparative fit index; GFI, the goodness of fit index; RMSEA, Root-mean-square error of approximation; SRMR, standardized root-mean-square residual; AIC, Akaike information criterion; BIC, Bayes information criterion; The numbers in bold meet the criterion set for good model fit.

model. The standardized factor loadings of CFA, ranging from 0.094 to 0.658, are shown in Table 4.

Reliability statistics

Table 5 depicts the internal consistency (Cronbach's a) indices and test-retest correlations for the RBS-R and each factor. The internal consistency for the total score on the RBS-R was 0.834, and the internal consistency for the factors ranged from 0.744 to 0.83. The aforementioned findings showed that this scale has strong internal consistency. A retest was conducted on 24 participants who had previously taken the RBS-R in order to evaluate the instrument's consistency and reliability The test-retest reliability was good to excellent for the total score of the RBS-R (0.749) and each factor (factor 1: 737, factor 2:0.829, factor 3:0.639, factor 4:0.754, factor 5:0.607, and factor 6:0.689). As for the reliability of the RBS-R, the results indicated a high internal consistency of all subscales and the total score of the RBS-R in this study.

Discussion

This study aimed to evaluate the psychometric properties of the RBS-R in patients with ASD in Jiangxi Province. If

TABLE 4 The standardized factor loadings of the confirmatory factor for RBS-R.

	Item	Factor-1	Factor-2	Factor-3	Factor-4	Factor-5	Factor-6
1	Body movements	0.346					
2	Head movements	0.414					
3	Finger movements	0.554					
4	Locomotion	0.437					
5	Object usage	0.201					
6	Sensory	0.501					
7	Hits w/body		0.429				
8	Hits against surface		0.461				
9	Hits w/object		0.470				
10	Bites self		0.552				
11	Pulls hair/skin		0.449				
12	Rubs/scratches		0.158				
13	Inserts finger/object		0.521				
14	Picks skin		0.374				
15	Ordering			0.409			
16	Completeness			0.401			
17	Washing			0.324			
18	Checking			0.412			
19	Counting			0.497			
20	Hoarding			0.502			
21	Repeating			0.172			
22	Needs to touch/tap			0.261			
23	Eating/mealtime				0.251		
24	Sleeping/bedtime				0.331		
25	Selfcare routine				0.347		
26	Transportation routine				0.619		
27	Play/leisure routine				0.658		
28	Communication				0.094		
29	Placement of objects					0.338	
30	No new places					0.365	
31	No interruption					0.396	
32	Walks certain way					0.502	
33	Sits certain place					0.368	
34	Appearance/behavior of others					0.284	
35	Uses certain door					0.575	
36	Videotapes					0.381	
37	Difficult transitions					0.515	
38	Insists on routine					0.275	
39	Insists on time					0.446	
40	Preoccupation with subject						0.506
41	Attached to object						0.397
42	Preoccupied with part of object						0.221
43	Preoccupation with movement						0.362

the RBS-R has strong validity and reliability, it will be used to gauge the severity of RRBs in patients with ASD and our findings confirmed the utility of the RBS-R as a measure of a wide range of RRBs seen in 2–4 years old children with ASD from Jiangxi province of China. This study supports Li's (21) assertion that the original RBS-R items may be modified to fit the Chinese context.

The Self-Injurious behaviors (33.86%) exhibited the lowest frequency behaviors compared to other subscales in our study, which was consistent with most studies from other countries such as China (21) and America (20). However, the frequencies were much lower for each item of this subscale compared to the results of He et al. (21) with 12 (rubs/scratches) of 1.84% in our study and 13.5% in other Chinese participants (21), 18.5% in Spanish participants (19), 16.3% in United States participants with ASD (20), and 2.7% in Italian participants with ASD (18). These behaviors of item 9 (hits w/object), item 11 (Pulls hair/skin), item 12 (rubs/scratches), and item 13 (inserts finger/object) were infrequent during the assessment of CARS according to the reports from parents or caregivers. The highest frequency of item was item 4 (locomotion), which was 77.17% in the study. Among Spanish-speaking patients with ASD, the highest frequency was 50.2% from item 28 (communication) (19). The frequency of item 28 (communication) in our report was 65.62%, higher than the former. In an American study, the highest frequency was 67.7%, from item 40 (preoccupation with one subject) (20), suggesting that different samples and cultures can explain the differences in frequencies.

We proposed the structural models with three to six factors according to some literature (16). In accordance with the statistical indices' guidelines for the structural equation model, these models were all reasonably good fits for our sample. The best model that fit well was the 6-factors model with the ratio between $\chi 2$ and the degrees of freedom ($\chi 2/df$) of 1.68, the CFI of 0.754, the GFI of 0.852, and the RMSEA of 0.042. The standardized factor loading ranged from 0.094 to 0.658, some items of which were lower than the cutoff. According to Kline's criterion, a factor loading cutoff of $\geq$0.35 was applied (28). However, the index did not become much better after removing these three items, therefore, we reserved item 28 (communication), item 12 (rubs/scratches), and item 21 (repeating), which would be found in ASD children. Additionally, several of the items on this scale overlapped with one another. For example, parents were puzzled by item 31 (no interruption) and item 37 (difficult transitions) as item 31 came along with item 37. Coincidentally, scholars (21) found there were duplicates among the content of certain RBS-R items among the ritualistic subscale and sameness subscale.

TABLE 5 The internal consistency and test-retest reliability of the RBS-R.

	Internal consistency	Test-retest
Factor1	0.830	0.737
Factor2	0.765	0.829
Factor3	0.744	0.639
Factor4	0.783	0.754
Factor5	0.751	0.607
Factor6	0.811	0.689
Total	0.834	0.749

The structure validity and internal validity of this scale were accessed using a confirmatory factor in accordance with the theoretical framework proposed by Bodfish (15) and other works of research. Concerning the reliability, the internal consistency for the subscales of the original structure ranged from 0.744 (Compulsive Behavior) to 0.83 (Stereotypic Behavior); the internal consistency for all RBS-R items was 0.834. Test-retest of the RBS-R ranged from 0.607 (Self-injurious subscale) to 0.829 (Self-injurious subscale); the test-retest for all RBS-R items was 0.749. These results indicated an acceptable internal consistency of all subscales and the total score of the RBS-R in this study. The internal consistency estimated by Cronbach's alpha value was lower than a study by He et al. (21) and higher than another study by Li et al. (24). The diverse samples used in the experiments may be the cause of the inconsistent outcomes. But according to these three investigations, the RBS-overall R's score and all of its subscales demonstrated acceptable levels of internal consistency.

Strengths and limitations

The first of the two strengths of our study is that we recruited 381 ASD children from 2 to 4 years old to ensure the sample size was sufficient to support the use of CFA, which was more than fivefold the number of items. Second, we collected these data of RBS-R while the assessment of CARS, so that evaluators were able to assess RRBs of ASD children face-to-face and we would check the scale to ensure that caregivers had finished the questionnaire, leading to reduced reporting errors from caregivers. The first limitation of our study was that we only obtained children 2–4 years old, in which, the behaviors from the compulsive subscale and sameness subscale may not function effectively. Secondly, we had not determined how this scale's psychometric characteristics varied according to factors like age, gender, cognitive capacity, and so on, since is known to be age-related and dependent on the intellectual functioning of individuals with ASD (29).

Conclusion

In conclusion, using the original framework of RBS-R to assess the severity of repetitive and stereotypic behaviors in young Chinese children with ASD from Jiangxi Province would

enable a better differential diagnosis. Future studies should expand the sample, focusing in particular on Chinese school-aged children with ASD.

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 the Ethics Committee of Jiangxi Provincial Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

XL, XZ, and SZ designed the study. XL, YX, MG, LH, and ZL performed the study. XL wrote and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Vitamin D status in children with a psychiatric diagnosis, autism spectrum disorders, or internalizing disorders

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Background and objective: Multiple studies suggest that children with Autism Spectrum Disorders (ASD) have significantly lower vitamin D3 levels than typically developing children. However, whether vitamin D3 deficiency is more common in children with ASD than in children with other psychiatric disorders remains unclear. This study was conducted to explore the prevalence of vitamin D3 in children with a psychiatric diagnosis including children with ASD or with internalizing disorders (mood and anxiety disorders). In addition, this study investigated the potential associations between vitamin D3 and Body Mass Index (BMI).

Materials and methods: Clinical data, including BMI and vitamin D3 levels, of 93 children (6–18 years; n = 47; 51% female) with ASD (n = 58) and internalizing disorders (n = 37) were retrospectively analyzed.

Results: In the overall sample, the prevalence of vitamin D3 deficiency (<50 nmol/L) was 77.4%. Additionally, 75.9% of the children with ASD and 79.5% with internalizing disorders had vitamin D3 deficiency. BMI was inversely related to vitamin D3 in the total group (p = 0.016). The multiple regression model for the total group significantly predicted vitamin D3 (p = 0.022). Age contributed significantly to the prediction. Stratified for sex and primary diagnosis, multiple regression models showed that for boys with ASD, higher BMI levels were associated with lower vitamin D3 levels (p = 0.031); in boys with internalizing disorders and in girls, no relation was found between BMI and vitamin D3 levels.

Conclusion: In this this cross-sectional, explorative study high rates of vitamin D3 deficiency in children with different psychiatric disorders were found. The results showed an inverse relation between BMI and vitamin D3 levels in the total group. Vitamin D3 deficiency was particularly common in boys with ASD

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and obesity. Lifestyle factors may contribute to the association between high BMI and low vitamin D3 levels in boys with ASD. Vitamin D3 deficiency is common in patients with psychiatric disorders and it is highly recommended to increase clinicians' awareness of this common and remediable risk factor.

KEYWORDS

autism spectrum disorders, vitamin D, BMI, obesity, internalizing disorders, mood and anxiety disorders, child and adolescent psychiatry

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder caused by a complex interaction between genetic and environmental risk factors (1–3). The combined data from several studies suggests an association between ASD and lower vitamin D3 status (4, 5). Vitamin D3 deficiency has been reported in a range of neuropsychiatric disorders across the lifespan, including for instance mood disorders, psychosis and different types of dementia (3–7). Reversely, in children with ASD lower vitamin D3 levels have been found than in typically developing (TD) children, possibly due to lifestyle factors such as unhealthy diet, being overweight or obese and less time spent outside (4, 5). However, the underlying associations of vitamin D3 deficiency in ASD are still poorly understood.

Two major forms of vitamin D exist, including vitamin D2 or ergocalciferol and vitamin D3 or cholecalciferol, which is more relevant in humans (4, 6, 8). Vitamin D3 plays a major role as prohormone and controls the expression of about 3% of our genes (3, 6). Chemically, vitamin D3 is a secosteroid that is strongly involved in the regulation of calcium levels. Synthesis of vitamin D3 in the skin from cholesterol following exposure to ultraviolet B (UVB) in sunlight is the major natural source (for 90%), and only a limited amount of vitamin D3 is derived from the diet or dietary supplement (9). To become activated, vitamin D must first be converted through hydroxylation in the liver into calcidiol, or 25-hydroxy vitamin D [25(OH)D] (4, 6, 8). Next calcidiol is converted in the kidneys into calcitriol or vitamin D [1,25 $(OH)_2D$], the potent neurosteroid that helps control brain cell growth, and acts on vitamin D receptor molecules found in most brain cells (3, 10).

Recommendations for vitamin D3 serum levels vary. With a cut-off of serum 25(OH)D < 50 nmol/L, it is estimated that about 30% of adults have vitamin D3 deficiency and about 60% have insufficiency [serum 25(OH)D 50–75 nmol/L] (8). In contrast to older guidelines, the consensus is now that a minimum <75 nmol/L is a reasonable cut-off. Actually, the discussion in the field is more toward even higher cut-off criteria than lower (4, 6).

Vitamin D3 plays a major role in calcium and bone metabolism. Research over the last decade has demonstrated the diverse functions of vitamin D3 throughout the brain (3–7). For instance, vitamin D3 is involved in brain proliferation, differentiation, neurotrophism, neuroprotection, neurotransmission, myelination, and neuroplasticity (3, 4, 6, 7). As such, reduced levels of vitamin D3 may lead to suboptimal brain development and deficiency may result in increased risk of developing psychopathology. In adults, vitamin D3 deficiency is associated with depressive disorders, schizophrenia, Parkinson's disease and Alzheimer's disease (4, 7, 10).

Most studies in children and adolescents with psychiatric disorders analysing vitamin D status have investigated an association with ASD; while only a limited number of children with other psychiatric disorders have been studied with respect to vitamin D3 status so far. These have been assembled in a recent systematic review indicating that vitamin D is indeed associated with a broad area of mental health problems in children (11). For children with ASD, two recent meta-analyses demonstrated that children and adolescents with ASD had significantly lower vitamin D3 blood levels than TD children (4, 5). Moreover, it has been hypothesized that vitamin D3 deficiency may increase risk of ASD by altering brain development (3–7).

Additionally, lifestyle factors predisposing for reduced vitamin D3 levels such as poor diet quality, being overweight and reduced exposure to sunlight by an imbalance between indoor and outdoor activities may be more common in children with psychiatric disorders. In fact, multiple studies, mostly in adults, have reported associations between psychiatric disorders and unhealthy lifestyles (12). Children with ASD show higher-than-average rates of obesity and are often less active outdoors (13–16) which may have a negative effect on vitamin D3 status. A meta-analysis of vitamin D deficiency and obesity in adults and children in general population revealed inverse associations between BMI and vitamin D3 levels, regardless of age group (17). However, thus far, little is known about clinical associations between vitamin D3 and BMI in children with different psychiatric disorders.

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; ASD, autism spectrum disorder; Calcitriol, 1,25(OH)D2; BMI, body mass index.

Here, we aim to explore the prevalence of vitamin D3 in children with a psychiatric diagnosis including children with ASD or with internalizing disorders (mood and anxiety disorders). In addition, we will further investigate potential associations between vitamin D3 and effects of being overweight or obese in this population. We hypothesize that: (1) vitamin D3 deficiency is similarly present across diagnostic groups; and (2) BMI is inversely related to vitamin D3 levels in children with psychiatric disorders.

Materials and methods

As part of ongoing explorative assessment on somatic comorbidity and lifestyle in children and adolescents with psychiatric disorders, we retrospectively reviewed documents and medical records between October 2020 and October 2021 including physical and laboratory measurements from patients diagnosed with ASD or internalizing disorders (mood and anxiety disorders) carried out between October 2017 and October 2020 at the outpatient clinic of Karakter University Centre for Child and Adolescent Psychiatry. Karakter UC provides psychiatric care for children and adolescents with an average or high intelligence level. In the Netherlands, referral to a University center, such as Karakter is only done if severe forms of psychopathology are present.

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The local Medical ethics committee approved based on the principal of good clinical practice.

Study sample

Study sample included children aged 6–18 with ASD (n = 54) and children with internalizing disorders (n = 39). Internalizing disorders included mood and anxiety disorders (i.e., depressive disorder, agoraphobia, generalized anxiety disorder, obsessive compulsive disorder, panic disorder, posttraumatic stress disorder, separation anxiety disorder, social phobia, and specific phobia). We included children for whom data on vitamin D3 status and BMI were available. Exclusion criteria were restrictive eating disorders with poor overall nutrition status and children who were supplemented with vitamin D3 at the time of assessment.

Measures

Physical assessment

Physical examination included measurement of weight and height. The prevalence of children being underweight, overweight or obese was defined by the cut-off values of BMI references per age group according to the International Obesity Task Force (18). Ethnicity of the child was based on the parental country of birth. If both parents were non-Dutch, mother's ethnicity determined the ethnicity of the child. Vitamin D3 levels were measured in a venous blood sample, which was processed at local laboratory facilities. We used the vitamin D3 reference values from the American Academy of Pediatrics Committee on Nutrition and The Institute of Medicine. Vitamin D3 insufficiency was defined as levels of 50–75 nmol/L; and deficiency as levels below <50 nmol/L (19–21).

Psychiatric assessment

During the first appointment a psychologist and child and adolescent psychiatrist interviewed the child and caregivers. Next, a mental state examination and physical examination was carried out by the child and adolescent psychiatrist or a nurse practitioner supervised by a child and adolescent psychiatrist. The psychiatric diagnosis was based on DSM-IV-TR (22) and/or DSM 5 criteria (23). Consensus diagnoses were established by a multidisciplinary team consisting of two child and adolescent psychiatrists, psychologists and two nurse practitioners.

Data analysis

Baseline characteristics of patients were reported using frequency distributions for the categorical variables and mean ± standard deviation (±SD) for the continuous variables. We reported frequency distributions for Vitamin D3 deficiency (levels below <50 nmol/L) and BMI categories according the classification tables by TNO (International Obesity Task Force) (18) for the different diagnostic groups as well as the total group. A chi-square test was conducted between the outcome variables and primary diagnosis. Next, following tests for normal distribution, we ran univariate linear regressions separately for boys and girls and according to the different diagnostic groups to assess the effect of BMI on vitamin D3 levels. To assess linearity a scatterplot of BMI against vitamin D3 was plotted separately for boys and girls and according to the different diagnostic groups. A p value < 0.05 (two-sided) was considered statistically significant. All analyses were conducted using SPSS version 24 (IBM Corporation, Armonk, NY, United States).

Results

Of the 99 patients, three patients were excluded because of eating disorders (anorexia nervosa with food refusal), and three because of supplementation with vitamin D3 at moment of laboratory assessment (**Figure 1** flowchart).

Table 1 shows the baseline demographic and clinical characteristics of the participants. Of the 93 patients included, 47 were female (50.5%) with mean age of 14.4 years (range

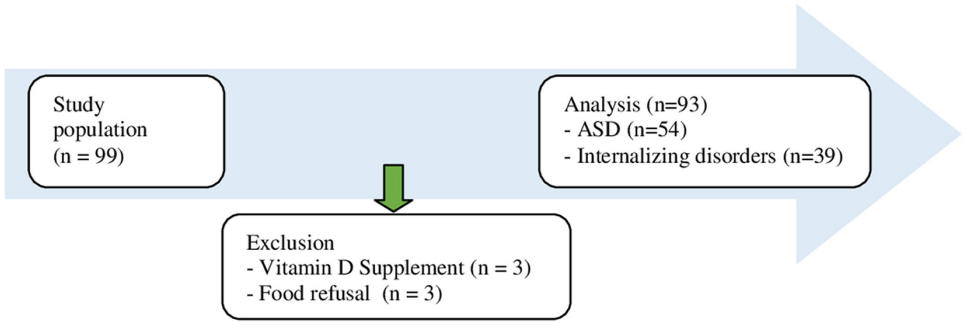

FIGURE 1
Flowchart of study inclusion.

6–18, SD 2.5). Over 50% of the children were 15–18 years. A total of 92.5% of the children were Dutch (Northern European descent). Psychiatric diagnoses were categorized in two main groups with ASD in 58.1% and internalizing disorders (mood and anxiety disorders) in 41.9% of the children as primary diagnoses. In total, 72 children were treated with psychiatric medication (77.4%) at moment of physical and laboratory assessment, comprising second generation antipsychotics (n = 53), antidepressants (n = 22), or stimulants (n = 27). Physical assessment displayed that 25.8% of the children were overweighed and 10.8% obese, according to BMI. Blood testing showed lower vitamin D3 deficiency (<50 nmol/L) in 77.4% (72/93) in the total group of children.

Table 2 presents BMI outcome, vitamin D3, sex and psychiatric medication separately for children with ASD and children with internalizing disorders. There were significantly more boys with ASD (72.2%) than boys with internalizing disorders (17.9%). Children with a primary diagnoses of an internalizing disorder more often had vitamin D3 deficiency (79.5%) compared to children with ASD (75.9%), however, these differences were not significant. Furthermore, 14.8% of children with ASD were classified as having severe overweight, whereas 5.1% of the children with ID were classified as having severe overweight. However, these differences were also not significant.

A multiple regression analysis was run to predict vitamin D3 levels from BMI and age in the total group and to determine how much of the variation in the dependent variable (vitamin D3 level) could be explained by the independent variables (BMI and age). As presented in Table 3, in the total group in model 1, higher BMI levels predicted lower vitamin D3 scores significantly ($p < 0.016$). In model 2, the multiple regression model including age, significantly predicted vitamin D3, F(2, 90) = 5.888, $p < 0.004$, adj. $R^2 = 0.096$. Age was the only factor contributing significantly to the prediction ($p = 0.022$).

Because our sample included more boys with ASD than internalizing disorders, the role of vitamin D3 and BMI stratified for sex was further explored. For boys, the prediction equation was: Vitamin D3 = 86.2–0.9 × BMI − 1.8 × AGE. BMI levels predicted vitamin D3, F(2, 43) = 4.410, $p = 0.018$, accounting for 17.0% of the variation in vitamin D3 with an adjusted R^2 = 13.0%, a small effect size according to Cohen (24). For girls, the prediction equation was: Vitamin D3 = 69.8 − 0.4 × BMI − 1.3 × AGE. BMI levels, however, did not significantly predict vitamin D3 levels, F(2, 44) = 1.63, $p = 0.205$. Regression coefficients and standard errors can be found in Table 3. The regression slopes for boys and girls are graphically presented in Figure 2.

TABLE 1 Descriptive of the study population (n = 93).

		n	%
Age	6–11 year	8	8.6
	12–14 year	32	34.4
	15–18 year	53	57.0
Sex	Female	47	50.5
	Male	46	49.5
Dutch	Yes	86	92.5
	No	7	7.5
Primary psychiatric diagnosis	ASD	54	58.1
	ID	39	41.9
Psychiatric medication	Yes	72	77.4
	No	21	22.6
BMI[1]	(Severe) underweight	2	2.2
	Normal	57	61.3
	Overweight	24	25.8
	Severe overweight (obesitas)	10	10.8
Vitamin D3	<50 nmol/L	72	77.4
	≥50 nmol/L	21	22.6
		Mean	**SD**
Length (cm)		167.2	15.0
Weight (kg)		62.1	18.4
BMI (kg/m^2)		21.9	4.6
Age (years)		14.4	2.5
Vitamin D3 (nmol/L)		40.9	16.6

BMI, body mass index; ASD, autism spectrum disorder; ID, internalizing disorders (depression and anxiety); SD, standard deviation. [1]Underweight, overweight and obesity prevalence for the children, were defined by the cut-off values on BMI references and age according to the International Obesity Task Force (18).

TABLE 2 Body mass index outcome, Vitamin D3, sex and psychiatric medication for children with ASD and children with internalizing disorders.

		Primary psychiatric diagnosis				*p*-Value[2]
		ASD (*n* = 54)		ID (*n* = 39)		
		n	%	*n*	%	
Sex	Female	15	27.8	32	82.1	0.000
	Male	39	72.2	7	17.9	
Vitamin D3	<50 nmol/L	41	75.9	31	79.5	0.685
	≥50 nmol/L	13	24.1	8	20.5	
Psychiatric medication	No	16	29.6	5	12.8	0.056
	Yes	38	70.4	34	87.2	
BMI[1]	(Severe) underweight	1	1.9	1	2.6	0.519
	Normal	32	59.3	25	64.1	
	Overweight	13	24.1	11	28.2	
	Severe overweight (obesitas)	8	14.8	2	5.1	

BMI, body mass index; ASD, autism spectrum disorder; ID, internalizing disorders. [1]Underweight, overweight, and obesity prevalence for the children, were defined by the cut-off values on BMI references and age according to the International Obesity Task Force (18). [2]Chi-square tests.

TABLE 3 Multiple regression results for Vitamin D3 level for boys and girls separately.

		B	SE B	ß	*F*	R^2	Adj. R^2
Total group							
Model 1					6.054*	0.062	0.052
	(Constant)	60.56	8.16				
	BMI	−0.90*	0.37	−0.25			
Model 2					5.888*	0.116	0.096
	(Constant)	78.17	10.98				
	BMI	−0.65	0.37	−0.18			
	Age	−1.60*	0.690	−0.24			
Boys							
Model 1					4.995*	0.102	0.082
	(Constant)	66.69	11.88				
	BMI	−1.19*	0.53	−0.32			
Model 2					4.410*	0.170	0.132
	(Constant)	86.17	15.52				
	BMI	−0.91	0.54	−0.24			
	Age	−1.81	0.96	−0.27			
Girls							
Model 1					1.376	0.030	0.008
	(Constant)	54.15	11.35				
	BMI	−0.60	0.51	−0.17			
Model 2					1.628	0.229	0.101
	(Constant)	69.78	16.07				
	BMI	−0.38	0.53	−0.11			
	Age	−1.39	1.02	−0.21			

ASD, autism spectrum disorder; BMI, body mass index; Model, "Enter" method in SPSS Statistics; B, unstandardized regression coefficients; SE B, standard error of the coefficient; ß, standardized coefficients; R^2, coefficient of determination; Adj. R^2, adjusted R^2. *$p < 0.05$.

Also, the primary diagnosis was stratified, due to the higher percentages of children with ASD and obesity (Table 4 and Figure 3). For boys with ASD, higher BMI scores partially predicted lower vitamin D3 levels: vitamin D3 = 94.3–1.2 × BMI − 1.9 × AGE. BMI and age predicted significantly vitamin D3, F(2, 36) = 5.13, p = 0.011 accounting for 22.2% of the variation in Vitamin D3 with an adjusted R^2 = 17.9%. For the other

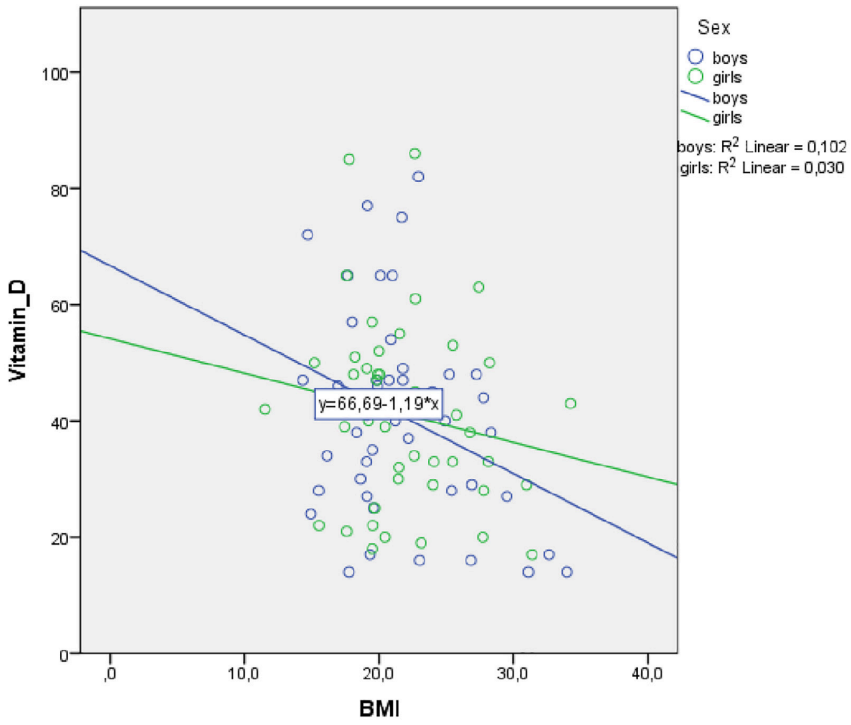

FIGURE 2
Scatterplot of vitamin D3 body mass index (BMI) for girls and boys separately.

models, the variables did not add significantly to the prediction.

Discussion

In this study high rates of vitamin D3 deficiency were found in children with psychiatric disorders, predominantly ASD and internalizing disorders. Additionally, the results showed that BMI was inversely related to vitamin D3 in the total group of children and more specifically in boys with ASD. Our findings overall indicate that children with a variety of psychiatric disorders are at risk of vitamin D3 deficiency, especially in co-occurrence with overweight and obesity.

Vitamin D3 deficiency is associated with a range of adverse somatic and psychiatric outcomes (cardiovascular, diabetes, depression, neurobiological disorders, neurodegenerative diseases, cancer, and psychosis) and is of major interest, considering its role in fundamentally biological processes (9, 24). Previous studies on the role of vitamin D3 in the pathogenesis of ASD in childhood have been performed, however, specific mechanisms are still unclear (3–5). In our study high percentages of low vitamin D3 levels were not specific for children with ASD but also observed in children with internalizing disorders. The results of this study showed a prevalence of vitamin D3 deficiency (<50 nmol/L) in the total group of children with severe psychiatric disorders of 77%; in ASD 76% and in internalizing disorders 80%. This is much higher compared to a study in the general population of Dutch children where a prevalence of 30% was previously described (25). However, studies vary in reports of vitamin D levels in general population worldwide (26).

Prevalence of vitamin D3 deficiency (<50 nmol/L) in a study of 307 adolescents (11–18 year) in the United States was 42% (27). In a study in the United Kingdom (n = 1102), 35% of the 4–8 year old children were vitamin D deficient (<50 nmol/L) (28). In Beijing China, a prevalence of vitamin D (<50 nmol/L) 57.8% was found (29). The Canadian Health Measures Survey reported a prevalence of vitamin D insufficiency (<50 nmol/L) of 13% in children aged 6–11 years from April to October (30). In Germany in 10.015 children (1–17 years) prevalence of vitamin D < 30 nmol/L was 12.5% for boys and girls; and vitamin D 30 to <50 nmol/L was 32.7% in boys and 33.5% in girls (31). A general population study of 1,829 adolescents in India reported a high prevalence of vitamin D deficiency (<50 nmol/L) of 97% (32). Also, within our study population, we observed that older children had a higher risk of vitamin D deficiency. Although infants are at risk for disease such as vitamin D-associated rickets, previous studies in higher-resource countries reported lower vitamin D levels in adolescents compared to toddlers (26, 31). Different, possibly combined explanations are possible; such as

TABLE 4 Results of a linear regression analysis of BMI and Vitamin D3 level for boys and girls and for ASD and internalizing disorders separately.

		ASD							Internalizing disorders						
		B	SE B	ß	*F*	R^2	Adj. R^2			B	SE B	ß	*F*	R^2	Adj. R^2
Boys								Boys							
Model 1					6.494*	0.149	0.126	Model 1					2.68	0.349	0.219
	(Constant)	74.02	12.91						(Constant)	5.617	17.65				
	BMI	−1.47*	0.58	−0.39					BMI	1.353	0.83	0.59			
Model 2					5.131*	0.222	0.179	Model 2					1.11	0.357	0.035
	(Constant)	94.29	16.71						(Constant)	1.398	27.26				
	BMI	−1.18*	0.58	−0.31					BMI	1.216	1.1	0.53			
	Age	−1.89	1.03	−0.28					Age	0.473	2.12	0.11			
		ASD							Internalizing disorders						
Girls								Girls							
Model 1					0.146	0.011	−0.065	Model 1					2.001	0.063	0.031
	(Constant)	47.08	18.49						(Constant)	63.36	15.66				
	BMI	−0.33	0.85	−0.11					BMI	−0.98	0.69	−0.25			
Model 2					1.784	0.229	0.101	Model 2					1.051	0.068	0.003
	(Constant)	71.19	21.45						(Constant)	73.52	30.14				
	BMI	0.49	0.9	0.16					BMI	−0.99	0.7	−0.254			
	Age	−3.04	1.65	−0.54					Age	−0.65	1.64	−0.071			

ASD, autism spectrum disorder; BMI, body mass index; Model, "Enter" method in SPSS Statistics; B, unstandardized regression coefficients; SE B, standard error of the coefficient; ß, standardized coefficients; R^2, coefficient of determination; Adj. R^2, adjusted R^2. *$p < 0.05$.

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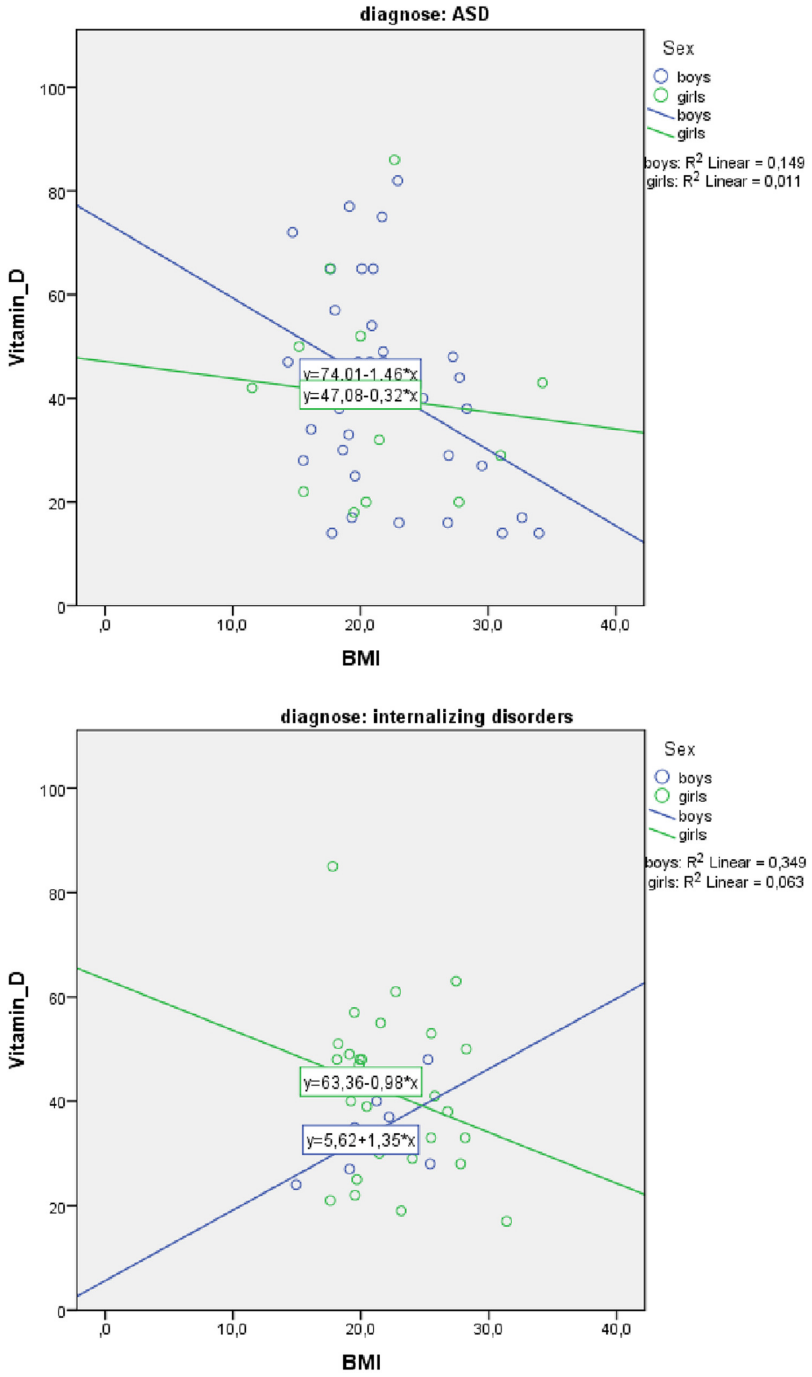

FIGURE 3
Scatterplot of body mass index (BMI) and Vitamin D3 level for boys and girls separately for autism spectrum disorder (ASD) and internalizing disorders.

less supplementation during adolescence and or an imbalance between in- and outdoor activities (in favor of indoor activities) (25, 28).

The variations in vitamin D level among studies could be attributed to difference in study design, inclusion of different age groups, definition of insufficiency or deficiency, and geographical differences (26). Populations at increased risk for vitamin D deficiency include immigrant/refugee children moving to higher-latitude countries (33, 34), dark skin color or skin pigmentation (25), children with chronic diseases that

decreases fat absorption (35), children receiving anti-epileptic medications (36), and obese children (37). According to our study, children with psychiatric disorders also appear to be at high risk for vitamin D3 deficiency.

Moreover, high levels of obesity were found in children with ASD and internalizing disorders. As obesity is an important risk factor for cardiovascular disease and increased morbidity rates during later life, it is important to be aware off the risk factors for obesity, such as some types of medication, the psychiatric disorder itself, genetic variations associated with obesity and last but not least lifestyle related factors (e.g., disordered sleep, insufficient physical activity, diet and eating behavior) (38, 39). A significant inverse association between vitamin D level and BMI in this study is in line with studies in typically developing children (17, 26). The underlying mechanisms associating low vitamin D in obesity include volumetric dilution, sequestration into adipose tissue, limited sunlight exposure, and decreased vitamin D synthesis in the adipose tissue and liver (40). For example, it is speculated that adipose tissue is responsible for lower circulating 25(OH)D levels due to a greater pool of distribution for the fat soluble vitamin (4, 40, 41).

Interestingly, in the present study, vitamin D3 deficiency was particularly common in boys with ASD with higher BMI. It could be hypothesized that life style factors (unhealthy diet and limited physical activity outdoors) may contribute to the association between high BMI and low vitamin D3 levels in boys with ASD more than in girls, but other mechanisms may play a role. For instance, sexual hormones, estrogen and testosterone, appear to have different effects on vitamin D metabolism, which may explain sex differences of vitamin D status (3, 42–44). However, further studies need to explore sex specific differences in vitamin D3 levels and obesity.

There are several limitations regarding this study. First, this study was performed to investigate whether lower levels of vitamin D3 are predominantly associated with ASD, or are related to a broad spectrum of psychopathology in a broader sense, e.g., across disorders. While the latter appeared to be the case, no conclusions can be drawn about more average or less severe psychopathology. Another limitation is that, although we have included children with a variety of internalizing child psychiatric diseases at different ages, the diagnostic subgroups were relatively small and did not allow further analysis. Also, there could be an overlap between children with ASD and internalizing problems (anxiety or mood symptoms). However, children diagnosed with internalizing disorders did not have a neurodevelopmental disorder (e.g., ASD). Our main focus was to explore if vitamin D3 is associated with a broad area of psychiatric disorders in children. It would be interesting for future research to investigate whether vitamin D3 deficiency is more present in children with ASD in combination with internalizing symptoms. A large cohort in ASD children in which two groups of internalizing and externalizing symptoms are dichotomized is preferably. Third, we did not include a control group with typically developing children to compare vitamin D3 levels. Fourth, the cross-sectional and exploratory design does not allow further exploration of intricate relationships between BMI and vitamin D3 levels in time, which clearly will be an important topic to address in future studies. Unfortunately, the study was underpowered. We calculated the achieved power in a *post hoc* analysis for the main linear multiple regression in boys with ASD. With a α of 0.05, a sample size of 39 (boys with ASD), two predictors and a partial R^2 of 0.149, the achieved power was 0.60. We therefore advice to increase the population size in future studies in order to replicate the results. Furthermore we did not have sufficient data to explore relation with different ethnicity, socio-economic status and to determine seasonal variations in serum vitamin D3 levels and effects on mental health.

Future research into vitamin D3 in child and adolescent psychiatric populations should take the following recommendations into account:

1) The role of vitamin D3 in psychiatric and somatic diseases needs further exploration, for instance how it relates to different factors (such as BMI, age, sex, diet, ethnicity, season, etc.), but also what the effects of supplementation are in psychiatric diagnoses. With respect to vitamin D3 supplementation many questions remain present. What is the optimal dosage for supplementation and how should vitamin D3 be administered? To what extent are follow up measures of vitamin D3 status necessary? How long should supplementation be provided?
2) A trans-diagnostic approach, including younger children and children with intellectual disability may provide additional insights. Ideally, larger diagnostic groups should be included allowing a higher degree of statistical evidence.
3) A longitudinal study design is preferable, which allows insights in developmental trajectories related to vitamin D3 status, diagnostic status, effects of sex differences and BMI.
4) At a broader public health level, further research is required into the effectiveness of interventions that promote a healthy lifestyle, especially in child psychiatric populations.

In conclusion, this study demonstrated high rates of vitamin D3 deficiency in children with psychiatric disorders, and an inversely relation between BMI and vitamin D3 in the total group of children, most pronounced in older boys with ASD. Vitamin D3 deficiency is common in children and adolescents with psychiatric disorders and it is vital to increase clinicians' awareness of this common and remediable risk factor. Identifying the specific role of vitamin D3 in psychiatric and somatic comorbidities may potentially provide better insights in disease etiology and may have important implications for interventions and future research.

Addressing lifestyle factors such as diet and time spent outdoors may improve physical and mental health in children and adolescents with psychiatric disorders.

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 Radboudumc. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

JM conceived and developed an initial draft manuscript in consultation with JZ, MD-B, and WS who regularly provided extensive feedback. HK and JM carried out the analyses and interpretation. All authors contributed to the final version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Repetitive transcranial magnetic stimulation for insomnia in patients with autism spectrum disorder: Study protocol for a randomized, double-blind, and sham-controlled clinical trial

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Background: Insomnia is the most common comorbidity in children with autism spectrum disorder (ASD) and seriously affects their rehabilitation and prognosis. Thus, an intervention targeting insomnia in ASD seems warranted. Repetitive transcranial magnetic stimulation (rTMS), a potentially effective treatment for improving sleep quality and optimizing sleep structure, has already been demonstrated to alleviate insomnia symptoms and sleep disturbance in different neurological and neuropsychiatric conditions. This trial aims to investigate the effects of rTMS on insomnia in patients with ASD.

Method : This study is designed to be a double-blind, randomized, and sham-controlled trial with a target sample size of 30 participants (aged 3–13 years) diagnosed with ASD comorbid with insomnia. The intervention phase will comprise 20 sessions of rTMS or sham rTMS applied over the right dorsolateral prefrontal cortex (DLPFC) within four consecutive weeks. The effect of rTMS on insomnia and other symptoms of ASD will be investigated through home-PSG (two consecutive overnights), sleep diary, CSHQ, CARS, ABC, SRS, RBS-R, and metabolomics analysis at baseline and posttreatment. A follow-up assessment 1 month after the intervention will examine the long-term effects.

Discussion : The results of this study may address an important knowledge gap and may provide evidence for the use of rTMS to treat insomnia in ASD. Furthermore, it will elucidate the potential mechanism and link between sleep disorders and clinical symptoms.

Clinical trial registration: The study is ongoing and has been registered at the Chinese Clinical Trial Registry (ChiCTR2100049266) on 28/07/2021.

KEYWORDS

autism spectrum disorder, repetitive transcranial magnetic stimulation, clinical trial, insomnia, neuromodulation

Introduction

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders with significant clinical heterogeneity characterized by social communication deficits and restricted, repetitive sensory–motor behaviors (1, 2). The prevalence of ASD has increased to 1/59, and over 70% of individuals with autism have developmental problems (intellectual disability, etc.), general medical issues (sleep disturbances, gastrointestinal problems, etc.), psychiatric conditions (anxiety and depression, etc.), or personality disorders (schizoid personality disorder, etc.) to varying degrees (2, 3).

Among these comorbidities, sleep disturbances, especially insomnia, are the most common conditions, and their prevalence in ASD is 40–80%, which is two to three times higher than that in the general population (4–6). Previous studies have found a bidirectional association between ASD and sleep disturbances (7). Furthermore, like the core symptoms of autism, insomnia may persist throughout life and can not only predict the severity of core symptoms and related daily behavior problems, such as tantrums and aggression, but also affect the health and wellbeing of parents (6, 8). Given the significant adverse impacts, treating insomnia has therefore been a primary therapeutic goal.

Over the years, lifestyle modifications, behavioral interventions, and pharmacological therapies have been adopted for treating sleep disturbances in ASD (9, 10). Among them, cognitive behavioral therapy for insomnia (CBT-I), as a first-line treatment to manage chronic insomnia, has also been used to treat sleep disturbances in children with ASD. One study of school-aged children with high-functioning ASD demonstrated that CBT-I was a feasible and promising treatment to improve the sleep and function of children and parents (11). Nevertheless, poor treatment responses (25%) were reported in ASD with parent-directed behavioral sleep interventions due to numerous ASD children having atypical cognitive profiles, such as impaired social cognition and atypical perceptual and information processing, which led to poor cooperation during the treatment (12, 13). As a stepwise approach to sleep disturbances, pharmacotherapy is often provided when behavioral intervention fails. Nevertheless, there are no medications with regulatory approval by the Food and Drug Administration (FDA) for the treatment of insomnia in ASD (9). Considering the severity of sleep problems, drugs including antidepressants, antipsychotics, benzodiazepines, and melatonin have been used off-label in the clinic with some potential side effects (14, 15). From the perspective of the parents, behavioral and nonpharmacological interventions are generally the first choice over equally effective medication (9). However, existing treatments are less effective. Therefore, identifying a safe and effective nonpharmacological treatment for insomnia in children with ASD is an urgent matter.

Repetitive transcranial magnetic stimulation (rTMS) is a noninvasive therapy that produces a magnetic field and introduces excitatory or inhibitory cortical excitability in the brain below the magnetic coil by modulating different stimulus frequencies (16). rTMS has been used to treat major depression and treatment-resistant depression (17). Recently, numerous studies have adopted high- or low-frequency rTMS protocols with positive results in in hemiparesis due to pediatric stroke, mild traumatic brain injury, and tics/Tourette syndrome (18, 19). However, despite the benefits demonstrated for adult migraine, rTMS has been insufficiently studied for migraine in children. In addition, evidence for rTMS in childhood epilepsy and ADHD remains mixed (19). In ASD, the results confirmed that by stimulating different brain regions, different clinical symptoms were correspondingly improved: stimulating the dorsal lateral prefrontal cortex (DLPFC) can improve irritability, repetitive behaviors, and executive functioning; stimulating primary motor cortices can improve motor behavior; stimulating the medial prefrontal cortex can improve mentalizing; and stimulating the premotor cortex can improve speech production and eye-hand coordination (20). Regarding the adverse effects of TMS in children, studies found that the overall rate of the adverse effects, including headache, facial discomfort, irritability, pain at the application site, headedness, and dizziness, is 25% (18, 19, 21). However, all these adverse effects are mild and transient and can be resolved after rest or medication (21).

Moreover, meta-analysis and systematic reviews have indicated that low-frequency rTMS applied to the dorsolateral prefrontal cortex is safe and has the potential to alleviate insomnia symptoms and sleep disturbance in different neurological and neuropsychiatric conditions (22–24). Studies have found that rTMS not only improves sleep quality and optimizes sleep structure but also maintains therapeutic efficacy over pharmacological treatments and cognitive behavioral therapy and has emerged as a promising tool for treating sleep disturbances (22). Moreover, after 10 daily sessions of rTMS treatment in patients with primary insomnia, the concentrations of serum GABA and brain-derived neurotrophic factor (BDNF) were significantly higher than baseline (25). As we already know, GABA and BDNF both have a close relationship with sleep regulation (26–28). The change in metabolomics may be the potential mechanism of rTMS treatment in alleviating insomnia (28).

However, the implications of rTMS for insomnia in ASD patients have not been fully established. Only one study indicated that rTMS can alleviate sleep disturbances in ASD, which were assessed by the Children's Sleep Habits Questionnaire (CSHQ) (29). No randomized, double-blind, controlled studies were conducted to evaluate the efficacy of rTMS for insomnia through objective assessments such as PSG or actigraphy. Therefore, we aim to conduct a randomized, double-blind, sham-controlled trial to explore the effect of

rTMS on insomnia with an objective assessment of ASD. Additional objectives include explorations of the effects of rTMS on ASD symptoms and the association between insomnia and ASD symptoms. We hypothesize that 1) rTMS applied to the right dorsolateral prefrontal cortex (DLPFC) can alleviate sleep quality and ASD symptoms and 2) metabolic changes may play a mediating role in sleep quality and ASD symptoms.

Methods and analysis

Study design and settings

This is a double-blind, sham-controlled pilot trial to examine the efficacy of rTMS on insomnia in ASD. Participants will be randomized to the rTMS group or sham rTMS group for 4 weeks (5 days/week). Assessments of the primary and secondary outcomes will be performed at baseline, after the 4-week treatment, and at the 4-week follow-up with rTMS or sham treatment (Figure 1). The protocol was approved by the Biomedical Research Ethics Committee West China Hospital of Sichuan University and is registered at the Chinese Clinical Trial Registry (ChiCTR2100049266).

Participants

Participants will be recruited from the special education schools, nursery and primary schools, the second outpatient department of the West China Mental Health Center of Sichuan University, and online. Measurements and interventions will be carried out at the second outpatient department of the West China Mental Health Center of Sichuan University.

Sample size

Previous studies in patients with insomnia have shown that rTMS treatment can decrease sleep onset latency by at least 17.88–44.8 min (30, 31). According to previous studies, the minimally important difference of the primary outcome (sleep onset latency) is assumed to be 10 min (SD, 8.4 min) (32). To detect this difference with a two-sided significance level of 0.05 and a power of 80%, and account for the drop rate of 10% according to existing rTMS studies in autism (33, 34), a total of 30 participants will be included (PASS, version 15).

Inclusion/exclusion criteria

Participants eligible for the trial must comply with all of the following:

1) A preliminary clinical diagnosis using DSM-5 in the Mental Health Center by a child psychiatrist will be confirmed by two well-trained child psychiatrists using the Autism Diagnostic Interview-Revised (ADI-R) and Autism Diagnostic Observational Schedule (ADOS-2) (35, 36).
2) Age between 3 and 13 years.
3) Patients with ASD must meet the following diagnostic criteria for insomnia in the International Classification of Sleep Disorders–Third Edition (ICSD-3) (duration > 3 months: difficulty sleeping (sleep latency > 20 min or wake-up time > 20 min after falling asleep) at least 3 nights per week, confirmed by sleep diary (at least 7 days) and portable home-PSG (2 consecutive nights, based mainly on the second-day monitoring data) (37).
4) Not taking any drugs that affect sleep or no change in the drug dose and not taking new drugs during the trial period.
5) Two living parents who are able to cooperate in signing the informed consent form and documents pertaining to related scales.

Participants will be excluded from the trial if they comply with one of the following:

1) Those who have a history of epilepsy or severe head trauma, who have medical device implants (such as pacemakers, etc.).
2) Participants cannot cooperate.
3) Severe neurologic or psychiatric disorders or medical conditions (e.g., skull defect and craniotomy) or other disorders likely to have an impact on sleep.

Participants who cannot tolerate the side effects (headache, etc.), develop any adverse events (seizure, etc.) during the treatment, or miss more than two rTMS treatment sessions in a row, or miss more than four sessions in total, will be withdrawn from this study.

Randomization and blinding

For participant allocation, a computer-generated random list will be used, which is based on the permutation block method with a random number generator and allocated at a ratio of 1:1. A research assistant concealed the allocation sequence using sealed, opaque envelopes that contain the treatment information (A or B) before officially entering the study. After completing the consent form and baseline measures, the corresponding envelope for a given participant ID will be given to the rTMS therapist prior to the first rTMS session. The rTMS therapist is the only person who determines the treatment method (rTMS or sham treatment) for A or B. Once the treatment information is decided, no change will be accepted. Others, such as study investigators, parents, patients and statistical analysts,

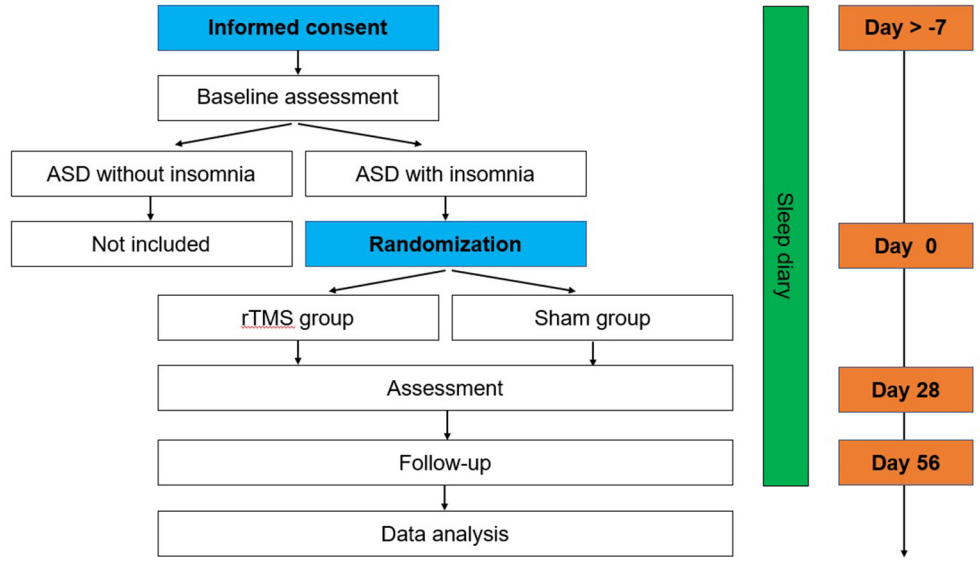

FIGURE 1
Time schedule of screening, interventions, assessments and visits of participants.

will be completely blinded to the treatment condition. Any adverse effects or body discomfort will be recorded after each rTMS treatment session. The aforementioned individuals will be unblinded to information about the treatment only when a participant experiences serious adverse events, and then an independent investigator will reveal the current treatment of this participant.

Intervention

We will perform rTMS with the CCY-I magnetic field stimulator (Wuhan Yiruide Co., Ltd.), which can adjust the stimulation frequency from 0 to 100 Hz through a circular coil. Before the first treatment, single-pulse TMS will be administered to the primary motor cortex to elicit a discernible hand muscle response in at least three of five consecutive pulses to determine the resting motor threshold (RMT). The stimulation site of rTMS is the right DLPFC, which is located 5 cm anterior to the site of maximal stimulation of the first dorsal interossei in a parasagittal plane. To ensure better positioning of the TMS coil, we further use the scalp region used for F4 EEG electrode placement in the 10–20 International System (38, 39). The right DLPFC is known as one part of the socially related brain areas, plays a crucial role in the integration of goals and the reward system, has been found the weak responses in ASDs and selected for treating primary insomnia with good effects (23, 24). In addition, another study using 25% MT for the treatment of ASD found that it could alleviate sleep disturbances with little adverse effects (29). In this ongoing clinical trial, 1 Hz rTMS at 50% RMT (50 trains of 30 pulses, with a 3-s interval between each, leading to a sum of 1,500 pulses) will be applied over the right DLPFC. The treatment time will be determined according to the specific conditions of the subject, but the daily treatment time needs to be consistent (afternoon, the error cannot exceed 1 h). The operation process of the sham group will be the same as that of the rTMS treatment group, with no magnetic field passing through the skull. There will be no difference between the appearance and sound of the devices in the rTMS group and the sham group.

Assessment

The assessments at baseline, after the 4-week treatment period, and the 4-week follow-up are summarized in Table 1.

The clinical and sleep assessment

Through the self-designed questionnaire, demographic information, including race, sex, age, weight, height, past and current illnesses and medications, family income, the parent's educational level, mother's pregnancy disease, and childbirth, will be obtained.

Sleep studies

The sleep diary will be sent to parents to record their child's sleeping habits, including the child's bedtime, the time at which the child left his or her bed in the morning, the time to falling asleep, the number of times the child woke up and fell back asleep, and the total sleep time, for at least 7 consecutive days

TABLE 1 Summary of the assessments.

Assessments	Randomization		
	Baseline	After 4 weeks of rTMS treatment	Follow-up at 4 weeks
Informed consent	√		
ADI-R	√		
ADOS	√		
Home-PSG	√	√	√
CSHQ	√	√	√
CARS	√	√	√
ABC	√	√	√
SRS	√	√	√
RBS-R	√	√	√
Blood	√	√	√

rTMS, repetitive transcranial magnetic stimulation; ADI-R, Autism Diagnostic Interview-Revised; ADOS, Autism Diagnostic Observational Schedule; PSG, polysmongraphy; CSHQ, Children Sleep Habits Questionnaire; CARS, Childhood Autism Rating Scale; ABC, Aberrant Behavior Checklist; SRS, Social Responsiveness Scale; RBS-R, Repetitive Behavior Scale-Revised. Home-PSG will be measured in two consecutive nights. Sleep diary will be record for at least 7 consecutive days before treatment, during the treatment and follow-up period. Adverse events report will be filled every day after randomization.

before treatment. These are also recorded during treatment and follow-up.

The Children Sleep Habits Questionnaire (CSHQ) is a parent-reported questionnaire (40). It consists of 41 questions that can reflect the common sleep problems in a typical recent week at 8 different levels: bedtime resistance, sleep onset delay, sleep duration, sleep anxiety, night waking, parasomnias, sleep-disordered breathing, and daytime sleepiness. A total score higher than 41 indicates sleep disturbance. The scale has adequate internal consistency, acceptable test-retest reliability, and acceptable validity to be widely used to evaluate sleep problems among different ages of ASD patients.

To objectively assess the change in sleep parameters and overcome the first-night effect, we will bring portable equipment to the patient's home for two consecutive overnight polysomnography examinations. Sleep data will be collected and scored *via* Somté PSG (Compumedics, OR). Measures will include six electroencephalographic (EEG) leads (F3-M2, F4-M1, C3-M2, C4-M1, O1-M2, O2-M1), two electrooculographic leads (ROC-M2, LOC-M2), 2 x Chin electromyography (EMG), 2 x Limb EMG, airflow from the pressure transducer and thermistor, thoracic and abdominal inductive RIP band, position, finger pulse oximetry, and electrocardiogram. Recording techniques and estimation of sleep parameters will follow the American Academy of Sleep Medicine guidelines (V.2.3).

Clinical assessment and questionnaires

The four scales below will be used to assess the changes in clinical symptoms from different aspects (severity, aberrant/repetitive behavior, social responsiveness) at baseline, after treatment and at the one-month follow-up (Figure 1).

The Childhood Autism Rating Scale (CARS) will be used to diagnose ASD by clinicians (41). Based on the levels of social, emotional, adaptive, communicative, and cognitive aspects, the CARS can divide ASD patients into three different severity groups. ASD severity will be graded by CARS as none to minimal symptoms (15–30 points), mild to moderate symptoms (30–37 points), and severe symptoms (38–60 points). We will use this scale to assess the severity of changes at the above three time points.

The Aberrant Behavior Checklist (ABC) will be used to evaluate the main ASD symptoms, including irritability, lethargy/social withdrawal, stereotypic behavior, hyperactivity/noncompliance, and inappropriate speech (42). ABC in Chinese has satisfactory psychometric properties and has been widely used in the evaluation of treatment efficiency.

The Social Responsiveness Scale (SRS) is a parent rating scale that is used to measure the severity or quantities of social impairment in clinical samples, including ASD (43). It consists of 65 items including five theoretical subscale scores (labeled social awareness, social cognition, social communication, social motivation, and autistic mannerisms). The total raw score ranges from 0 to 195, with higher scores indicating increased social impairment. We will use this scale to assess changes in social responsiveness.

The Repetitive Behavior Scale-Revised (RBS-R) is used to measure the various repetitive behaviors among individuals with ASD (44). There are 43 items rated for severity on a 4-point Likert scale. The total raw score ranges from 0 to 172, and higher scores indicate serious repetitive behaviors.

Untargeted metabolomics analysis

We will collect whole blood using a heparin sodium anticoagulant tube and perform plasma separation as soon as possible. Briefly, the sample will be centrifuged at 3,000 rpm at 4°C for 10 min, the upper layer will be collected, and 0.2 mL/tube will be transferred into 2 mL centrifuge tubes. After the sample is labeled, it will be quick-frozen in liquid nitrogen for 15 min and stored at −80°C. Then, the sample will be incubated on ice for 5 min and centrifuged at 15,000 rpm for 5 min at 4°C. Some of the supernatant will be diluted to a final concentration containing 53% methanol with LC–MS grade water. The sample will be subsequently transferred to a fresh Eppendorf tube and then centrifuged at 15,000 g for 10 min at 4°C. Finally, the supernatant will be injected into the LC–MS/MS system for analysis. The raw data files generated by UHPLC–MS/MS will be processed using Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform peak alignment, peak picking, and

quantitation for each metabolite. These metabolites will be annotated using the KEGG database (http://www.genome.jp/kegg/), HMDB database (http://www.hmdb.ca/), and Lipidmaps database (http://www.lipidmaps.org/).

Outcomes

The primary outcome of this study will be the difference in sleep onset latency between baseline and the end of treatment. Secondary outcomes will be differences in other parameters derived from polysomnography, the sleep diary, CSHQ, CARS, ABC, SRS, RBS-R, and metabolomics analysis.

Safety outcomes

Family members will also be asked to complete an adverse event report every day, and this report will include information about potential side effects, including headache, nausea, vomiting, and seizures. If the above symptoms occur during treatment, treatment will be immediately stopped to see if these symptoms subside. If status epilepticus occurs, the patient will be sent to the emergency room for symptomatic treatment. Safety outcomes and treatment-related side effects will be assessed and reported in the publication.

Follow-up

Four weeks after the last rTMS or sham treatment, parents and patients will be asked to return for a reevaluation of symptoms using the above scale. The CSHQ and home-PSG will be then used to assess sleep quality. In addition, whole blood samples will be collected again.

Data management

A standardized case report form (CRF) was developed for the current study. After the clinical symptoms of the patients are evaluated at the specified time points, the data will be entered into the Excel database immediately. To ensure the accuracy of the data, another researcher will double-check.

Statistical analysis

We will use SPSS software version 26.0 (IBM Corp, Armonk, New York, US) to perform data analysis. Demographic and baseline data will be analyzed with standard descriptive statistics. Normally and non-normally distributed data will be presented as means (SDs) and medians (quartiles), respectively. The comparison of basic data between the rTMS group and sham rTMS group will be performed using Student's *t*-test, the chi-squared test, or the nonparametric test according to the data type. To verify the efficacy of our intervention, the final analysis will be conducted according to the intent-to-treat (ITT) principle, and missing data will be replaced by means of the last observation carried forward rules. Changes in quantitative variables over time (evaluation of the therapeutic effect of rTMS on variations in sleep latency) will be analyzed using random-effect models allowing us to take into account repeated measurements. Time (baseline, post, and 1-month follow-up) and group (rTMS or sham) effects will be estimated and tested, along the interaction with the group. According to the same strategy, changes in other quantitative variables over time will be investigated. To further analyze the relationship between the change in the parameter of sleep and clinical symptoms, logistic regression, general linear correlation or regression will be taken into consideration between various types of outcomes. The number and proportions of participants with side effects, adverse events, and serious adverse events will also be reported. All statistical analyses will be two-sided, and statistical significance will be set at $p<0.05$. For untargeted metabolomics analysis, principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) will be performed in metaX (a flexible and comprehensive software for processing metabolomics data). We will apply univariate analysis (*t*-test) to calculate the statistical significance (*P* value). Metabolites with VIP $>$ 1 and *P* value$<$ 0.05 and fold change (FC)$\geq$ 2 or FC$\leq$ 0.5 will be considered to be differential metabolites.

Discussion

This study protocol presents the design of an RCT investigating the therapeutic effects of rTMS over the right DLPFC for insomnia in ASD by using a combined approach of home-PSG, sleep diary and clinically relevant scores from participants and caregivers. The results of this study will provide preliminary data on whether rTMS can alleviate insomnia in patients with ASD.

Although the cause of sleep disturbances in children with ASD is not clear, it has been found that the hyperarousal state also exists in ASD with insomnia (45, 46). Apart from that, there are several other possible hypotheses to explain it. First, the underlying abnormal pathophysiological mechanism of ASD may be the direct cause of sleep disturbances (47). Abnormal neural connectivity in the brain plays a pivotal role in ASD. The DLPFC, an essential part of integrating distributed brain networks that support the operation of verbal/auditory and spatial information in the brain, is dysfunctional according to proton magnetic resonance spectroscopy data in ASD (48, 49). Furthermore, abnormal sleep-promoting transmitters such as melatonin and GABAergic circuits have been found in ASD children with sleep disturbances (50–52). Second, the important role of cortical excitation/inhibition balance

has been confirmed in sleep-stage-specific regulation (53). Increased cortical excitability has been found in different types of insomnia patients (54). In ASD, studies have found that cortical inhibition decreases and that the balance is disrupted, a noted predictor of autistic phenotypes (55). Third, there is a bidirectional association between ASD and sleep disturbances. Sleep disturbances may be related to the clinical phenotype and core symptoms of ASD (6, 56). Fourth, sleep problems may be associated with psychiatric comorbidity and independent of ASD (47).

rTMS is a noninvasive technique that produces a magnetic field by passing a brief electric current and introduces excitatory or inhibitory cortical excitability in a small area of the brain below the magnetic coil by modulating different stimulus frequencies (>1 Hz or <1 Hz) (57). Recently, rTMS has been used in ASD. Two double-blind, randomized trials found that high-frequency rTMS (5 Hz and 20 Hz) applied to the DLPFC or bilateral dorsal medial prefrontal cortex can improve anxiety and executive function performance in patients with ASD (33, 34). High-frequency rTMS (10 Hz) to the left DLPFC can also alleviate depressive symptoms among ASD patients with depression (58). Low-frequency rTMS can increase cardiac vagal control and reduce sympathetic arousal, alleviating behavioral problems post-TMS improvement (59, 60). One study adopted low-frequency rTMS in children with low-functioning autism and found that it can increase the peak alpha frequency in different brain regions and alleviate behavioral symptoms assessed by the ABC (61). Furthermore, meta-analyses and systematic reviews indicated that rTMS could help treat some dimensions of ASD, such as repetitive and stereotyped behavior, sociability, or some aspects of executive and cognitive functions (62, 63). One study adopted theta burst stimulation (TBS), a patterned repetitive transcranial magnetic stimulation (rTMS) protocol over the bilateral posterior superior temporal sulcus and proved that it had an immediate effect on parent-rate autistic symptoms in adults with ASD (64). For glutamate and GABAergic circuits that are abnormal in ASD, one study found that rTMS (1 Hz) treatment for 2 weeks effectively alleviated the acquired autistic-like symptoms in a rat model by regulating synaptic GABA transmission (65). Furthermore, in adults with ASD, one study found that rTMS to the DLPFC may modulate local glutamate levels (66).

Considering its potential role, we hypothesize that rTMS can alleviate insomnia symptoms in ASD. However, no study has explored the effect of rTMS on insomnia symptoms in objective ways (such as PSG) in ASD. Therefore, the results of this study may address an important knowledge gap and may provide evidence for noninvasive and nonpharmaceutical interventions to treat insomnia in ASD.

Trial status

The described trial is ongoing, and recruitment commenced in July 2021. However, an overall slow rate of progress is due to COVID-19 restrictions. The data collection will continue until 30 participants completed all intervention sessions and the corresponding pre- and post-measurements. Follow-ups will be conducted 1 month after the stimulation sessions to investigate long-lasting effects.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee on Biomedical Research, West China Hospital of Sichuan University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

JJ, LT, YZ, TL, and XT designed the trial. JJ, LT, and XT designed the stimulation model and defined the stimulation parameters. YZ and TL planned the data analysis. JJ is responsible for the recruitment of the participants and the data collection during the study and drafted the manuscript. YZ, LT, and XT edited and revised the paper. All authors approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Salivary oxytocin in autistic patients and in patients with intellectual disability

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Background: Assessing the role of oxytocin (OT) in the regulation of social interaction is a promising area that opens up new opportunities for studying the mechanisms of developing autism spectrum disorders (ASD).

Aim: To assess the correlation between the salivary OT level and age-related and psychopathological symptoms of children with intellectual disability (ID) and ASD.

Methods: We used the clinical and psychopathological method to assess the signs of ASD based on International Classification of Diseases (ICD-10), the severity of ASD was specified by the selected Russian type version "Childhood Autism Rating Scale" (CARS). Patients of both groups had an IQ score below 70 points.

Results: The median and interquartile range of salivary OT levels in patients with ID and ASD were 23.897 [14.260–59.643] pg/mL, and in the group ID without ASD - Me = 50.896 [33.502–83.774] pg/mL ($p = 0.001$). The severity of ASD on the CARS scale Me = 51.5 [40.75–56.0] score in the group ID with ASD, and in the group ID without ASD—at the level of Me = 32 [27.0–38.0] points ($p < 0.001$). According to the results of correlation-regression analysis in the main group, a direct correlation was established between salivary OT level and a high degree of severity of ASD Rho = 0.435 ($p = 0.005$). There was no correlation between the salivary OT level and intellectual development in the group ID with ASD, Rho = 0.013 ($p = 0.941$) and we have found a relationship between oxytocin and intellectual development in the group ID without ASD,

Rho = 0.297 (p = 0.005). There was no correlation between salivary OT and age, ASD and age.

Conclusion: The results of this study indicate that patients in the group ID with ASD demonstrated a lower level of salivary OT concentration and a direct relationship between the maximum values of this indicator and the severity of autistic disorders, in contrast to patients in the group ID without ASD.

KEYWORDS

oxytocin, intellectual disability, behavior, childhood age, autism spectrum disorders

Introduction

Oxytocin (OT) is a neuropeptide with two variants of hormonal effects: central and peripheral. OT has received much attention recently and has been called the "most studied neuropeptide" (1). OT is synthesized in the hypothalamus and released to the brain *via* distributed neural pathways and to the systemic circulation *via* the posterior pituitary (2). The peripheral effect of OT, associated with the regulation of labor and lactation is the most studied and actively used in medical practice.

The actions of oxytocin in the brain are attracted the attention of researchers with its influence on social behavior, parent-child relationships, stability of intra-group relationships, and communications (3–6). Plasma OT concentrations have been associated with gender, age, anxiety, attachment behavior, and cognitive abilities (7). There are studies that have shown that children who receive enough maternal care, physical, non-verbal and verbal contact, demonstrate higher levels of OT (8). The results of the study of low oxytocin levels support the impression that this neuropeptide actively affects the manifestations of mental disorders. There was a significant decrease in OT in women with postpartum depression (9, 10) with manifestations of alexithymia and anorexia nervosa (11, 12).

Nowadays, OT is a new biomarker and indicator of mental illness or the severity of the disease. Obtaining central OT levels is challenging, therefore researchers acquire peripheral OT levels through blood plasma or saliva, thereby offering an accessible way to assess on central OT circuitry. The meta-review of 2021 showed the significance of the dysfunction of the OT system in the mental illness (13). Another meta-analysis of 2021 has investigated whether there are differences in endogenous OT concentrations in individuals with ASD compared to matched neurotypical controls across all ages. It was showed OT levels are lower in children with ASD as compared to neurotypical controls, but this effect seems to disappear in adolescent and adult populations. OT levels of individuals with vs. without ASD seems to be only present in the studies with male participants. It was supposed that differences of OT levels were slightly more pronounced for blood samples as compared to saliva samples. Authors discussed that further research employing more homogeneous methods is necessary to explore the possible use of OT level measurement as a diagnostic marker of ASD (14). These results indicate the need to consider the age of patients when planning a research study to determine the level of endogenous oxytocin, giving preference to children of preschool and primary school age. Based on these data, a further study with more focus on pre-pubertal children on a cohort is therefore suggested. The same review drew attention to the important issue of comparing OT concentration levels in different environments and the significance of such differences for the diagnosis of mental disorders.

Oxytocin level in central nervous system, saliva and plasma

The OT level is a very dynamic indicator with a rapidly changing concentration in the biological environments of the body. The oxytocin molecule has a very short half-life period [3–6 min on the periphery and 19 min in the central nervous system (CNS)], and the baseline level of OT in adults is quite low (<8 pg/ml) under initial conditions (15).

One of the studies, conducted using the method of liquid chromatography-mass spectrometry (LC-MS), showed that the maximum concentrations of this neuropeptide in urine, saliva and plasma were reached 15–30 min after intranasal administration with a sharp decrease over the next 2–3 h (16).

The information on the basic level of OT varies significantly in publications depending on gender, age and type of activity (cooking, playing different games individually or in groups etc.). In 31 students, the concentration of the hormone in saliva was recorded before the study 45.96 (33.27) – 77.93 (74.46) pg/ml (17); there was a difference in the level for different variants of activity (individual and group) from 60 ± 8 to 234 ± 35 pg/ml in women and from 63 ± 15 to 207 ± 24 pg/ml in men (18).

For children with typical development, the OT level is 90–100 pg/ml, which differs from the OT level in children with the consequences of abuse (19). Some authors show normalized values of the OT level, which makes it difficult to find information about absolute indicators of the OT level in saliva (20).

Recent investigations show that research opportunities in reliably assessing the effect of OT on humans are significantly limited by the inaccessibility of CNS structures. In this regard, it becomes relevant to use alternative samples as a source for assessing the activity of the hormone in the human brain, which makes it possible to eliminate the risks associated with the intake of cerebrospinal fluid (CSF). Urine and saliva are the preferred mediums for OT analysis because of their non-invasive collection methods (21). Although there are many publications indicating that it is necessary to clarify the correspondence of neuropeptide levels in various biological fluids (22) due to conflicting views on the relationship between central and peripheral OT activity. There are studies about the degree of correlation of OT in plasma, saliva and CSF (23–25). A study of the OT concentration in biological samples of patients with neurological and neurosurgical pathology showed a significant correlation between saliva and CSF, thereby confirming that saliva OT can help assess the level of OT in the CNS. On the contrary, the OT level in plasma did not correspond to its level in the CSF (13, 22).

Oxytocin and autism spectrum disorders

In accordance with the present results, previous studies have demonstrated OT role in the mechanisms of regulation of social behavior, adaptability and emotional response in patients with ASD. This finding broadly supports the work of other studies in this area and indicate that OT can be considered a good diagnostic marker in patients with severe autism. Changes in plasma levels of neuropeptides, including OT and mRNA, correlated with the severity of disorders, have been reported in children with ASD (26).

It was shown that OT levels (44.72 ± 36.1 pg/ml) in children with ASD were significantly lower ($p < 0.05$) than in age-matched (102.1 ± 34.31 pg/ml) and sex-matched (53.05 ± 38.38 pg/ml) children of the control group. In the same study, a noticeable correlation was noted between the severity of developmental disorders and the concentration of the hormone in saliva: for mild degree—59.22 ± 27.32 pg/ml, for moderate degree—47 ± 25.47 pg/ml, for severe degree—27.92 ± 10.23 pg/ml (26).

The level of endogenous OT is associated not only with the severity of ASD, but also with changes in the functioning of brain regions. The identified associations between the oxytocinergic system and amygdala–hippocampal pathways are anticipated to be of relevance for understanding the role of OT in modulating appropriate neural and physiological responses to stress and restoring homeostasis. It was revealed that in adult men with ASD, higher levels of salivary OT are associated with higher expressions of secure attachment but lower degrees of interregional functional coupling between the amygdala and hippocampal regions (27).

Measurement of the OT level in patients with ASD was performed to assess its role in the systematic regulation of socialization, monitor the results of the use of intranasal OT spray, study the impact on cognitive abilities and emotional attachment (28–30). The significant correlation in the whole-group analysis was presumed to reflect the fact that the ASD subgroup showed abnormal peripheral OT levels. Based on the spectrum concept described above, the salivary OT level reflects the ASD characteristics in the whole-group analysis. Conversely, the significant correlations between salivary OT level and AQ (The Autism Spectrum Quotient) were not observed in the within-group analysis (31).

The plasma levels of these neuropeptides reflected different aspects of autistic behaviors, with OT correlated with verbal communication. Thus, the dependence of the OT concentration and the manifestations of the verbal communication violations were noted in a group of 84 patients with ASD compared with a group of 85 participants without ASD. Correlation analysis showed that children with ASD with higher plasma OT concentrations tended to have less impairment in verbal communication (Rho = –0.22, $P = 0.076$) and had lower plasma OT levels than neurotypical children of the corresponding gender ($P = 0.028$) (32).

Working with young patients does not always allow taking blood for research; therefore saliva sampling is a non-invasive method for obtaining a biological sample. The concentration of neuropeptides in saliva is no less informative and reliable than level of neuropeptides in blood plasma. It was found that salivary OT increased in children with ASD during interaction with parents and decreased 15 min after the end of contact (20).

Importantly, pretreatment blood OT concentrations also predicted treatment response, such that individuals with the lowest pretreatment OT concentrations will be showed the greatest social improvement. It was found that the response to therapy was directly related to the initial OT level. So OT treatment enhances social abilities in children with ASD and that individuals with pretreatment OT signaling deficits may stand to benefit the most from OT treatment. These results indicate that peripheral OT levels may reflect the degree of social deficit, and that it is important to understand how CNS and plasma, plasma and salivary OT levels are related to the severity of ASD. This rather contradictory result may by due to bias and cannot be extrapolated to all patients. Results should be interpreted with caution. So in one study of the use of intranasal OT in children with ASD, an association was shown

between social improvement and an increase in the level of the hormone in plasma in children who received placebo (33). Another study reported similar OT levels in healthy people and autistic people [ASD 36.2 ± 13.2 pg/mL, TD 43.6 ± 17.0 pg/mL, t(38) = 1.45, p = 0.154, Cohen's d = 49] in studying the relationship between attention to social information and peripheral levels of OT (31).

All this demonstrates the need to refine the options for implementing the effects of OT and to search for factors that create conditions for obtaining more reliable results.

Oxytocin and autism spectrum disorders with intellectual disability

There is practically no information about the level of OT in people with intellectual disability (ID). At the same time, a large number of people with ASD have ID (34). There is evidence that the functions of OT differ in men and women with severe mental retardation and that OT partially affects autism and is associated with some repetitive actions and non-verbal communication in patients with ASD with severe mental retardation (35).

Some research results show that oxytocin can improve social interaction in a group of patients suffering from ASD with concomitant ID (36). It remains unclear whether the presence of mental retardation affects the level of OT in ASD. This is important to understand for the development and application of treatments for ASD based on changes in OT levels.

Hypothesis

We hypothesized based on existing evidence that patients with ID with ASD and patients with ID without ASD would have different levels of OT in saliva. This hypothesis is based on the fact that a reduced level of OT is characteristic of ASD (26), that OT in saliva reflects the level of OT in the CNS better than OT in plasma (22).

The aim of the study: to assess the correlation of salivary OT levels with age and psychopathological characteristics of children suffering from ID and ASD.

Materials and methods

Patients

The study included 121 children hospitalized for the first time in the Pediatric Department of the Krasnoyarsk Regional Psychoneurological Early Treatment Center, Krasnoyarsk, Russia, which was an approved platform for participation in the research of Krasnoyarsk State Medical University.

The children underwent inpatient treatment between 2018 and 2021 for aggressive, self-harming behavior, and emotional problems. The respondents were selected by the consent of legal representatives to participate in the study, the age of 4–13 years, the presence symptoms of ID, of ASD, the ability to complete tasks (Human figure drawing test and spitting saliva into a test tube). Patients over the age of 13 were excluded due to the high probability of the influence of hormonal characteristics. We included children with disabilities (the patients are given a disability group) and orphans in the study in compliance with all the rules for obtaining consent and monitoring compliance with their rights (**Tables 1**, **2**).

In the ID with ASD (Pervasive developmental disorders F84) group, there were 8 children with Childhood autism (F84.0), 12 children with Atypical autism (F84.1), 9 children with Mental retardation with autistic features (F84.1), 1 with Rett syndrome (F84.2), 4 children with Other childhood disintegrative disorder (F84.3). In the group ID without ASD, there were 68 children with Mild mental retardation (F70.0–F70.1), 19 children with Moderate mental retardation (F71.0–F71.1).

TABLE 1 Descriptive statistics of quantitative variables.

Indicators	Me		Q_1 – Q3		Min		Max		*ETH*
	ID with ASD (n = 34)	**ID without ASD (n = 87)**	**ID with ASD (n = 34)**	**ID without ASD (n = 87)**	**ID with ASD (n = 34)**	**ID without ASD (n = 87)**	**ID with ASD (n = 34)**	**ID without ASD (n = 87)**	
Age	7.0	9.0	5.75–10.0	6.0–11.0	4	3	12	13	p = 0.098
IQ[a]	61.0	63.0	45.8–70.0	50.0–68.0	37	37	70	70	p = 0.589
CARS[b]	**51.5**	**32.0**	**40.8–56.0**	**27.0–38.0**	33	13	59	51	**p < 0.001**

ID with ASD—a group of patients with intellectual disability and autism spectrum disorders; ID without ASD—a group of patients with intellectual disability without autism spectrum disorders.
IQ[a] indicators of the intellectual level, determined using the test "Human figure drawing test".
CARS[b] Childhood Autism Rating Scale.
Me [Q1—Q3]-Median [25–75% percentile], the first quartile, Q_1, is equal to the 25th percentile, the third quartile, Q_3, is equal to the 75th percentile.
Min, max—minimum, maximum values of indicators. The bold values indicate values with $p < 0.001$.

TABLE 2 Descriptive statistics of categorical variables.

Indicators	Abs.		%			
	ID with ASD (n = 34)	ID without ASD (n = 87)	ID with ASD (n = 34)	ID without ASD (n = 87)	χ^2	P
Sex male/female	27/7	66/21	79.4/20.6%	75.9/23.1%	0.173	$p = 0.677$
Orphancy	**1**	**26**	**2.9%**	**29.9%**	**10.238**	$\mathbf{p = 0.001}$
The patients are given a disability group	**16**	**16**	**47.16%**	**18.39%**	**10.329**	$\mathbf{p = 0.001}$

ID with ASD—a group of patients with intellectual disability and autism spectrum disorders; ID without ASD—a group of patients with intellectual disability without autism spectrum disorders. The bold values indicate values with $p = 0.001$.

Most of the patients from both groups received antipsychotics, antidepressants, nootropic drugs, according to clinical guidelines and treatment protocols. None of the study participants received psychostimulants.

Almost all children received help from a psychiatrist at their place of residence in specialized outpatient clinics for children and adolescents before hospitalization. There was no information about receiving specialized psychiatric care before admission to the hospital in 1 child from the group ID with ASD and in 9 children from the group ID without ASD. The organization of treatment both in the polyclinic at the place of residence and in the hospital was necessarily accompanied by psychological work and psychoeducation of the patients themselves, their parents, teachers to improve socialization, facilitate adaptation in the children's collective, and reduce suffering.

Informed consent

The parents of the patients signed a written consent prior to inclusion on the basis of complete information in accordance with the requirements of the "Law of the Russian Federation on July 2, 1992 N 3185-1 on psychiatric care and guarantees of the rights of citizens in its provision."

In the absence of parents, consent was obtained from legal representatives:

– close relatives who have the official status of guardians appointed by the court of the Russian Federation to represent the interests of a child left without parental care;

– an official from the institution of residence of a child authorized by the state to monitor the observance of the rights and interests of an orphan child who is on full state support and has neither parents nor guardian relatives.

Children under the age of 13 gave their verbal consent.

Separately, written consent was obtained for medical intervention to determine the level of OT and consent to the processing of personal data. The study was conducted in compliance with the modern biomedical ethics and ethical standards developed in accordance with the World Medical Association Declaration of Helsinki (Protocol No. 86/2018 of the local ethical committee of Krasnoyarsk State Medical University of 08.11.2018).

Methods

The analysis of the clinical picture of disorders based on diagnostic criteria (ICD-10) was performed in all children. The signs of mental disorder were studied by two psychiatrists. The first was an employee of the hospital, the second—an employee of the Medical University. Further, the results of the clinical examination were supplemented with materials of experimental psychological examination and psychometric methods. This stage was implemented by the staff of the psychological service of the hospital.

Assessment of the severity of autistic disorders

The method "Childhood Autism Rating Scale (CARS)" (37, 38) made it possible to assess and compare the degree of social and communicative deficit in patients with soma of various pathologies. In the works devoted to the study of the validity of this test, its reliability was demonstrated. The Cronbach's alpha coefficient for CARS was 0.772, and the degree of correlation with the Autism Behavior Checklist (ABS)—0.732 (39). Information that CARS may not provide a cross-cultural valid assessment of ASD (40), pointed out the need to use a proven adapted version of the test (41). The adapted Russian version showed fairly high reliability values of $r = 0.9$ ($p < 0.001$) and correlations with the scale of the CARS $r = 0.8$ ($p < 0.001$) (41).

In addition, the CARS technique could not be used independently for the diagnosis of ASD. Many of the signs

listed in it could be observed in other diseases. This type of examination required a mandatory assessment of the compliance of the condition with the criteria of ICD-10 by a psychiatrist.

Determining the degree of intellectual disability

We used the "Human figure drawing test" to assess the intellectual level, which made it possible to correlate the degree of development of cognitive functions with a 100-point IQ-test system. To date, this diagnostic procedure is quite unified, widely used for intellectual assessment, is readily used in medical institutions, schools, medical schools, and social rehabilitation centers (42, 43). There is an option to compare the results of autism research conducted in various fields of health and education. When studying the validity of the "Human figure drawing test" in children with mental disorders, a general correlation of the Full Scale Intelligence Quotient (FSIQ) from the standard intellectual level test and standard Goodenough-Harris scores corresponding to 0.813 ($p < 0.01$) was established. For patients with an intelligence quotient (IQ) of less than 70 points, the validity of the test was 69.2% (44). The "Human figure drawing test" offered an effective way to the tasks of our study. In some patients included in the survey, it was impossible to determine the intellectual level using a full-scale version of the IQ-test due to the autistic characteristics of children or their behavioral disorders. The drawing test was convenient, performed within a few minutes, well understood, did not provoke protest emotional and behavioral reactions in the survey participants (45).

Saliva sampling and oxytocin measurement

Saliva sampling was performed to assess the hormonal status and determine the level of OT. Saliva sampling was carried out on the 7–10th day of hospitalization, in the morning from 8 to 9 o'clock, before meals. Saliva samples (0.3–0.8 ml) collected in paper cups were immediately transferred to plastic microtubes (1.5 ml). They were frozen and stored at –20°C to be centrifuged twice at 4°C at 1,500 × g for 15 min 3 days later. The samples were kept at –20°C until assayed.

Determination of salivary OT was performed using a 96-plate commercial OT-ELISA kit (Clode-Clone Corp., China), as described previously (46). Recent studies have shown that OT values are reliable when measure in saliva by enzyme immunoassay (46, 47). Measurements were performed in duplicate. Samples (100 μl) were treated according to the manufacturer's instructions. The optical density of the samples and standards was measured at wavelengths of 450 nm by a microplate reader. Sample concentrations were calculated according to the relevant standard curve (48).

Several studies have shown that OT in saliva, determined by immunoassay, is reliable, stable over time results, and correlates with the behavioral processes (49, 50).

Statistical analysis

We carried out the mathematical analysis using the SPSS medical statistics software package for Windows version 24. The significance of the results was established at $p < 0.05$ and $p < 0.01$. The normality of the distribution was checked using the Shapiro-Wilk test.

Analyzing the deviation from the normal distribution of features, the descriptive part of statistical processing was carried out by non-parametric methods. Medians and interquartile range (Me [25%; 75%]) were calculated for indicators of quantitative variables of age, the severity of autism, the severity of ID, the salivary OT level. Categorical variables of indicators of gender, disability, and orphanhood were evaluated using the Pearson Criterion (χ^2).

Spearman's correlation coefficients (Rho) were determined in each group to assess the correlation of ASD symptoms with age, OT and the ID degree.

We used multivariate regression analysis to predict the degree of influence of independent variables (constant—study group, gender, age, intelligence, CARS, orphanhood, disability) on the dependent variable OT (salivary oxytocin level). Regression analysis was carried out both for the combined database and separately for the group ID with ASD and for the group ID without ASD.

The sample size of ID patients with ASD $n = 34$ for cross-group comparisons was determined with an estimated effect size of 0.35; alpha = 0.05; degree = 0.84 (G*Power3.1.9.7).

Results

We did not find any significant differences between the groups in the ratio of sex ($p = 0.678$), age ($p = 0.098$) or IQ level assessed by the "Human Figure drawing test" ($p = 0.589$). Differences were found in the levels of OT concentration in saliva and indicators of the severity of autism between the groups (**Figure 1**).

Median indicators and interquartile range (ME [Q_1-Q_3]) of OT level in saliva of patients in the group ID with ASD were 23.897 [14.260–59.643] pg/ml, and in the group ID without ASD were Me = 50.896 [33.502–83.774] pg/ml. The difference between the indicators has reached the required level of significance ($p = 0.001$). Our results of assessing the severity of ASD on the CARS scale showed Me = 51.5 [40.75–56.0] score in the group ID with ASD. For patients from the group ID without

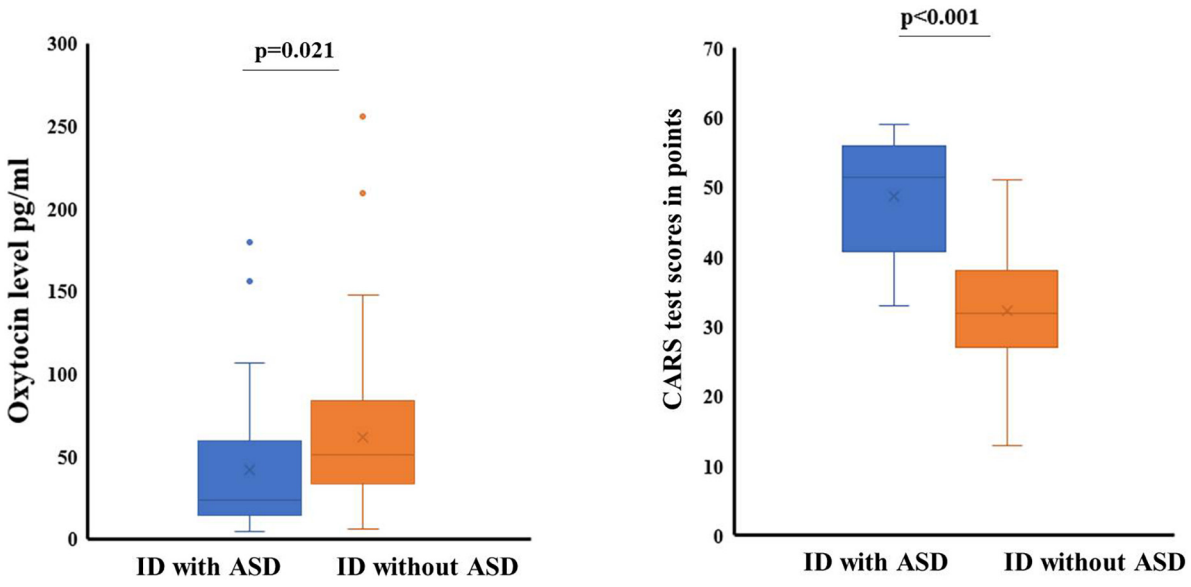

FIGURE 1
The concentration of OT in saliva, CARS test score and the level of severity of ASD. ID with ASD—the group of patients with intellectual disability and autism spectrum disorders; ID without ASD—the group of patients with intellectual disability without autism spectrum disoders.

ASD, the severity of ASD was at the level of Me = 32 [27.0–38.0] points, difference ($p < 0.001$).

Our results revealed a significant difference between the groups in terms of the number of orphans and the disabled. 29.9% of children from the group ID without ASD showed signs of social distress due to orphanhood. In the group ID with ASD, only 1 child (2.9%) was an orphan ($p = 0.001$). Children from group ID with ASD had given a disability group in 47.1% compare with 18.4% of patients with ID without ASD ($p = 0.001$).

The results of the correlation analysis performed by us showed that patients from the group ID with ASD maintained a direct relationship between indicators of severe severity of ASD and high levels of OT concentration in saliva (Figures 2A,B). Spearman's correlation coefficient in this group was Rho = 0.435 ($p = 0.005$). In the second group of ID without ASD, this relationship was not significance Rho = 0.104 ($p = 0.338$). At the same time, we did not find a correlation between the level of OT concentration in saliva and the level of intellectual development for the ID group with ASD, Rho = 0.013 ($p = 0.941$) and we have found of a relationship in OT indicators with intellectual development for the ID group without ASD, Rho = 0.297 ($p = 0.005$) (Figures 2C,D).

There was no significant correlation between age and the salivary OT (Figures 3A,B) in both groups. The correlation coefficient in the ID group with ASD was Rho = –0.087 ($p = 0.623$), and in patients and the ID group without ASD - Rho = –0.076 ($p = 0.481$). The results of assessing the relationship between the severity of autistic signs and age did not reach the required level of significance (Figures 3C,D). The correlation coefficient in the ID group with ASD was Rho = –0.182 ($p = 0.302$), and in patients and the ID group without ASD – Rho = 0.013 ($p = 0.903$).

To clarify the degree of influence of factors, a multiple regression analysis was carried out. We assessed the degree of change in the dependent variable (indicators of OT concentration in saliva), under the influence of several factor signs (gender, age, severity of autism, severity of intellectual disability, the state of orphanhood and the presence of disability).

The regression model we constructed showed a moderate Chaddock scale correlation (weak—from 0.1 to 0.3; moderate — from 0.3 to 0.5; noticeable—from 0.5 to 0.7) for the combined groups between the concentration of oxytocin in saliva and other factors ($R = 0.376$). This model will be correct for 14% of cases.

The calculation of a separate model for each group showed the differences between them. For the group ID with ASD, a significant Chaddock scale correlation was established between the salivary OT concentration and other factors ($R = 0.586$), and for patients from the group ID without ASD, a moderate correlation was established ($R = 0.33$). The probability of error in predicting results in this model also differed between groups. For the group ID with ASD, the constructed model is correct in 34% of cases ($R^2 = 0.343$), and in the group ID without ASD, our model of the influence of several factors on the concentration of OT in saliva is correct only in 10% of cases ($R^2 = 0.108$).

According to the values of the coefficient B, we found that of all the indicators used in the model, a significant effect on the dependent variable (the indicator of the saliva OT

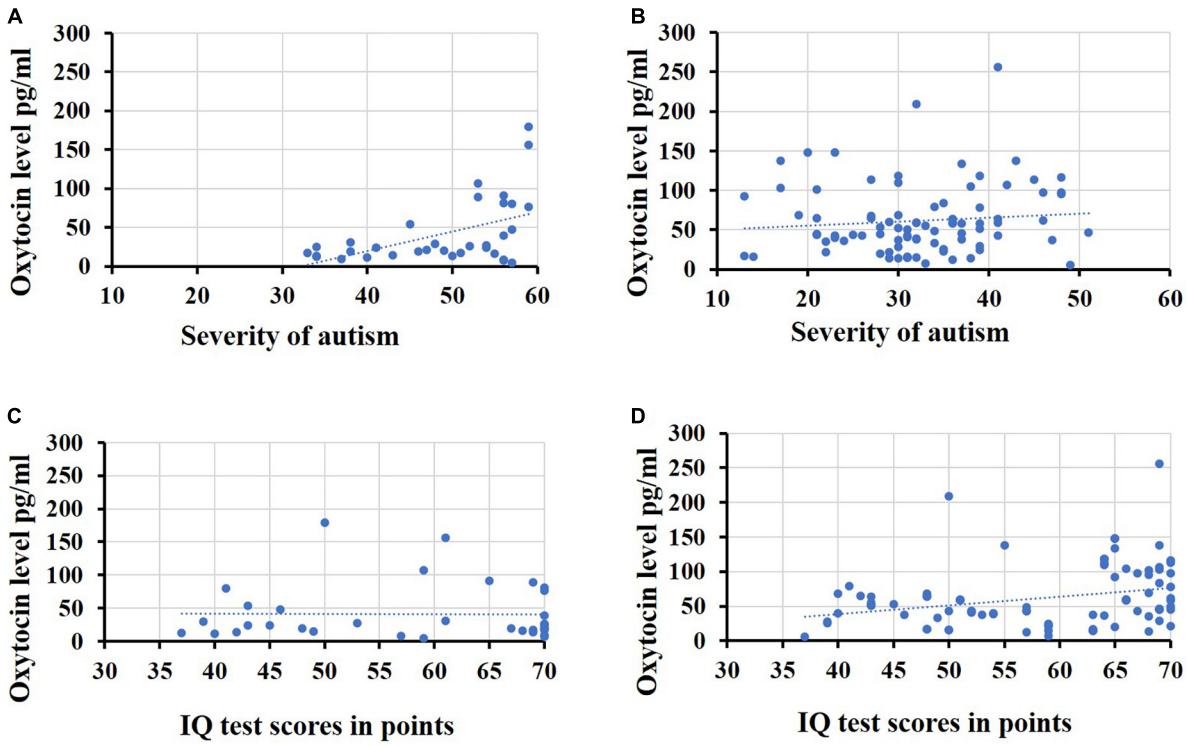

FIGURE 2
Indicators of the oxytocin level. **(A)** The oxytocin level in saliva and severity of autism in the group ID with ASD, **(B)** the oxytocin level in saliva and severity of autism in the group ID without ASD, **(C)** the oxytocin level in saliva and the level of intellectual development patients in the group ID with ASD, **(D)** the oxytocin level in saliva and the level of intellectual development patients in the group ID without ASD.

concentration). This has been confirmed for the severity of ASD on the CARS scale, B = 0.987 (p = 0.039) in the model with combined groups. In the model with the group ID with ASD, a reliable level of indicators of the influence of the severity of ASD on the concentration of OT in saliva remained, as in the model with the combined groups, B = 2.959 (p = 0.002). In the model with the group ID without ASD, reliable indicators of the effect on the concentration of OT in saliva were found for IQ indicators, B = 1.167 (p = 0.018). The results of multiple regression analysis are presented in **Table 3** and **Figures 4**, **5**.

Discussion

The data we have obtained require discussion and further study. The results of our research, which showed a lower saliva OT level in children grope ID with ASD, correspond to the results of other studies studying the nature of the dysfunction of the OT system in this pathology (51).

At the same time, the direct correlation that we established between the indicators of severe autistic disorders and high levels of saliva OT concentration in the group ID with ASD does not correspond to the materials of scientific publications on the reduced concentration of this hormone in autistic pathology (52). Such a result may be associated with different levels of adaptive abilities of patients, or it may be due to the peculiarities of the response to stress and the more significant than expected role of OT in the regulation of response to emotional and intellectual stress.

The stressful situation of the examination can provoke the release of OT due to the fear and anticipation of subsequent difficulties (53), which contributes to the reduction of harm from stress due to cardioprotective mechanisms of OT that effect the magnitude and hemodynamic parameters of cardiovascular stress responses (54).

It is likely that OT can increase physical and cognitive resilience, preventing the consequences and improving the clinical outcomes of stress-related traumatic disorders after acute or prolonged traumatic experiences (55). At the same time, there is information that OT improves impaired social adaptation by normalizing hyper- or hypo-brain activity (56) and demonstrates an adaptive effect in both autistic and neurotypical study participants (52).

It is necessary to plan the application of methods for assessing adaptability and stress resistance in further studies.

The low rates of orphanhood and elevated OT levels that we found in the group of ID with ASD with more severe variants of developmental disorders that required social support in the

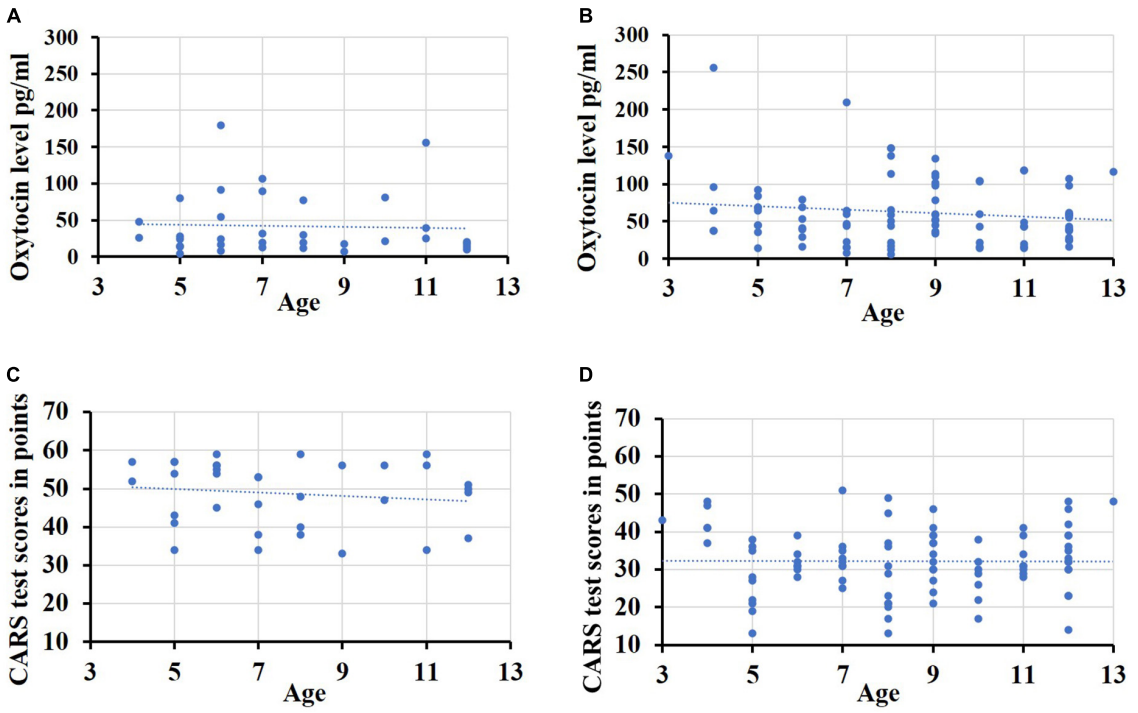

FIGURE 3
Indicators of severity of autistic disorders, oxytocin levels and age. **(A)** The oxytocin-levels and age in the group ID with ASD; **(B)** the oxytocin levels and age in the group ID without ASD; **(C)** the severity of autistic disorders and age in the group ID with ASD; **(D)** the severity of autistic disorders and age in the group ID without ASD.

TABLE 3 Summary of hierarchical regression analysis for patients in both groups.

	Combined indicators		ID with ASD (n = 34)		ID without ASD (n = 87)	
	Unstandardized coefficients B	p	Unstandardized coefficients B	p	Unstandardized coefficients B	p
Constant	−78.723	0.051	−70.937	0.161	−1.280	0.971
Group	42.931	**0.001***	–	–	–	–
Gender	−5.617	0.562	−24.527	0.182	−2.307	0.840
Age	−1.757	0.287	4.433	0.233	−1.956	0.309
CARS	0.987	**0.039****	2.959	**0.002****	0.427	0.452
IQ	0.711	0.073	−1.065	0.180	1.167	**0.018****
Orphanhood	−11.891	0.259	−41.247	0.330	−8.098	0.467
Disability	5.898	0.530	5.510	0.695	3.470	0.778
R—determination coefficient	0.376		0.586		0.330	
R^2—the value of the coefficient of determination	0.141		0.343		0.108	
F	2.661		2.35		1.627	

*p = 0.001, **p < 0.05. The bold values indicate values with significant difference.

form of the given a disability group, deserve attention and additional study. The reliable difference between the groups in these parameters, established by using descriptive statistics methods, was significantly limited during further processing. Multiple regression analysis did not reveal the correlation and mutual influence of these indicators. However, the presence of significant differences between the groups and the assumption that it is the increase in the content of this hormone in

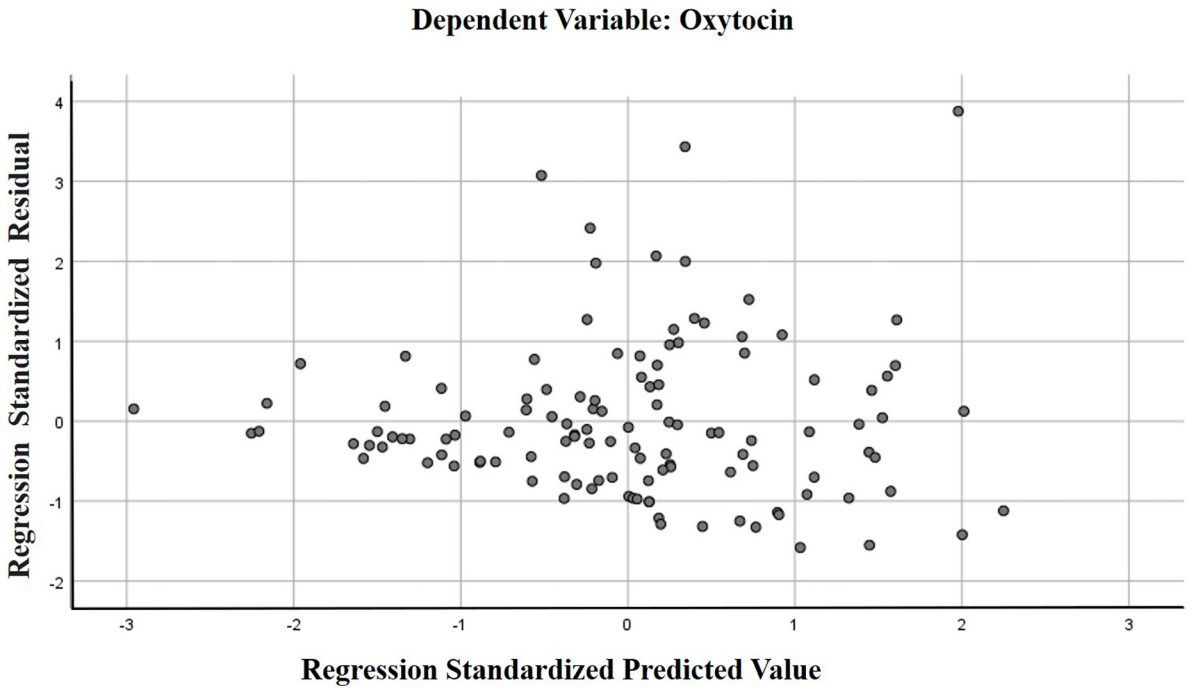

FIGURE 4
Scatter plot of standardized predicted values against standardized residuals for the regression model with combined groups.

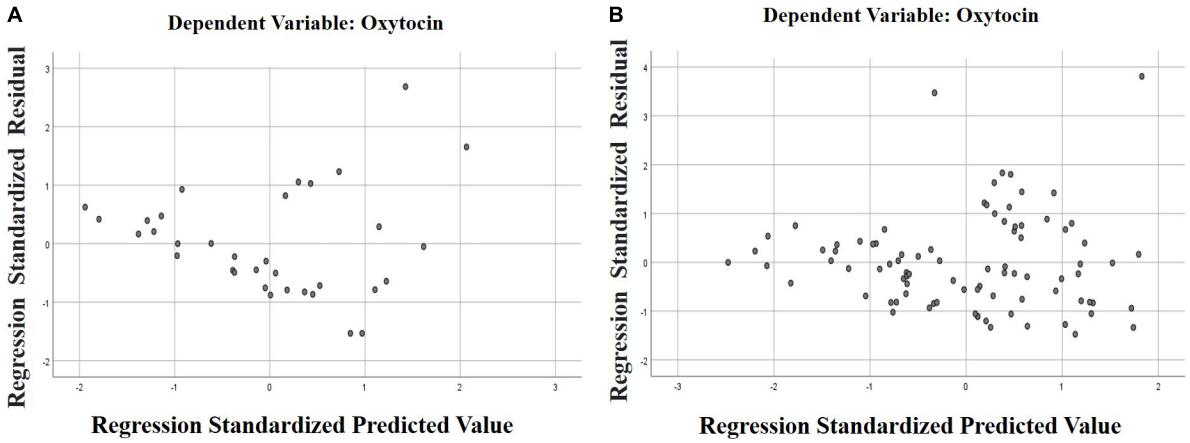

FIGURE 5
Scatter plot of standardized predictive values versus standardized residuals for each group's regression model. **(A)** ID with ASD (the group of patients with intellectual disability and autism spectrum disorders); **(B)** ID without ASD (the group of patients with intellectual disability without autism spectrum disorders).

biological environments that affects the stability of emotional ties, reducing the risk of parents abandoning a sick child, will remain relevant to research in this area.

Based on the identified cases of high OT levels in the group ID with ASD in severe variants of ASD, we can safely assume that the level of OT indicates not so much the severity of disorders as the preservation of adaptive mechanisms. This assumption is supported by the materials of studies on the influence of OT on parent-child relationships, for the implementation of protective mechanisms in painful procedures (57), on attention to social signals (58).

While OT significantly affects the mechanisms of social adaptation (56), it seems that the effects of OT strongly depend on the context and experience gained, especially at an early age (59).

Patients with higher indicators of intellectual development in the group ID without ASD are easier than other participants to record and use positive experiences, realizing it through mediated mechanisms and reactions accompanied by an increase in the level of OT in saliva (60).

In addition, other hypotheses and explanations are acceptable.

Taking this into account, in further studies it is necessary to plan the use of methods for assessing adaptability and stress resistance. The use of medication by our participants is a limitation of the study. Published articles contain indications of such limitations (33). Today, it is not known how drugs interact with OT and change its concentration. The necessary conditions were ensured by the fact that during the study the drug intake did not change, remained stable and did not differ between groups.

There are practically no patients with ASD and high intellectual level in the hospital. As a rule, such patients receive the necessary assistance and support at their place of residence and study. The hospital approved for the study is referred by a psychiatrist from the place of residence. These are patients with the most severe variants of pathology. We formed the study groups from such patients.

The study of neurotypical children and children with ASD and high intellectual level requires the organization of work in other conditions, for instance, at school. This is beyond our capabilities and leads to limitations in research.

The relatively small sample size is also a potential limitation. We conducted a study among patients and their relatives who have a higher motivation in organizing treatment due to fairly severe disorders. We cannot directly transfer the results obtained to all patients with ASD or ID.

The research outcomes and the limitations described indicate the relevance of organizing studies with the required number of patients. Despite the complexity and rather high cost of such work, which make it difficult to create a large sample, it is necessary to conduct research on more powerful cohorts to deepen our understanding of the mechanisms of oxytocin's influence on the pathogenesis of autism spectrum disorders.

Conclusion

As a result of the study, we found that children with ASD, who were admitted to the hospital for the first time, revealed a lower salivary OT level compared to the group of patients with ID. Children with autistic features showed a more noticeable tendency to increase OT levels in severe ASD than children with ID. For patients of the group ID without ASD, a higher level of OT concentration in saliva and a direct relationship of the maximum values of this indicator with a milder degree of ID were established. Although these results are correlational and preliminary, it raises several intriguing ideas that deserve further study.

Data availability statement

The original contributions presented in this 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 Local Ethical Committee of Krasnoyarsk State Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

YP: literature collection and analysis, concept and design development, data interpretation and analysis, verification of critical content, and preparation of the manuscript. IM and ET: data interpretation and analysis and preparation of the manuscript. MB and DD: data interpretation and manuscript substantiation. NA: collecting material. AP: collecting material, data processing, and analysis. AS: verification of critical content. OL: concept and design development, literature collection and analysis, data processing, and verification of critical content. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Eye Movement Desensitization and Reprocessing in adolescents with autism; Efficacy on ASD symptoms and stress

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Introduction: Eye Movement Desensitization and Reprocessing (EMDR) is a well-established and thoroughly researched treatment method for posttraumatic stress symptoms. When patients with an autism spectrum disorder (ASD) are treated with EMDR for their Posttraumatic Stress Disorder (PTSD), they sometimes report a decrease in the core symptoms of ASD. This explorative pre-post-follow up design study is designed to investigate whether EMDR with a focus on daily experienced stress, is effective in reducing ASD symptoms and stress in adolescents with ASD.

Methods: Twenty-one adolescents with ASD (age 12 to 19) were treated with ten sessions EMDR, focusing on events of daily experienced stress.

Results: No significant decrease of ASD symptoms was found on the total score of the Social Responsiveness Scale (SRS) as reported by caregivers from baseline to end measurement. However, there was a significant decrease on total caregivers SRS score comparing the baseline to the follow-up measurement. On two subscales, Social Awareness and Social Communication, a significant decrease was found from baseline to follow-up. On the subscales Social Motivation and Restricted Interests and Repetitive Behavior, no significant effects were found. On pre- and posttest scores of total ASD symptoms measured by the Autism Diagnostic Observation Schedule (ADOS-2), no significant effects were found. On the contrary, scores on self-reported Perceived Stress Scale (PSS) showed a significant decrease from baseline to follow-up. Also, 52% of adolescents showed a significant improvement of global clinical functioning at endpoint measurement on the Clinical Global Impression Improvement, rated by an independent child psychiatrist.

Discussion: In sum, these results of this uncontrolled study suggest a partial effect of EMDR in adolescents with ASD on ASD symptoms, rated by their caregivers. In addition, the results of this study show that EMDR treatment on daily experienced stress significantly reduce perceived stress as reported by the participants, and improves global clinical functioning.

The results also suggest a 'sleeper effect', since no significant effects were found between baseline- and post- treatment measurements, but only between baseline- and follow up three months after the treatment. This finding is in line with other studies investigating psychotherapeutic effects in ASD. Implications for clinical practice and suggestions for future research are discussed.

KEYWORDS

autism spectrum disorder, eye movement desensitization and reprocessing (EMDR), stress, adolescents, children

Introduction

Autism spectrum disorder (ASD) is a common neurodevelopmental disorder with an average estimated prevalence of around 1% (1, 2).

For most individuals core ASD symptoms (i.e., deficits in communication, social-emotional reciprocity and restricted and repetitive behavior and interests) are present throughout life and place a great mental burden on individuals and their families (3, 4). Often, individuals with ASD report a lower quality of life (5), show impaired family-functioning due to for example elevated stress levels (6), a limited social network, and less meaningful relationships (7).

Adults with ASD perceive more stress in their daily life, and have less capacity to cope with this stress than mentally healthy individuals (8). This stress may cause lower social functioning. In addition, the stress itself may contribute to social disfunctioning of adults with ASD (9–11). Also, in childhood elevated stress levels may increase the risk of social communication impairments by triggering avoidance behavior, and than may further complicate development of the already hampering social skills (12–14). Focussing on reducing stress may be a new gateway to treatment options on social and emotional functioning in ASD. In general, psychotherapeutic interventions focussing on reducing stress in children and adolescents diagnosed with ASD, show positive effects. For instance, a meta-analysis on the effectiveness of mindfulness based therapies in reducing psychological distress in children with ASD and their caregivers, showed significant gains in subjective wellbeing and decrease of psychological distress, after treatment, and at three month follow-up. (15). Also, studies using a prepost design on the effects of Acceptance and Commitment Therapy (ACT) showed a reduction of stress, more pro-social behavior, more social awareness and more social cognition in children with ASD (8, 13, 16).

Among others, Eye Movement Desensitization and Reprocessing (EMDR) may be considered as a way to increase social skills by reducing stress in individuals with ASD. EMDR is a well-established treatment for adults and children with post-traumatic stress disorders (PTSD) (17–20). Different studies suggest that EMDR may be effective for several other conditions than PTSD; for instance in groups with extreme stress as a result of painful medical examination, spinal cord injury or extreme stress in pregnant women with a history of stillbirth (21). In these studies only a few EMDR sessions diminished perceived stress or anxiety.

One of the main hypothesized working mechanism of EMDR is that EMDR reduces the vividness of images of unpleasant memories held up in the working memory capacity by eye movements that concurrently use up processing resources in the working memory capacity (22–24). Working memory has been supposed to be an important fundamental higher-order function, underlying other executive functions (25) and is found to be impaired in individuals with ASD (26–29). A possible hypothesis for reducing core symptoms of ASD when treating an individual with ASD with EMDR, may be that stressful thoughts and images no longer occupy the already impaired working memory and so more mental space is left for social interactions.

First case studies in adults with ASD treated by EMDR for their PTSD complaints, reported significant progress in overall functioning (30, 31). Lobregt van Buuren et al. (32) showed in adults with ASD and a history of adverse events and trauma, that on one hand after 7 sessions EMDR a reduction was reported in core ASD symptoms, measured by the Social Responsiveness Scale-Adults and on the other hand a significant reduction of trauma-related symptoms and psychological distress. Furthermore, Leuning et al. (33) presented a case study in which an adolescent with ASD was treated with 10 weekly sessions of EMDRfor daily stress and confusion. Although measurements did not show a significant increase of social responsiveness or decrease in symptoms of ASD, a decrease was found in caregiver-reported restricted interest and repetitive behavior. In addition, caregivers reported better daily functioning in personal hygiene, improved school attendance and a greater ability to share feelings and thoughts. Comparing caregiver-based scores and self-reported scores on the SRS, the youngster in this case study reported no significant reduction in ASD symptoms. In research literature it is described that especially adolescents may have difficulties in properly reporting their own ASD symptoms (34).

In order to search for a novel entrance to improve social functioning in ASD, this explorative uncontrolled study was designed to investigate whether EMDR on daily experienced stress is effective in perceived stress, reducing ASD core symptoms and/or improving global functioning in adolescents with ASD.

Materials and methods

Participants

This study with a pre-post-follow up design was conducted at the outpatient care department of Karakter University Centre Nijmegen, an academic center for child and adolescent psychiatry in The Netherlands between November 2017 and July 2020. All adolescents were formally diagnosed with ASD by an experienced and multi-disciplinary team and classified by the criteria from DSM-IV-TR (35) or DSM 5 (3). Comorbid psychiatric disorders were allowed, except PTSD. Other inclusion criteria were a full-scale IQ of 80 or higher and the ability to understand and speak

Dutch. Patients were excluded when they had PTSD complaints like flashbacks, reliving's, nightmares and strong avoidance tendencies, reported in an interview based on the Clinician Administered PTSD Scale for Children and Adolescents (36). No other treatments were allowed during the study except pharmacotherapy with stable dosages of medication.

Measures

Primary outcome

The total score of the Social Responsiveness Scale (SRS-A) (37) completed by one of the caregivers was used to assess the change in ASD symptoms prior, during, and after treatment. The SRS-A assesses the ability in adolescents to engage in reciprocal social behavior in natural social settings, measured in four subscales: (1) Social Awareness, (2) Social Communication (3) Social Motivation, and (4) Restricted Interests and Repetitive Behavior. In total, the four subscales comprise 65 items that are answered on a 4-point scale (never true to almost always true). The total score of the caregiver-rated SRS served as the primary outcome measure. Because of doubts in the research field as to whether young people are able to properly report their complaints themselves, the parent score was chosen as the primary outcome measure (34).

Cronbach's alpha for SRS-A completed by caregivers was 0.95. Based on earlier research with a same research population on Pivotal Response Treatment (38, 39) a clinical responder on the SRS-A was defined as a reduction of > 25% on the total SRS score.

Secondary outcomes

The total score of SRS-A of the adolescents self-report was used as a secondary outcome measure. Also based on earlier research with a same research population on Pivotal Response Treatment (38, 39) a clinical response on the SRS-A was defined as a reduction of > 25% on the total SRS score.

Cronbach's alpha for the SRS-A completed by adolescents, was 0.96. SRS-A total score had a sensitivity of 0.85 and a specificity of 0.83 for ASD versus typically developing participants. Correlations with established ASD scales were moderate to high (r = 0.25 −0.83) (40).

Another secondary outcome measure is the Autism Diagnostic Observation Schedule 2 (ADOS-2) (41), which is a semi-structured observation schedule in which the clinician elicits social, communicative, stereotyped and play behavior to observe symptoms of ASD. Observations of the clinician, who is blinded for baseline outcomes, are categorized and a score is assigned for each domain of ASD symptoms. Calibrated Severity Scores (CSS) (42) are compared pre-treatment and post-treatment.

Calibrated severity scores on the ADOS-2 showed a good discrimination between clinical ASD and non-spectrum classifications (42). These calibrated severity scores are used earlier in a similar population of individuals with ASD (38, 39). For these scores, a change was computed between baseline and endpoint (43).

The Trauma Symptom Investigation Form in Autism Spectrum Disorders (TIF-ASD) (44), translated into Dutch, measures behavioral aspects of ASD related to traumatic events. The TIF-ASD assesses the impact of traumatic events on five core symptoms of autism: (1) social and (verbal) communication skills; (2) behavioral problems; (3) stereotypical & ritualistic behaviors; (4) self-care skills; (5) vegetative symptoms. The total scale consists of 20 items which are completed by parents. Items are answered on a 5-point scale ranging from never true to always. Cronbach's alpha for the TIF-ASD was 0.92. Information on validity of the TIF-ASD is not available from the developers (44). Clinical responding on the TIF-ASD was defined as a reduction of > 25% on the total score.

The Clinical Global Impressions (CGI) scale is a well-established research rating tool applicable to all psychiatric disorders that can easily be used by the practicing clinician to meet this need.

Change in clinical global functioning was assessed at endpoint and follow-up using the Clinical Global Impression-Improvement (CGI-I) (45), rated on a 7 point scale (*very much improved-score 1- to very much worse-score 7*) by experienced child psychiatrists who were unfamiliar with the participant. Ratings were based on information about the clinical status of functioning, symptoms, and well-being in major areas of the participants life (i.e., home, school, relations). This information was provided by the coordinating therapist of the participant, who was instructed not to provide details on the treatment phase. Based on earlier research on Pivotal Response Treatment (38, 39), a clinical responder was defined as being *much improved (score 2)* or *very much improved (score 1)* on the CGI-I.

Experienced stress was measured by the Perceived Stress Scale-10 (PSS-10) (46) that assess the degree in which individuals rate their lives as unpredictable, uncontrollable, and overwhelming. The 10 self-report items were answered on a 5-point scale ranging from never applicable to very often applicable. Normscores are only available for adult participants (47), where a score above 14 is indicated as above average stress. Cronbach's alpha for the PSS was 0.92. Convergent validity ranges from 0.56 to 0.71. (48). In the research of Hirvikoski & Blomqvist (8) 25 adults with ASD showed significantly higher scores (mean score 29, 38) on the PSS than 28 typically developing adults (mean score 19, 69). Clinical responding status was defined as a reduction of > 25% on the total score in post- treatment and/or follow up measurement compared to baseline.

Intervention

EMDR is a protocolized treatment (49), in which negative and adverse memories are stripped of their negative charges and become neutral in remembrance. This study used the Dutch standard EMDR protocol for children and adolescents (19).

At the beginning of every session, the participant was asked: 'In this recent week, were there any occasions or events which gave you stress or made you upset?' In case the participant did not come up with an event, parents were asked to assist in choosing events to focus on. With this upsetting memory, the original EMDR protocol was followed: making a stationary picture of the most upsetting moment, targeting a negative cognition which caused most distortion or stress, find out the most appropriate positive cognition, determining the Validity of Cognition (VoC, on a scale from 1 to 7)), determining the Subject Units of Disturbance (SUD, on a scale from 0 to 10) and indicating the body awareness of the tension. Then desensitization was started by offering a distracting stimulus, in which bilateral eye movements were the most frequently used distracting working memory tasks. Repeatedly the participant was asked to report about his emotions, his cognitions, his bodily awareness's, while being distracted. When SUD decreased to zero, and the positive cognition was installed, the participant was asked: 'Does this event reminds you of things you have experienced earlier in your life?' If so, this earlier experience was also treated with the EMDR protocol. Reason for this question to be asked is to be sure that the past drivers of daily

stress were also treated. The session ended with installing a positive competence of the participant, conform the Dutch EMDR protocol.

In the next session the therapist asked the participant if there was any SUD resting on the images of last week's remembrance. If so, EMDR treatment was continued. If not, the participant was asked for upsetting moments over the past week.

EMDR was provided by four trained EMDR therapists. They all completed supervision of the Dutch EMDR society and were trained in extra skills for providing EMDR to children and adolescents with ASD. Supervision was given by a certified supervisor of the Dutch EMDR society[1].

Procedure

After being referred to the study, a screening was done to determine if the adolescent had a PTSD diagnosis (as exclusion criteria) and met the inclusion criteria. If no PTSD diagnosis was present and all other inclusion criteria were met, the adolescent and his/her caretaker were asked for informed consent. Written informed consent was obtained from all participants and caregivers that were included in the study.

Before initiating the treatment, the ADOS and CGI-I were administered. In the four weeks prior to starting the treatment (baseline), the adolescent and one of his caregivers completed the SRS-A (adolescent and caregiver), TIF-ASD (caregiver), and PSS (adolescent) on a weekly basis. After a baseline phase of four weeks, the 10-week protocol for the EMDR treatment started, with sessions planned weekly. At each (weekly) time point, the SRS-A, TIF-ASD and PSS-10 were completed to assess change in these measures over the course of EMDR. When the EMDR was finished, the SRS-A, TIF-ASD, PSS-10, ADOS-2 and CGI-I were administered as endpoint measures. In addition, the SRS-A, TIF-ASD, PSS and CGI-I were administered at 3-month follow-up.

Committee of 'experts by experience'

In order to set up the research in collaboration with the relevant population, a committee of experts by experience was conducted. This committee consisted of four former patients and a mother of one of them. All patients had a diagnosis of ASD and were treated with EMDR for their former PTSD complaints. They advised the research team on approach of participants, i.e., about formulation of patient information and the desirability of using rewards for participants. This committee was consulted before the study started, in the middle of the study and was informed of results afterward.

Statistical analysis

Baseline descriptive statistics were obtained for all 21 participants. All available data was used and no imputation was conducted for any missing data since the explorative nature of this study. Outliers were identified by using density plots or boxplots and were adjusted to quartile (Q)1 – 1.5 * interquartile range (IQR) and Q3 + 1.5 * IQR to obtain normal distribution if necessary. Outcomes on the four measurements with the SRS-A, TIF-ASD and PSS-10 prior to start of the treatment were averaged to obtain a baseline score for each of these measures. Paired samples *t*-tests (or Wilcoxon signed ranks tests when adjusting outliers did not contribute to obtaining normal distribution) were conducted in SPSS version 25 (50) to explore changes from baseline to endpoint and from baseline to follow-up on the SRS-A, ADOS-2 (only endpoint), TIF-ASD, PSS-10 and CGI-S. Furthermore, percentages of clinical responders on the SRS-A total score and CGI-I were explored by obtaining descriptive statistics and change scores.

In addition to the baseline versus endpoint and baseline versus follow-up comparisons, linear mixed-effect models, using the lmer function of the lme4 package (51) in R (version 4.0.3; R Core Team) (52) were used to assess the change over time during the EMDR treatment on the weekly administered SRS-A total score and subscales, PSS and TIF-ASD. A per-participant random adjustment to the fixed intercept as well as the slope over time were included in the models besides the fixed effect of time. Confidence intervals (95% CI) were derived from the function *confint* in R (R Core Team) (52), with CIs that exclude zero indicating significant estimates. Also correlational analyses in SPSS were conducted to explore the relationship between (1) change in the caregiver- and self-reported total SRS-A scores over the course of EMDR and (2) participants' age, total IQ, gender and baseline severity of ASD symptoms. A paired-samples *t*-tests was conducted to compare the baseline CSS of the ADOS-2 to the endpoint score. For all outcomes, sensitivity analyses were conducted excluding participants for which the EMDR treatment was interrupted due to the restrictions resulting from the COVID-19 pandemic ($N = 3$).

The study was approved by the Local Ethics Committee (CMO Arnhem-Nijmegen, NL60026.091.16).

Results

Participants

Thirty adolescents with ASD were screened for inclusion, of which 21 (12 males, 9 females) were finally included in the period from November 2017 until January 2020. Reasons for exclusion were not adhering to one of the inclusion criteria ($N = 7$) or anxiety that led to no commitment for the EMDR treatment ($N = 2$).

Table 1 shows the descriptive characteristics at baseline for the participants. On the CGI-S at baseline, the majority of participants had a score between 5 (markedly ill) and 7 (very severely ill), two participants received a score of 4 (moderately ill) and one participant received a score of 3 (mildly ill). Of the included participants, most participants received 75% of the treatment protocol.

Five young adolescents were unable to complete the SRS self-report. They did not understand the questions on the report form or had severe stress while answering the questions. For them, only caregiver-reported measures were included.

Most frequent comorbidity existed of: Attention Deficit Hyperactivity Disorder (in five cases), anxiety disorders (in five cases) and depression (in six cases). Three participants had comorbid ADHD ánd depression, two participants had a comorbid depression ánd a (social) anxiety disorder. These comorbid disorders were diagnosed by well trained child psychiatrists.

From the patients that were included in the study, fourteen participants used medication on a daily basis. Among them, three used stimulants (i.e., methylphenidate, dexamfetamine, and lisdexamfetamine), four antipsychotics (i.e., risperidon and aripiprazol), two antidepressants (i.e., fluoxetine and sertraline)

1 www.emdr.nl/supervisoren ven

TABLE 1 Baseline descriptive characteristics.

	Mean (SD)/N (%)	Range
Gender		
Male	12 (57.1)	
Female	9(42.9)	
Age	15.0 (2.0)	11.9-19.3
TIQ	104.6 (9.2)	88-125
VIQ	105.4 (11.7)	85-126
PIQ	99.9 (11.4)	70-117
ADOS CSS	4.8 (2.9)	1-10
CGI - Severity	5.3 (0.9)	3-7

TIQ, total intelligence quotient; VIQ, verbal intelligence quotient; PIQ, performance intelligence quotient; ADOS CCS, autism diagnostical observation schedule calibrated severity score; CGI-Severity, clinical global impression scale - severity.

and five used a combination of either an antidepressant and an antipsychotic or a combination of a stimulant and an antipsychotic.

Further exploration of the data reveals that participation varies between 5 and 10 sessions. Sixteen participants (76%) completed 9 or 10 sessions of the program. One participant who received five sessions, was an "early completer," indicating recovery from stress full events and negative mood states over last few weeks. The total response rate was therefore 81%.

Primary outcome measure

Caregiver-reported social responsiveness score (SRS-A) change from baseline to endpoint and baseline to follow-up

In Table 2 the results of the paired samples *t*-tests for change in SRS scores from baseline to endpoint and baseline to follow-up are shown with means and standard deviations. For the caregiver-reported SRS-A, no significant change was found from baseline to endpoint for the total score or any of the subscale scores (all $p > 0.05$). However, a lower score at follow-up compared to baseline was found for the total caregiver-reported SRS-A score ($p = 0.010$) and for the caregiver-reported subscales Awareness ($p = 0.002$) and Communication ($p = 0.021$). No significant change from baseline to follow-up was found for the caregiver-reported subscales Social Motivation and Restricted Interests and Repetitive Behavior of the SRS-A (all $p > 0.05$).

Caregivers social responsiveness score (SRS-A) change over the course of EMDR

Mixed model analysis showed no significant main effect of time for the total caregiver-rated SRS score, Estimate = 0.87 (1.37), 95% CI: −1.84–3.75. Mixed model analyses for each of the caregiver-rated SRS subscale scores indicated no significant main effect of time for the Social Awareness (Estimate = 0.24 (0.37), 95% CI: −0.49-1.02), Social Communication (Estimate = 0.43 (0.53), 95% CI: −0.62-1.55) Social Motivation (Estimate = 0.24 (0.31), 95% CI: −0.36-0.89) or Restricted Interests and Repetitive Behavior (Estimate = 0.22 (0.27), 95% CI: −0.31-0.78) subscales. No change over the course of EMDR treatment was found in caregiver-reported Social Responsiveness.

Sensitivity analyses excluding participants of which the EMDR treatment was interrupted due to the restrictions resulting from the COVID-19 pandemic did not alter conclusions for the primary outcome measures.

Secondary outcome measures

Self-reported social responsiveness score (SRS-A) change from baseline to endpoint and baseline to follow-up

On the self-reported SRS-A, no significant changes from baseline to endpoint and from baseline to follow-up were found for the total score and for any of the subscales (all $p > 0.05$). At endpoint and follow-up respectively, percentages clinical responders were 4.76 and 9.52% on the caregiver-reported SRS-A and 9.52 and 14.29% on the self-reported SRS-A.

Self-reported social responsiveness score (SRS-A) change over the course of EMDR

As for the caregiver-rated SRS, no significant main effect for time was found for the self-reported SRS total score in the mixed model analysis (Estimate = 0.42 (1.79), 95% CI: −3.18-4.12) and for the Social Awareness (Estimate = 0.09 (0.41), 95% CI: −0.71-0.96) Social Communication (Estimate = 0.13 (0.69), 95% CI: −1.25-1.57), Social Motivation (Estimate = 0.08 (0.48), 95% CI: −0.88-1.07) or Restricted Interests and Repetitive Behavior (Estimate = −0.04 (0.26), 95% CI: −0.56–0.51) subscales. No change over the course of EMDR treatment was found in Social Responsiveness reported by the adolescent.

There was no significant relationship between the change estimates on the caregiver- and self-reported SRS-A and participant's age, total IQ, gender and severity of ASD symptoms. See Table 3 for correlations between mean changes in SRS-A score and participant characteristics.

Severity of autism (ADOS-2)

Paired samples *t*-tests indicated no significant change from baseline to endpoint on the ADOS-2 CSS (see Table 2), indicating no change in severity of ASD symptoms after the EMDR compared to before the EMDR.

Trauma symptom investigation form in autism spectrum disorders (TIF-ASD)

Table 2 shows the results of the paired samples *t*-test comparing the total score on the TIF-ASD from baseline to endpoint and from baseline to follow-up. No significant change on the TIF-ASD was found from baseline to endpoint ($p > 0.05$), but a significant reduction was found from baseline to follow-up ($p = 0.011$).

Mixed model analysis showed no significant main effect of time for the TIF-ASD (Estimate = 0.98 (0.67), 95% CI: −0.34-2.40) indicating no significant change in impact of traumatic events related to ASD over the course of EMDR treatment.

Clinical global impression and improvement (CGI-I)

Non-parametric Wilcoxon signed rank tests on the CGI-S scores (see Table 2) showed a significant decrease from baseline to endpoint

TABLE 2 Means and standard deviations and results of paired samples tests.

	Baseline	Endpoint	Follow-up	Baseline to endpoint		Baseline to follow-up	
	M (SD)	M (SD)	M (SD)	*t* (df)	*p*	*t* (df)	*P*
SRS-A caregiver-report							
Total score	80.88 (22.82)	72.90 (19.85)	76.08 (22.17)	1.21 (9)	0.257	3.10 (11)	**0.010****
Awareness	24.06 (6.44)	20.60 (6.11)	21.08 (7.26)	1.96 (9)	0.082	4.06 (11)	**0.002****
Communication	25.56 (7.89)	22.70 (5.48)	22.83 (7.40)	0.88 (9)	0.401	2.69 (11)	**0.021***
Motivation	17.51 (6.29)	16.90 (6.32)	17.58 (5.45)	0.65 (9)	0.533	1.31 (11)	0.218
Restricted Interests and Repetitive Behavior	13.75 (5.78)	12.70 (6.63)	14.58 (6.16)	0.51 (9)	0.623	1.29 (11)	0.231
SRS-A self-report							
Total score	70.65 (26.46)	59.63 (29.16)	61.33 (29.96)	1.15 (7)	0.286	0.86 (8)	0.416
Awareness	19.92 (8.01)	18.13 (8.79)	17.44 (9.62)	0.89 (7)	0.402	0.76 (8)	0.470
Communication	22.47 (9.43)	17.25 (10.35)	17.44 (10.21)	1.31 (7)	0.232	0.95 (8)	0.372
Motivation	15.78 (7.00)	13.00 (6.59)	14.67 (6.06)	1.16 (7)	0.283	0.39 (8)	0.705
Restricted Interests and Repetitive Behavior	12.48 (5.90)	11.25 (8.24)	11.78 (7.93)	0.23 (7)	0.825	0.62 (8)	0.553
ADOS-2							
Calibrated Severity Score	4.80 (2.93)	5.11 (2.87)	–	−0.74 (17)	0.470	–	–
TIF-ASD							
Total Score	50.39 (12.20)	50.88 (15.01)	46.75 (10.75)	0.41 (7)	0.691	3.05 (11)	**0.011***
PSS-10							
Total Score	18.92 (7.00)	21.00 (8.81)	13.25 (8.42)	−1.05 (6)	0.332	2.72 (11)	**0.020***
CGI							
Severity Scale	5.32 (0.65)	3.95 (1.51)	4.33 (1.30)	−3.01[a]	**0.003****	−2.41[a]	**0.016***

*$p < 0.05$, **$p < 0.01$, [a]represents z-statistic from Wilcoxon signed ranks test; ADOS-2., autism Diagnostic Observation Schedule second edition; CGI, clinical global impression; df, degrees of freedom; M, mean; *p*, *p*-value (two-tailed); PSS-10, perceived stress scale ten item edition; SD, standard deviation; SRS-A, social responsiveness scale for adults; *t*, test statistic resulting from paired samples *t*-test; TIF-ASD, Trauma symptom investigation form in autism spectrum disorders.

($p = 0.003$) and from baseline to follow-up ($p = 0.016$). On the CGI-I, 52.4% of participants showed a clinical significant response at endpoint. At follow-up, 23.8% of the participants showed a significant response on the CGI-I. Chi-square analyses did not indicate a significant relationship between responder status on the CGI-I and participant's age (11-15 y; 12-18 y), gender, IQ (below average, average, above average) or severity of ASD symptoms (low, moderate, and severe).

Perceived stress (PSS)

In Table 2 results are shown from the paired samples *t*-tests comparing the total scores on the PSS-10 at baseline with the total scores at endpoint and follow-up. Total score of participants is above norm score for typical adults (14) at baseline (18.92) and at endpoint (21.00). At follow up, the score is reduced to 13.25, which is below the norm score of 14. No significant change in the PSS-10 was found at endpoint compared to baseline ($p > 0.05$), but a significant reduction on the PSS-10 was found from baseline to follow-up ($p = 0.020$).

Mixed model analysis showed no significant main effect of time on the PSS, (Estimate = –0.29 (0.21), 95% CI: −0.70-0.16) indicating no significant decrease in perceived stress by adolescents over the course of EMDR treatment.

As for the primary outcome measures, sensitivity analyses excluding participants of whom the EMDR treatment was interrupted due to the restrictions resulting from the COVID-19 pandemic did not alter conclusions for any of the secondary outcome measures.

TABLE 3 Correlations between mean change in SRS-A score and participant characteristics.

	Age	TIQ	Gender	Severity of ASD symptoms
SRS-A				
Caregiver-report	0.28	0.02	−0.36	0.03
Self-report	0.22	0.25	0.18	0.03

None of the correlations showed significance at α = 0.05 (two-tailed). ASD, autism spectrum disorder; SRS-A, social responsiveness scale for adults; TIQ, total intelligence quotient.

Discussion

This explorative study is, to the best of our knowledge, the first to investigate the effectiveness of EMDR in reducing core symptoms of ASD in adolescents, diminishing experienced stress, and improving global clinical functioning. In contrast to our main hypothesis, no significant reduction of core ASD symptoms was found on the

total score of the Social Responsiveness Scale (SRS) as reported by caregivers and participants, after 10 sessions EMDR on daily stress. However a significant reduction in the severity of total score of ASD symptoms and the subscales Social Awareness and Social Communication was found between baseline and follow up based on caregivers' report. On self-reported SRS, no significant reduction on the ASD symptoms was found. Results of pre- and posttest scores of total ASD symptoms measured by the ADOS-2, showed no reduction on ASD symptoms. On the contrary, on the secondary outcome measures for self-reported stress and global clinical functioning, clear improvement is shown. Participants indicated less perceived stress from baseline to follow-up, as hypothesized. Also in line with our expectations, significant global clinical improvement was shown in 52% of adolescents on the Clinical Global Impression Improvement Scale from baseline to end-measure and 24% from baseline to follow up measure.

Findings regarding the severity of autism symptoms in this study are partly in line with the outcome of the earlier study of Lobrecht van Buuren et al. (32), as these both indicated a decrease in severity of ASD symptoms. Lobregt van Buuren et al. found significant effects on total self-report SRS-scores in adult patients with ASD and PTSD, whereas our study found no decrease in self-reported ASD symptoms, but on caregiver-reported ASD symptoms at the SRS-A, from baseline to follow up. Possibly there may be a difference in the reliability of self-reported ASD symptoms between adolescents and adults with ASD. Results from earlier studies are mixed regarding the ability of individuals with ASD to identify experienced symptoms of ASD in themselves (53–55). This may be caused by limitations in formulating thoughts about feelings and emotions, especially for adolescents with ASD (56).

In this study, effects were only found from baseline to follow-up measurement. In other research trials this tendency of delayed onset of treatment effect is called a 'sleeper effect', due to the time that is needed to incorporate change and gain benefits (39, 57). Discussion on whether or not a "sleeper effect" can be detected, is ongoing (58). Future research is warranted to explore the presence of a sleeper effect in (EMDR) treatment in patients with autism.

Significant global clinical improvement (52.4% was very much improved or much improved on CGI measurement) was shown in this study. This means that patients showed progress in functioning in every daylife. This might be due to stress reduction, and so led to partial decrease of ASD symptoms, and improved global clinical functioning. This finding is in line with previous research on effects of Quality of life (QoL) on treatment effect (5, 59).

Although a methodological strength of this study is the inclusion of different outcomes measures to identify treatments gains (semi-structured therapist-child interaction, caregiver-ratings, self-reports, and clinician ratings), this explorative study has also some major limitations that need to be addressed: First, due to the uncontrolled study design, the results have to be interpreted with caution, because of the possibility of interference with non-specific treatment effects. Second, this study has a small sample size, which may have introduced the risk of false negative and false positive findings.

A third limitation concerns the relatively high number of missing data, which is mainly caused by the fact that severely affected participants were included, who sometimes felt unable to carefully respond to all the weekly report forms. Also, it is important to mention that, in research literature, questions are raised about the usefulness of SRS subscales to measure treatment effects (38, 39, 60). Multiple studies using the SRS compared to other instruments, have shown that ASD symptom clusters are highly correlated (61, 62). The reliability of interpreting scores on subscale level is therefore debatable and should be done with caution (37). Furthermore, although all EMDR therapists were trained and supervised during the study, therapist adherence (as in the extension to which all therapists used the same therapeutic techniques) was not officially monitored. Lastly, no longer term follow-up assessment was performed in this study. It is therefore not possible to reflect on treatment effects on the longer term, in the light of the earlier mentioned "sleeper effect."

In general, the results of this exploratory study provide meaningful insights, that substantiate further investigation of the application of EMDR treatment for stress reduction in adolescents with ASD.

Preferably, future studies should use a randomized control study design, but also multiple case studies designs could be of interest. It is of great importance to collect information on several follow up periods, minimize the number of missing data, monitoring therapist adherence, assessing the usability of more generic outcome measures, and looking for applicability of the treatment on a younger age. Additionally, experienced based sampling methods using modern technology (e.g., app's) which allows almost realtime measurements of stress levels would be of interest. Also it can be valuable to collect qualitative and quantitative data about adolescents' and parents satisfaction about method and results. This may give us more detailed information about what works in helping children with ASD to experience less stress in their daily life, and thereby hopefully increase their possibilities in social functioning.

Conclusion

This exploratory uncontrolled study indicates that EMDR on daily stress and confusion decreases perceived stress in adolescents with ASD, and may improve their global clinical functioning (i.e., the impact of the symptoms on the patient's ability to function). EMDR treatment has a partial and delayed effect on decreasing core symptoms of ASD.

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 Medisch Ethische Commissie Oost Nederland (Radboud University Nijmegen). Research was conducted in accordance with the World Medical Association's Declaration of Helsinki. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

EL wrote the first draft of the manuscript. IB-S supervised and wrote the data analyses. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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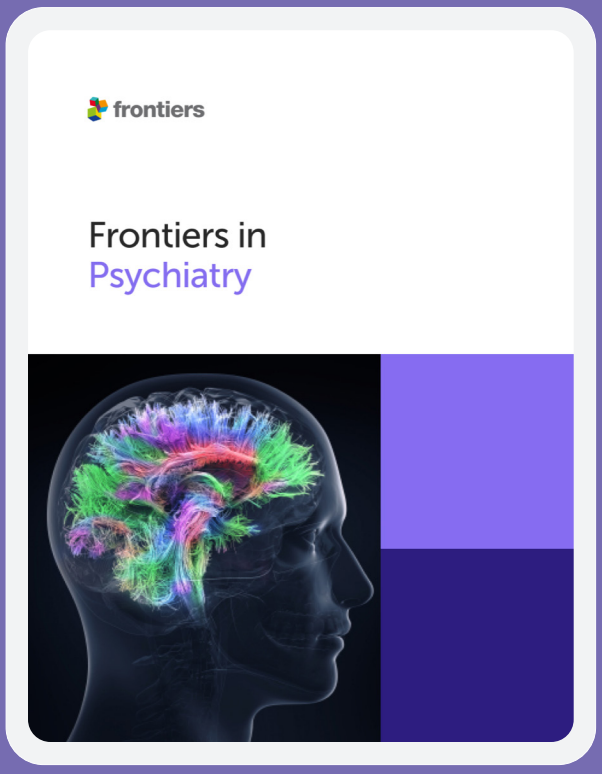

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