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Autism Spectrum Disorder

Profile, Heterogeneity, Neurobiology and Intervention

Edited by Michael Fitzgerald

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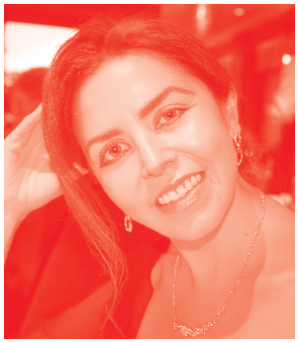

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Autism Spectrum Disorder - Profile, Heterogeneity, Neurobiology and Intervention
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Edited by Michael Fitzgerald

Contributors
Maria Rhode, Kate Grayson, Shaheen Akhter, Michael Beenstock, Harumi Jyonouchi, Lee Geng, Astrid Vicente, João Xavier Santos, Celia Rasga, John Rothman, Yulia Furlong, Michael Fitzgerald

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Meet the editor

Professor Michael Fitzgerald was the first Professor of Child and Adolescent Psychiatry in Ireland, specialising in autism spectrum disorders (ASDs). He has diagnosed more than 5000 persons with ASDs. He has written many peer-reviewed publications and authored, co-authored and co-edited thirty-four books, some of which have been translated into Japanese, Dutch, and Polish. Professor Simon Baron-Cohen described one of Professor Fitzgerald's books on autism as, "The best book on autism", and described him as an "exceptional scholar". He has lectured extensively throughout the world, including at The Royal Society/British Academy and the British Library in London. He was the overall winner of the "Excellence in Psychiatry" Award in 2017 and was nominated as one of the top four psychiatrists by Hospital Professional News Ireland. Professor Fitzgerald recently retired to spend more time in Brussels and continues to write on autism.

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Preface

Autism spectrum disorder (ASD) is one of the most heterogeneous conditions in psychiatry and clinical psychology. To begin to understand autism, one must embrace complexity and ever-increasing complexity. This makes it difficult for professionals to keep up to date with all aspects of autism, thus the need to bring together various aspects of this condition in new books. Controversial problems include the lack of biomarkers and the lack of sub-types, as discussed in Chapter 1 by Michael Fitzgerald. This chapter also focuses on the problem with truly scientific screening and diagnostic instruments and indeed the 'gold standard diagnosis' in autism is a clinical diagnosis by an expert in autism.

Co-morbidity is common and central to the presentation of autism in clinical practice. In relation to co-morbidity, epilepsy is an important comorbidity in the presentation of autism as demonstrated in Chapter 2 by Akhter Shaheen. Autism without some co-morbidity is uncommon. Factors associated with this co-morbidity are many and include multiple genetic and environmental factors. Shaheen also explores genomic copy number variants and metabolic disorders and concludes on the importance of metabolic factors in the pathogenesis of the ASD–epilepsy connection.

Chapter 3 by Yulia Furlong covers the 'hot topic' of autism and gender, which has been much discussed by psychiatrists and psychologists, particularly those in the justice system. This chapter provides the most current thinking on this particularly important topic with a focus on diagnosis, occurrence rates, aetiology, informed consent, legal issues, and treatment.

Section 2 examines the neurobiological aspects of autism.

Chapter 4 by Michael Beenstock examines parenting and reproductive stoppage in ASD. The author distinguishes between absolute non-stoppage when parents have no further children and relative non-stoppage when they have fewer children. Both types of non-stoppage vary with the age of diagnosis.

Chapter 5 by Harumi Jyonouchi and Lee Geng focuses on the association between monocyte cytokine profiles and co-morbid conditions in ASD. The authors point out that the presence of co-morbid medical conditions may hold a key to assessing pathogenesis in markedly heterogeneous ASD conditions. Unfortunately, these are not properly sought for in routine ASD evaluation of children. The authors describe their research in this area with a focus on GI symptoms, allergic rhinitis, sleep disorders, and paediatric acute-onset neuropsychiatric syndrome, among other conditions. They concluded that there is an association between monocyte cytokine parameters and specific co-morbid medical conditions existing in ASD subjects studied.

Chapter 6 by John Rothman examines the role of catecholamines in the aetiology of autism and a proposed therapy. The author notes a growing body of evidence supporting the role of catecholaminergic dysfunction in the core symptoms of ASD.

He suggests that exerting a presynaptic effect to inhibit tyrosine hydroxylase and thus the synthesis, storage, and release of all catecholamines Li-79 (a tyrosine hydroxylase inhibitor) may diminish neurotransmitter release and its associated growth factors, exerting a therapeutic effect on ASD.

Chapter 7 by João Xavier Santos, Célia Rasga, and Astrid Moura Vicente examines exposure to xenobiotics and gene-environment interactions in ASD. The xenobiotics include air pollutants, persistent organic pollutants, pesticides, and so on. This systematic review suggests neuropathological mechanisms including oxidative stress and dysregulation of signalling pathways. This line of research opens novel and important perspectives to future prevention and personalised interventions for ASD.

Section 3 covers interventions in ASD.

Chapter 8 by Maria Rhode and Kate Grayson describes an observationally and psychoanalytically informed parent/toddler intervention for young children at risk of ASD. The chapter discusses a recent randomized controlled trial on a parent-mediated intervention that demonstrates that supporting parental confidence is essential to improvement. Study samples were between eighteen and twenty-four months old and were in the high-risk category of the Checklist for Autism in Toddlers (CHAT). This study showed that a significantly lesser proportion of treated children were later diagnosed than the CHAT would predict and that the treatment merits further study, with higher numbers.

I would like to thank the Author Service Manager, Maja Bozicevic, who was always most helpful.

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Section 1

Profile, Controversial Issues, Gender and Co-Morbidity in Autism

Chapter 1

Introductory Chapter: Controversial Issues in Autism - Research and Practice

Michael Fitzgerald

1. Introduction

The major reason why there are so many controversial issues in autism is because it is such a heterogeneous condition. There are no sub-types of autism at this time, despite almost fifty years of searching for them. This quest continues but there is doubt whether it will succeed. There are no diagnostic biomarkers in autism or indeed, in psychiatry as a whole. This in a way, is what distinguishes psychiatric diagnoses from medical diagnoses like diabetes. Of course, there are many conditions associated with autism like tuberous sclerosis, but that is quite a different matter to having a biomarker specifically for autism. There is also a great deal of heterogeneity in relation to aetiological factors. There are a large amount of genes involved of small effect, associated with autism and the number is continually increasing, but there is no specific pattern of genetic findings for autism and unlikely to be again because of heterogeneity. We need new research paradigms for research in psychiatry. The medical model will not suffice.

2. Heterogeneous issues in autism

2.1 Validity

Validity is probably the biggest problem in psychological and psychiatric research on autism. Validity of diagnostic categories is probably the weakest link in research in this area.

2.2 Screening and diagnosis in autism

Charman and Gotham [1] state that, 'we're limited by the lack of a true test for autism spectrum disorder'. the same goes for screening instruments. One of the most widely used screening incidents, the M-CHAT [2] (modified checklist) according to Peter Hess [3], 'misses a majority of autistic children at eighteen months, and this failure was particularly in those with average IQ'. Hackethal [4] stated that the M-CHAT, 'misses the majority of children with ASD'. In terms of diagnostic instruments for autism, Charman and Gotham [1] state correctly that, 'expensive ASD-specific diagnostic instruments will not always be appropriate'. Indeed, NICE [5] recommend no specific instrument for clinical diagnosis and state that clinical diagnosis of autism should be done by a clinical expert in autism, which usually will mean a psychiatrist, psychologist or specialist paediatrician. Charman and Gotham [1] state that, 'professionals must be realistic about

the limitations of diagnostic instruments: ultimately, these measures cannot solve a difficult diagnostic decision, and they may not be universally necessary. Experienced clinical judgement is essential for a correct diagnosis'. This would be in line with the NICE [5] Guidelines. The ADI-R (Autistic Diagnostic Instrument Revised) [6] and ADOS (Autism Diagnostic Observation Scale) [6] criteria to define autism are very narrow concepts of the disorder. I see many parents who come to me in great distress knowing that their child has autism and that the school also observed this, but having been told that their child did not have autism according to the ADI-R. This instrument is not appropriate to making a sole diagnosis of autism in clinical practice. It not uncommonly misses high functioning autism. In addition, Ventola et al., [7] have shown that the ADI-R was significantly under-diagnosing toddlers. How biased and unrepresentative the patients in this survey can be seen by Professor Gillian Baird's work [8] on autism in the general population. Indeed, using these narrow criteria gives a prevalence of autism of 25 per 10,000. When you use the broader autism spectrum, you get a truer rate of 116 per 10,000. One of the problems also is that the National Institute for Health Care Excellence, [5] Guidelines on the diagnosis of autism which are accepted throughout the world, are not followed. Professor Dorothy Bishop, Professor of Developmental Neuropsychology at the University of Cambridge told Adam Feinstein, [9] that, 'if it could be shown that there were real benefits in accuracy of diagnosis from adopting this lengthy procedure, then I would be happy to say 'okay', but the originators of the instrument have never demonstrated this – it is more an article of faith with them' [9].

The problems with different criteria for diagnosis was demonstrated by Fitzgerald et al., [10] from a sample of 309 persons referred with, 'autistic tendencies', found that 272 met criteria for autism on the Autistic Disorder Diagnostic Check List by Lorna Wing [11] which was the predecessor of the DISCO (Diagnostic Interview for Social Communication). 144 met ICD 10 criteria [10]. Kanner and Eisenberg, [12] five criteria gave a number of 24 with autism while Kanner and Eisenberg's two criteria gave a diagnosis of 220 [10].

Steven Hayman, former Director of The National Institute of Mental Health in the United States stated that the diagnostic and statistical manual enterprise was, totally wrong... an absolute scientific nightmare. Many people who got one diagnosis, got five diagnoses, but they don't have five diseases – they have one underlying condition', [12]. Insel [12] said that the psychiatric diagnosis, 'have no reality. They are just constructs', and he wanted a, 'diagnostic system based upon biological foundations'. While females with autism are more likely to be missed, there is the paradox that women are more likely to be given a psychiatric diagnosis with less physical tests done on them, then males and more physical diagnosis to be missed [13]. ASD diagnosis, 'lacks biological and construct validity' [14].

Replication of research findings in autism is and continues to be quite a problem. Replication is central to our understanding of science. Indeed, we usually require multiple replications before we would feel that a finding is secure. Ioannidis [15] stated that, 'there is increasing concern that most current published research findings are false'. There are problems with study power, sample size, data mining and hypothesis being tested at the end of a study which weren't there in the beginning. Bottema-Bentel et al. [16] studied autism treatment studies in one hundred and fifty papers and found that only 6% of them with genuine conflicts of interest admitted to them in the original paper and could not complete their statistical analysis on these papers because there was insufficient numbers of high quality papers. The authors of this paper were equally concerned about the lack of mention of adverse events, and only 7% of the one hundred and fifty studies examined adverse events [16].

The publication of only positive findings and the more important point, the non-publication of negative findings has been a serious journal problem for a long time. In science, negative findings are as important as positive findings.

2.3 Psychology of autism

There are problems with TOM (problems with theory of understanding other peoples' minds) and autism. TOM deficits have been a dominant theory of autism for many decades. It is mentioned endlessly by psychologists and psychiatrists and has been presented as a, 'fact'. The situation is much more complicated. It is not as universal as the theory proponents have suggested and there is considerable non-replication. This has been reviewed by Gernsbacher and Yergeau [17]. They pointed out that this stereotyping has been particularly damaging for persons with autism. It has stigmatised them. The neurodiversity movement has challenged this stigma [18]. The deficit in TOM undermines persons with autism's independence, truthfulness and trustworthiness and makes them unsuitable for many jobs. This is unhelpful and isolates them and leads to further social exclusion.

2.4 Vaccines and autism

The false association between the MMR vaccine and autism has been by far, the most damaging research carried out in this area. It was published in a prestigious journal, The Lancet [19]. It led to a crisis in vaccination with many children not being vaccinated and an increase in deaths.

2.5 Problems with peer reviewed papers

Peer reviewed papers are polished up at the writing stage to make them more persuasive then they are in reality. The rhetoric of a paper is very important, and style of writing does matter, but has been given little attention in the literature. A professional scientific editor can make quite a difference to a paper because it will then come across much better. If the paper is on a current, 'Hot Topic', it will arouse more editorial interest and will probably be more likely to be published. Of course, funding bodies for research are also likely to have a special interest in these, 'Hot Topics', and many of the same persons who are on the funding organisation will later review the papers at the publication stage.

3. Conclusion

The greatest problem in autism research and indeed, psychiatry and clinical psychology generally is heterogeneity. It is impossible to get two samples of subjects completely identical and therefore, there are problems with generalisations to the general population. There was an attempt to establish, 'pure autism', with diagnostic instruments like ADI, but this was never possible to do and was never likely to be accomplished. The concept of, 'pure autism', does not exist. We have to develop more psychiatric and psychological models to deal with research in autism.

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Chapter 2

Epilepsy: A Common Co-Morbidity in ASD

Shaheen Akhter

Abstract

ASD and epilepsy, two common co-occurrent conditions, may appear in a developing brain in various genetic and non- genetic syndromes. The fact that multiple genetic and epigenetic factors, metabolic diseases, environmental factors and epileptic encephalopathies are related to the causation of both ASD and epilepsy indicate the presence of some common underlying pathophysiologic mechanisms. Although many questions are yet to be answered, recent studies suggest that synaptic aberrant connectivity and disruption of the delicate balance between neuronal excitation and inhibition (E/I imbalance) leads to various aspects of neuronal dysfunction. The presence of intellectual disability increases the likelihood of co-morbid ASD and epilepsy and all these associations greatly affect the quality of life of these children as well as their families. Therefore, understanding the genetic, cellular and molecular basis of relationship between these common co-morbid conditions is fundamental in planning appropriate and prompt management of these children. Future researches will as such continue to address the pathophysiology underlying the genetic, chromosomal, metabolic-mitochondrial disorders and environmental factors related to these co-morbidities as well as preventing them. Thus, it will lay the base of focused investigations and targeted management in this field.

Keywords: ASD, epilepsy, co-morbidity, intellectual disability, genetics, metabolic

1. Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by deficits in social-communication interaction and restricted and repetitive behaviors [1]. It covers a wide variation in clinical presentation, symptom severity, and cognitive ability. These symptoms are present from early years of life.

Epilepsy is characterized by an enduring tendency to produce epileptic seizures and practically defined as having two untriggered seizures occurring at least 24 hours apart [2]. Associated comorbidities are very common in ASD. Among them, epilepsy is an important medical condition that could affect the lives of persons with ASD. Kanner's original paper in 1943 describing 11 children with "autistic disturbances of affective contact," included one child with history of seizures and abnormal electroencephalogram (EEG) [3]. Since then, the relationship of autism to epilepsy has been an area of interest for scientists for decades.

Association between autism and epilepsy has now been recognized and well established. Prevalence of epilepsy in autism ranged from 5–46% in quite a large number of studies [4–10] which exceeds than that of the general population

(0.6–1%) [11–12]. This wide range is likely due to heterogeneity of groups being studied, particularly with regard to cognitive functioning of the participants, sample age range, sex and inclusion or exclusion of other co-occurring medical conditions [8].

In addition, methodological differences used for diagnosing ASD also lead to these differences in prevalence rate. In the earlier studies, only severe autism associated with a high rate of intellectual disability (ID) and a high rate of epilepsy were included, whereas more recent studies that have used the current broader "ASD" criteria (DSM-5) might lead to a lower rate of ID and a lower rate of epilepsy.

Several factors have been associated with a greater risk for developing epilepsy. Among them, ID is the single most common risk factor. Amiet et al., in a meta-analysis on epilepsy in autism encompassing articles from 1963–2006 demonstrated a relationship between epilepsy in autism with ID and gender. Here epilepsy was present in 21.5% of subjects with autism who also had ID and 8% of subjects without ID [9].

Previous studies in the ASD population have found that idiopathic ASD (i.e., no known cause) had a lower risk of developing epilepsy than those with syndromic autism (i.e., associated with underlying neural/genetic abnormalities) [13]. Pavone et al. found epilepsy in only 7.4% with idiopathic ASD compared to 55% of patients with syndromic ASD [14]. These findings match with the hypothesis that one abnormal neural dysfunction may make the brain more susceptible to another neurological dysfunction [15]. This also supports the hypothesis that epilepsy, ID and ASD may all be the result of a mutual underlying neurological condition. Equally, children with epilepsy also have an increased risk for being diagnosed with ASD [8, 16].

Besides, on average, autistic adults with epilepsy have, less cognitive ability and weaker daily living skills than their autistic peers who do not have seizures [17, 18]. There is also a strong influence on the quality of life and well-being in children with epilepsy.

Because of these facts, clinicians and researchers have worked to understand how epilepsy and ASD can relate to each other. Studying the two disorders in combination may help in understanding their genetic, molecular, and cellular mechanisms that are critical in the field of appropriate management of the both [19, 20].

2. Age of onset of epilepsy

Differing results have been found regarding the age of onset of epilepsy in ASD. Most of the researchers found seizure onset during early childhood [5, 21, 22]. However, Bolton et al. in a long-term follow-up study of 150 individuals with autism, found epilepsy onset in the majority of cases over 10 years and some in adulthood [7], this also agrees with few other studies [6, 23]. While others found, two peaks in the age distribution of seizure onset in autism, one in early childhood and a another in adolescence [13, 24]. Epilepsy persists in adulthood in up to 80%, with remission in about 16% in ASD.

3. Sex

Reports have suggested that females with autism have higher rates of epilepsy than males [6, 8, 13, 16, 22, 25, 26]. Amiet's study reported prevalence of epilepsy, in autistic females 34.5% versus 18.5% in autistic males [9]. Studies have reported a more frequent association of epilepsy in persons with ID than without ID [6, 9]. Since females

with autism tend to have more severe ID compared to males [9, 27], greater ID severity might be a possible cause for this high prevalence of epilepsy in autistic females [28].

4. ID and epilepsy in autism

Intellectual disability (ID), referring to general intelligence and adaptive functioning below −2 standard deviations for population norms, occurs in about 38% of children with ASD [29].

A key concept that has developed during the past 40 years is the strong association between intellectual disability and a higher prevalence of epilepsy in individuals with ASD [25]. Amiet et al. carried out a meta-analysis from published reports between 1963 and 2006 on autism and epilepsy to assess the relative risk of epilepsy in autism with respect to ID [9]. The pooled prevalence of epilepsy with and without ID was highly significant (21.5% versus 8% respectively). This study also highlighted on the association of increasing ID severity on the prevalence of epilepsy in autism [9, 30].

Other authors also have argued that intelligence mediates the relationship between autism and epilepsy [17, 26, 30–34], and lower the intellectual ability, the higher the prevalence of epilepsy and autism. According to Viscidi et al. al, low IQ is the best predictor of epilepsy in children and also commented that the presence of ID can guide prognosis and alert physicians regarding who are at increased risk for epilepsy [6]. A recent study on 6975 children by Jasua et al. with ASD found ID alone as an independent predictor for the increased prevalence of epilepsy [19, 20].

The high rate of co-occurrence of ID, epilepsy and ASD suggests potentially shared underlying mechanisms. All three could result from the same pathophysiologic mechanisms. Therefore, it may be more likely to occur in genetic conditions that lead to abnormal excitability and disrupted synaptic plasticity, such as fragile X syndrome, neuroligin 2 mutations, Rett syndrome, tuberous sclerosis complex, cyclin-dependent kinase-like 5 (CDKL5) mutations, and "interneuronopathies" resulting from aristaless-related homeobox, X-linked (ARX), all of which include ASDs, IDs, and epilepsy [35].

5. Etiology and pathogenesis

The high co-occurrence of autism and epilepsy has led to the speculation that there are some common mechanisms linking these two types of disorders. But a singular *pathophysiological* mechanism responsible for the seizures and autistic phenotype is unlikely. Scientists have stressed mainly upon the genetic factors as the most common contribution for this co-occurrence followed by environmental and metabolic conditions.

Buckley and Holmes have conceptualized ASD and epilepsy both as disorders of aberrant connectivity caused by multiple genetic and environmental factors [36]. Chromosomal abnormalities [37], metabolic conditions [38, 39], environmental factors, e.g., maternal rubella during pregnancy [40], and brain damage via neonatal jaundice are examples that have been recognized as predisposing to both epilepsy and autism [41].

5.1 Genetic factors and syndromes

ASD and epilepsy are both described in various genetic syndromes, which includes single and common gene mutations as well as undiscovered rare mutations

and copy number variations [36, 42]. Both ASD and epilepsy can be understood as disorders of synaptic plasticity, where the same pathological mechanisms result in developmental imbalances of excitation and inhibition in the developing brain.

This genetically-derived abnormal plasticity can result in both ASD and epilepsy. Examples are fragile X, Rett syndrome, tuberous sclerosis complex (TSC), CDKL5 mutations, neuroligin mutations, "interneuronopathies" that results from X-linked aristaless-related homeobox (ARX) and Neuropilin 2 (NRP2) gene mutations. Moreover, the process of epileptogenesis and/or spontaneous seizures may result in maladaptive synaptic plasticity and produce imbalances of excitation and inhibition. All these processes might contribute to behavioral and learning difficulties. Alterations in receptors, signaling molecules or neurotropins may also result in synaptic abnormalities. Early-life seizures due to genetic conditions may be associated with both ASD and epilepsy (**Figure 1**).

Synaptic plasticity is the process whereby, the connections between 2 neurons of the synapses, get strengthened by experiencing or practicing. When these connections (i.e., synapses) are activated, AMPA receptors mediated by depolarization blocks release of magnesium and helps in entry of calcium through the NMDA receptors. This stimulates calcium dependent activation of kinases and other signaling pathways and enhances gene transcription and trafficking of receptors. This results in faster and stronger synaptic connections. This is known as long-term

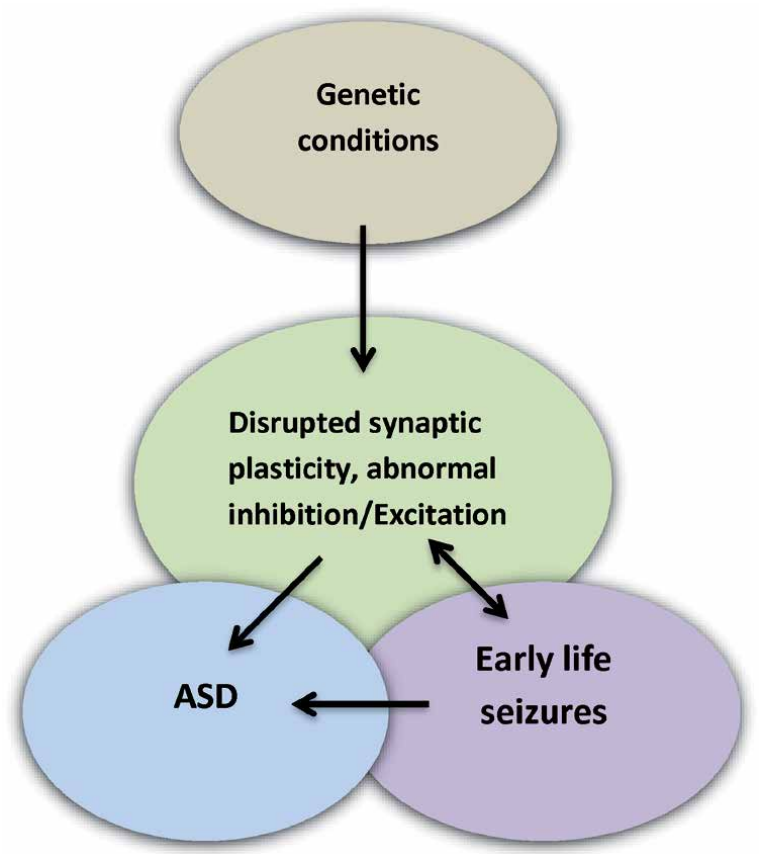

Figure 1.
Abnormal excitability and disrupted synaptic plasticity in the developing brain result in both ASD and Epilepsy. This abnormal plasticity can result from different genetic conditions. Early life seizures during early post-natal development may also alter synaptic plasticity and results in ASD. Mechanisms lie in alterations in receptors, signaling molecules or neurotrophins.

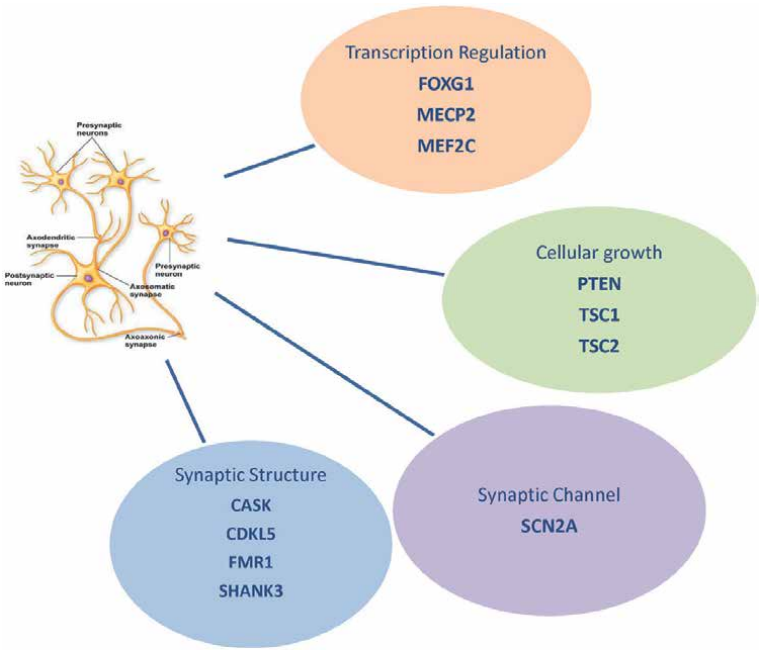

Figure 2.
Four important biological pathways for neuronal development and function common to autism spectrum disorder and epilepsy, that includes transcriptional regulation (FOXG1, MECP2 and MEF2C), cellular growth (PTEN, TSC1, and TSC2), synaptic channels (SCN2A), and synaptic structure (CASK, CDKL5, FMR1, and SHANK3).

potentiation and, is believed be the cellular basis of learning. In some of the genetic conditions associated with autism and epilepsy, variety of genes are disrupted upon which synaptic plasticity depends. These include cyclin-dependent kinase-like 5 (CDKL5) in West syndrome, MeCP2 in Rett syndrome, FMRP in fragile X mental retardation syndrome, mTOR in tuberous sclerosis, and reelin in lissencephaly.

Knowledge of copy number variation and single gene disorders that are disturbed in these two developmental disorders include gene transcriptional regulation; cellular growth and proliferation; and synapse development, stability, and function. An overview of biological common pathway of ASD and epilepsy are shown in **Figure 2**.

5.1.1 Single gene disorders

5.1.1.1 Fragile X syndrome

Fragile X syndrome (FXS) is the most frequent form of genetic disorder causing ID and often presents with ASD and epilepsy. It occurs when a triplet repeat (CGG) expansion leads to inactivation of the FMR1 gene which is responsible for coding of FMRP- fragile X mental retardation protein. FMRP is associated with and regulates various mRNA related to development and functions of dendritic spines, axons and synapses, formation and wiring of neuronal circuits and plasticity of brain. It also regulates metabotropic glutamate receptor (mGluR)-induced long-term depression (LTD). As the "mGluR theory of fragile X" postulates that FMRP and group I metabotropic glutamate receptors (mGluRs) play oppositional roles at the level of synaptic function, loss of FMRP function and activation of mGluRs lead to excessive AMPA receptor internalization, exaggerated LTD and therefore, disrupted synaptic activity. Bianchi et al. provided compelling evidence that a voltage-gated

inward current, ImGluR (V), is the cellular basis for the epileptogenic behavior induced by activation of the mGluR5 receptor [43, 44]. In addition, dysregulation of glutamergic neurons in FXS can disrupt the normal actions of inhibitory GABAergic neurons, and downregulation of GABA receptor subunits and altered expression of a number of enzymes involved in the metabolism of GABA. Identification of this mechanism could contribute to hyperexcitability and epilepsy in the fragile X syndrome [45].

Physical features include prominent ears, long face, macrocephaly, and macroorchidism. The cognitive profile includes hyperactivity, anxiety, tactile defensiveness, gaze avoidance, and socialization difficulties. Epilepsy is reported in approximately 10–20% of individuals with FXS [46]. Seizure patterns in FXS typically resemble benign focal epilepsy of childhood (BFEC). Moreover, 23% of individuals with FXS without clinical seizures demonstrated centrotemporal spikes on EEG.

5.1.1.2 Tuberous sclerosis complex

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder that results from mutations in the *TSC1* or *TSC2* genes [47]. Although skin, kidney, heart, eye, and lung can be affected, involvement of the brain is associated with most significant morbidity. Central nervous system is consistently involved, with 90% of individuals affected showing structural abnormalities, and almost all having some degree of CNS clinical manifestations [48].

TSC1 and *TSC2* genes, found in chromosomes 9 and 16, are responsible for encoding two proteins namely hamartin and tuberin respectively. They bind together to form a protein complex which in turn regulates the mammalian target of rapamycin (mTOR). The loss of function mutation in either of the two genes results in overactivity in mTOR signaling cascade with consequent disinhibition of protein synthesis and cell growth. A simplified diagram in **Figure 3** shows the activation of mTOR cascade [48]. This shows the underlying brain dysfunction resulting in susceptibility to epilepsy, autism and cognitive impairment.

Cortical tubers constitute the hallmark of the disease and are pathognomonic of cerebral TSC. The number and localization of cortical tubers may account for the variability of the neurological phenotype observed in TSC patients [49]. Autism appears to be more common in infants with frontal and temporal tubers, and it has been suggested that an early dysfunction in the associative areas owing to the location of cortical tuber may be responsible for the autistic features [49]. Tuberin, the product of TSC2 gene is expressed to a large extent in frontal and temporal regions of brain- the areas that are responsible for the behavioral phenotypes of the autistic disorder [50].

Epilepsy is the most common presenting symptom in tuberous sclerosis complex. In up to 80% to 90% of persons with TSC, seizures will develop during their lifetime, with the onset most frequently in childhood. Approximately one-third develop infantile spasms. Almost all seizure types can be seen in persons with tuberous sclerosis complex, including tonic, clonic, tonic–clonic, atonic, myoclonic, atypical absence, partial, and complex partial. Only "pure" absence seizures are not observed [51].

Epilepsy in TSC is often medically intractable. The treatment of seizures in TSC is often difficult but efficacy of Vigabatrin in children has proved to have best results.

Although mutations in both TSC1 and TSC2 are associated with development of autism, TSC2 mutation has greater likelihood of developing ASD [52]. Again, early-onset and difficult to control infantile spasms, especially if there is an epileptic focus in a temporal lobe, carry an increased likelihood of getting ASD in a

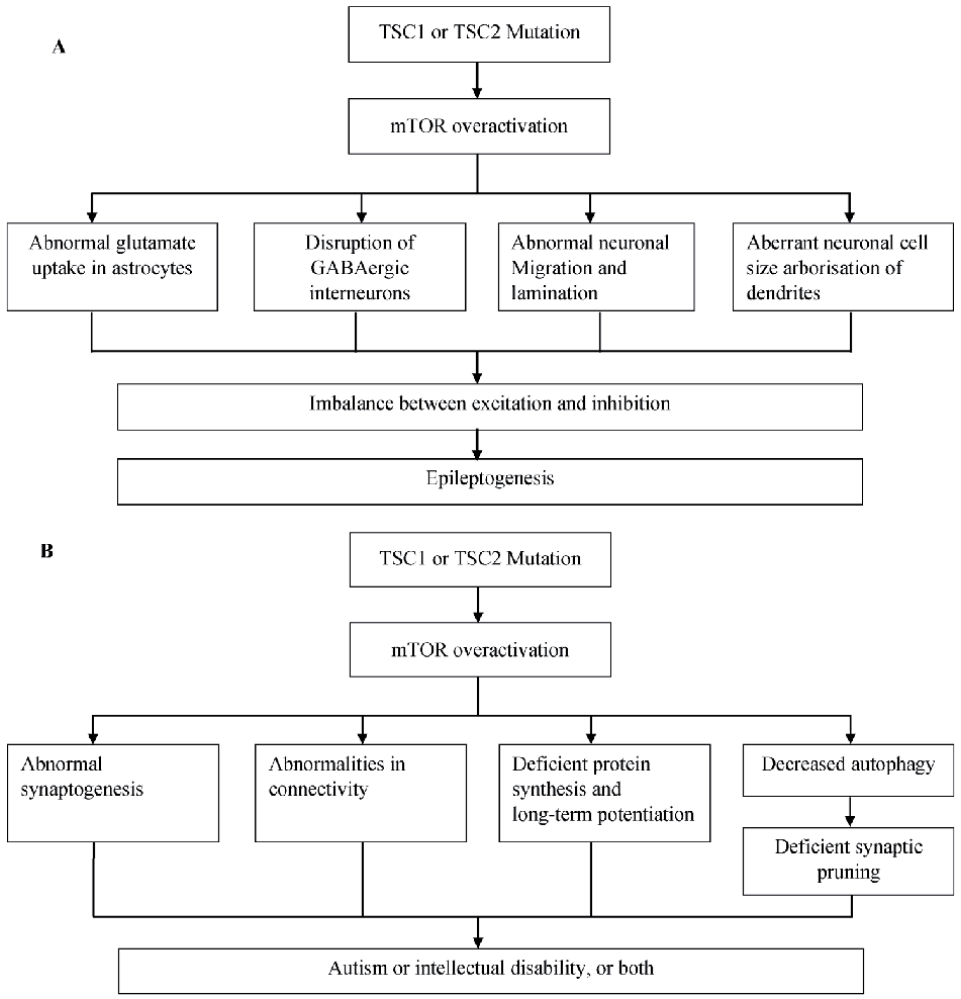

Figure 3.
Schematic representation of the potential roles of mTOR overactivation in determining the neurological and neuropsychiatric manifestations of tuberous sclerosis. (A) mTOR overactivation can dysregulate the balance between neuronal excitation and inhibition, leading to epileptogenesis. (B) mTOR overactivation can alter synaptogenesis and synaptic pruning, connectivity, and long-term potentiation, leading to an increased susceptibility to autism or intellectual disability, or both. mTOR=mammalian target of rapamycin. Courtesy of: Curatolo P, Moavero Vries P J. Neurological and neuropsychiatric aspects of tuberous sclerosis complex. Lancet Neurol 2015; 14: 733–45.

child. Because early onset of infantile spasms and associated hypsarrhythmia may have a malignant effect on brain development in infants with TSC, the importance to search for ways to anticipate the onset of infantile spasms before they become apparent as seizures is very important [53].

Rapamycin normalizes the dysregulated mTOR pathway, and recent clinical trials have demonstrated its efficacy in various TSC manifestations, suggesting the possibility that rapamycin may have benefit in the treatment of TSC brain disease.

5.1.2 PTEN

PTEN is a tumor suppressor gene that encodes a phosphatase affecting G1 cell cycle arrest and inhibiting the PI3K–AKT–mTOR pathway, which has roles in controlling cell growth, survival and proliferation [54, 55]. ASD and macrocephaly and have been reported in children with germline PTEN mutations. PTEN-related

ASD is, therefore, emerging as one of a group of megalencephaly disorders associated with dysregulation of the PI3K–AKT–mTOR pathway [56]. In patients with PTEN mutations, seizures have been reported, in whom focal cortical dysplasia has also been reported [57]. Epilepsy seems to be a part of the phenotype for many of the megalencephaly disorders that are associated with impaired regulation of the PI3K–AKT–mTOR pathway [56] but the exact roles of mutations in these specific genes with and their relation to seizures and ASDs are not clarified.

5.1.2.1 MECP2-related disorder (Rett syndrome)

MECP2-related disorder, result of an X- linked loss- of- function mutation of MECP2, starts presenting with regression typically at 6 to 18 months of age after a period of apparently normal development. Females are predominantly affected with this disorder which is manifested with ID, postnatal microcephaly, loss of spoken language, and stereotypic hand movements. Besides autistic symptoms individuals with MECP2-related disorder may present other symptoms like respiratory rhythm abnormalities, gait impairment, and cardiac complications as well. Approximately 50–90% of children are reported to have seizures, the type of which is variable [58]. The age of onset of seizure is rarely before 2 years of age, and the severity appears to decline after adolescence.

MeCP2 acts, at least in part, as a transcriptional repressor during brain development. And it may be required to reduce aberrant transcriptional events, thus allowing the transcriptional machinery to function efficiently. In addition, it has been suggested to have a function in synaptogenesis or maintenance of neuronal function. The onset of Rett syndrome at 6 to 18 months, coincides with a period of widespread synaptogenesis in the human brain [59], which is compatible with the view that RTT could be caused by failure to form synapses appropriately. Evidence supporting a role for MeCP2 in synapse formation includes altered glutamatergic synapse numbers in vitro and in vivo and changes to neuronal morphology in some brain regions. These findings suggest that long-term changes occur in neuronal networks in the MeCP2-deficient brain [60].

5.1.2.2 CDKL5-related disorder

CDKL5-related disorders are X-linked condition, manifest early in life with epilepsy, usually infantile spasms, postnatal microcephaly and severe neurodevelopmental problems. Girls with mutations in CDKL5 display various ASD features including abnormal social interactions, repetitive movements, and absent speech. However, the developmental disability and the epilepsy phenotype associated with this condition are much greater than those typically seen in children with classical forms of ASD.

CDKL5 is a key-limiting factor in regulating synapse formation. To exert its role CDKL5 binds and phosphorylates the cell adhesion molecule NGL-1. This phosphorylation event ensures a stable association between NGL-1 and PSD95 (key candidates in ASD pathogenesis) in glutamatergic post synapses during dendrite spine development and generates significant role in stabilizing the postsynaptic membrane [61].

5.1.2.3 FOXG1-related disorders

FOXG1-related disorders are associated with epilepsy, severe ID, absent speech with autistic features. Children may present with duplications on chromosome 14q12 or mutations of FOXG1. Children with duplication of 14q12 often present

with infantile spasm followed by ID with autistic features [62]. These patients may also present postnatal microcephaly, morphologic abnormalities of corpus callosum and choreiform movements. The mean age at epilepsy onset for children with deletions/loss-of function mutations of FOXG1 is 22 months. FOXG1 is a brain-specific transcriptional repressor protein that regulates neurogenesis.

5.1.2.4 MEF2C-related disorder

These are extremely rare genetic disorder caused by a in the *MEF2C* gene. This mutation, often a deletion, leads to the dysfunction of MEF2C protein which is essential to the proper functioning of the neurological system in addition to other systems.

Patients with mutations and deletions of MEF2C on chromosome 5q14.3 may present with severe ID, epilepsy, and stereotypic movements. Autistic features have been recognized with some overlap with features found in MECP2-related disorder with a very small deletion encompassing the MEF2C gene [63]. This The epilepsy found in individuals with MEF2C-related disorder can be variable, with 20% presenting with infantile spasms, 33% presenting with infant-onset myoclonic epilepsy, 24% presenting with childhood onset generalized epilepsy. *MEF2C* is essential for early neurogenesis, neuronal migration and differentiation.

5.1.2.5 CASK-related disorders

CASK-related disorders are genetically defined neurodevelopmental syndromes that includes ASD, ID, ADHD as well as epilepsies. CASK encodes for calcium/calmodulin-dependent serine protein kinase (CASK), located on chromosome Xp11.4, in which pathogenic variants underlie a range of NDDs.

Mutations affecting CASK were first described in cases with microcephaly with pontine and cerebellar hypoplasia (MICPCH), followed by the identification in cases with X-linked ID (XL-ID), developmental delay (DD), and ASD. But ASD diagnosis here is difficult because of the presence of the severity of impairment and ID.

CASK is expressed with high expression in the developing human brain and has a role in synapse formation and cortical development. Reduced CASK protein levels affect presynaptic development and decrease inhibitory pre-synapse size, which might have consequences to E/I balance in developing neural circuitries. Aberrant E/I balance, and synaptogenesis are two common biological pathways that underlies the NDDs of different genetic origin.

5.1.2.6 Other conditions with genetic abnormalities

There are few syndromes which are not always present with autism and epilepsy both. But where, genetic mutation in combination with environmental risk factors can result in the appearance of autism and epilepsy. The responsible genes are CNTNAP2, RELN, SYNGAP1, SYN1, NRXN1, BCKDK, RBFOX1 and SCN1A, SCN2.

5.1.3 Genomic copy number variants

5.1.3.1 15q11-q13 duplication syndrome

15q11-q13 duplication syndrome is characterized by developmental delay (DD), epilepsy, and autism.

Individuals with this syndrome have features of both PWS and AS which are caused by deletions spanning this region. Muscle hypotonia is observed in almost all individuals with Dup15q syndrome, and can be severe. ID and feeding difficulties are common. Joint hyperextensibility and drooling accompanies the hypotonia in most individual.

Seizures affect approximately 60% of children with Dup15q syndrome, with the typical onset occurring before age 5 years. A high incidence of infantile spasm with later progression to Lennox–Gastaut syndrome (LGS) has also been reported [64]. However, multiple seizure types including tonic, atonic, tonic–clonic, myoclonic, complex partial, and atypical absence have also been reported. These seizures can be intractable.

A majority of individuals with Dup15q syndrome meet the diagnostic criteria for autism. Expressive language is typically severely impacted, and may even be absent. Behavioral difficulties like ADHD, anxiety, and frustration leading to tantrums are sometimes associated in some affected individuals.

Dup15q syndrome is caused by presence of at least one extra maternally derived copy of the Prader-Willi/Angelman critical region (PWACR) within chromosome 15q11.2-q13.1. Duplications may vary in size but must contain the PWACR to be causative of dup15q syndrome. This duplicated region encodes for GABRA5, GABRB3, and GABRG3 of the GABA receptor subunit allow one to hypothesize the inhibitory-synapses mediated dysregulation as the pathogenesis of the epilepsy and ASD phenotypes found in this disorder [65].

5.1.3.2 Trisomy 21 (Down syndrome)

Trisomy 21 or Down syndrome (DS) is a genetic condition in which a child is born with an extra copy of their 21st chromosome. It is usually associated with characteristic facial features, mild to moderate ID, and few associated congenital anomalies. Previous thinking held that autism is rare in DS. But the fact is that, it is estimated that autism in individuals with Down syndrome is 10–25 times more common than in the typical population [66]. However, this diagnosis often comes much later than it would for an otherwise typical child. This might be due to the presence of associated ID. The prevalence of epilepsy in patients with DS is approximately 1–13%. Infantile spasm (IS) is most frequently found seizure and represents 4.5–47% of these children [67]. Lennox–Gastaut syndrome (LGS), reflex seizures and others such as partial and generalized tonic clonic seizures have also been described in children with DS. A high rate of EEG abnormalities has been reported in DS, even among children without epilepsy [67].

Important mechanisms of epileptogenesis in DS are due to alteration of neuronal or synaptic anatomy resulting from fewer inhibitory inter-neurons, decreased neuronal density and membrane channel dysfunction due to altered membrane potassium permeability, decreased voltage threshold for spike generation.

5.1.3.3 Other copy number variants (CNVs)

Certain pathogenic copy number variants are highly associated with ASD and epilepsy. Deletions of 15q11.2, 16p11.2, and duplication of 16p13.11 have been detected with high frequency in individuals with ASD [68].

5.1.3.4 Phelan–McDermid syndrome (22q13 deletion syndrome)

Phelan-McDermid syndrome (PMS) is a rare genetic condition caused by deletion of 22q13.3 containing the SHANK3 gene. The genetic changes that cause PMS

vary from person to person and so do the clinical features. PMS can appear de novo or be inherited from a parent (20%) who carries a related genetic defect. A broad spectrum of medical, intellectual and behavioral challenges can arise from the symptoms of PMS; however, ID at varying stages, delayed or absent speech, motor delays, low muscle tone, symptoms of ASD and epilepsy have been found to be some of the most regularly observed traits of people with PMS. Some have reported a benign course of generalized tonic–clonic or myoclonic seizures with typical EEG features.

Current research specifies the inability of the single functioning copy of *SHANK3* to produce sufficient Shank3 protein for normal functioning. This may be responsible for most of the neurologic symptoms associated with this disorder. A larger series found seizures to be three times more common when the de novo deletion occurred on the maternally rather than paternally inherited chromosome 22 [69].

5.2 Environmental and epigenetic

5.2.1 Environmental fctors

Although genetic factors are clearly involved in ASD risk, they cannot fully account for all the cases. It is likely that a combination of autism-related genes and specific environmental factors might act as risk factors that triggers the development of autism. A population-based case–control study done in India found several environmental factors for example, the living conditions of family members, infection during pregnancy and preeclampsia, that could trigger development of the autism disorder [70]. Schmidt 2014 reviewed the environmental factors associated with autism, some of which may also be associated with epilepsy [71]. They reported consistent results for an association of higher maternal intake of certain supplements with reduction in ASD risk, with the strongest evidence for folic acid supplements [71, 72]. If a mother is exposed to a relevant environmental toxin and her offspring has a genetic predisposition, the combined effect might result in development of ASD, and carries a risk of epilepsy as well.

Intrauterine infection, e.g., maternal rubella during pregnancy has long been associated with a high risk of ID, autism and epilepsy in the offspring [40]. Use of antiepileptic drug sodium valproate during pregnancy can also affect brain development of the fetus, leading to ID and autism [71]. Rybakowski et al., emphasized that factors occurring already before conception like age of the parents, family autoimmune factors and maternal metabolic factors like obesity, diabetes, hypertension play important roles [72]. Many authors reported of factors occurring during pregnancy, such as bleeding throughout the pregnancy, multiple pregnancy, intrauterine infections (TORCH, bacterial, other) and maternal hypothyroidism. Arterial hypertension during pregnancy seems important, along with pre-eclampsia and eclampsia, severe anemia, smoking during the pregnancy [71, 73], maternal stress during pregnancy etc. Among other factors preterm delivery, low birth weight, intrauterine growth retardation and hypoxic ischaemic encephalopathy are mentioned. Among neonatal factors, the most commonly mentioned are: intraventricular bleeding, hyperbilirubinemia and congenital defects. While all these are responsible for development of ASD, more research is needed to know the association of epilepsy in these cases. However, environmental risk factors do not solely cover the exposure to toxins but include all changes other than those on a DNA–level, such as maternal nutrition, infection during pregnancy, and prematurity as well as parental age at conception.

5.3 Metabolic disorders associated with epilepsy in ASD

Many metabolic disorders may be associated with ASD and epilepsy. Among them, conditions like mitochondrial disease and dysfunction and abnormalities in cerebral folate metabolism are the common associations. Many of these conditions can lead to brain damage if inadequately treated. Frye provided a strong argument for treating any underlying metabolic disorders, both for ameliorating autism and epilepsy [74]. He also added the importance of understanding metabolic and genetic biomarkers. If these disorders can be detected early in life or even prenatally, treatment can be started at the earliest possible time. Identifying metabolic defects might help using standard known or novel treatments in children with epilepsy.

These metabolic disorders have diverse classic presentations, so basing a diagnostic strategy on the search for one or two specific key symptoms is inappropriate.

However, mitochondrial disease is of particular interest in children with ASD since it is being increasingly recognized as a cause of epilepsy in individuals with ASD [74, 75].

5.3.1 Disorders of energy metabolism

Several disorders affecting energy metabolism have been documented in ASD, including mitochondrial disorders and creatine deficiency syndromes. The prevalence of mitochondrial abnormalities appears to be very high in ASD [75].

Disorders of creatine metabolism have also been reported in children with ASD and epilepsy [76].

The general presentation of children with disorders of creatine metabolism includes developmental delay, regression, ASD features, ID, receptive and expressive language disorders, and seizures.

5.3.2 Disorders of cholesterol metabolism

Smith–Lemli–Opitz syndrome (SLOS) is a congenital disorder of cholesterol metabolism caused by mutations in both DHCR7 genes. Metabolically, children with SLOS demonstrate elevated concentrations of 7-dehydrocholesterol and reduced cholesterol concentrations in the blood. Interestingly, 50%–75% of children with this disorder meet the criteria for ASD [77]. This disorder is may be associated with seizures along with their other clinical presentations [78].

5.3.3 Disorders of vitamin metabolism

These include disorders of folate, pyridoxine, biotin, and carnitine metabolism. Children with cerebral folate deficiency (CFD) are commonly diagnosed with epilepsy and/or ASD [78].

Since the folate transport system is energy-dependent, a wide variety of mitochondrial diseases and novel forms of mitochondrial dysfunction related to ASD [79] have been associated with CFD.

Pyridoxine and its active form pyridoxal-5-phosphate play major roles in metabolism of glutamic acid to GABA acting as a cofactor. Pyridoxal-5-phosphate depletion reduces glutamic acid decarboxylase activity, resulting in a reduction in GABA synthesis. In children with ASD, several studies have reported significant improvement in behavior and cognition attributable to combined therapy with magnesium and pyridoxine [80].

5.3.4 Disorders of γ-aminobutyric acid metabolism

Succinic semialdehyde dehydrogenase deficiency is a rare disorder of GABA metabolism that results from a mutation in both ALDH5A1 genes. Neurological manifestations may include seizures, and ASD features among others.

5.3.5 Disorders of pyrimidine and purine metabolism

Children with ASD and comorbid seizures have been described to have disorders of purine and pyrimidine metabolism. Patients show a variable combination of mental retardation, epilepsy, ASD features, and cerebellar vermis hypoplasia.

5.3.6 Disorders of amino acid metabolism

Disorders in the metabolism of phenylalanine, have been described in children with ASD and comorbid epilepsy. Phenylketonuria is an autosomal recessive inborn error of phenylalanine metabolism resulting from deficiency of phenylalanine hydroxylase secondary to a mutation in the PAH gene on chromosome 12q23.2. Children with PKU who go untreated or who do not adhere to the diet adequately may demonstrate poor growth, poor skin pigmentation, microcephaly, seizures, spasticity, ataxia, aggressive behavior, hyperactivity, ASD features, global developmental delay, and/or severe intellectual impairment. Recently an inactivating mutation in the branched-chain ketoacid dehydrogenase kinase was described to be associated with autism, epilepsy, and intellectual disability in three families with two children each who were products of first-cousin consanguinity.

5.3.7 Mitochondrial dysfunction associated with epilepsy in ASD

A recent meta-analysis found that 5% of children with ASD met the criteria for classic mitochondrial disease, while as many as 30% of children with ASD may manifest mitochondrial dysfunction.

Prevalence of abnormal mitochondrial function in immune cells derived from children with ASD is exceedingly high.

A meta-analysis found that, overall, 41% of children with ASD and documented mitochondrial disease are reported to have seizures.

Mitochondrial dysfunction has also been reported in many genetic syndromes associated with ASD and epilepsy. For example, in Rett syndrome, Phelan–McDermid syndrome, 15q11-q13 duplication syndrome, Angelman syndrome and Down syndrome, mitochondrial dysfunction may underlie the phenotype of ASD with epilepsy, regardless of the underlying cause**.**

Abnormalities in mitochondrial function can lead to abnormal development in brain circuits, resulting in both neurodevelopmental disorders and epilepsy through several mechanisms. Abnormalities in mitochondrial biomarkers have also been found in the brains of individuals with ASD. Thus, it is very likely that changes in mitochondrial function in the brain affect neural transmission and function in children with ASD. Neural synapses that are areas of high energy consumption and are especially dependent on mitochondrial function may be one of the mechanisms for developing these developmental disorders. Recent studies have suggested that oxidative stress may be involved in the development of epilepsy.

Studies have found connection between reactive oxygen species and mitochondrial dysfunction in brain tissue from individuals with ASD. This may be another mechanism where mitochondrial dysfunction can lead to the development of epilepsy in ASD.

Immune dysfunction is found to be implicated in the development of epilepsy, and evidence of cellular and humoral immune dysfunction has also been implicated in ASD. Thus, studies suggest abnormalities in immune cell function result in seizures in ASD.

Another physiological abnormality that is becoming increasingly recognized in both ASD and epilepsy is the dysregulation of calcium [81]. On the other hand, epilepsy may be a common symptom of metabolic disorders and be a clue that a metabolic disorder may be the underlying etiology of the neurodevelopmental abnormalities in children with epilepsy and ASD. One advantage of investigating and diagnosing metabolic disorders is that treatments for many of these metabolic disorders are available.

6. Epilepsy syndromes with ASD as frequent neurodevelopmental sequelae

Several specific epilepsy syndromes with early onset epilepsy show an autistic behavior, some also appear to be the risk factor for later diagnosis of ASD. If they are identified appropriately and treated, behavioral improvement which is radical in some of the cases can be seen. In these cases, epilepsy originates in the brain networks responsible for communication and interactions. These include infantile spasms and Lennox–Gastaut syndrome. More recently, clinical overlap has been observed in cases with continuous slow waves during sleep (CSWS) and Landau–Kleffner syndrome and ASD [82].

6.1 West syndrome (WS)

WS is an epileptic encephalopathy characterized by infantile spasm/epileptic spasms, an EEG pattern of hypsarrhythmia and cognitive stagnation or regression. They usually occur before 2 years of age. Genetic causes are present in many of them and abnormalities in several brain developmental pathways are noted.

ASD may develop in a few of them. However, an association between TSC and duplications of FOXG1 have been reported consistently.

6.2 Lennox–Gastaut syndrome (LGS)

LGS is a childhood-onset epilepsy, which is characterized by constellation of several distinct types of seizures and electroclinical features of diffuse slow spike waves and generalized paroxysmal fast activity in sleep. Prevalence of ASD in LGS is rare, although ASD has been reported in patient with LGS resulting from duplications of maternal 15q11q13 [83].

6.3 Landau–Kleffner syndrome (LKS)/continuous spikes and slow waves during slow sleep (CSWS)

LKS is an epilepsy-aphasia syndrome that is characterized by regression in language and characteristic CSWS on EEG-termed as electrical status epilepticus of slow sleep (ESES). Several children who had been diagnosed with ASD were noted to have a predominant language deficit. Stereotypies and withdrawal are also common in LKS, but whether these children also have deficits in social reciprocity is not clear. The association may be more related to severe receptive language deficit. Copy number variants have been detected in patients with LKS who also have associated ASD [84], and, most recently, GRIN2A mutation have been identified in patients with epilepsy-aphasia phenotypes [85].

7. Regression in Autism

Developmental regression is present in approximately one-third of children with ASD [86] and is believed to have an association with epilepsy. The relationship between regression and epilepsy in ASD has therefore long been of interest because of the hope that some developmental regression in idiopathic ASD could be caused by epilepsy and be reversible through using anti-seizure therapies [87].

The overlap of language and autistic regression to epilepsy, EEG epileptiform activity, sleep, and to epileptic encephalopathies such as LKS continue to be controversial areas of research and of clinical interest because of the close clinical resemblance to autism.

LKS or acquired epileptic aphasia may present as a developmental language regression followed by autistic-like social-communicative phenotype. LKS usually presents between 3 and 7 years of age with loss of language skill in children who were previously normal and most but not all affected children have convulsive seizures. In case on early LKS, it becomes difficult to differentiate it from ASD clinically and the diagnosis is done mainly on EEG finding and response to anti-seizure treatment. The EEG in the awakened state often has a normal background with seizures of various types in LKS. During sleep, it is characterized by epileptic discharges throughout the sleep-electrical status epilepticus (ESES) on EEG that may affect cognitive processing. In children with autistic regression, both language and behavior in association with significant social deficits occur between 18 and 24 months compared to usually only language regression in LKS, which is more dramatic and the social deficits are less severe than those with autism [88]. McVicar et al. in their study reported that children with isolated language regression have a higher frequency of epileptiform discharges and seizures than children with both language and autistic (i.e., social and behavioral) regression [89].

An extensive review, in 2002 on epileptiform neurocognitive disorders linked with speech/language deterioration concluded that "acquired epileptiform aphasia (AEA) can be conceptualized on a spectrum with other epileptiform neurocognitive disorders that may share pathophysiological features". They also added that "without better documentation of potential factors around the time of the regression, it will be difficult to identify the fundamental factors that differentiate these conditions, their response to treatment and long-term prognosis" [90].

CSWS, an epileptic syndrome of that is associated with EEG pattern of ESES may present with regression in global skills that overlaps with autism [91]. However, the differences in age of regression, type of regression, frequency of epilepsy and EEG abnormalities suggest that these are distinct phenotypes.

Nonconvulsive status epilepticus (NCSE) may also have features that have a strong similarity to autism. The child may exhibit poor reciprocal social interaction, poor verbal and nonverbal communication. But with effective treatment, the features of autism disappear.

Magnetoencephalography has identified precise location of the source of these epileptic EEG discharges. The finding from this investigation shows that focal spike waves (FSW) in the perisylvian region and located in the superior temporal gyrus may cause auditory and verbal agnosia (LKS). When FSW predominate in the prefrontal regions, a cognitive regression with features of CSWS, when in cortical areas like the superior temporal sulcus or the fusiform gyrus involving the networks relating emotions to higher-level visual representations could interfere with the developing capacity to recognize the emotional signals of faces, that are typically deficient in autism.

Other epileptic disorders, like refractory partial epilepsies of frontal or temporal origin like the Benign epilepsy with centrotemporal spikes (BECTS)also interfere

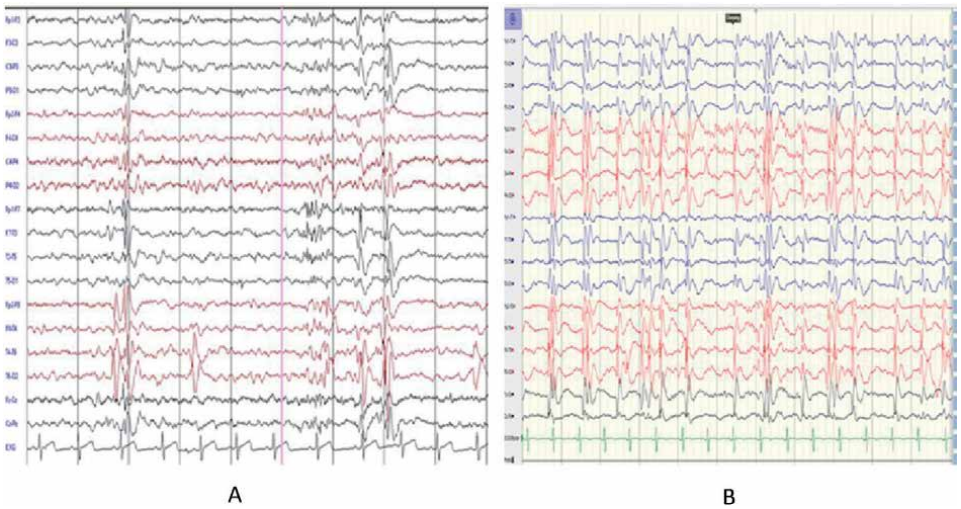

Figure 4.
Electroencephalogram finding of a (A) 4 years 6 months old boy with LKS, and regression of speech for one year showing spike and waves over frontal central and temporal regions (B) 7 year old girl with regression of speech and cognitive function in a case of CSWS showing ESES, generalized spike waves in all the channels.

with developing language networks and/or other circuits involving the "social brain" such as the amygdala, cingulate and orbitofrontal cortex and account or contribute to language and autistic regression. Other examples are Focal dysplasias, hippocampal sclerosis, congenital tumors or tuberous sclerosis and hypothalamic hamartomas.

In addition, an increasing number of genetic and metabolic encephalopathies with severe developmental problems are now recognized with autistic regression where epilepsy may aggravate the regression.

It is also found that approximately 20% of children with autistic regression without epilepsy may have an abnormal EEG, the majority with spikes or spike-and-wave discharges [92]. Although this abnormal EEG is found usually after the regression, there is no evidence of a causal relationship between the epileptiform abnormalities and the regression [93].

Figure 4 shows EEG of LKS in awake and CSWS in sleep with ESES.

8. Autism in epilepsy

A meta-analysis of 19 studies showed a pooled ASD prevalence of 6.3% in individuals with epilepsy, which is considerably higher than the reported prevalence of 0.75% to 1.1% in the general population [94]. Tuchman et al. reported approximately 30% of children with epilepsy have autism and/or intellectual or developmental disabilities [95]. Several studies have shown that children with epilepsy have an increased risk of being diagnosed with ASD [8, 16, 22]. A higher prevalence was found for studies with younger age groups, ID, and specific epilepsy syndromes (West syndrome, Dravet syndrome).

Epilepsy and autism both can arise from abnormal excitability and disrupted synaptic plasticity in the developing brain. This abnormal plasticity can also result from genetic conditions.

Early-life seizures can produce a variety of cellular and molecular changes in the hippocampus, including short-term enhancement of excitation and long-term enhancement of inhibitory neurotransmission and reductions in excitatory

neurotransmission [35]. All these early seizures also have numerous disruptive effects on neural development, including abnormal synaptic reorganization, and cortical interneuron dysfunction [96, 97]. This in turn disrupts the construction of cortical networks necessary for acquiring certain skills during development, and may predispose an individual towards developing ASD [15]. Further, risk of both epilepsy and ASD is elevated in numerous genetic disorders as mentioned previously, such as Rett syndrome, fragile X syndrome, and tuberous sclerosis complex [37].

Lukmaji et al. emphasized the importance of screening for autism in persons with epilepsy, and vice versa, to appropriately tailor treatment decisions and improve patient outcomes [28]. As in their systematic review revealed the occurrence of autism in persons with epilepsy and epilepsy in persons with autism to be higher than the previously reported independent occurrence of each of these conditions in the general population.

9. EEG abnormalities

ASD are associated with increased incidence of EEG abnormalities. EEG epileptiform abnormalities were found at a range of 35% to 86% in ASD individuals with epilepsy [9, 21, 98] and up to 28.6% [21] to 60% [9] in individuals without epilepsy. These discharges are often more common when there is history of autistic regression, even if there is no history of seizures or epilepsy [99]. In addition to epileptiform discharges, non-epileptiform discharges were also found in ASD, these were- disorganized and slowing of background rhythm, asymmetry etc. [100]. But epileptiform EEGs seemed to be more common than nonepileptiform abnormalities in most of the studies [101–105].

Abnormal EEG is considered as a biomarker of cortical dysfunction [8, 100] and provide evidence that autism is a neurobiological disorder [106]. Interictal discharges are thought to interfere with normal neural processing which may further impair cognitive function [17, 18, 100]. The clinical importance of epileptiform discharges without overt seizures are not clear, but they may also cause behavioral and cognitive problems [99].

EEG should be considered in children with clinical or suspected seizures and, in all the children where autism is questionable and a clinical suspicion of LKS is present. Performing a sleep-EEG was highly recommended by Pacheva et al. in all patients to prevent underdiagnosis of ESES and LKS [21]. The authors also mentioned the need of timely treatment to get improved behavior and cognition in patients with ESES. Fernandez et al. also concluded in that a treatment trial with AED is justified in patients with epileptic encephalopathies and cognitive dysfunction/regression, that could be related to epileptiform discharges [107]. Children with LKS may also have an autistic-like regression that extends to behaviors beyond language [87]. Presence of epileptiform EEG abnormalities even in the absence of clinical seizure found in LKS and ESES is a controversial problem [108, 109].

10. Management of individuals with both epilepsy and autism

Both the conditions should be managed individually. This depends upon the causes if present, especially in case of epilepsy. First of all, the cause of epilepsy in autism should be investigated appropriately. It has to be ensured that the autistic features are not the result of ESES or frequent epileptiform discharges [110].

The diagnosis of epilepsy become more difficult in autism, especially if there is accompanying intellectual disability because history taking in these cases becomes more difficult.

The treatment of epilepsy in ASD is based on the general principles of treatment of epileptic seizures with traditional antiepileptic drugs (AEDs). Usually, valproate, lamotrigine and levetiracetam are used as the most effective and tolerable AEDs for individuals with ASD [74]. But levetiracetam can have negative effects on mood and behavior and be associated with deterioration in children, whereas lamotrigine tends to be a mood-leveling antiepileptic drug and Topiramate can be associated with word-finding difficulties [110].

The discovery of the role of neuronal autoantibodies has been one of the most exciting developments in the recent years. These antibodies can result in seizures, loss of skills, (sometimes a dramatic loss), behavioral changes and even psychosis. Effective immunotherapy can, in at least some cases, reverse all these changes [111, 112]. This is an area which requires further consideration.

New therapeutic options were suggested for ASD and epilepsy, based on the opinion that gene defects could determine all the symptoms of these disorders. This also includes modulators of GABA A receptors, GABA agonists, modulators of GABA metabolism, glutamate receptor antagonists, insulin-like growth factor 1 and m-TOR inhibitors for ASD-epilepsy comorbidity [113]. M-TOR inhibitors like Everolimus (Rapamycin) and Sirolimus have positive results in patients with TSC, ASD and PTEN-related disorders. After treatment with conventional GABAnergic agonists, a paradoxical result was reported in ASD [101]. In addition, ASD with epilepsy having 15q11.2 duplication, effectiveness of benzodiazepines was reported to be lowered [114].

In addition to the treatment, the usual management of autism should be continued.

10.1 Management of additional comorbidities in the presence of both epilepsy and autism

10.1.1 Attention deficit hyperactivity disorder

ADHD is common in children with autism and in children and adolescent with epilepsy. Diagnosing ADHD in epilepsy sometimes becomes difficult, because some of the children with epilepsy present with features of ADHD. This is due to frequent epileptiform discharge. In those cases, treating these epileptic discharges with AEDs will alleviate these symptoms. Few children with epilepsy may have inattention, hyperactivity and distractibility as a result of antiepileptic medication, as for example, treatment with phenobarbitone, benzodiazepine or vigabatrin [110]. So, review of antiepilepetic medication is very important before diagnosing the child as having ADHD.

Epilepsy is a highly variable condition and after treatment with AED, there might be is no change in seizure or even there is high frequency of seizure and since ADHD medication is started, it may be incorrectly concluded that this increase in seizures is because of the ADHD treatment.

There are two groups of medication currently used to treat ADHD: stimulants (methylphenidate, amphetamine) and non-stimulants (Atomoxetine, alpha-2 agonists). When children with symptoms of ADHD require medication, current guidelines recommend starting with a trial of a stimulant like methylphenidate. If this first stimulant does not prove to be effective, the alternative stimulant is then used [115]. If stimulants are not effective or cause intolerable adverse effects, then nonstimulants like atomoxetine, alpha-2 agonists, and antidepressants are used.

Methylphenidate is the most commonly used medicine for ADHD. Large observational studies conducted in children and adolescents with epilepsy have found that ADHD medications in general and stimulants like methylphenidate are not associated with increased risk of seizures [116]. Use of low and moderate doses of methylphenidate has been observed in reduction of seizure frequency and severity along with improved quality of life in a Brazilian study done in 2015 [117]. However, it is important to monitor seizure frequency in the first few weeks and months after prescribing methylphenidate [116] as there are still questions regarding use of this drug in epilepsy.

Guidance for identification and treatment of individuals with attention deficit/hyperactivity disorder and ASD based upon expert consensus in the UK in 2020 [118] emphasized on non-pharmacological interventions and care management, including psychoeducation, carer interventions, behavioral/environmental and Cognitive Behavioral Therapy (CBT) approaches and educational interventions, followed by pharmacological treatments. They have commented that in children, pharmacological intervention should be preceded by behavioral observation and psychological intervention as the first-line treatment. And if psychological/environmental interventions fail in children, then ADHD medication may be helpful for treating symptoms of inattention. Medication should be used in a 'low and slow' approach as people with both ADHD with epilepsy may be more treatment resistant and more prone to develop side effects to medication. Medication should be given for the shortest time possible and side effects should be monitored carefully.

Researchers also mentioned about the relative little evidence, on using other ADHD treatments, such as atomoxetine, guanfacine and clonidine in children with autism, having both epilepsy and ADHD [110]. However, several research papers and reviews found no clear evidence about exacerbation of seizures with these medications. Besag et al., in their paper summarized, about 30% of children with epilepsy had ADHD and about 70% among those with epilepsy and ADHD benefited from treatment of their ADHD symptoms with methylphenidate [119].

10.1.2 Anxiety

Anxiety is commonly found in young people with autism and epilepsy. Children with epilepsy with co-morbid psychiatric disorders like — ADHD, depression, and anxiety disorders, end up with significant compromise in academic performance and social skills, leading to deterioration in the Quality of life [120].

Risperidone and Aripiprazole in low dose can improve the behavior in children with autism. The mechanism is probably through decreasing anxiety. But the dose used should be very low because of the risk of seizure exacerbation with high doses of these antipsychotic drugs. On the other hand, the antiepileptic drugs like carbamazepine, phenobarbital and phenytoin may decrease the blood levels of antipsychotic drugs and a larger dose of antipsychotics may be required who are taking these AEDs. The management goals in pediatric epilepsy with anxiety disorders are — adequate seizure control, optimization of the functioning of the child and keeping the patient in best and simple pharmaco-therapeutic regimen [121].

The clinician should avoid an antiepileptic drug which is having side effects like behavioral problems.

Behavioral therapy should be the first-line approach to managing anxiety. Despite the effectiveness of selective serotonin reuptake inhibitors in decreasing anxiety in adults and teenagers there is a lack of evidence for a beneficial role of these drugs in treating anxiety in children with autism, according to a Cochrane review.

10.1.3 Sleep

Sleep disturbances are very common in children with autism. Epilepsy and sleep have reciprocal relationships. In some of the cases, sleep facilitates seizures and, in some seizures, adversely affects sleep architecture. If sleep problems are present, possibility of nocturnal seizures should it is eliminated. In that case careful history and if required antiepileptic medication should be tried. And if the sleep disturbance is not due to nocturnal seizures, melatonin is the drug of choice [110]. There is no good evidence of exacerbating seizures using melatonin. Animal work suggests that melatonin might have an antiepileptic effect. Identification and management of sleep disorders may improve seizure control and challenging behaviors of autism

11. Future direction

No single unifying ASD–epilepsy phenotype is there till now but understanding possible commonalities in subgroups of children with an ASD– epilepsy phenotype should help us in understanding the pathophysiology of both ASD and epilepsy [110].

Prospective, population-based studies are recommended, whenever there is any history of regression [110]. These studies should include investigations like genetic and chromosomal studies, searching for metabolic/mitochondrial disorders, EEG including sleep EEG and also testing for possible neuronal antibodies.

Environmental factors, prenatal factors such as maternal exposure to infection, toxic chemicals, pollution, alcohol and drugs should be searched for as these are the risk factors of autism and they might also cause epilepsy. A history of exposure to antiepileptic drugs like maternal valproate and learning problems/probable autism in the offspring and thorough obstetric and neonatal factors should also be an essential part of the history-taking.

12. Conclusions

Epilepsy is a common co-occurrence in children and persons with ASD. Determining the bidirectional prevalence of autism and epilepsy is important. Understanding the specific effects of the genes/metabolic/environmental pathways affected may give a better insight into the pathogenesis of the developmental problems. ID is also an important association in epilepsy and ASD. Multiple interrelated factors are there in the pathogenesis of ASD-epilepsy connection. Further, understanding these comorbidities will have a profound effect on the management of these challenging patient populations. For example, it has been found that autistic symptoms can be minimized when epilepsy is being treated in patients with both the conditions. Well-managed epilepsy, autism, and associated comorbidities can significantly improve the quality of life in both patient and caregiver. Further population-based studies and investigations including genetic, and, metabolic, in addition to EEG are needed, especially in case of regression in order to detect both these conditions in the early years of life.

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Chapter 3

Autism and Gender Identity

Yulia Furlong

Abstract

Since the turn of the century, we are witnessing a dramatic surge in the numbers of children and adolescents referred to gender clinics, this is happening in the context of general increase in numbers of individuals identifying as non-binary. The chapter ahead will initially address the shifting landscape of gender dysphoria (GD), and provides a comprehensive overview of the latest findings in the fields of autism and GF. The higher rates of autism' diagnosis among gender diverse samples prompted the development of several hypotheses that attempt to explain the link between autism spectrum and gender spectrum, as well as development of relevant clinical guidelines that contain strong advocacy for adolescents with neurodiversity not to be precluded from gaining access to gender-related services. In the public arena, a highly publicised UK High Court's case that is commonly referred to as Bell v Tavistock highlighted the growing concerns regarding the unexplained surge in the number of adolescents identifying as having GF, as well as pointed to the lack of evidence that hormones and surgery improve long-term outcomes. The chapter explored the recommendations that came out of this ruling and highlighted the implications for Australian jurisdiction by illustrating medico-legal changes on Perth-based gender services.

Keywords: gender identity, gender dysphoria, autism spectrum disorder, sex differences, clinical guidelines, gender reassignment, consent to treatment, puberty suppression, cross-hormone treatment

1. Introduction

The empirical and research interest in the topic of gender identity and gender diversity in children and adolescents has increased exponentially over the last two decades. Such trend has been evident from inspired research activity in the field of transgender youth, as illustrated by **Figure 1** depicting the steady increase in the volume of relevant publications from 1997 to 2016 [1]. Since the turn of the century, we are witnessing a dramatic surge in the numbers of children and adolescents referred to gender clinics, this is happening in the context of general increase in numbers of individuals identifying as transgender. Internationally, this trend is well documented in the Western countries, for example in Canada between the periods 2000–2003 and 2008–2011 [2], in United States [3], the Netherlands [4], in the United Kingdom [5] and in Australia [6]. To address this gap of unmet clinical needs, the dedicated gender identity clinical services have been set up out of existing child and adolescent mental health services in collaboration with paediatric endocrinology services in order to deliver specialised gender affirming care.

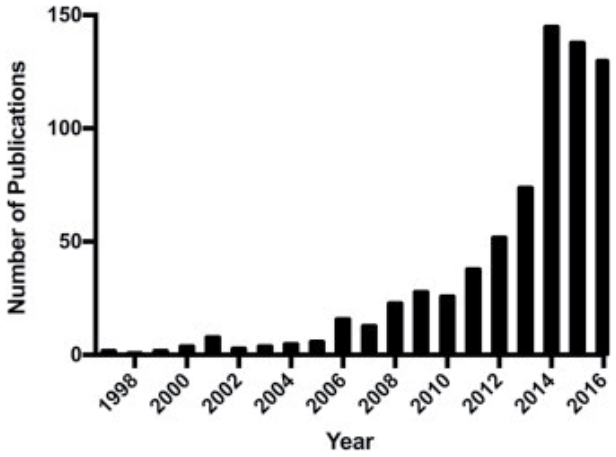

Figure 1.
PubMed indexed publications from 1997 to 2016 using the search term "transgender youth". Turban and van Schalkwyk [1]. Copyright © 2021 Elsevier Inc. except certain content provided by third parties.

In the public arena, a well-publicised UK High Court's case that is commonly referred to as Bell v Tavistock [7] highlighted the growing concerns regarding the unexplained surge in the number of adolescents identifying as having gender dysphoria, as well as pointed to the lack of evidence that hormones and surgery improve long-term health outcomes, including suicidal risk. This well-publicised court decision caused ripple effect across community of trans activists, gender and legal scholars, advocacy groups and service users who are still reeling from the ruling. There are loud voices on both sides of the argument with popular opinion that this landmark judgement will result in a fundamental transgression of trans and adolescent rights. On the other hand, the ruling was welcomed by traditionalists and more conservatively inclined "as a victory for common sense and safeguarding" [8].

2. Gender identity

Gender dysphoria (GD) has been defined in the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5), as a characteristic of the individuals presenting with incongruence between their natal sex (sex at birth) and their experienced gender [9]. The diagnosis is characterised by intense and persistent cross-gender identification, which is often associated with significant distress of one's own assigned biological and social characteristics. The key elements of GD' diagnostic construct evolved from DSM-IV-TR diagnostic criteria for Gender Identity Disorder [10] by shifting the focus towards the dysphoria associated with the incongruence and moving away from the notion of identity disturbance.

The International Classification of Diseases, 10th Edition [11] describes the desire to live and be accepted as a member of the opposite sex as transsexualism, under the "disorders of adult personality and behaviour" which limits the use of this diagnostic category for children and adolescents. We are looking forward to the rolling out of ICD-11 [12] that will come into effect on January 1, 2022, as it contains new taxonomies of Gender Incongruence of Childhood (2- year duration) and Gender Incongruence of Adolescents and Adults ("at least several months" duration, criteria otherwise similar to Gender Dysphoria.) These new diagnostic categories moved out of Chapter V and no longer under the "Mental and

behavioural disorders" section, instead they can be found under the "Conditions related to sexual health", chapter 17. Such move is justified based on the notion that the broader spectrum of gender identity issues is increasingly recognised as part of normal human diversity and should not be classified as a mental illness [13].

Gender diversity is an umbrella term that reflects the growing recognition that being transgender is part of the continuum of gender spectrum. It is used to describe different gender identities in a non-stigmatising way, similar to the way researchers use 'neuro-diverse' to describe variations in cognitive style that are characteristic of autism and attention deficit hyperactivity disorder (ADHD). The diversity in

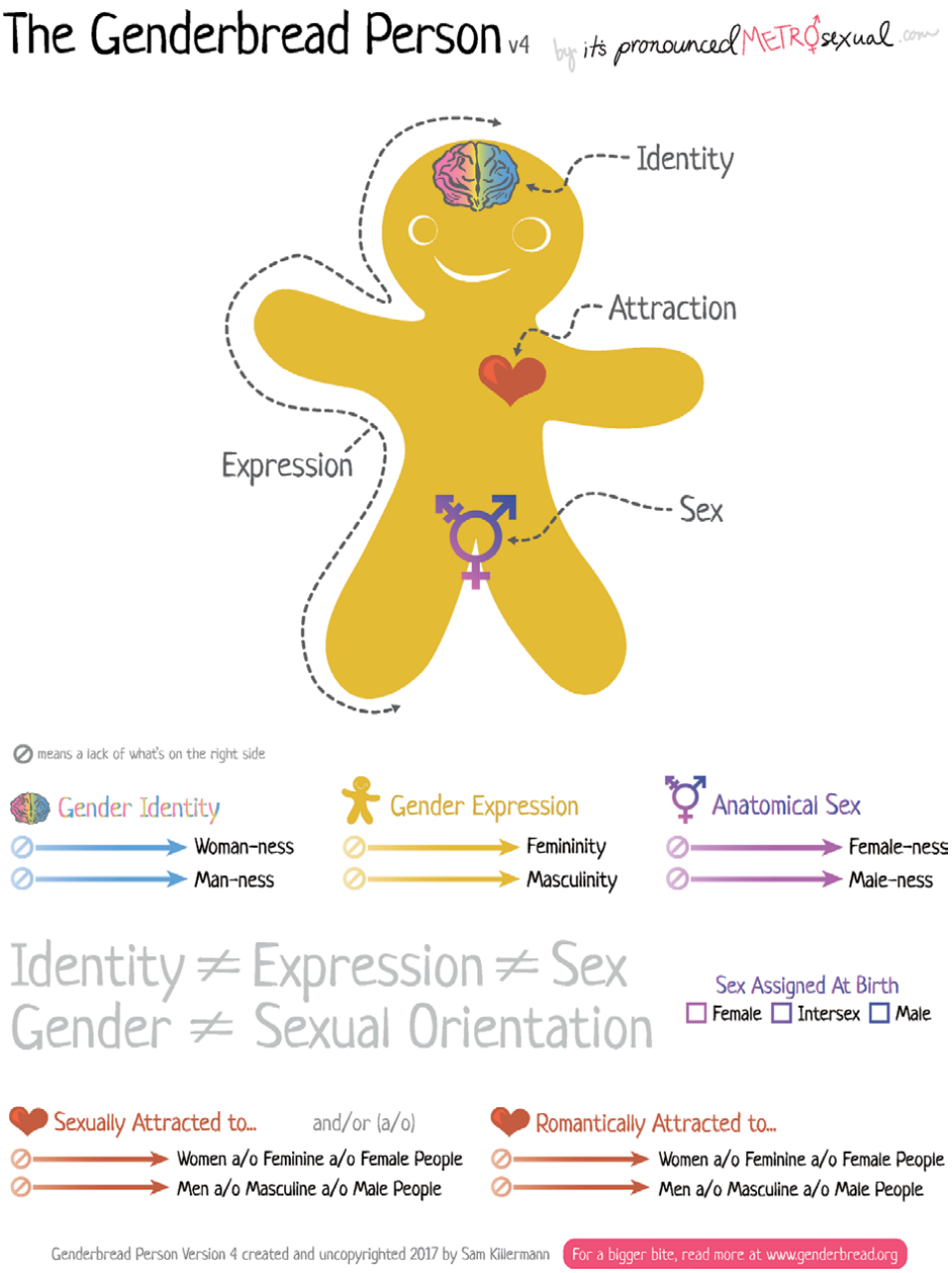

Figure 2.
Genderbread Person v4.0. A teaching tool for breaking the big concept of gender down into bite-sized, digestible pieces (author Sam Killermann, [16]).

gender expression encompasses a range of descriptions, including 'non-binary', 'transgender', 'gender nonconforming' - that individuals may adopt when their gender identity, expression or behaviours do not conform to the expected norms and stereotypes of their natal sex [14]. There is a renewed freeing sense that gender is more fluid than it was ever thought to be before, and either that gender of self and others are less deterministic of who we are or that it is seen as natural that a person's sense of their gender fluctuates. The narrative of suffering and gender dysphoria are not universal to Trans' population and not every gender diverse person hates their body, hence it's important to avoid misleading assumptions. Views about gender and sexuality are influenced by multitude of factors, including one's own orientation and identity, personal experiences and upbringing, religious and moral beliefs, as well as popular cultural stereotypes. At any given time, gender identity belongs to the intersubjective field where possibilities for evolving gendered roles may be created, for example, in a situation where women and men may experience the impact of hierarchical structures in a workplace that can trigger the identity of dominance to emerge as a way of healthy adaptation process.

Two-spirit is a contemporary term adopted by some Native American Nations, and Aboriginal peoples to signify their spiritual, sexual, gender, cultural, and community identities, and the use of this term has been known to facilitate an individual's reconnection with the tribal understandings of non-binary sexual and gender identities [15]. Some traditional Diné Native Americans acknowledge a spectrum of four genders: feminine woman, masculine woman, feminine man, and masculine man. The term "third gender" has been used to describe the hijras of India (male at birth choosing a female identity) who have gained legal identity in 2014. Third gender also applies to fa'afafine of Polynesian Samoa's population and to 'sworn virgins' of Albanian Alps. In Thailand one can find up to 18 different gender roles, identities and diverse visual markers of masculinity and femininity.

Gender, psychosexual development and identity formation are all intertwined. One way to integrate the many components of gender identity and gender expression is by utilising the so-called 'Genderbread Person' model [16], see **Figure 2** above.

3. Psychodynamic underpinning of gender

Psychoanalytic contribution to the study of gender issues first of all belongs to Sigmund Freud and his theory of psychosexual development and the recognition of Freud's understanding of the ego as body-ego, which is under the influence of the id [17]. Freud's theories are echoed by the contemporary gender theories that propose that the id, like genetic material, has male and female impulses [18]. Freud also speaks of "psychical hermaphroditism" and human ability to produce a different core gender identity under certain conditions, the notion debated by Myra J. Hird who argues that there is, at some level, a refusal to allow a person to transgress the boundaries of traditional gendered identities as represented in the visual 'cultural genitals' [19]. Freud saw nature as masculine and coined the phrase "anatomy is destiny" [20], that can be summed up as - one's gender determines one's main personality traits. Nevertheless, with the rise of the feminism, the Freudian father-centred theory rooted in the concepts of the 'penis envy' and the 'castration complex' had been gradually replaced by maternal or more gender neutral primary caregiver' determinants [21–23]. Anne Fausto-Sterling suggests that the critical aspects of pre-symbolic gender embodiment occur during infancy as part of the synchronous interplay of caregiver-infant dyads. Around the time children begin to speak, they recognise themselves as distinct entities in the mirror and commence their transition to symbolic representation and achieving accurate gender labelling

of self and others by the age of three [24]. "Gender is about affinities"– does it feel "like me", or different from me? Gender may remain stable or evolve and change, the author urges that clinicians take a dynamic developmental view of gender identity formation after into account [24]. Finally, the Lacanian perspective on gender difference is rooted in the original concepts that situate in the space between life and death and propose that a gender transition is more about strategy of being than about sexuality [25].

4. Mental health comorbidities

Review of the recent literature suggests that 0.17% to 1.3% of adolescents and young adults identify as transgender [26]. Transgender individuals experience disproportionately high rates of negative mental health outcomes, as compared with their cisgender heterosexual peers, as well as their gender-normative lesbian, gay and bisexual peers [27]. Recent studies have offered a deeper understanding of the prevalence of depression among trans- and gender-variant youth, providing evidence that rates of depression are 2.4 to 3.5 times higher than in their cisgender peers, 50.6% vs. 20.6% in a retrospective matched cohort (n = 360) of 12–29 years old patients at community health centre in Boston [28] and 41.3% vs. 11.8% in a high school-based sample (n = 8,166) from New Zealand [29]. A study by Veale et al. measured stigma-related experiences, social supports, and mental health (self-injury, suicide, depression, and anxiety) among a sample of 923 Canadian transgender adolescents and young adults aged 14 to 25; they reported that over two-thirds (68.3%) of the sample experienced a major depressive episode in the past year [30].

The large scale 2015 U.S. Transgender Survey (USTS) with 27,715 respondents with a median age of 26 years, found that 40.4% of respondents reported attempted suicide in their lifetime, 81.7% of respondents had seriously thought about killing themselves in their lifetimes, and 48.3% had done so in the past year while 7.3% had attempted suicide in the past year; respondents who reported having a disability had higher prevalence on all suicide-related measures than those without disabilities [31].

Another large survey that was primarily capturing an eating-related pathology revealed that transgender students had increased rates of eating disorder diagnosis compared to cisgender heterosexual women (15.8% vs. 1.85%), this set of data was collected from 289,024 students via the American College Surveys from 233 U.S. universities [32].

5. Autism spectrum disorders and gender identity

Among the general population, the prevalence of autism spectrum disorder (ASD) in children is estimated at 1% with a ratio of 1:42 for boys and 1:189 for girls respectively [33]. The evidence that suggests an overrepresentation of ASD in gender diverse samples, particularly in children and adolescents [34, 35], is robust and largely accepted by scientific community. The association between GD and ASD has been of great clinical interest because it has implications for diagnosis and treatment.

5.1 Co-occurrence rates

Trans Pathways study conducted in Australian large sample of trans and gender-diverse young people (n = 859; mean age = 19.4), found that 22.5% of the sample

reported having received a formal ASD diagnosis, while more than one third (35.2%) had highly suspected but undiagnosed ASD [34]. This type of large epidemiological study is difficult to construe in view of diagnostic imprecision of gender dysphoria and heterogeneity of both, GD and ASD constructs and therefore studies in this field significantly vary in methodology and chosen diagnostic constructs. A focus on diagnosis is less sensitive to the presence of subthreshold or mild autistic symptoms, which is why some studies utilised the Broader Autistic Phenotype (BAP) that is defined as a collection of sub-diagnostic autistic traits more common in families of individuals with ASD than in the general population. Evidence of an intermediate phenotype and a latent construct in autism was first reported in the landmark twin study of Folstein and Rutter [36]. Jones et al. [37] used Autism Spectrum Quotient – AQ [38] to measure BAP in a sample of adults with GD, typical adults and adults diagnosed with ASD, and found that 17.5% of the GD sample had a score above the AQ cut-off for BAP. Interestingly, more females with GD scored above the cut-off than males with GD, which is in contrast to the recognised male - female distribution in ASD.

The first systematic study into the incidence of autism diagnosis in young people referred to a specialised gender clinic via the use of a diagnostic interview, reported an ASD' higher than expected prevalence rate of 7.8%, and an overrepresentation of ASD diagnoses in boys compared to girls with a ratio of 3:1 [39]. While, overall, this study was methodologically sound, it sadly lacked a clinical control group for comparison. Contrary, the study by Pasterski et al. [40] has shown no difference in relation to ASD' prevalence rate between trans people and the general population by utilising the threshold for a potential diagnosis with an ASD-rate of 5.5%. The disparity of these findings and the difference in prevalence rates could be as a result of the chosen study populations with recognised difference in presentation between children and adults, study design and methodology, utilised diagnostic categories and assessment tools.

Skagerberg et al. [41] reported ASD scores that fell, on average, in the mild/moderate range in a sample of children and adolescents with GD with no significant difference between boys and girls with GD, and scores that fell in the normal range in a control sample of typically developing young people. Skagerberg et al. measured autistic symptoms using a quantitative measure - the Social Responsiveness Scale [42], that was also used in another controlled study of children with GD [43] with 44.9% of GD' sample scoring within the clinical range for autistic traits with, on average, moderate scores. This Canadian study also examined risk factors for ASD with an overlap of only high birth weight, but not the other risk factors, with both, raised gender nonconformity and autistic traits among children with GD [43].

Glidden et al. [44] systematically appraised 19 out of 58 available articles regarding the co-occurrence of gender dysphoria and ASD from Medline, PubMed, PsycINFO, and Embase databases in the period from 1966 to July 2015. The authors of this systematic literature review concluded that the research in to the co-occurrence between gender dysphoria and ASD is limited, especially for adults. The literature investigating ASD in children and adolescents with gender dysphoria showed a higher prevalence rate of ASD compared with the general population. Since Gidden's systematic review, recent well-designed Dutch study confidently confirmed an over-representation of symptoms of ASD in children and adolescents with GD [45]. Their estimated prevalence of ASD was 14.5%, which is approximately four times higher than the 3.5% in the normative sample and much higher than the prevalence estimate of 1% found in the general population [33]. Their GD sample showed elevated levels of autistic symptomatology on all subdomains, not just on stereotyped behaviour and resistance to change' measures. van der Miesen and colleagues [45] found that young people with GD had more reported autistic

symptoms compared to typically developing children and adolescents, but less reported autistic symptoms compared to children and adolescents with ASD.

There seems to be less studies that took an alternative root and investigated GD symptoms within an ASD population. Australian survey by George and Stokes [46] aimed at measuring prevalence of "gender variance" in ASD and found that individuals with ASD of all ages report increased homosexuality, bisexuality, and asexuality, but decreased heterosexuality. Sexual Orientation was surveyed using the Sell Scale of Sexual Orientation in an international online sample of 309 young adults with ASD that were screened with Autism Quotient (M = 90, F = 219, M = 32.30 years, SD = 11.93) which was compared to sexual orientation of 310 controls that were represented by aged-matched neurotypically developing individuals (M = 84, F = 226, M = 29.82 years, SD = 11.85). In the group with ASD, 69.7% identified as non-heterosexual, while in the control group, 30.3% identified as non-heterosexual.

Strang et al. [47] found that children with ASD were 7.59 times more likely to express gender variance by expressing "wishes to be of the other gender" as per Child Behaviour Checklist [48] compared to their neurotypical peers and established equal sex distribution for the gender variance. Similarly, Janssen et al. [49] found that children with ASD were 7.76 times more likely to express gender variance than children from the non-referred comparison group, with no significant difference between boys and girls. There is a consensus that in most cases, gender diverse identities and behaviours are stable and not secondary to ASD but co-occur as an aspect of personal identity' development [34].

The exact numbers accounting for the overlap between autism and gender variance has a wide degree of variation, ranging between 6% and 26% of for ASD among gender-variant people, while the rate of gender variance among people with ASD is estimated between 4% and 8% [39, 49–51].

5.2 Hypotheses attempting to explain ASD/GD association

Gender diverse behaviours, including crossdressing, tomboyism and paraphilias in children and adolescents with ASD may be considered as part of the ASD phenotype and representations of unusual, restricted interests and the development of atypical gender identity in autism could relate to the developmental rigidity that is characteristic of autism. This hypothesis focusing on individual psychological characteristics and obsessional interests suggesting that gender could be among the preoccupations or obsessions often seen in ASD was not fully supported by van der Miesen and colleagues [52]. The study of VanderLaan et al. [53], which suggested that specifically intense obsessional interests are one of the hypothesised mechanisms underlying the possible GD-ASD co-occurrence. Van der Miesen's findings highlight several subdomains of the autistic spectrum that might be involved in this possible association, including social and communication difficulties, but not obsessional interests [52].

5.3 Developmental hypothesis

The individuals with ASD might not reach normative flexibility in gender development that will equip them with necessary skills to deal with gender variant feelings, which might explain the overrepresentation of ASD in GD. Furthermore, Robinow [54] suggested that neurobiological abnormalities associated with reduced social functioning in ASD, such as those found for frontal and temporal regions, might make it difficult for some children to acquire concepts regarding gender norms. Clinicians have observed that at least some children with GID misclassify

their own gender, even at ages beyond those in which correct self-labelling is expected [55]. Social communication deficits might, therefore, underlie the cognitive "lag" that many GD children exhibit in terms of their gender constancy development [55].

Erik Erikson described eight stages of psychosocial development through which a neurotypically developing adult should pass from infancy to adulthood [56]. As articulated by Erikson, Identity versus Role Confusion represents the fifth stage of psychosocial development that take place during adolescence between the ages of 12 and 19. It has been hypothesised that individuals with ASD become acutely aware of their uniqueness and differences compared to others during their formative years, and, as a result, may develop confusion of identity and identity crisis which could include gender nonconforming behaviour and GF.

5.4 Social perception and preoccupations hypotheses

This theory implies that core ASD symptoms of social deficit will likely influence child's ability to interpret social cues when it comes to gender conforming behaviour and navigating nuanced social interactions with same and opposite peer groups. Specific neuropsychological profiles with deficits in "theory of mind," the ability to attribute mental states (beliefs, intents, desires, etc.) to oneself and others and recognise that these are different from one's own, may affect development of the "self" in general. When expressing their gender variance and sexuality young people with ASD may be less inhibited by the social norms or even more oppositional to social restrictions when expressing their gender variance. It could be theorised that excessively rigid cognitive style or dichotomous thinking pattern could predispose a child with ASD to interpret slight gender nonconforming inclination as total and fundamental preference.

5.5 Neurodevelopmental masculinisation

The theory of the extreme male brain (EMB) stipulates that individuals with autism may develop an extreme variant of the typical male pattern of behaviours and cognitions originating from high levels of foetal testosterone [57]. While prenatal testosterone is linked to the the association between ASD and GD in assigned girls, explaining the male pattern of their identity and behaviour, same theory cannot applied to assigned boys. Adolescent girls with ASD had a significantly higher prevalence of endorsement of item 'the Wish to be of the Opposite Gender' compared to adolescent boys with ASD [52]. Thus, Van der Miesen et al. [52] partly supports Neurodevelopmental Masculinisation hypothesis but found no significant differences in CSBQ total score between boys and girls with GD, and diverging gender differences on the subdomains of ASD, which are not all consistent with the EMB theory, rendering it highly unlikely [52]. In a comparison sample of birth-assigned females diagnosed with GD, Jones and colleagues [37] established increased rates of ASD symptoms, while birth-assigned males diagnosed with GD did not have increased levels of ASD symptoms. Jones and colleagues hypothesised that elevated levels of foetal testosterone may lead not only to reduced empathy, reduced social interest, reduced social skills, and more ASD, but also contribute to developing GD via neurodevelopmental masculinisation pathway [37]. Among adults with ASD, the symptoms of tomboyism and bisexuality were commoner in females with ASD, while assertiveness and leadership, the aspects that are considered to be typically masculine were reportedly weaker in both, females and males with ASD, compared with typically developing controls [57]. This data signifies that an extreme male pattern might not apply to all aspects of gender roles and sexuality.

A brain MRI study in individuals with ASD also found attenuated typical gender differences in white matter tracts [58], providing support for gender atypicality as one of the potential underlying mechanisms for co-occurring GD–ASD.

5.6 ADHD comorbidity theories

Evidence suggests that core ADHD symptoms and associated externalising disorders are overrepresented in both groups of interest, young people with neurodiversity and young people with gender variance. One large retrospective study had a surprising finding of a significant overrepresentation of gender variance, occurring 6.64 times more frequently among children with ADHD, than among a non-referred comparison group [47]. This study determined that parental report of gender variance was significantly greater in two groups of children, ASD group (5.4%) and ADHD group (4.8%) that collectively represent children with neurodevelopmental disorders, while the proportion of children with gender variance among combined medical group (1.7%) and non-referred comparison group (0–0.7%) were statistically different from ASD and ADHD groups; gender variance occurred equally in girls and boys [47]. In ADHD, impulse control difficulties are essential criteria for diagnosis and could potentially affect gender expression by reducing ability to inhibit primary gender impulses in spite of societal pressure to conform to gender stereotype [47]. Among transgender youth with ASD, children and adolescents may be less aware of the social stereotypes, hence the ASD/ADHD cohort are likely to ignore the societal influences against cross-gender expression and express their gender inclinations more freely or even parade their feelings of gender incongruence as an oppositional response to unaccepted societal rules.

6. Clinical guidelines for co-occurring autism spectrum disorder and gender dysphoria or incongruence in adolescents

The initial clinical guidelines for ASD-GD [59] in adolescents have been developed using Delphi method [60] and contain strong advocacy for adolescents with ASD to gain equal access to gender-related services and not be precluded from gender affirming care when diagnostic criteria of GD are met. The guidelines emphasise the need for carrying out gender assessments in tandem with an in-depth consideration and accommodation of ASD-related factors that may impact gender-related exploration and identify broader needs of neuro-diverse adolescents [59]. Since the initial guidelines' have been publicised, more recent study by Strang et al. [61] reported on a proposed community-driven clinical model to attend to the broader care needs and preferences of adolescents with ASD/neurodiversity and GD. It is also important to engage young people with a lived experience of ASD/GD and their carers into a productive dialogue about further services and interventions as they hold unique insights into how services can best respond to the complex needs of affected individuals and promote many other related domains including education, employment, housing and family services. Certainly, there are reports that the education and community outreach programs as part of gender diversity services are very important to the service users and have grown exponentially in a way that was not initially anticipated [62].

Adolescents with ASD may not embody a binary transgender presentation, while some may conflate sexuality with gender and need affirming education. In the course of psychological therapy, one may wish to explore whether traits of ASD such as intense/obsessional interests or social communication deficits contribute to a child's gender schema (e.g. wishing to be a specific anime character) and, eventually

determine their gender-nonconforming identity. It is especially valuable to discriminate if ASD' influence is long-lasting with no alternative gender preferences, especially in light of reports that highlighted the desistence pattern of gender variance among young people with ASD [54, 63]. The results of these studies should be viewed with caution owing to the lack of robust evidence underpinning the conclusions.

Gender transition is a complex multi-stage procedure that could be difficult to achieve by individuals with neurodevelopmental disorders, including autism and ADHD, as their treatment decisions, planning and follow through may be compromised due to a deficit at a higher-level executive functioning. Typical ASD' cognitive profile also implies a certain level of inflexibility and a highly selective hyper-focus; these qualities are likely to reduce an individual's ability to set and complete goals [64] and may compete with their care needs and treatment priorities. Having a rigid cognitive set may make it hard for a young person with ASD to recognise gender fluidity and to see gender expression as a spectrum; rigid thinking may also lead some to assume that having gender incongruence means that they must seek affirming medical treatment. These more vulnerable individuals may need additional help in navigating the care system and deciding on appropriate service and management plan, its important to ensure that they understand that gender affirming treatment is optional.

7. Bell v Tavistock and consensus on treatment

More recently, gender affirming models of care have come under the medico-legal scrutiny and now the issues of consent to gender altering treatments are being regular debated as part of legislative agendas in English, European, American, Canadian, Australian and New Zealand's' family courts.

7.1 Court case details

This came on foot of highly publicised UK-based Bell v Tavistock high court ruling in December 2020 that found against Tavistock NHS trust in relation to their gender affirming clinic providing "potentially misleading" advice around hormone therapy and therefore jeopardising the integrity of informed consent' gathering, thus making consent process legally invalid [7]. This case was brought by Keira Bell, a 23-year-old woman who was commenced on puberty blockers at the age of 16 before desisting and de-transitioning, and who was joined by the unnamed mother of a 15-year-old girl with autism who is on the waiting list for gender affirming treatment [7]. In their decision, the Rt Hon Dame Victoria Sharp, Lord Justice Lewis and Mrs. Justice Lieven, ruled that it unlikely that children under the age of 16 who were considering gender reassignment treatment were mature enough to give informed consent to be commenced on puberty-blocking drugs [7].

7.2 Specific findings

More specifically, the high court determined that:

1. informed consent in the legal sense cannot be given by young persons under the age of 13.

2. the court was also doubtful that a young person aged 14 or 15 could fully understand the immediate and long-term consequences of the treatment in physical and psychological sense.

3. trans persons under the age of 16 will likely need a court authorisation before starting treatment with puberty blocking drugs [7].

7.3 Examining outcomes

In a more nuanced examination of evidence on treatment outcome, the court highlighted the finding that the overwhelming majority of patients taking puberty blocking drugs proceed to the first step of actual gender reassignment by taking cross-sex hormones, in some cases 100% of eligible transgender individuals who received puberty suppression proceeded to cross-hormone treatment [65]. Reflecting on this trend, some critiques of puberty suppression treatment have even suggested that puberty blockade 'locks' a child into a permanent state of gender incongruence [66]. Therefore, describing puberty blockers as simply a "pause button," "completely reversible" or "life-saving" is misleading to young patients and their families.

7.4 Gillick competence

In the context of gender reassignment treatment, the Bell v Tavistock ruling takes a different view of the Gillick competence and reassesses how a consenting right of a person under 16 years of age operates in practice. While considered fundamentally progressive and encompassing the right to self-determination and autonomy, the Gillick competence could be detrimental to minors and to, so-called, vulnerable populations. The high court was also critical of what it characterised as the Tavistock's "surprising" lack of investigation into the steady rise in referrals of native girls and of individuals with autism spectrum disorder [8]. Social factors, in particular peer influence, social contagion, parent–child conflict, and maladaptive coping mechanisms may be significant contributing factors in cases of adolescent onset gender dysphoria in natal females, recently termed 'rapid onset gender dysphoria', a socially mediated subtype, the validity of which was disputed by scientific community [67, 68].

7.5 Lack of consensus

The issue of a lack of consensus on current early medical treatment was another issue of concern highlighted by the court. Indeed, the clinical guidelines for the management of adolescents with GD differ widely with no clear agreement that has the backing of the colleges of psychiatry or other leading medical colleges. The Royal College of Psychiatrists in the UK takes a conservative view by stating the following: "The College acknowledges the need for better evidence on the outcomes of prepubertal children who present as transgender or gender-diverse, whether or not they enter treatment. Until that evidence is available, the College believes that a watch and wait policy, which does not place any pressure on children to live or behave in accordance with their sex assigned at birth or to move rapidly to gender transition, may be an appropriate course of action when young people first present" [69]. The Royal Australian and New Zealand College of Psychiatrists is currently reassessing their position statement by engaging with a working group of relevant experts and representative groups, which provides little in the way of direction right now.

7.6 Desisters

In addition to raising the consent issues, the premise of the Bell v Tavistock court case also shines the light on the transgender youth who choose to de-transition, as

the complainant Keira Bell is certainly not alone in the so-called 'desisters' camp. According to the Amsterdam outcome study of 77 individuals who were followed up from a young age of approximately 9 years (mean age 8.4 years) until adulthood (mean age 18.9 years), most children with gender dysphoria will not remain gender dysphoric after puberty [70]. This is represented by 43% of original cohort that belonged to desistance group who no longer had a desire for gender reassignment, as opposed to 27% of persistence group who remained cross-gendered [70]. Many children who experience GD will not continue to experience dysphoria into adolescence and adulthood.

The qualitative data that was generated in the same study sample was analysed by Steensma and colleagues and represents insightful interpretation of influences that determined gender identification for desisters and persisters. Interestingly, all subjects representing both groups pointed towards the changes in their social environment, physiological and biological changes that were either anticipated or took place, and their first experiences of falling in love and developing sexual attraction as major influences in their gender related interests and behaviour [71]. Taken together, the prior research supports the notion that persistence of childhood GD is most closely linked to the intensity of early GD, as well as the amount of gender diverse behaviour and body discomfort as a result of the feeling of the incongruence between the bodily characteristics and gender identity. There are also recognisable differences in motives or cognitive constructs of the dysphoria. Although, both persisters and desisters in Steensma et al. study [71] reported a desire to be the other gender during their childhood years, the underlying motives of their desire differed between persisters who explicitly indicated that they believed to be the "other" sex and the desisters who only wished to be the "other" sex. Interestingly, the desisters also indicated that their incongruence was more likely to be caused by the perceived mismatch of their bodily representations and the desired social gender role. In line with these findings, Drummond et al. [72] found that girls with persisting GD recalled significantly more gender-variant behaviour and GD during childhood than the girls classified as having desisting GD. Another study of 139 natal boys with gender identity disorder by Singh [73] confirmed the link between the intensity of childhood GD and adolescent and adult persistence of GD; Singh also linked the desistence of GD with a higher social class.

7.7 Seven points of difference

The high court judgement reads like a cautionary tale remining us that overconfidence in new treatments is dangerous. With this ruling, the High Court has set up an expectation of accountability of the health professionals engaged in the provision of paediatric and adolescent medical transition. The issue of consensus on best treatment was explored by the recent Dutch empirical ethical study that generated seven points of difference that needs resolving before the views on treatment could be unified [74]. Among these seven contentious points are following themes:

1. a sound explanatory model for GD

2. the heterogeneity and the diagnostic construct' stability of GD

3. the role of comorbidity

4. the recognition of normal gender variation

5. an issue of social contagion

6. the implications of medical treatment, and finally

7. the validity of consent, recognising complexities around parental rights to consent and ability to understand where the person is on their transgender journey, believing in people trusting themselves and being in charge of their bodies and their own destiny.

Most of these queries remain either unexplained or fraught with controversy at present, hence, we require more strict system of checks and balances to deliver the right treatment for GD's sufferers.

8. Perth experience

The Gender Diversity Service (GDS) located at Perth Children's Hospital (PCH) in Western Australia, has been specifically designed as a dedicated tertiary specialist service for children and adolescents who present with clinically significant forms of GD. The author's first-hand experience of working in GDS gives her additional insight into the benefits and challenges of operating of such Tier-4 clinical service. GDS is a state-wide service that that has been set up in 2015 as part of local child and adolescent mental health services to provide assessment, consultation and gender-affirming treatment for young people under 18. From the outset, this recently established public service has been committed to embedding research in everyday care by developing a longitudinal cohort database "The GENder identiTy Longitudinal Experience" (GENTLE) and attracting research staff to their multi-disciplinary team [13]. GDS has been conceptually developed with so-called Dutch model of care in mind after the process of comprehensive national and international consultation and developing adequate skill base.

For the purpose of this publication, the author wanted to use Western Australian example in order to illustrate how does influential Bell v Tavistock ruling affect other jurisdictions. The Australian legal system historically takes a very respectful view of advances in English law, and it is assumed that an Australian judge would be expected to give significant weight to the Bell v Tavistock ruling. Australian services, such as PCH GDS, are following the recommendations from Bell v Tavistock ruling and there is a sense that Australian health practitioners would be viewed in a negative light if they were aware of the Bell v Tavistock ruling but continued to practice in a way that is not consistent with court's recommendations. The Court authorisation is intended to take into consideration the young person's best interests as well as the court's view of their capacity and will provide some validation to the consent gathering process and lend some additional support and security for treatment to be considered medico-legally sound.

From now on, all young people under 18 who wish to start new puberty suppression treatment or cross sex hormone treatment will need to go through the Family Court via Legal Aid and gender service providing necessary mental health and endocrinology reports to the court. Regardless, if both parents consent, or/and if the gender clinician determines that the young person does have capacity to consent, the decision to treat is to be taken to the Family Court to provide a further level of authorisation. On a positive note, it could be opportunistic that a new body of case law, arising from young people's cases, may establish Australian precedents which could support young people in accessing gender affirming health care in Australia and internationally.

9. Conclusion

In conclusion, taking into account latest research findings and vast body of information generated and shared by specialists and researchers in both fields, autism and gender identity, the evidence is supportive of higher rates of clinical diagnoses of ASD among gender diverse samples, including social deficit and various degree of restricted and repetitive behaviours, thus confirming a link between autism and gender incongruence. The scientific community is concerned that dismissing gender variance as another manifestation of autism may place affected individuals at risk and delay people with ASD accessing gender diversity services.

With regard to diagnostic process and increased recognition of heterogeneity that exists within transgender youth populations, there is a growing concern that the current diagnostic categories do not adequately differentiate the children with true forms of GD from those who show merely gender nonconforming behaviour. Clinically, the intensity of early GD is an important predictor of persistence of GD and may help clinicians to accurately discriminate between persisters and desisters before the start of puberty. Some experts believe that the clinical recommendations should be separated between natal boys and girls, as their presentation of GD is distinct with different predictive factors for the persistence of GD.

The international commitment to rigorous clinical research in the area of gender diversity and running more naturalistic studies that are unaffected by a clinical context, will hopefully provide us with reliable and safe treatment options for children and adolescents experiencing gender dysphoria. Until that time we will be exploring all available options with young people and their families before undergoing invasive interventions with unknown long-term implications. For now, the enormous, growing body of knowledge on gender dysphoria requires synthesis, integration, and generation of sound clinical recommendations. There is a call for more systematic and adequately powered multi-centre studies that are expected to move beyond a confirmation of the existing overlap between ASD and GD and towards a translational research into the underlying causes of the overlap between these two spectrums of autism and gender diversity, as well as offering insight on how autism presents in gender-diverse people. I would like to end with a notion that a gender curiosity, "fantasy that one can change one's gender on demand" [75] sometimes is just that; and at least for some, this fantasy is better than reality of gender reassignment. Knowing that may open our minds to more sensitive and exploratory approach that informed by therapeutic listening, mentalisation, non-judgmental positive regard with empathy, respect and ability to tolerate uncertainty.

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Section 2

Neurobiology of Autism

Chapter 4

Parenting and Reproductive Stoppage in the Psychopathology for Recurrence Risk of Autism Spectrum Disorder

Michael Beenstock

Abstract

During 1950 to 1975 autism was considered to be psychopathological in origin, brought on by 'bad' mothering in particular. Subsequently, research into the etiology of autism spectrum disorder (ASD) has been dominated by the neurodevelopmental paradigm according to which ASD is genetic or biological in origin. In the present paper population cohort data for Israel are used to show that recurrence risk of ASD (when more than one child has ASD) depends on three parent-related phenomena. First, it varies inversely with the 'veil of ignorance' defined as the period of time younger siblings were raised before their elder sibling was diagnosed. Second, it varies inversely with the 'shadow of ASD' defined as the period during which parents raised their child with ASD before younger siblings were born. Third, recurrence risk is greater if parents knew the ASD status of their child before conceiving their next child. These three effects, which are shown to be consistent with a behavioral theory of ASD, are inconsistent with neurodevelopmental theory. They suggest that what parents know or do not know about the ASD status of their child is salient for recurrence risk in their subsequent children.

Keywords: reproductive stoppage, natural experiment, recurrence risk, psychopathology of ASD, neurodevelopmental theory, population cohort data, diagnostic timing

1. Introduction

> *"Many people made a mistake in going from a statement which is undoubtedly true – that there is no evidence that autism has been caused by poor parenting – to the statement that it has been disproven. It has not actually been disproven. It has faded away simply because, on the one hand, of a lack of convincing evidence, and on the other hand, an awareness that autism was a neurodevelopmental disorder of some kind."*
>
> *Sir Michael Rutter [1].*

Rutter, a pioneer of the neurodevelopmental paradigm for autism spectrum disorder (ASD), was referring to the early belief that the etiology of autism was behavioral, induced by "refridgerator" mothers in particular and poor parenting in

general. His reference to "poor parenting" was intended as a criticism of theories due to Kanner and Bettelheim, who claimed that bad parenting plays a key role in the etiology of autism. Kanner [2], who identified autism as a separate pathology, observed that few of his 11 patients had warm-hearted parents, and subsequently noted that his patients "were exposed from the beginning to parental coldness, obsessiveness and a mechanical type of attention to material needs only." Moreover, it is as if they had been "kept in refridgerators which did not defrost." [3] Bettelheim [4] took this argument further, and attributed autism exclusively to the behavior of parents in general, and to "refridgerator mothers" in particular. Indeed, psychoanalytical theory continues to inform the treatment of ASD in some parts of the world, especially in France, Argentina and South Korea [5].

Kanner eventually took exception to Bettelheim's position, noting that "at no time have I pointed to parents as the primary post-natal source of pathogenicity." [6] Subsequently, this developmental psychopathology was discredited following the scandal which broke out after Bettelheim's death [7, 8] and was abandoned in scientific research. Indeed, behavioral research into autism disappeared altogether.

Rutter was also referring to the dominance of the neurodevelopmental model, pioneered by Rimland [9], in the empirical study of ASD, according to which its etiology is mainly genetic or biological and is also environmental. However, environmental factors exclude parents and what occurs within families, and refer instead to exposure to pollution and related factors that might harm brain development [10].

In the present paper we report empirical results for the recurrence risk of ASD, which are inconsistent with the neurodevelopmental model, and for which behavioral interpretations are suggestive. These results are generated by a natural experiment [11] in which the age at which children with ASD are diagnosed serves to randomize their parents' state of mind at the time they decided to have further children. Some parents had further children before their previous child was diagnosed with ASD, while other parents had further children after diagnosis. The former parents could not have engaged in reproductive stoppage [12] because they did not know (for sure) that their child had ASD. The latter parents, by contrast, consciously refrained from reproductive stoppage.

The two types of parents are different in other ways too. Parents who could not have engaged in reproductive stoppage raised their next child under a "veil of ignorance", which lasted until their previous child was diagnosed with ASD. By contrast, parents who refrained from stoppage raised their next child in the "shadow of ASD", which lasted from when previous children were diagnosed until their younger siblings were born. Neurodevelopmental theory attaches no importance to the veil of ignorance and the shadow of ASD, or whether parents conceived younger siblings before or after their previous children had been diagnosed. Parents under the veil of ignorance might be less stressed than other parents. On the other hand, parents in the shadow of ASD gained experience in raising children with ASD. If recurrence risk depends empirically on the durations of the veil of ignorance and the shadow of ASD, this begs a behavioral interpretation in which stressed parents may be more likely to raise children with recurrence risk, when experienced parents are less likely.

The main hypothesis of interest is whether recurrence risk of ASD depends on phenomena such as the veil of ignorance and the shadow of ASD. Since recurrence risk is only observed if parents do not engage in reproductive stoppage, these phenomena are to some degree self-selected. If so, their causal effect on recurrence risk would not be identified. To establish causality an auxiliary hypothesis is proposed in which reproductive stoppage depends on when elder siblings are diagnosed with ASD. If the latter is independent of recurrence risk, it serves to

randomize the veil of ignorance and the shadow of ASD, which are related to reproductive stoppage, and thereby identify their causal effects on recurrence risk.

We use population cohort data for Israel to study reproductive stoppage and to show that the risk of ASD recurrence among younger siblings of children diagnosed with ASD depends causally on the durations of the veil of ignorance and the shadow of ASD.

2. Theory

2.1 Reproductive stoppage

Let C_{it}^{*} denote a latent or index variable ([13], p. 888), which measures parents' desire in family i in the general population to have a further child when their previous child is aged t. C_{it}^{*} is hypothesized to depend on a vector of observable covariates (X_i) including the existing number of children, their gender mix, the age of mothers and perhaps fathers, their ethnicity, schooling and economic status etc. Parents have unobserved preferences for children denoted by c_i. Parents may penalize small birth gaps in the interest of birth-spacing, but the penalty, denoted by $g_i(t)$, tends naturally to zero with the birth gap (t).

The latent variable model for the general population may be written as:

$$C_{it}^{*} = X_i\beta + c_i - g_i(t) \tag{1}$$

where β is a vector of parameters to be estimated. Let C_{it} denote a zero–one dummy variable, which equals 1 if parents conceive their next child when their previous child is aged t. This event occurs when C_{it}^{*} turns positive as illustrated in **Figure 1** where C* is measured on the vertical and the age of the previous child (t) is measured on the horizontal axis. Schedule A plots the relation between C* and t in Eq. (1), and is drawn for positive c and Xβ = 0. Schedule A is naturally negative at the origin unless parents wish to conceive straight away, and it tends to c as the birth gap (t) increases. Parents conceive when C* turns positive, when their child is aged t_0, which varies inversely with their preference for children (c) and directly with g(t). If c is negative they will not conceive at all because C* remains negative.

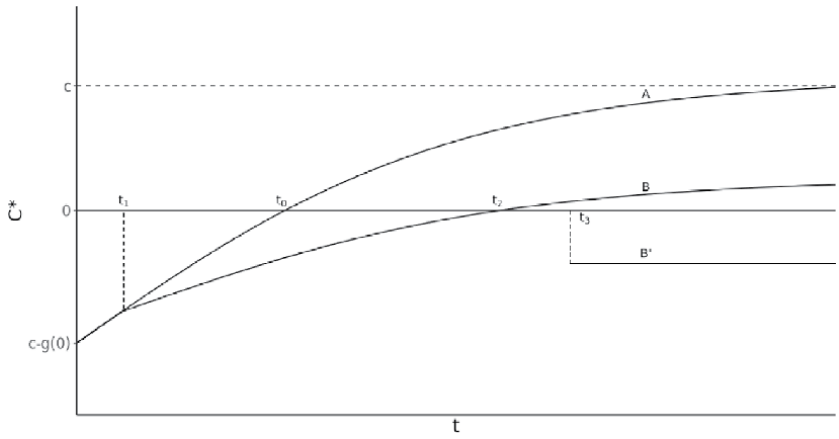

Figure 1.
Conception timing of younger siblings.

Eq. (1) applies to the general population, which we adapt for parents of children with ASD. We introduce two new unobservable phenomena in addition to c and g, which apply specifically to parents of ASD children. When their index child is born, parents are unaware that they are no longer part of the general population. However, they gradually realize that their child has developmental difficulties, denoted by d(t), which varies directly with age (t). Even before their child is diagnosed with ASD, they might consider reproductive stoppage. Parents also vary by their resilience [14], or their ability to cope with crises denoted by r, which may be positive or negative. We add to Eq. (1) $r_i - d_i(t)$, which may be positive for resilient parents.

Schedule B in **Figure 1** refers to parents of ASD children. At first, it is congruent with schedule A, but after their child is aged t_1, at which parents begin to worry about their child's developmental problems, it lies below schedule A, where the vertical difference between the schedules equals r – d(t). This distance naturally increases with t. In **Figure 1** schedule B becomes positive at t_2, so the parents of children with developmental difficulties will tend to delay conception relative to the general population. Of course, schedule B might never become positive, in which case parents engage in reproductive stoppage.

Suppose that the child is diagnosed with ASD when he or she is aged t_3. This would induce a discontinuous increase in d, which lowers schedule B as in schedule B′. The parents depicted in schedule B, who conceived when their child was aged t_2, regret their decision. However, it is too late and they could not have known. Matters would have been different had their child been diagnosed prior to t_2. Note that if parents are sufficiently resilient and their desire for children is sufficiently strong, schedule B′ may lie above schedule B, in which case parents conceive further children despite the ASD status of their child. We refer to this by "informed" non-stoppage, and the solution at t_2 by "uninformed" non-stoppage.

This theory implies that observationally similar parents (with the same X) in the general population will have different probabilities of natural stoppage. It also implies that observationally similar ASD parents will have larger probabilities of reproductive stoppage than in the general population. Finally, it implies that observationally similar parents of ASD children have different probabilities of non-stoppage because they differ by their resilience (r), their desire for children (c), their reaction to developmental difficulties and to diagnoses of ASD (d). It also means that observationally similar parents in the general population cannot be compared with the parents of ASD children, because β in the general population may differ, and because r and d do not apply to the general population. Finally, the probability of non-stoppage varies directly with the age at which ASD is diagnosed, and informed non-stoppage is less probable than uninformed non-stoppage.

In summary, the probability of non-stoppage is predicted to depend through β upon the observable covariates (X) including age at diagnosis. Eq. (1) is estimated using data for families with ASD children only; data for the general population are not used.

2.2 The veil of ignorance and shadow of ASD

Suppose A and B are two observationally similar families. Their first children have ASD, and their second children were born three years afterwards. The only difference is that ASD was diagnosed in family A at 2 years and in family B at 8 years. This gives rise to three differences between families A and B. First, when family A decided to have their second child, they already knew about the ASD status of their first child. They decided against reproductive stoppage in having their second child. Matters are obviously different in family B; they had their second child without knowing about the ASD status of their first child. Second, the

younger sibling in family A was raised in the 'shadow of ASD'. His parents had a year's experience raising a child with ASD before their second child was born. Family B obviously had no such experience before their second child was born. Third, the second child in family B was raised until 5 years under a 'veil of ignorance', which ended when his elder sibling was diagnosed. During the veil of ignorance family B might have been concerned about developmental delays in their child, but they did not know for sure that their child would eventually be diagnosed with ASD. In family A the veil of ignorance is zero and the shadow of ASD is a year. In family B the veil of ignorance is 5 years and the shadow of ASD is zero.

According to the neurodevelopmental paradigm of ASD, recurrence risk should be the same for families A and B, because parents' knowledge about the ASD status of their index children plays no role in the neurodevelopmental model. Neurodevelopmentalists might argue, however, that family A was more genetically predisposed to ASD recurrence than family B. Family A's child was diagnosed sooner because his ASD were more severe than B's. That is why A's child was diagnosed more quickly. This argument would predict that recurrence risk should be greater in family A than in family B. Suppose, however, that their recurrence risk differs, and that recurrence risk in B- type families is greater than in A-type families. We suggest three behavioral reasons why this might arise. First, family A is positively self-selected because it decided against stoppage. Parents in family A decided to go ahead despite the risk, either because they were more resilient and self-confident of coping with this risk, or because they suspected that the risk of recurrence is relatively low in their family. Either way, this reduces recurrence risk in family A relative to family B. Second, family B had five years to raise its second child under the veil of ignorance, whereas family A raised its second child entirely in the shadow of ASD. If knowledge of ASD empowers family A to mitigate the risk of recurrence, this would further reduce recurrence risk in A-type families relative to B-type families. On the other hand, if knowledge imperils rather than empowers, A-type families who are fearful of ASD might raise their second child less successfully relative to B-type families who are unaware of ASD. This might increase recurrence risk in A-type families relative to B-type families.

The empower-imperil dichotomy is related to self-fulfilling and self-defeating theories in social psychology [15], dating back to Thomas' Theorem [16]. Family A's knowledge of ASD may become a self-fulfilling expectation if parents believe and fear that ASD will recur in their younger child. If, instead, family A uses its knowledge and experience with ASD to mitigate recurrence risk, the expectation of ASD is self-defeating. During the veil of ignorance, family B has no knowledge of ASD. If knowledge empowers, recurrence risk among B-type families is expected to vary directly with the veil of ignorance. The converse is expected if knowledge imperils.

3. Methodology

3.1 Reproductive stoppage

Two empirical methodologies are considered for estimating β in Eq. (1). If $r + c - d - g = u$ is assumed to have a logistical distribution, β may be estimated by logit using data for C. For informed non-stoppage, the relevant population consists of parents who conceived further children after the date of diagnosis of their index child. For uninformed non-stoppage, the relevant population consists of parents who conceived further children before this date of diagnosis. These two populations may be combined by controlling for the age of diagnosis of the index child. Parents

are less likely to have further children if their index child is diagnosed sooner rather than later.

The second methodology is based on survival analysis focusing on the age of the index child when and if parents conceived their next child. Specifically, a Cox proportional hazards model may be estimated for these purposes. The second methodology [17, 18] is more ambitious than the first [19], because it professes to explain the timing of conception or birth and not merely whether stoppage occurred or not. We prefer the first method to the second because more ambitious methods are generally less robust. For example, Hoffmann et al. [17] assume that birth hazards are strictly proportional to all the covariates in their model, even though this assumption is not essential for testing hypotheses about non-stoppage. They also compare parents of ASD children with parents in the general population, a between-group comparison, instead a within-group comparison in which the parents of ASD children who had further children are compared with parents who had no further children.

Because the data used in the present study end in December 2012, fertility is right-censored; parents of index children who had no further children by December 2012 might have had children subsequently. Hence, censoring artificially increases stoppage even controlling for age of mothers in December 2012. If mothers' age in December 2012 exceeds 45 years, fertility is ascertained and is not censored. A radical solution to the censoring problem would be to ignore diagnoses made after 2004 under the assumption, for example, that parents must have stopped if younger siblings are not born within 8 years. An alternative solution, which avoids discarding data, is to assume that the probability of censoring varies inversely with mothers' age in December 2012. Since this probability is likely to vary nonlinearly with age in December 2012, we estimate this censoring effect as a spline ([13], p. 199). We also use splines to estimate other potentially nonlinear time related variables, such as mothers' age and the age at diagnosis of index children.

If C equals one (non-stoppage), the number of children may be larger or smaller. Just as observationally similar families might stop or not depending on what is not observed (u), so might they choose to have different numbers of children if they do not stop absolutely. Since the number of younger siblings of index children has the character of count data, which take discrete but limited values such as 0, 1, 2, etc. we suggest the use of count data methods [20] to test hypotheses about relative stoppage in which the dependent variable is C_i = 0, 1, 2, etc. Specifically, we use "zero-inflated" Poisson regression (ZIP) where the probability of absolute stoppage (C = 0) is enlarged according to a complementary log log (CLL) model for the probability of absolute stoppage, and where u is assumed to have a Poisson distribution ([13], p 861; [21]). ZIP embodies the intuition that to have any further children is a harder decision than to have more or fewer further children. This specification combines absolute and relative stoppage, where the former is expressed through zero inflation, and the latter by count data regression.

According to ZIP, the probability of having no further children is:

$$P_i(0) = \lambda_i + (1 - \lambda_i)\exp(-\mu_i) \qquad (2)$$
$$\lambda_i = 1 - \exp[-\exp(X_i\gamma)].$$
$$\mu_i = \exp(Z_i\theta).$$

where λ denotes the CLL probability of having no further children, X are covariates in the CLL model, exp.(−μ) is the Poisson probability of having no further children, and Z are covariates in the Poisson model. Since P(0) is the probability of absolute stoppage, it varies directly with λ and inversely with μ. CLL is a nonlinear transform of the logit model since exp.(Xγ) equals the log odds ratio.

Relative stoppage occurs when parents who refrain from stoppage have fewer further children. The ZIP probability of having positive numbers of children (C > 0) is:

$$P_i(C>0) = (1-\lambda_i)\mu_i{}^C \, exp\,(-\mu_i)\frac{1}{C!} \tag{3}$$

Suppose for family i the CLL and Poisson probabilities of absolute stoppage are 0.305 (= λ) and 0.223 (= exp.(−μ)) respectively so that the probability of absolute stoppage, P(0), is 0.46 (as in our data). These probabilities imply that μ = 1.5, i.e. family i is expected to have 1.5 further children. The ZIP probability of having one further child is 0.232 and having two further children is 0.174. Hence, ZIP has inflated the probability of having no further children from 0.223 to 0.46, and it has deflated the Poisson probability of having positive numbers of further children by a factor of 1 - λ.

The expected value of the number of further children given that it is positive equals:

$$E(C>0) = \frac{(1-\lambda)\mu}{1-P(0)} = \frac{\mu}{1-\,\exp\,(-\mu)} \tag{4}$$

which varies directly with μ and does not depend on λ. In summary, absolute stoppage varies directly with λ and inversely with μ, and relative stoppage varies inversely with μ.

3.2 Recurrence risk

As in Sandin et al. [22] and Beenstock et al. [23], we use population cohort data to estimate logit models for ASD recurrence in which the covariates include standard variables, such as the ages of parents and their ethnicity. We supplement these variables by three additional variables. The first is a dummv variable ('informed') that equals 1 if the younger siblings of index children were conceived or born after the index child was diagnosed, and zero otherwise. If parents who refrain from reproductive stoppage are positively selected, the coefficient of 'informed' is expected to be negative (smaller recurrence risk). If they are negatively selected, the coefficient is expected to be positive. According to the neurodevelopmental model, the coefficient is expected to be zero.

The second variable is the duration of the veil of ignorance, which is measured by the age of younger siblings when index children were diagnosed. The veil of ignorance is zero, of course, if 'informed' = 1. If knowledge imperils, recurrence risk is expected to vary inversely with the veil of ignorance; ignorance is bliss. According to the neurodevelopmental model, the coefficient on the veil of ignorance is expected to be zero.

The third variable is the duration of the shadow of ASD, which is measured by the date of birth of younger siblings minus the data of diagnosis of the index child. If knowledge empowers, experience in raising children with ASD may help parents raise their further child more effectively, in which case recurrence risk is expected to vary inversely with the shadow of ASD. If, instead, knowledge imperils, recurrence risk is expected to vary directly with the shadow of ASD. Knowledge is expected to imperil when parents who refrain from stoppage are negatively selected. According to the neurodevelopmental model, the coefficient on the shadow of ASD is expected to be zero.

If parents who refrained from stoppage are negatively selected, it might be expected that for them knowledge imperils, in which case recurrence risk would vary directly with the shadow of ASD. If, instead, they are positively selected, their knowledge might be expected to empower them to mitigate the risks of ASD recurrence. Therefore, estimates of the coefficients on 'informed' and the shadow of ASD are unlikely to be independent.

The study of recurrence risk has typically focused on immediate younger siblings. In the present study, we also attach importance to higher order siblings. ASD may not recur among immediate younger siblings, but it may recur among higher order siblings. Inevitably, estimates of recurrence risk and its determinants, may be biased if the incidence of ASD recurrence among higher order siblings is ignored. This bias will be smaller in countries where fertility is low. The bias would be zero if parents limited their fertility to two children. Matters are different in our empirical application for Israel where fertility is high. In our study, families supply more than one observation for estimating recurrence risk. For example, a family with 8 children supplies 7 observations if their firstborn is diagnosed with ASD.

The use of data for all younger siblings raises two statistical concerns. First, the outcomes of younger siblings from the same family are unlikely to be independent; they certainly cannot be treated as the independent outcomes of younger siblings from different families. They share the same parents, the same index child, and they share each other. Consequently, we cluster standard errors of parameter estimates by family ([13], p. 586). Second, we estimate family specific effects that capture familial phenomena that might induce recurrence risk ([13], chapters 11 and 17). These phenomena may be neurodevelopmental or genetic, but they may also be behavioral. Whereas clustering picks up interactions between siblings, specific effects pick up patterns related to families.

Another difference is that, as in the case of Eq. (1), we use censoring methods instead of discarding observations, which are potentially censored. Our data are obtained from administrative records in Israel up to December 2012. Younger siblings born, for example, in 2009 might not have been diagnosed with ASD by December 2012. However, their contribution to recurrence risk estimates is censored since they might have been diagnosed with ASD in 2013 and beyond. Some investigators assume that it takes 8 years for ASDs to be diagnosed [24], and would exclude younger siblings born after 2003. This radical solution to censoring typically discards many observations. In any case, we show below that 8 years is not long enough. Instead of discarding data, we assume that the probability of censoring varies inversely with younger siblings' age in December 2012. Siblings who were teenagers in December 2012 are uncensored.

Apart from censoring there are several covariates that are related to time, e.g., the age of mothers when their index child was born, age at diagnosis of index children, year of diagnosis, veil of ignorance and shadow of ASD. These time-related variables are not expected to have linear effects. For example, mothers' fecundity at age 40 is naturally smaller than at age 30. Also, the probability of censoring is expected to vary nonlinearly with the age of younger siblings in December 2012. Therefore, we estimate these relationships as splines ([13], p. 199).

4. Population cohort data

The study group comprises the younger siblings of children diagnosed with ASD in Israel during 1984 to 2012. The outcome of interest is whether ASD recurred among these younger siblings. Since 1981, families of children diagnosed with ASD have been eligible for benefit from Israel's National Insurance Institute (NII).

Applications for benefit are processed rapidly (within about two months) and benefits are back-dated to the date of diagnosis, provided the application was lodged within 12 months of diagnosis. Consequently, the date of diagnosis is recorded. These data have been matched using the Population Registry and personal id numbers to the parents (and step parents) and siblings (and half siblings) of the children diagnosed with ASD. Dates of conception are approximated by dates of birth minus 9 months. Hence, we are able to determine whether index children were diagnosed before the conception of their younger siblings, during their pregnancy, or after their birth.

Details regarding administrative data sources, diagnostic criteria, the study population, as well as data tabulations etc. may be found in Beenstock, Levine and Raz [23], who used these data in a previous study. The study population comprises 9572 cases of ASD diagnosed during 1984 and 2012, involving 9117 families. Hence, there are 455 cases of recurrence risk. However, in 88 recurrences younger siblings were diagnosed before their older siblings, leaving 367 recurrences according to birth order. Judging by the proximity in diagnoses of these 88 cases, we suspect that attention was drawn to elder siblings once their younger siblings were diagnosed. Since almost all cases of ASD in Israel are known to NII [25], these data constitute population cohort data, which in contrast to survey data and clinical samples, are likely to be free of sample selectivity.

Table 1 shows that 4219 parents of children with ASD had no further children by December 2012. However, many families had several further children, reflecting the high rate of fertility in Israel. The same applies to the birth orders of index children, of which 4076 were firstborns. In many families, however, index children are not firstborns. Indeed, **Table 1** shows that ASD may suddenly occur after the birth of several children. These data may be unique in enabling the estimation of birth order effects on stoppage and related phenomena. Finally, **Table 1** reports years in which the diagnoses were made, and the number of cases for population subgroups, of which ultra-orthodox Jews account for 12 percent of the population, and non-Jews (mainly Arabs) who account for 20 percent of the population. See Raz et al. [25] for further discussion of the incidence of ASD among these sub-groups.

The age at which autism spectrum disorders (ASD) are diagnosed has a wide variance. Some children are diagnosed quickly, before they are 3 years old, while others are diagnosed in their teens. **Figure 2** shows that in Israel although 40 percent were diagnosed by the age of four, the age distribution has a long tail, and more than 10 percent were diagnosed after they were ten years old. This means that many parents raised the younger siblings of children who are eventually diagnosed with ASD without being aware of the ASD status of the latter. It also means that many parents did not engage in reproductive stoppage because they were unaware of the ASD status of their index child when their subsequent children were conceived or born. **Figure 2** also shows that initially girls are diagnosed more quickly than boys.

The first column of **Table 2** refers to the proportion of parents who refrained from stoppage by ethnicity and year of diagnosis. The second and third columns refer to the proportions of children whose parents were informed or not when they were born and conceived. Note that because parents who refrained from stoppage had several further children (**Table 1**), they might have been informed for some of these children, especially higher order siblings, and uniformed for others, especially immediate siblings.

Figure 3 plots the distribution of the duration of the veil of ignorance for uninformed parents. It has a mode at 2.5 years with a long right-hand tail. For some, the veil of ignorance exceeds ten years. During this period, parents raised their further children without knowing that their index child would eventually be diagnosed with ASD.

Number diagnosed	9572
Number of families	9117
Number of younger siblings	
0	4219
1	2974
2	1222
3	378
4	151
5	91
6	33
7	28
8+	21
Index Year of Diagnosis	
1989–1995	217
1996–2001	1622
2002–2006	2534
2007–2012	4744
Index Birth Order	
1	4076
2	2588
3	1336
4	563
5	265
6	131
7	66
8	30
9	23
10	18
11+	21
Ethnicity	
Jews	8539
Ultra-orthodox Jews	935
Non-Jews	500
Mixed-marriages	28

Table 1.
Study group characteristics.

Figure 4 plots the distribution of the shadow of ASD. It has a mode of a year and long right-hand tail. Some parents reared their children with ASD for as long as ten years and more before their younger siblings were born. Indeed, there is much similarity between the distribution of the shadow of ASD in **Figure 4** and the veil of ignorance in **Figure 3**. Both distributions have natural minima at zero, and do not

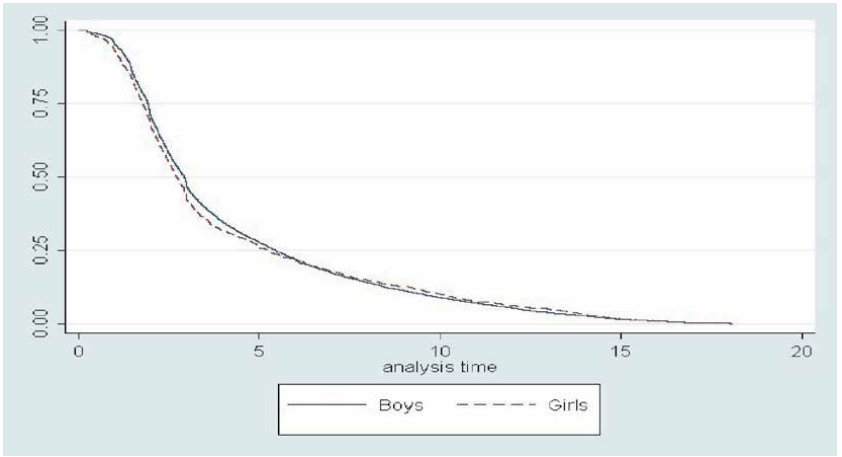

Figure 2.
The age distribution of ASD diagnoses in Israel.

No-Stoppage	Total		Informed	Uninformed
All	54%			
Jews	4618	54%	2690	2818
Non-Jew + Half	280	53%	174	179
Ultra-Orthodox	706	76%	466	528
Not Ultra-Orthodox	4192	52%	2398	2469
Year of diagnosis				
1989–1995	124		96	56
1996–2001	1019		815	436
2002–2006	1428		1016	741
2007–2012	2327		937	1764

Table 2.
Non-stoppage: Informed and uninformed.

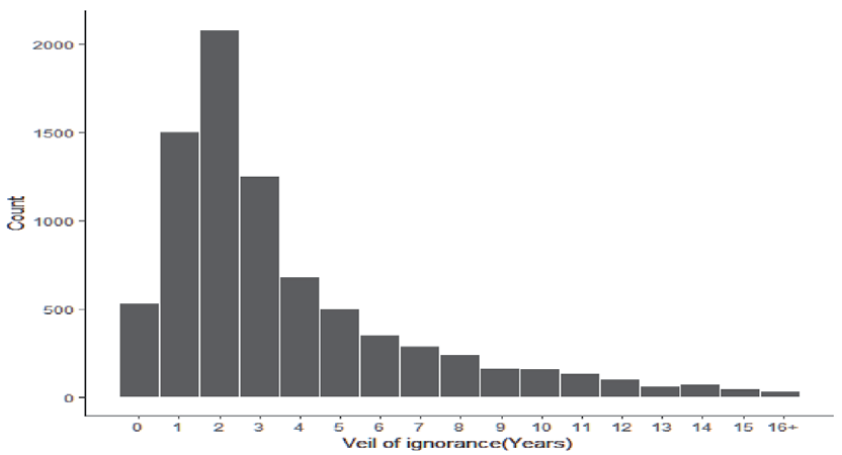

Figure 3.
The distribution of the veil of ignorance.

overlap. If the veil of ignorance is zero, the shadow of ASD must be positive. If the shadow of ASD is zero, the veil of ignorance must be positive by definition.

Rates of recurrence risk are reported in **Table 3**. Overall recurrence risk is 4.53 percent. However, for diagnoses of index children made prior to 2000 recurrence risk was lower (3.8%). Recurrence risk among the ultra-orthodox is smaller (3.2%)

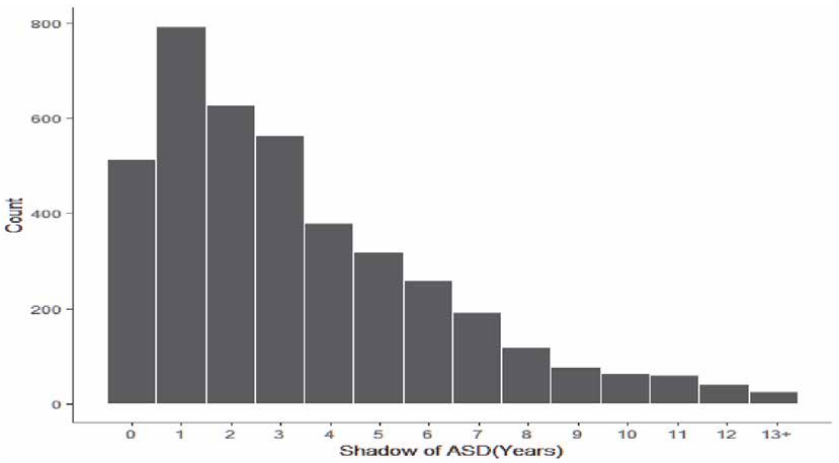

Figure 4.
The distribution of the shadow of ASD.

	Recurrence Risk
All	0.0453
Before 2000	0.0380
Ultra-orthodox	0.0320
Boy - boy	0.0642
Girl –girl	0.0321
Girl - boy	0.0862
Boy- girl	0.0185
Informed	0.0367
Informed – from conception	0.0350
Uninformed	0.0476
Uniformed – from conception	0.0468
Veil of Ignorance	
< 1.25 years	0.0669
1.25–3.5 years	0.0572
> 3.5 years	0.0447
Shadow of ASD	
< 1.32 years	0.0439
1.32–3.33 years	0.0572
> 3.33 years	0.0278

Table 3.
Rates of recurrence risk.

because (as explained below) their fertility is higher. Recurrence risk also depends on gender mixes. The largest risk (8.62%) occurs when the index is a girl and her younger sibling is a boy. The smallest risk (1.85%) occurs when the index is a boy and his younger sibling is a girl.

Recurrence risk is a percentage point larger if parents are uninformed. For example, using conception as a reference point, recurrence risk for the informed is 3.5% and for the uninformed it is 4.68%, which seems to suggest that knowledge empowers more than it imperils. Recurrence risk also appears to vary inversely with the veil of ignorance, and perhaps to vary inversely with the shadow of ASD. The former appears to suggest that ignorance is bliss, and the latter appears to suggest that experience in raising children with ASD helps parents mitigate recurrence risk.

5. Results

5.1 Absolute stoppage

The results in **Table 4** refer to the probability of parents of children with ASD having further children (non-stoppage) by the end of the study period in December 2012. Model 1 refers to all parents regardless of being informed or not. It shows that non-Jews (Arabs) and ultra-orthodox Jews are more likely to engage in non-stoppage than Jews in general, while mixed couples (Jews and Arabs) behave similarly to Jews in general. A number of variables capture the effect of target family size. Non-stoppage varies inversely with the birth order of the index child. If the index child has a twin, the probability of non-stoppage decreases by more than what is implied by birth order. Several studies have shown that there is male preference in Israel [26, 27]; parents are more likely to have further children if their children are all girls. **Table 4** suggests that male preference does not apply to ASD families. Finally, the presence of other disabled siblings in the family increases stoppage, but this effect is not statistically significant.

Several time-dependent variables in **Table 4** have been estimated by splines, all of which are statistically significant. The direction of their effects are indicated by +/− signs in **Table 4**. For example, older mothers are less likely to engage in non-stoppage. Mothers who were older in 2012 were more like to have not stopped, implying that the fertility of younger mothers in December 2012 is right-censored, as expected. Finally, the probability of non-stoppage varies directly with age of diagnosis, implying that ignorance about the ASD status of their children reduces the probability of stoppage.

Models 2 and 3 decompose the non-stoppage models for informed (at birth) and uninformed parents. Models 2 and 3 refer to the probability of informed and uninformed stoppage respectively in the population as a whole. The covariates that are statistically significant (or not) in Model 1 are also statistically significant in Models 2 and 3. However, their odds-ratio coefficients are different. On the whole, their deviations from unity are larger for the uninformed than the informed. For example, the OR coefficient for non-Jews is 1.62 in Model 3 and 1.34 in Model 2, and the coefficients for twins are 0.67 and 0.25 respectively. Age at diagnosis is omitted from Model 2 because it is not relevant for informed parents, but it is extremely statistically significant in Model 3. This effect ranges between −2 at two years to 1.8 at five years and 2 at ten years. Therefore, the size effect of age at diagnosis on the odds ratio for stoppage is large and negative, especially over the range of 2–5 years.

Model 4 refers to uninformed parents according to age at conception rather than age at birth. The OR coefficients in Model 4 should therefore be compared with their counterparts in Model 3. On the whole, the OR coefficients are similar in terms

	1 Informed + Uniformed		2 Informed from birth		3 Uninformed from birth		4 Uninformed from conception	
	OR	**P-value**	**OR**	**P-value**	**OR**	**P-value**	**OR**	**P-value**
Intercept	8.5079	0.0380	0.5022	<0.0001	0.2999	0.2987	0.6654	0.6901
Mixed	0.5434	0.1742	0.4403	0.1190	0.5861	0.3036	0.7826	0.5832
Non-Jew	1.4855	0.0007	1.3376	0.0107	1.6157	0.0001	1.5044	0.0004
Ultra-Orthodox	4.4353	<0.0001	2.5784	<0.0001	3.6689	<0.0001	3.9408	<0.0001
Twins	0.3482	<0.0001	0.6697	0.0007	0.2470	<0.0001	0.2456	<0.0001
Index birth order	0.7557	<0.0001	0.8333	<0.0001	0.8525	<0.0001	0.8176	<0.0001
Disabled sibling	0.7791	0.1420	0.9239	0.6515	0.8296	0.3430	0.8593	0.3987
No males	1.0261	0.7550	1.0280	0.7238	1.0076	0.9329	1.0523	0.5370
year of diagnosis	0.9156	0.1567			1.0165	0.8142	1.0060	0.9228
Mother age at birth of index	Spline -	0.0308	Spline -	<0.0001	Spline -	<0.0001	Spline -	<0.0001
Mother age in 2012	Spline +	<0.0001	Spline +	< 0.0001	Spline +	0.0217	Spline +	0.0126
Age at diagnosis	Spline +	< 0.0001			Spline +	<0.0001	Spline +	< 0.0001
Log likelihood	−4929.6		−4886.6		−4053.7		−4722.3	
Observations	9087		9087		9087		9087	

Note: OR odd ratio. Direction of splines indicated by +/−.

Table 4.
Logit models for absolute non-stoppage.

Age at diagnosis	Model 1	Model 3	Model 4
2.5	0.45	0.19	0.29
5	0.55	0.62	0.73
7	0.63	0.66	0.77

Notes: Jews excluding ultra-orthodox, dummies = 0, year of diagnosis 2010, mother age = 30. Model numbers refer to ***Table 4****.*

Table 5.
Non-stoppage and age at diagnosis.

of their p-values and their size effects. However, because these estimates are precise, the differences between them are statistically significant.

In **Table 5** we use the results in **Table 4** to calculate the probability of non-stoppage for observationally similar families, which differ by the age at which their index child was diagnosed. According to Model 1, the probability of non-stoppage varies directly with age at diagnosis, as expected. The probability of non-stoppage increases from 0.45 when age at diagnosis is 2.5 years to 0.63 at 7 years. For uninformed parents at birth (Model 3) these probabilities are initially much smaller

(0.19 instead of 0.45) but are slightly larger at 7 years (0.66 instead of 0.63). For uninformed parents at conception the probabilities of non-stoppage are larger as expected. Model 2 does not feature in **Table 5** because for informed parents age at diagnosis does not matter.

5.2 Relative stoppage

We use the zero-inflated Poisson model to distinguish between absolute and relative stoppage. As in **Table 4**, we compare families with ASD children who stopped or not, and who had more or fewer further children if they did not stop. The first column in **Table 6** refers to the complementary log log (CLL) component of the ZIP model in which λ refers here to the probability of non-stoppage, and the covariates refer to the X variables hypothesized to affect the logit probability of engaging in absolute non- stoppage, i.e. the probability of having further children after the index child. For example, Non-Jews and ultra-orthodox Jews are more likely to engage in absolute non-stoppage (less likely to engage in absolute stoppage). The second column refers to the Poisson probability (μ) of having 0, 1, 2, etc. further children after the index child, and the covariates refer to the Z variables hypothesized to affect the number of further children. For example, the ultra-orthodox are likely to have exp.(0.8299) = 2.3 further children more than other parents. This means that the ultra-orthodox are less likely to engage in absolute stoppage and less likely to engage in relative stoppage.

In **Table 6** the X and Z covariates for Eq. (2) are almost identical. Covariates that are statistically significant, carry the same signs in the CLL and Poisson models (non-Jews, ultra-orthodox, age of mother, age at diagnosis). An exception is birth order of the index child, which reduces absolute stoppage, but increases relative stoppage. This means, for example, that parents of second children diagnosed with ASD are more likely to stop than parents of firstborns, but the former are likely to have more further children than the latter. Another exception is twins, which increases absolute stoppage but does not significantly affect relative stoppage.

	CLL model		**Poisson model**	
	Estimate	**p-value**	**Estimate**	**p-value**
Intercept	−0.7409	<0.0001	0.0378	0.2791
Non-Jew	0.3889	<0.0001	−0.4153	0.1892
Mixed			0.2787	0.0001
Ultra- orthodox	0.9184	<0.0001	0.8299	<0.0001
Twins	−0.1186	0.2450	−0.7496	<0.0001
Birth order	0.0800	<0.0001	−0.2051	<0.0001
Disability	−0.0778	0.4457	−0.1729	0.1325
Mother age	Spline -	<0.0001	Spline -	<0.0001
Age at diagnosis	Spline +	0.0084	Spline +	<0.0001
Mother age 2012	Spline +	<0.0001	Spline +	<0.0001
Observations	9087			
Log likelihood	−9664			

Table 6.
Zero-inflated Poisson model for absolute and relative stoppage.

Age at diagnosis is specified in the CLL and Poisson models as is appropriate. The former implies, as in **Table 4**, that the probability of absolute non-stoppage varies directly with age at diagnosis. The latter implies that the probability of having more than one subsequent child also varies directly with age at diagnosis. Hence, both absolute and relative non-stoppage vary directly with age at diagnosis.

In **Table 7**, we use the results in **Table 6** to calculate the effect of age at diagnosis on the probabilities of absolute and relative non-stoppage, where the former refers to the probability of non-stoppage, and the latter refers to the expected value of the number of further children. As expected, both outcomes vary directly with age at diagnosis. The probability of absolute non-stoppage increases from 0.48 when the age at diagnosis is 2.5 years to 0.61 when the age at diagnosis is 10 years, and relative non-stoppage as measured by the expected value of the additional number of children increases from 0.37 to 0.7. Recall that the latter is defined by the probability of having one further child multiplied by one plus the probability of having a second further child multiplied by two etc. Hence, the expected number of children are weighted probabilities, which may be fractions as in **Table 7**.

5.3 Recurrence risk

Our main results are reported in **Table 8** where Models 1 and 2 are logit models for the probability of recurrence risk with common effects and random effects specifications. The latter hypothesizes that individual families have different recurrence risks, whereas the former hypothesizes that different families share common recurrence risks, but recurrence risk for siblings from the same family are correlated. Hence, for Model 1 parameter standard errors are clustered. On the whole, clustered standard errors are smaller than their unclustered counterparts, suggesting that siblings are negatively correlated within families as far as recurrence risk is concerned. This means that ordinary standard errors under-estimate the significance levels of the results. The reported p-values refer to the clustered standard errors for model 1. Since the results of the two models are similar, we focus here on Model 1.

Because most of the variables in **Table 8** were featured in a previous study [23], we focus here on the three new parental variables, which are highlighted in italics. The results of the previous study focused on birth gaps and birth orders of index children and their younger siblings. We reconfirm that short birth gaps (less than 2 years) increase recurrence risk, that recurrence risk varies inversely with the birth orders of younger siblings and the birth orders of index children. Also, recurrence risk is smaller in the non-Jewish population, varies directly with mothers' disability, and younger siblings are censored, as expected, with the probability of censoring tending to zero at 9 years of age. We found no evidence that recurrence risk varies with mothers' age (when her index child was born), nor could we detect a time

Age of index at diagnosis	Probability of stoppage	Expected number of children
2.5	0.72	1.44
5	0.71	1.52
7	0.71	1.61
10	0.73	1.69

*Notes: Based on **Table 6**. See notes to **Table 5**.*

Table 7.
Age at diagnosis and absolute and relative non-stoppage.

Model	1 Common Effects			2 Random Effects	
	Odds Ratio	SD-cluster	P-value (clustered)	Odds Ratio	P-value
Intercept	0.1706	0.1661	< 0.0001	0.1693	<0.0001
Female sibling	0.2851	0.1274	< 0.0001	0.2847	<0.0001
Birth order after index	0.8344	0.0704	0.0102	0.8374	0.0226
Index birth order	0.9244	0.0488	0.1077	0.9251	0.0999
Birth gap < 2	1.4297	0.1363	0.0087	1.4350	0.0090
Female index	1.6112	0.1324	0.0003	1.6094	0.0003
Mother disability	1.6995	0.2273	0.0196	1.7016	0.0217
Ultra-Orthodox	0.7682	0.1674	0.1153	0.7675	0.1058
Non-Jew	0.2294	0.4222	0.0005	0.2294	0.0005
Veil of ignorance	*0.8552*	*0.0286*	*< 0.0001*	*0.8547*	*<0.0001*
Shadow of ASD	*0.9300*	*0.0350*	*0.0387*	*0.9830*	*0.0593*
Informed	*0.6364*	*0.1621*	*0.0053*	*0.6355*	*0.0069*
Censor	Spline +		<0.0001	Spline +	0.0024
Observations	8164			8164	
Log likelihood	−1376			−24864	

Table 8.
Logit model for recurrence risk: Informed at birth.

trend in recurrence risk that might have reflected the positive time trend in the incidence of ASD in the general population [25].

We turn now to the three highlighted behavioral variables in **Table 8**. 'Informed' is a dummy variable, which equals 1 if index children were diagnosed before their younger siblings were born and is zero otherwise. The relative risk of ASD recurrence when parents are informed is reduced by slightly more than 30 percent. This effect is statistically significant and its p-value is 0.0053. 'Shadow of ASD' refers to the experience (in years) that informed parents had in raising their children with ASD before their younger siblings were born. The estimated coefficient implies that the relative risk of recurrence decreases by approximately 7 percent for each additional year of experience. This effect is very statistically significant since its p-value is almost zero. Finally, 'veil of ignorance' refers to the period of time (in years) during which uninformed parents raised the younger siblings of index children before the latter were diagnosed. The estimated coefficient implies that the relative risk of recurrence decreases by about 15 percent for each year of ignorance. This effect is very statistically significant too. Indeed, a likelihood ratio test overwhelming supports the retention of all three variables. However, their inclusion does not significantly affect the parameter estimates of the other variables in **Table 8**.

As mentioned, results for Model 2 are similar to those for Model 1, suggesting that Model 1 is robust with respect to random effects. Family random effects are hypothesized to be normally distributed with mean normalized to zero. The standard deviation of these effects is estimated at 0.588, which implies that recurrence risk for families at the lower 95 percentile is 1.47 percent, and it is 13 percent at the upper percentile. Mean recurrence risk is 4.5 percent. The asymmetry stems from the fact that the standard deviation refers to the log odds ratio. These results suggest that recurrence risk differs widely among families. **Figure 5**, which plots family specific effects expressed as odd ratios, suggests that there are two types of family.

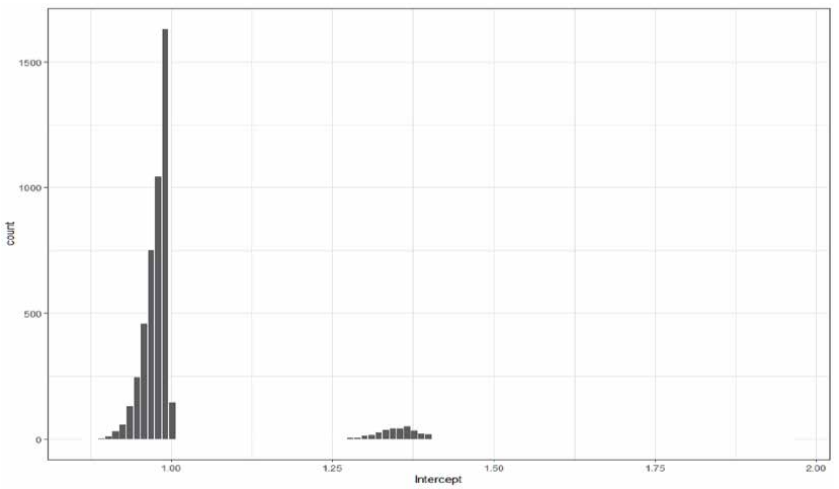

Figure 5.
Distribution of family specific odds ratios.

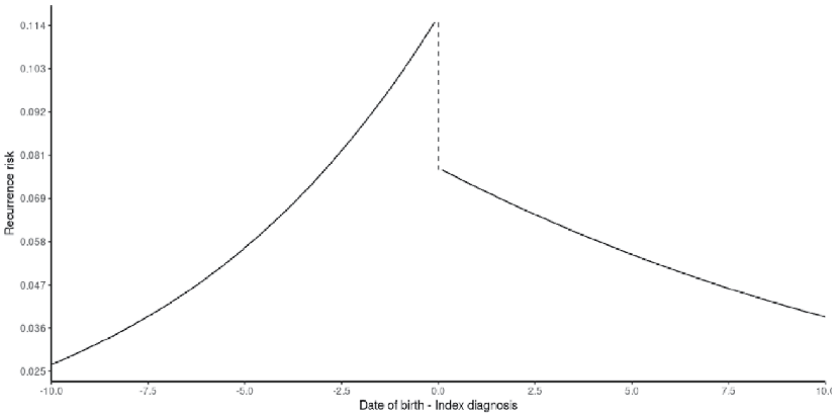

Figure 6.
Recurrence risk and shadow of ASD or veil of ignorance.

The first group has odds ratios that are slightly less than 1, while the second group, which is smaller, has odds ratios that are about 1.3.

In **Figure 6** we plot the relationship, implied by **Table 8**, between recurrence risk (on the vertical axis) and the difference between the birth dates of younger siblings and the dates at which index children were diagnosed (on the horizontal axis). Parents are 'informed' if this difference is positive because younger siblings were born (or conceived) after their index siblings were diagnosed. If this difference is negative, parents were 'uninformed'. Therefore, the shadow of ASD increases to the right of 0 on the horizontal axis, and the veil of ignorance increases to the left. The baseline for recurrence risk is 4.5 percent as in the data because logit models replicate sample means. Notice that the origin for the shadow of ASD is 2.91 percent because informed parents have lower odds ratios according to **Table 8**. **Figure 6** shows that recurrence risk varies directly with the gap between dates of birth and dates of diagnosis. **Figure 6** also shows that recurrence risk varies inversely with the shadow of ASD and the veil of ignorance. At one year, the veil of ignorance reduces recurrence risk from 4.5 percent to 3.87 percent and to 2.03 percent after 5 years. At one year, the shadow of ASD reduces recurrence risk from

4.5 percent to 2.71 percent and to 2.09 after 5 years. The results in **Table 8** refer to "informed" at birth. Since the results are similar for informed at conception, we do not present them here.

6. Conclusion

The opening quotation from Rutter, made in 2010, applies also today. In this paper, we respond to Rutter's challenge by reporting empirical evidence of three behavioral phenomena in the determination of recurrence risk. Our interpretation of these phenomena in terms of empowerment and imperilment theory, and self-selectivity into reproductive stoppage is less important than their statistical salience. Perhaps other interpretations exist. However, behavioral theory provides axioms, which predicted these effects. By contrast neurodevelopmental theory does not.

The result that recurrence risk is smaller among informed parents is consistent with them being more resilient. This does not mean that they are better parents. It simply means that parents who knowingly or consciously decided against reproductive stoppage are different to parents who conceived before their index child was diagnosed. Nor do we claim that the neurodevelopmental model is false. Indeed, our results are consistent with this model. However, they are not exclusively so. We find that the three behavioral parental phenomena significantly improve predictions of recurrence risk when they are added to the neurodevelopmental model. However, standard neurodevelopmental covariates such as birth gaps and birth orders continue to be statistically significant; they are not superseded by the three behavioral parental phenomena.

Can these behavioral results be confounded by neurodevelopmental effects? This would be the case if the difference between the dates of diagnosis of elder siblings and the dates of conception or birth of younger siblings happened to be correlated with neurodevelopmental phenomena. This difference is positive for uninformed families and negative for informed families. This difference also equals age at diagnosis minus sibling age gaps. There is no reason to suspect that sibling age gaps are directly or indirectly correlated with neurodevelopmental genotypes. However, age at diagnosis might be negatively correlated, if severer cases of ASD are diagnosed more quickly. If so, recurrence risk should vary inversely with age at diagnosis, and birth gaps should have no effect on recurrence risk. Since our results reject both of these predictions, we do not think that they are an artifact of confounding.

Standard neurodevelopmental covariates, such as birth order, might also bear behavioral interpretations. The neurodevelopmental interpretation is that birth order is naturally larger in families that have had more regular children. These families are presumed to be genetically less susceptible to ASD recurrence. A behavioral interpretation might be that parents who have had more experience in raising children are more resilient, which is why recurrence risk varies inversely with birth order. The same applies to covariates such as the ages of parents, which have behavioral as well as neurodevelopmental interpretations. In observational studies results are inevitably ambiguous. On the other hand, whereas most neurodevelopmental covariates have behavioral interpretations, the three behavioral phenomena studied here do not have neurodevelopmental interpretations.

We make a methodological contribution by exploiting the randomness in the timing of diagnoses as a source of natural experimentation. Randomized trials are obviously not feasible because parents cannot be assigned into treatment groups who are informed and controls who are uninformed. By contrast, natural experimentation induced by the timing of diagnosis most probably reveals the same

information with greater reliability provided recurrence risk is sufficiently independent of the timing of diagnosis.

In summary, the Bettelheim Affair blighted behavioral research into the etiology of ASD. However, the distinction should be made between discredited psychoanalytical theories and untested psychopathological theories that are behavioral. We close with some further quotations from Rutter [28]. "At first sight, it might seem that autism is the diagnostic category least likely to require a developmental psychopathology perspective." However, in reference to grand discredited theories they add, "There is a continuing need to remain skeptical about the new evangelisms that have come to take their place, but equally the imperative must be to replace doubt with programmatic research that truly tests competing hypotheses." Hopefully, the present paper will be judged in this light.

Notes/thanks/other declarations

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Chapter 5

Associations between Monocyte Cytokine Profiles and Co-Morbid Conditions in Autism Spectrum Disorders

Harumi Jyonouchi and Lee Geng

Abstract

Autism spectrum disorder (ASD) is a behaviorally defined syndrome with frequent co-morbidities. Evidence indicate a role of innate immunity in ASD pathogenesis. This study addressed whether innate immune abnormalities are associated with ASD co-morbid conditions and/or other clinical co-variables when assessed as changes in monocyte cytokine profiles. This study included 109 ASD (median 11.5 year) and 26 non-ASD subjects (median 11.4 year). Monocyte cytokine profiles were evaluated in association with age/ethnicity, ASD severity, medications, and co-morbidities present in >15% of ASD subjects [gastrointestinal (GI) symptoms, epilepsy, allergic rhinitis, specific antibody deficiency (SAD), and fluctuating behavioral symptoms resembling pediatric acute-onset neuropsychiatric syndrome (PANS)]. ASD severity did not affect frequency of co-morbid conditions. GI symptoms, epilepsy, SAD, and PANS like symptoms revealed associations with changes in production of tumor necrosis factor-α (TNF-α)/soluble TNF-receptor II (sTNFRII), interleukin-1ß (IL-1ß)/IL-6/IL-10, and IL-6, respectively, mostly independent of other co-variables. ASD severity was associated with changes in multiple cytokines but frequently affected by other clinical co-variables. Our findings revealed associations between specific monocyte cytokine profiles and certain co-morbid conditions in ASD subjects, independent of other clinical co-variables. Our findings will aid in assessing treatment options for ASD co-morbidities and their effects on ASD behavioral symptoms.

Keywords: autism spectrum disorder (ASD), co-morbid conditions, innate immune memory (IIM), monocyte cytokines, trained immunity

1. Introduction

Autism spectrum disorder (ASD) is a behavioral defined syndrome except for a small subset of patients with defined gene mutations such as MECP2 (Rett syndrome) and TSC1/TSC2 (tuberous sclerosis) [1, 2]. However, core ASD symptoms may be the results of the effects of various genetic and environmental factors that may also affect organs other than central nervous system (CNS). Consequently, medical conditions affecting other organs may also affect ASD symptoms, partly through pain and discomfort. This makes it difficult to have reliable objective

diagnostic measures universally applicable for ASD children. This assumption is supported by the fact that ASD is characterized by multiple co-morbid medical conditions, with GI symptoms being the most common [3, 4].

Many co-morbid conditions reported in ASD subjects are associated with immune mediated inflammation in pathogenesis; GI symptoms found in ASD subjects have been in part implicated with chronic GI inflammation due to dysregulated gut immune responses to microbiota [5, 6]. Mounting evidence also indicates that many, but not all the ASD subjects show some immune abnormalities that affect almost every arm of the immune system [7, 8]. Moreover, given the fact that the immune system and the CNS interact closely interact [9], a role of neuroinflammation in ASD pathogenesis is highly suspected in a subset of ASD subjects.

In our previous studies, we focused on abnormalities of innate immunity, which plays a major role in the neuro-immune network including stress responses [10–12]. One of the reasons that we focused on innate immune abnormalities is based on findings from one of the most thoroughly studied animal models of ASD, maternal immune activation (MIA). MIA is induced by sterile stimulants of innate immunity in pregnant rodents [13]. MIA generates lasting effects on behavioral symptoms and the immune functions in offspring [13, 14]. It has been puzzling that how innate immunity, which lacks memory for specific antigens, can cause such lasting effects. However, discovery of innate immune memory (IIM) caused by initial stimuli through epigenetic regulations [15, 16], has helped us understand the lasting effects of dysregulated IIM in various inflammatory conditions. In fact, dysregulated IIM is now implicated in the pathogenesis of common neuropsychiatric diseases such as schizophrenia and depression [17, 18]. We found abnormalities of innate immunity in many, but not all the ASD subjects we studied by assessing cytokine profiles from purified monocytes [11].

Given both the considerable amount of abnormalities found in the monocyte cytokine profiles and the high frequency of co-morbid medical conditions in ASD subjects, questions understandably arise concerning the association of co-morbid conditions with changes in monocyte cytokine production. Many ASD subjects are treated with neurotropic medications including selective serotonin re-uptake inhibitors (SSRIs), anti-seizure medications used as mood stabilizers, neuroleptics, and medications for ADHD. These medications may also affect monocyte cytokine profiles. Therefore, this study addressed whether monocyte cytokine profiles differ depending on co-morbid conditions, ASD severity, and other clinical co-variables. The results indicate that co-morbid medical conditions are associated with changes in production of specific cytokines and such associated are not affected by other clinical co-variables.

2. Materials and methods

Study subjects: Study subjects were recruited following the study protocols (#17:53 and #19:53) approved by the institutional review. Signed consent forms were obtained prior to entering the study.

ASD subjects: ASD subjects (N = 109) were recruited from the Pediatric Allergy/Immunology Clinic at SPUH. Diagnosis of ASD was made at various autism diagnostic centers, including ours, based on the Autism Diagnostic Observation Scale (ADOS) and/or Autism Diagnostic Interview-Revisited (ADI-R), as well as other standard measures. ASD subjects were also evaluated for their behavioral symptoms and sleep habits with the Aberrant Behavior Checklist (ABC) [19] and the Children's Sleep Habits Questionnaires (CSHQ) [20], respectively. Information regarding cognitive ability and adaptive skills were obtained from previous school

evaluation records performed within 1 year of enrollment in the study; these results were based on standard measures such as the Woodcock-Johnson III test (for cognitive ability), and Vineland Adaptive Behavior Scale (for adaptive skills) [21].

Non-ASD controls: A total of 26 non-ASD subjects served as controls. These subjects were recruited in the Pediatrics Subspecialty and General Pediatrics Clinics at our institution. These subjects were typically growing and satisfied our exclusion/inclusion criteria.

Demographics of study subjects were summarized in **Table 1**.

Diagnosis of food allergy (FA): IgE mediated FA was diagnosed with reactions to offending food, by affecting the skin, GI, and/or respiratory tract immediately (within 2 hours) after intake with positive prick skin testing (PST) reactivity, and/or presence of food allergen-specific serum IgE. Non IgE mediated FA (NFA) was diagnosed if GI symptoms resolved, following implementation of a restricted diet (i.e., avoidance of offending food), and symptoms recurred with re-exposure to offending food [22]. NFA was also defined as being non-reactive to PFT and negative for serum IgE specific for food allergens [22].

Diagnosis of asthma and AR: AR and allergic conjunctivitis (AC) were diagnosed when subjects had corresponding clinical features along with positive PST reactivity and/or positive serum IgE specific to causative allergens [23, 24]. Asthma was diagnosed following the asthma guidelines from the Expert Panel Report 3 [25].

Diagnosis of Antibody deficiency syndrome: When the subject revealed protective levels of antibodies in less than 11 of 14 serotypes of *Streptococcus pneumonia* after the booster dose of Pneumovax® or PCV13®, he/she was diagnosed with SAD [26]. Antibody levels greater than 1.3 μg/ml were considered protective [26].

Diagnosis of PANS like symptoms and sleep disorders: Pediatric acute-onset neuropsychiatric syndrome (PANS) is a clinical diagnosis [27]. Most ASD subjects recruited to the study were diagnosed with PANS by other physicians. We attempted to validate the diagnosis based on the PANS diagnostic criteria [27]. In some ASD children, pre-existing neuropsychiatric symptoms made it difficult to apply the clinical diagnostic criteria of PANS. In this study, we categorized ASD patients with recurrent worsening behavioral symptoms following immune stimuli (typically microbial infection) more than 2×, not controlled by proper management of triggering stimuli as ASD subjects with PANS like symptoms. Diagnosis of sleep disorders that lasts at least more than 6 weeks is based on parental reports and results of CSHQ.

Sample collection: Venous blood samples were obtained by the physician in this study. We obtained one sample from each non-ASD controls. As for ASD subjects, we obtained multiple blood samples at different time points from select ASD subjects (N = 9), in order to assess variability of *monocyte* cytokine profiles. If parents

	ASD subjects (N = 109)	**Non ASD controls (N = 26)**
Gender	95 M, 15 F (Female 13.8%)	19 M, 7 F (Female 26.9%)
Age: Average ± SD (years) Median (Min to Max)	12.4 ± 5.9 11.5 (2.2–26.3)	12.2 ± 5.8 11.4 (3–28.8)
Ethnicity	84 W, 5 AA, 19 Asian, 1 Mixed	22 W, 2 AA, 2 Mixed

Abbreviations used: AA; African American, ASD; autism spectrum disorders, SD; standard deviation, W; Caucasian.

Table 1.
Demographics of study subjects.

or study subjects preferred, we applied a topical lidocaine/prilocaine cream (Emla cream®) to the site of venipuncture prior to blood sampling.

Cell cultures: Ficoll–Hypaque density gradient centrifugation was used for separating PBMCs. From PBMCs, PBMo were further purified using a column of magnetic beads labeled with anti-CD3, CD7, CD16, CD19, CD56, CD123, and glycophorin A (monocyte separation kit II – human, MILTENYI BIOTEC, Cambridge, MA, United States). Combination of these antibodies depletes T, B, natural killer, and dendritic cells from PBMCs.

Cytokine production by purified PBMo was induced by incubating cells overnight (2.5×10^5 cells/ml) with a panel of agonists of TLRs. This assay system was designed to reflect the effects of microbial byproducts commonly encountered in real life. Lipopolysaccharide (LPS), a TLR4 agonist, represents a signaling pathway activated in response to a gram negative [G (−)] bacteria. Zymosan, a TLR2/6 agonist, mimics an innate activation signal in response to G (+) bacteria and fungi. CL097, a TLR7/8 agonist, activates innate signaling pathways in response to ssRNA viruses that cause common respiratory infection. Candida heat extract as a source of ß-glucan, a dectin-1 agonist, was used as well as a C-lectin receptor agonist. PBMos were incubated overnight with LPS (0.1 μg/ml, GIBCO-BRL, Gaithersburg, MD, USA), zymosan (50 μg/ml, Sigma-Aldrich, St. Luis, Mo), C097 (water-soluble derivative of imidazoquinoline, 20 μM, InvivoGen, San Diego, CA, USA), and candida heat extract (HCKA, heat killed *Candida albicans* (10^7 cells/ml, InVivogen, San Diego, CA) in RPMI 1640 with additives as previously described [28]. Overnight incubation (16-20 h) was adequate to induce the optimal responses in this setting in previous studies [11]. Cytokine levels in the culture supernatant were then measured.

Levels of CCL2, IL-1β, IL-6, IL-10, IL-12p40, transforming growth factor-ß (TGF-ß), tumor TNF-α, and sTNFRII cytokines were measured by enzyme-linked immuno-sorbent assay (ELISA); 10–100 μl/well supernatants were used for ELISA. The OptEIA™ Reagent Sets (BD Biosciences, San Jose, CA, USA) were used for ELISA of IL-1ß, IL-6, IL-10, IL-12p40, and TNF-α. For CCL2, sTNFRII, and TGF-ß ELISA, reagents were obtained from BD Biosciences and R & D (Minneapolis, MN, USA). IL-23 ELISA kit was purchased from eBiosciences, San Diego, CA. Intra- and inter-variations of cytokine levels were less than 5%.

Statistical analysis: We used a two tailed Mann–Whitney test for comparison of two sets of numerical data. Kruskal-Wallis test was used for comparison of more than 2 sets of numerical data. When assessing differences in frequency between two groups, we used the Fisher exact test. For assessing differences in frequency among multiple groups, we used the Chi-square test and the Likelihood ratio. P value of less than 0.05 was considered nominally significant. Co-variance analysis was done with the use of analysis of variance (ANOVA) for a fixed factor or for a variable factor. NCSS2020 (NCSS, LLC. Kaysville, UT) was used for such statistical analysis.

3. Results

Clinical characteristics: Frequencies of co-morbid conditions among the recruited ASD subjects are summarized in **Table 2**. These results are consistent with the results of our previous studies [12, 29]. Age and gender were not associated with ASD severity (data now shown). Frequencies of co-morbid conditions and the use of neurotropic medications did not differ due to ASD severity in 108 ASD subjects who were verified ASD severity (**Table 3**).

Changes in monocyte cytokine production depending on ASD severity: We then examined whether monocyte cytokine profiles differed with ASD severity

Comorbid conditions	ASD subjects (N = 109)	Controls (N = 26)
GI[1] symptoms	71/109 (65.1%)	0
history of NFA	68/109 (63.4%)	2/26 (7.7%)
Seizure disorders	18/109 (16.5%)	0
Asthma	5/109 (4.6%)	0
Allergic rhinitis	17/109 (15.6%)	0
Specific antibody deficiency	26/109 (23.9%)	0
PANS like symptoms	62/109 (56.9%)	0
Disturbed Sleep	48/109 (44.0%)	0

[1]*Abbreviations used: ASD; autism spectrum disorder, GI; gastrointestinal, NFA; non-IgE mediated food allergy, PANS; pediatric acute-onset neuropsychiatric syndrome.*

Table 2.
Frequency of comorbid conditions in the study subjects.

ASD[1] severity	Level 1	Level 2	Level 3	Chi-Square
Co-morbid conditions				
GI symptoms	14/27[2]	20/32	36/49	p > 0.1
Seizure disorder	1/27	6/32	10/49	p = 0.081
Antibody deficiency	6/27	10/32	10/49	p > 0.1
PANS like symptoms	12/27	19/32	30/49	p > 0.1
Disturbed sleep	8/27	16/32	23/49	p > 0.1
Medications				
SSRIs	6/27	8/32	4/49	p > 0.1
Anti-seizure medications	3/27	9/32	8/49	p > 0.1
ADHD medications	3/27	3/32	3/49	p > 0.1
Neuroleptics	4/27	1/32	9/49	p = 0.079

[1]*Abbreviations used: ASD; autism spectrum disorder, GI; gastrointestinal, NFA; non-IgE mediated food allergy, ADHD: attention deficiency hyperactivity disorder, PANS; pediatric acute-onset neuropsychiatric syndrome, SSRI: selective serotonin reuptake inhibitor.*
[2]*One ASD subject was excluded from this analysis due to lack of validation of ASD severity.*

Table 3.
Frequencies of Co-morbid conditions and medication use did not differ due to ASD severity.

and if such changes were affected by other clinical co-variables. ASD severity was shown to be associated with changes in production of TNF-α, IL-1ß, IL-10, and CCL2, and TNF-α/sTNFRII ratios (**Table 4**). However, production of inflammatory monocyte cytokines (TNF-α and IL-1ß) under several culture conditions is affected by presence of co-morbid conditions and the use of ADHD medications (**Table 4**).

Changes in monocyte cytokine production depending on co-morbid conditions: Since associations between ASD severity and monocyte cytokine profiles were often affected by other co-morbid conditions and medication use (**Table 4**), we also evaluated whether changes in monocyte cytokine profiles in ASD subjects were affected by the presence of co-morbid conditions.

Co-morbid conditions with objective diagnostic measures: We evaluated changes in monocyte cytokine profiles in association with co-morbid conditions which were

Monocyte cytokine production	ASD[5] severity Level 1	ASD severity Level 2	ASD severity Level 3	Kruskal-Wallis test
	N = 33	N = 37	N = 52	
TNF-α (CLO97)[1]	2390.2 ± 1786.9	2720.2 ± 983.3	2073.0 ± 1171.6	$p < 0.01$
IL-10 (CLO97)	1318.1 ± 595.3	918.2 ± 576.1	1009.9 ± 607.2	$p < 0.05$
TNF-α/sTNFRII (CLO97)[2]	3.78 ± 3.34	8.43 ± 10.43	8.73 ± 17.27	$p < 0.05$
TNF-α (ß-glucan)[3]	1816.2 ± 1139.4	2360.4 ± 1249.9	1512.2 ± 883.9	$p < 0.005$
IL-1ß (ß-glucan)[4]	2578.7 ± 922.0	2809.7 ± 984.4	2062.9 ± 949.7	$p < 0.005$
TNF-α (ß-glucan+LPS)[1]	2338.0 ± 259.0	2658.4 ± 984.5	2000.2 ± 916.5	$p < 0.01$
CCL2 (ß-glucan+LPS)	2708.0 ± 2477.4	1758.6 ± 16681	1574.8 ± 1553.1	$p < 0.05$
CCL2 (zymosan)	9105.2 ± 6631.1	7668.4 ± 4973.2	5870.3 ± 4414.4	$p < 0.05$

[1]ANOVA co-variance analysis revealed an association with the use of ADHD medications ($p < 0.02$ and $p < 0.05$ under culture conditions stimulated with CL097 and ß-glucan+LPS, respectively).
[2]ANOVA co-variance analysis revealed an association with GI symptoms ($p < 0.05$).
[3]ANOVA co-variance analysis revealed an association with PANS like symptoms ($p < 0.05$).
[4]ANOVA co-variance analysis revealed an association with Disturbed sleep ($p < 0.05$).
[5]Abbreviations used: CCL2; C-C chemokine ligand 2, IL; interleukin, LPS; lipopolysaccharide, TNF; tumor necrosis factor.

Table 4.
Differences in monocyte cytokine production depending on ASD severity.

evaluated with objective measures as defined in the method section. We found that such co-morbid conditions were observed in more than 15% of our ASD study subjects; these include GI symptoms, seizure disorders, AR, and SAD. Our results revealed that there are significant associations between monocyte cytokine production and ASD co-morbid conditions as described above (**Table 5**). Presence of GI symptoms are mainly associated with changes in TNF-α production and the ratio of TNF-α/sTNFRII ratios under several culture conditions. Co-variance analysis showed that these parameters were mostly not affected by other clinical co-variables including medication use. The exceptions are TNF-α production and TNF-α/sTNFRII ratios under zymosan stimulated cultures, which are affected by ASD severity (**Table 5**). Diagnosis of seizure disorders was the most notably associated with changes in IL-1ß production and IL-1ß/IL-10 ratios under the CL097 stimulated cultures, independent of clinical co-variables that we assessed (**Table 5**). The AR diagnosis is mainly associated with changes in sTNFRII production. The SAD diagnosis is mainly associated with changes in IL-6 and IL-10 production. Most of these cytokine parameters were again not affected by the other clinical variables that we assessed.

Co-morbid conditions based on clinical diagnosis: Although assessment of PANS like symptoms and sleep disorder were diagnosed without objective measures, given the high frequency of these conditions, we also assessed differences in monocyte cytokine parameters in association with these two co-morbid conditions. Significant differences in certain monocyte cytokine parameters were found in the presence of PANS like symptoms and sleep disorders (**Table 6**). PANS like conditions were associated with changes in inflammatory cytokines (IL-6 and IL-1ß), as well as sTNFRII and CCL2. Only IL-1ß production was affected by other clinical covariables. As for sleep disorders, changes in TGF-ß levels were mainly associated with the presence of sleep disorders and changes in TGF-ß productions was independent of clinical co-variables.

Comorbid conditions	ASD[1] with comorbid condition	ASD without comorbid condition	Non-ASD Control	Kruskal-Wallis test
GI symptoms	**N = 81**	**N = 42**	**N = 26**	
TNF-α (LPS)	685.1 ± 744.8[3]	373.0 ± 366.5	474.4 ± 505.8	$p < 0.01$
TNF-α (zymosan)[2]	1498.8 ± 1055.6	1047.6 ± 709.6	1609.7 ± 748.3	$p < 0.01$
TNF-α (ß-glucan)	1961.6 ± 1108.2	1609.7 ± 1135.7	1931.1 ± 948.5	$p = 0.113$
TNF-α/sTNFRII (LPS)	0.69 ± 0.89	0.50 ± 1.27	1.28 ± 3.53	$p < 0.05$
TNF-α/sTNFRII (zymosan)[2]	2.98 ± 2.61	2.67 ± 5.68	3.54 ± 3.61	$p < 0.05$
TNF-α/sTNFRII (ß-glucan)	6.84 ± 6.79	3.88 ± 2.31	13.5 ± 18.0	$p < 0.01$
Seizure disorders	**N = 24**	**N = 99**	**N = 26**	
IL-1ß (CL097)	3732.3 ± 1092.8	4566.4 ± 1357.5	3715.5 ± 1367.9	$p < 0.01$
IL-1ß/IL-10 (CL097)	5.10 ± 7.98	8.98 ± 12.44	3.98 ± 2.53	$p < 0.001$
TNF-α/sTNFRII	3.89 ± 6.74	8.04 ± 13.74	4.31 ± 3.48	$p < 0.02$
(CL097) CCL2	18862 ± 19577	14588 ± 9672	11499 ± 7621	$p < 0.02$
(CL097) IL-1ß (ß-glucan)	1955.9 ± 996.9	2546.4 ± 972.1	21767.0 ± 999.9	$p = 0.056$
Allergic rhinitis	**N = 20**	**N = 103**	**N = 26**	
sTNFRII (LPS)	1538.1 ± 395.5	1284.0 ± 493.7	1172.5 ± 504.2	$p < 0.05$
IL-1ß (CL097)[2]	5038.3 ± 1368.3	4285.8 ± 1315.6	3715.5 ± 1357.9	$p < 0.01$
sTNFRII (ß-glucan)[2]	497.8 ± 225.2	414.1 ± 286.6	340.8 ± 257.7	$p < 0.05$
sTNFRII (LPS + ß-glucan)	488.8 ± 249.4	392.5 ± 297.3	371.6 ± 354.3	$p = 0.07179$
Antibody deficiency	**N = 31**	**N = 92**	**N = 26**	
IL-6 (medium)	2303.5 ± 1935.8	3869.2 ± 2581.629553 ± 18279	3373.3 ± 1562.9	$p < 0.01$
IL-6 (LPS)	19536 ± 9625	6309.2 ± 1974.7	20152 ± 12909	$p < 0.01$
IL-6 (zymosan)[2]	5510.5 ± 2085.6	388.8 ± 380.0	7538.5 ± 9310.0	$p = 0.106$

Comorbid conditions	ASD[1] with comorbid condition	ASD without comorbid condition	Non-ASD Control	Kruskal-Wallis test
IL-1ß (medium)	214.5 ± 253.3	646.6 ± 503.0	270.3 ± 185.9	$p < 0.05$
IL-10 (medium)	328.3 ± 336.4	1471.8 ± 417.9	597.1 ± 355.7	$p < 0.005$
IL-10 (LPS)[2]	1215.8 ± 539.2	620.1 ± 375.3	1200.0 ± 543.7	$p < 0.05$
IL-10 (zymosan)	506.3 ± 399.8	1150.0 ± 605.8	651.4 ± 380.2	$p = 0.128$
IL-10 (CL097)	863.2 ± 570.6	426.4 ± 354.6	1093.0 ± 579.5	$p = 0.07$
IL-12 (zymosan)	302.2 ± 290.3		360.7 ± 425.0	$p = 0.1206$

[1]Abbreviations used: ASD; autism spectrum disorder, GI; gastrointestinal, IL; interleukin, LPS; lipopolysaccharide, TNF; tumor necrosis factor.
[2]Co-variance analysis revealed that changes in TNF-α and TNF-α/sTNFRII (zymosan) production with GI symptoms are affected by ASD severity ($p < 0.05$). Changes in sTNFRII production (ß-glucan) with allergic rhinitis was affected with the use of anti-seizure medications ($p < 0.05$). Changes in production of IL-6 (zymosan) and IL-10 production (LPS) with antibody deficiency was affected with the use of neuroleptics/SSRIs and PANS like symptoms.
[3]The results were expressed as a mean ± SD. Cytokine levels were shown as pg/ml.

Table 5.
Differences in monocyte cytokine production in association with GI symptoms, seizures disorders, allergic rhinitis, and antibody deficiency in ASD subjects.

Comorbid conditions	ASD[1] with comorbid condition	ASD without comorbid condition	Non-ASD Control	Kruskal-Wallis test
PANS like symptoms	**N = 73**	**N = 50**	**N = 2**	
IL-6 (CL097)	6747.6 ± 1939.2[3]	7795.3 ± 1824.6	6325.5 ± 2011.2	$p < 0.005$
IL-6 (ß-glucan)	5502.2 ± 1725.7	6211.1 ± 1731.7	5230.4 ± 1706.7	$p < 0.05$
IL-6 (ß-glucan+LPS)	6505.7 ± 1654.5	7661.9 ± 1943.0	5756.6 ± 1875.3	$p < 0.00001$
IL-1ß (ß-glucan+LPS)[2]	3176.5 ± 1278.1	3706.3 ± 1029.4	2545.7 ± 987.1	$p < 0.001$
sTNFRII (zymosan)	618.2 ± 350.1	723.0 ± 293.6	663.7 ± 368.1	$p = 0.069$
CCL2 (zymosan)	6717.1 ± 5609.0	8055.4 ± 4899.3	6138.5 ± 6351.9	$p < 0.05$
Sleep disorders	**N = 56**	**N = 67**	**N = 26**	
IL-10 (LPS)[2]	1289.6 ± 499.9	1497.9 ± 422.6	1200.0 ± 543.7	$p < 0.05$
sTNFRII (LPS)[2]	1228.6 ± 473.4	1410.4 ± 484.6	1172.5 ± 551.9	$p < 0.05$
TGF-ß (medium)	535.1 ± 469.6	685.1 ± 443.1	398.0 ± 372.1	$p < 0.01$
TGF-ß (LPS)	545.7 ± 498.1	689.8 ± 436.2	372.8 ± 340.1	$p < 0.005$
TGF-ß (zymosan)	429.2 ± 415.4	542.8 ± 356.7	285.9 ± 287.0	$p < 0.001$
TGF-ß (CLO97)	459.3 ± 442.9	613.7 ± 421.0	325.8 ± 299.2	$p < 0.005$
TGF-ß (ß-glucan)	370.4 ± 335.7	518.4 ± 345.7	259.7 ± 280.8	$p < 0.0005$

[1]Abbreviations used include: IL; interleukin, LPS, lipopolysaccharide, PANS; pediatric acute-onset neuropsychiatric syndrome, TGF, transforming growth factor, TNF; tumor nectrosis factor, sTNFRII; soluble TNF receptor II.
[2]Changes in IL-1ß (ß-glucan+LPS) production with PANS like symptoms are affected with ASD severity. Changes in IL-10 and sTNFRII production with LPS was affected by ASD severity ($p < 0.01$) and Seizure disorder ($p < 0.05$). Changes in production of IL-10 (LPS) with sleep disorder is affected with the use of SSRIs ($p < 0.005$), and specific antibody deficiency and PANS like behaviors ($p < 0.05$) by co-variance analysis. sTNFRII production with sleep disoders are affected with the use of SSRIs ($p < 0.005$) by co-variance analysis.
[3]The results were expressed as a mean ± SD. Cytokine levels were shown as pg/ml.

Table 6.
Differences of monocyte cytokine profiles with presence of PANS like symptoms and sleep disorders in ASD subjects.

4. Discussion

ASD subjects suffer from multiple co-morbid conditions. However, we know little about how the presence of co-morbid conditions are associated with ASD pathogenesis. Core ASD symptoms used for diagnosis such as irritability, hyperactivity, self-injurious behaviors, etc. can be affected by discomfort and pain caused by co-morbid medical conditions. In addition, recently, mounting evidence indicates a pathogenetic association between GI symptoms and the onset/progress of ASD [5, 9]. This may also be true for other common co-morbid conditions such as seizure disorders.

Unfortunately, impaired expressive language in ASD subjects make it more difficult to diagnose co-morbid medical conditions. For example, sinus headache caused by untreated sinusitis and AR can aggravate head banging and aggression (pinching others, etc.). Too often, such behaviors are dismissed as just being autistic, and diagnostic and treatment measures for common childhood diseases may not be properly sought in ASD children [30]. Considering the fact current ASD diagnosis is based on behavioral symptoms, the presence of co-morbid medical conditions may hold a key to assess pathogenesis in markedly heterogeneous ASD subjects and their variable behavioral symptoms.

When addressing the importance of co-morbid conditions frequently seen in ASD subjects, the role of immune mediated inflammation likely needs to be

considered as a common denominator. The immune system has long been thought to play a role in neuroinflammation and is implicated with pathogenesis of ASD. One of the most extensively studied animal models of ASD is MIA, in which, ASD like behavioral changes in offspring are induced by sterile immune activation through stimuli of innate immunity given to pregnant rodents [13]. Discovery of IIM [15, 17] shed a light on the lasting effects of sterile, antigen non-specific inflammation generated in the MIA model. IIM is thought to be generated through epigenetic changes [17] and such changes created in fetal and early infancy could make such individuals more susceptible to common inflammatory conditions such as food induced enterocolitis syndrome (FPIES), a condition that were found frequently in ASD subjects in our clinic. Altered IIM skewed to pro-inflammatory responses may cause dysregulated responses to commensal microbiota in the gut, causing chronic GI inflammation, resembling inflammatory bowel diseases (IBD). Such changes in innate immune responses may lead to aberrant responses to respiratory microbes, resulting in altered clinical manifestations, as well. Such dysregulated innate immune responses to immune stimuli can also affect the brain, since many signaling pathways associated with innate immunity have roles in the nervous system [17].

Despite progress of our understanding of IIM, we do not know which innate immune parameters are associated with co-morbid medical conditions and how these parameters are associated with ASD severity. IIM is closely associated with changes in monocyte cytokine profiles [16]. Previously, we have found significant changes in monocyte cytokine profiles in a subset of ASD patients [10, 11]. Therefore, this study addressed whether the specific monocyte cytokine parameters are associated with ASD co-morbid conditions. In this study, we randomly screened monocyte cytokine profiles in ASD subjects recruited to the study. In our clinic, because of the allergy/immunology specialty, we likely recruited more ASD subjects with co-morbid medical conditions. However, we reasoned that such potentially skewed ASD study subjects may make it easier for us to find specific monocyte markers associated with co-morbid conditions.

We found changes in certain monocyte cytokine parameters had an association with ASD severity (**Table 4**). However, parameters associated with inflammatory responses (production of TNF-α and IL-1ß, and TNF-α/sTNFRII ratio) were also found to be affected by other clinical co-variables including GI symptoms, and PANS like behaviors (**Table 4**). This finding seems to support our initial assumption that associations between ASD behavioral symptoms and changes in monocyte cytokine profiles are affected by other clinical co-variables.

Therefore, we decided to assess changes of monocyte cytokine parameters in association with co-morbid conditions frequently found in ASD subjects. We found GI symptoms along with NFA or FPIES like conditions in ASD subjects at high frequency (>60%), which was consistent to our previous studies [10, 12, 29]. Most of the ASD patients with GI symptoms had a history of FPIES like symptoms (**Table 2**). In these patients, we found changes in production of TNF-α and TNF-α/sTNFRII ratios in association with GI symptoms, but to our surprise, we did not find any associations with other inflammatory markers typically associated with neuroinflammation. It may be that GI symptoms are mainly driven TNF-α mediated inflammation in these ASD subjects as seen in patients with IBD [31]. Our finding may indicate the possibility that treatment measures typically used for IBD patients may be applicable for treating GI symptoms in ASD. Interestingly, TNF-α production under zymosan mediated cultures was affected by ASD severity; this may provide further support of the gut-brain axis concept [5, 6].

As for seizure disorders, we found changes in IL-1ß production under the cultures stimulated with ß-glucan and CLO97 in ASD subjects with seizure disorders

(**Table 5**). This association was independent of any other clinical co-variables by co-variance analysis. IL-1ß has been implicated with a major inflammatory component in febrile seizures and is also implicated in the pathogenesis of seizures associated with neuroinflammation [32, 33]. These results may indicate utility of IL-1ß blockers for controlling seizures in ASD subjects, if control is not well achieved by the 1st line anti-seizure medications. This finding is also intriguing because we have found better control of seizures with the use of IL-1ß blockers in some ASD subjects previously [34].

The presence of AR appeared to be associated with an increase in sTNFRII levels which may be indicative of increase in counter-regulatory measures for allergic inflammation. However, since the numbers of AR patients in this study was relatively low, these results need to be validated in future studies.

Our ASD study subjects included a fair number of ASD subjects with SAD (**Table 2**). These ASD subjects revealed lower production of IL-6 and IL-10 under several culture conditions (**Table 5**). Two of these parameters were affected by the presence of PANS like symptoms. This may not be surprising, since in our experience, we often observe a high frequency of SAD in non-ASD PANS patients. Interestingly, ASD subjects with PANS like behavioral symptoms also revealed lower production of IL-6 (**Table 6**). IL-6 is associated with terminal differentiation of B cells and is reported to be lower in patients with antibody deficiency such as common variable immunodeficiency [35]. On the other hand, IL-6 has also be implicated with neuronal development, following neuronal insult during fetal and newborn periods [36, 37]. Reduced IL-6 production may reflect subsequent suppression, following prior IL-6 mediated neuroinflammation. If so, lowering IL-6 production may have evolved into impaired antibody production in some ASD subjects who had suffered from IL-6 mediated inflammation in their early years.

In subjects with autoimmune encephalitis refractory to rituximab, IL-6 blockers such as tocilizumab which is an inhibitor of the IL-6 receptor, are reported to be effective [38, 39]. However, in ASD subjects with lower IL-6 production, the use of IL-6 blockers may not be effective, even if the PANS like behavioral symptoms are attributed to AE. IL-10 production was also lower in ASD patients with SAD, however this is not associated with the presence of PANS like symptoms. Changes in IL-10 production is reported in patients with common variable immunodeficiency (CVID) [40]. Therefore, this finding may be associated with the pathogenesis of antibody deficiency.

When we assessed associations between sleep disorders and changes in monocyte cytokine profiles, we expected to see changes in inflammatory monocyte cytokines, since ASD subjects with PANS like symptoms often suffer from sleep disorders. However, we mainly found lower production of TGF-ß which is considered to be a counter-regulatory cytokine and associated with tissue repair, promoting fibrotic changes [41]. Our results may indicate a decrease in counter-regulatory measures in neuroinflammation in sleep disorders in ASD subjects. The etiology of and the role neuroinflammation plays in sleep disorders in ASD are not well understood. Our finding may indicate that impairment of TGF-mediated pathways may play a role in sleep disorders in ASD.

Our previous studies indicated that IL-1ß/IL-10 ratios can be general markers for dysregulatred innate immune responses in ASD subjects [12]. However, in this study, we did not find strong associations with this parameter to specific co-morbid medical conditions, except for seizure disorder. This parameter may be associated with general inflammation caused by immune mediated inflammation. However, in order to more fully assess treatment options for co-morbid medical conditions in ASD subjects, detailed analysis of monocyte cytokine profiles is likely required.

Our study may be limited by the relatively small sample side of ASD subjects who had specific co-morbid conditions. Findings in this study need to be validated by a study using a larger numbers of study subjects, in association with responses to specific treatment measures targeted to each co-morbid condition.

5. Conclusion

Our study revealed that associations between monocyte cytokine parameters and specific co-morbid medical conditions exist in the ASD subjects studied, independent of other clinical variables. This indicates that there is a possibility that monocyte cytokine profiles may be used for assessing treatment options in ASD subjects with specific co-morbid medical conditions.

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Conflict of interest

The authors have nothing to disclose.

Abbreviations

AA	African American
ABC	aberrant behavior checklist
AC	allergic conjunctivitis
ADHD	attention deficiency hyperactivity disorder
ADI-R	autism diagnostic interview-revisited
ADOS	autism diagnostic observation scale
AR	allergic rhinitis
ASD	autism spectrum disorder
CCL2	C-C chemokine ligand 2
CSHQ	children's sleep habits questionnaires
CNS	central nervous system
CVID	common variable immunodeficiency
FA	food allergy
FPIES	food induced enterocolitis syndrome
GI	gastrointestinal
IBD	inflammatory bowel disease
IIM	innate immune memory
IL	interleukin
LPS	ipopolysaccharide
MIA	maternal immune activation
NFA	non-IgE mediated FA
PANS	pediatric acute-onset neuropsychiatric syndrome
PBMo	peripheral blood monocytes
PST	prick skin testing

SAD	specific antibody deficiency
SD	standard deviation
SPUH	Saint Peter's University Hospital
TLR	toll-like receptor
sTNFR	soluble TNF receptor
TGF	transforming growth factor
TNF	tumor necrosis factor

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Chapter 6

L1-79 and the Role of Catecholamines in Autism

John Rothman

Abstract

A growing body of evidence supports a role for catecholaminergic dysfunction in the core symptoms of autism spectrum disorder (ASD). This paper reviews the direct and indirect role of catecholamines on the central and peripheral nervous systems in ASD. Catecholamines innervate every tissue in the body and almost all tracts of the brain, providing a common neurologic regulatory mechanism for all ASD symptoms. Because the morphology of the catecholaminergic synapse is regulated by growth factors that are released contemporaneously with neurotransmitters, an event that results in abnormally large catecholamine release, will also release high levels of growth factors, which can result in the budding and arborization of nerve terminals. Here, we hypothesize that a hypertrophic synaptic morphology can occur in catecholaminergic systems and increase catecholaminergic tone throughout the body, resulting in an imbalance between catecholaminergic neurologic mechanisms and those that oppose them, and consequently pathology. By exerting a presynaptic effect to inhibit tyrosine hydroxylase and thus the synthesis, storage and release of all catecholamines, L1–79 (a tyrosine hydroxylase inhibitor) may diminish neurotransmitter release and its associated growth factors exerting a therapeutic effect on ASD by reducing the hypertrophic morphology of the synapse and bringing catecholamines back into a homeostatic balance with oppositional neurologic and metabolic influences.

Keywords: autism, autism spectrum disorder, catecholamines, D, L-α-methyl-para-tyrosine, L1–79

1. Introduction

Childhood autism is more prevalent than childhood cancer, juvenile diabetes and pediatric AIDS combined, with an estimated prevalence of 3 M children in Europe, 1.5 M in the US, and tens of millions throughout the rest of the world. More disturbing is that for no explicable reason childhood autism appears to be increasing at a rate of 10–17% per year [1]. Typically displayed in early childhood, autism may be associated with many co-morbidities that include epilepsy, attention deficit/hyperactivity disorder (ADHD), abnormal sensory or motor responses, disturbed sleep, reduced cognitive functionality, anxiety and aggression [2–4], or none at all.

In 2019 the CDC reported the rate of autism in the US to be 1 in 59 children, with boys being 4 times more susceptible than girls [5]. This means that 1 in 42 boys are diagnosed with autism. There was an increase of about 30% since the assessment of autism prevalence conducted previously (**Table 1**), and more almost 3x the rate that was reported only 20 years ago. In New Jersey the observed rate of autism was

Identified Prevalence of Autism Spectrum Disorder				
ADDM Network 2000-2010				
Combining Data from All Sites				
Surveillance Year	**Birth Year**	**Number of ADDM Sites Reporting**	**Prevalence per 1,000 Children (Range)**	**This is about 1 in X children...**
2000	1992	6	6.7 (4.5–9.9)	1 in 150
2002	1994	14	6.6 (3.3–10.6)	1 in 150
2004	1996	8	8.0 (4.6–9.8)	1 in 125
2006	1998	11	9.0 (4.2–12.1)	1 in 110
2008	2000	14	11.3 (4.8–21.2)	1 in 88
2010	2002	11	14.7 (5.7–21.9)	1 in 68

Table 1.
CDC: Prevalence of autism in the US [5].

1 in 46 children, which means that 1 in 29 boys born in New Jersey are likely to be autistic [6, 7].

The lifetime cost of raising an autistic child was estimated in 2014 to be $3.2 M more than the cost of raising a non-autistic child [8], and this does not take into account the societal costs of maintaining these this population as adults once their families are no longer able to do so. The societal costs of autism are broad and deep, and many have never been explored. For example, it was only in mid-2017 that information was developed on the rate of healthcare utilization by autistics and it was found that their need for psychiatric care as well as care for the high incidence of autism associated comorbidities was far beyond that of the general population or other elements of the psychiatric patient population [9]. Similarly, it was not until September of 2017 that the rate of school suspension and expulsion was dramatically higher in the autistic population, and growing as the autistic population grew in numbers [10].

Recently, attention has been brought to bear on autism associated mortality rates. Although autism is not typically considered to be a fatal disease, several investigators have reported a significantly increased mortality in the autistic population with the major cause of death being suicide. A matched case cohort study based upon the Swedish National Patient Registry and the Cause of Death Registry looked at deaths between 1987 and 2009 and found a 256% greater death rate in autistic patients compared to the general population. The mean age at the time of death was 70.2 years for the general population and 58.39 for patients with autism, with suicide associated with better performing patients [11]. A review of 1706 children and adolescents reported an 18% increased risk of suicidal ideation or attempts in autism [12]. 35% of patients with Asperger's syndrome were reported in a Canadian study t have attempted suicide [13]. Similarly, in Japan [14], Australia [15], England [16], and Belgium [17]. In a French review of the PubMed literature it was found that overall 21.3% of autism patients reported suicidal ideation or had attempted suicide, with the noteworthy observation that "... the methods used are often violent" [18].

Autism is quite heterogenous and has a broad pallet of potential symptoms. These symptoms transcend established investigative disciplines including behavioral studies, developmental studies, neurology, pharmacology and so forth. No truly workable definition of autism has yet emerged to define this heterogenous constellation of symptoms. Theories regarding the causes of autism include impairments within the autonomic nervous system [19], cerebellar dysfunction [20], mitochondrial impairment [21], exposure to toxins [22], and many others.

2. Autonomic function and autism

The autonomic nervous system has been implicated in symptoms that resemble those seen in autism. ASD has been associated with abnormal findings in autonomic related structures including the insula [23, 24] and the amygdala [25–27]. Autonomic related changes such as increases in basal heart rate [28–31] and diminished heart rate variability due to psychosocial challenges [32, 33] are seen in autism. The autism-autonomic linkage is exemplified by the consequences of respiratory sinus arrhythmia (RSA) that includes difficulties with socialization [30, 34], language difficulties [34, 35], and delays in cognitive development [35].

Kushki [19] hypothesized a chronically over activated autonomic system is a correlate of autism based upon the exaggerated levels of anxiety that attend autism [33], physiologic hyperarousal [36–38], and other correlates. Anxiety is perhaps the greatest co-morbidity associated with autism which may drive other features of the disease [39, 40], and has been associated with central nervous system structures that are linked to autonomic function [41, 42]. Phenotypically autism and anxiety both present with stereotyped repetitive and limited interests, avoidance behaviors and speech problems [43–45]. The relationship between anxiety and reported autonomic symptoms of elevated heart rate, perspiration, and other sequelae of the "fight or flight" reaction reveal a role for the peripheral nervous system function in autism [36–38]. However, this may be secondary to central autonomic activation. Central functions may manifest as elevated emotional responsiveness and exaggerated threat perception or diminished inhibition of fear responses [36], which are associated with the central structures mentioned above in which autonomic responsiveness and emotional responsiveness overlap.

There is a considerable body of evidence, which will not be reviewed here, that associates autism with cholinergic function in the central nervous system, specifically with various α-subtype nicotinic receptors, notably in the cerebellum. However, as autonomic function is classically considered to be a balance of cholinergic and catecholaminergic systems, perceived increases or decreases in cholinergic function may be manifestations of change in the dynamic balance of these systems with catecholaminergic tone. It may be possible to effect therapeutic change through manipulation of either acetylcholine-based manipulations or the counterbalancing of dopamine, norepinephrine, or epinephrine mediated mechanisms.

3. Adrenergic CNS changes in autism

Recent work by Hamilton, et al. [46] who sequenced exomes of families with a history of autism found deficiencies in the human dopamine transporter gene (hDAT), a protein responsible for the presynaptic reuptake of dopamine. CNS dopamine is a crucial element in systems that mediate motor function, motivation, attention and reward [47–50]. As this system is known to be associated with ADHD, and approximately 45% of autistic patients manifest symptoms of ADHD [3, 51–53], there is reasons to suspect a common pathway underlying these two diseases. Moreover, dopamine related genes *DRD1, DRD3* and *DRD4* are associated with an increased risk for ASD [54] as well as repetitive stereotyped behavior [55–57], and defiant and anxiety disorders [56]. Males with multiple tandem repeats in the monoamine oxidase-A (MAOA) promotor gene responsible for degrading dopamine show in increased proclivity for autism [58]. Aside from changes in synaptic dopamine uptake and degradation, changes dopamine receptor function and avidity have been reported [59–61], as have changes in dopamine synthesis and DOPA decarboxylase. Additionally, it has been observed that pharmacologic

manipulation of dopamine has clinical efficacy in ASD [62, 63], for example with risperidone, a drug approved to treat ASD.

Other lines of support come from observations of lower levels of dopamine β-hydroxylase in the plasma of autistic patients [64, 65] and reductions in platelet [66] and urine dopamine [67]. Similarly, inferences have been published that the mesolimbic cortex and striatum may provide a neurologic substrate linked with the motor and behavioral symptoms seen in autism as a result of a dopaminergic imbalance in these structures [68, 69].

Nguyen et al. [70] used in silico methods to clarify the genetics underlying the contribution of dopamine the etiology and pathogenesis of autism and found genes implicated that regulate both Ca^{++} metabolism and dopaminergic neurotransmission. They found proteins implicated in ASD regulate dopamine signaling in multiple places including reuptake and catabolism, and they defined discrete molecular clusters that act on systems implicit in dopaminergic systems such as androgen receptors that stimulate DOPA decarboxylase. Another finding was the potential role of dopamine mediating the modulation of dendritic spines which determine synaptic strength and may be important in the developmental delays associated with ASD [71].

In an interesting cybernetic model, Kriete and Noelle [72] developed a sophisticated methodology to investigate the role of dopamine and the changes in executive function associated with autism. They showed that the intensely focused cognition associated with autism, as well as the pathognomonic lack of cognitive plasticity and inability to react with appropriate conscious focus to changes in the stimulus milieu could be modeled as changes in dopaminergic systems in the prefrontal cortex. By differentiating cognitive control from plasticity, and further by showing how developmental changes in younger brains can account for the timing of the manifestation of autistic symptoms, these authors findings support a causal adrenergic mechanism underling at least some of the symptoms associated with autism.

Taken together, a good case can be made for dopamine as a key mediator of the motor, speech, social behavior, behavioral perseveration, and reward aberrations that are typical symptoms of autism. The precise regulation of dopaminergic function of autonomic function appears to involve the projection of Purkinje cells to the medial prefrontal cortex (mPFC) and the ventral tegmental area (VTA) of the striatum. Atrophied Purkinje cells is one of the most consistent neuropathologies associated with autism [73–76], and MRI data indicates persons with autisticism have smaller than normal cerebellar vermal volume [77]. Mice with diminished Purkinje cell mass evidence numerous autistic symptoms such as repetitive behaviors and impaired executive function [78]. Cerebellar Purkinje cells project to the mPFC where it appears they modulate dopaminergic transmission in this region directly, and via a remodeling of the VMA and thalamic interactions with the mPFC, and it has been suggested that cerebellar deficits observed in autism result in cortical, thalamic, and striatal integration via dopamine mediated pathways [79].

An immunologic linkage of dopaminergic function in autism was reported by Kirsten et al. [80] when they prenatally exposed rat pups to lipopolysaccharide, a stimulator of innate and adaptive immunity. Autistic symptoms of impaired communication, deficits in learning and memory, and repetitive/restricted behavior were observed in the presence of impaired tyrosine hydroxylase (TH) function which was taken as a marker of reduced striatal dopaminergic function. Support for this concept was also found when rat pups were given poly I:C, an immunogenic stimulator, and upregulation of various genes associated with dopamine neural development were observed [81].

The phosphatase and tensin homolog on chromosome ten (PTEN) is tumor inhibitory gene that inhibits PI3K and MAPK pathways, and a germline mutation of

this gene has been associated with autism [82]. Mouse mutations of this gene have resulted in symptoms similar to autism [83], and PTEN deletions have been found to enhance the survival and the function of dopaminergic neurons [84]. Work in this area has shown that mice with PTEN mutations have elevated TH and DA2 receptors in the striatum and prefrontal cortex, that PTEN reduces TH phosphorylation via MAPK suppression, downregulates dopamine synthesis in PC12 (pheochromocytoma) cell cultures, and that a PTEN-TH pathway may function as a "core regulator of dopamine signaling". Moreover, this mechanism appears to be operative in autistic patients, as 3 PTEN mutants identified in autistic patients cannot suppress TH, which supports the concept of TH suppression as a potential mechanism for therapeutic intervention in autism [85].

Consistent with the finding that TH over activity might underlie the symptoms of autism is the finding by D'Souza et al. [86] that the commonly used model for autism in which symptoms in animals are induced by administering valproic acid is related to the ability of this agent to induce TH transcription at every concentration tested.

4. GI abnormalities in ASD

Autism is associated with gastrointestinal pathology from the esophagus to the colon [87–91]. The literature suggests that GI pathophysiology is an intrinsic component of autism in many patients and may be a central component to the etiology of the disease. GI problems have been reported in 42% of children with ASD and 12% of controls, with chronic diarrhea and constipation being the most prevalent problems. The severity of these problems correlates with the severity of ASD [92]. It is noteworthy that in both GI dysfunction and ASD imaging reveals abnormalities in brain regions associated with emotional and sensory functions [93, 94], and GI problems contribute to behavioral problems, attentional deficits, and self injury [95]. Gut bacteria influence intestinal permeability, mucosal immunity, the enteric nervous system, pituitary functions, and the modulation of pain (cited [96]).

There is increasing reason to believe that the interaction between gastric microbiota and the brain are contributory to the symptoms seen in ASD. This is mediated via the autonomic innervation of the intestine and the hypothalamic–pituitary axis which is innervated by catecholamines and which generates GI signaling molecules affecting enteroendocrine and mucosal immune cells. The "Gut Brain Axis" is comprised of central and peripheral nervous systems as well as the neuroendocrine and immune systems, and communication is bidirectional, with vagal inputs to the brain as well as endocrine and neuroendocrine signaling [97]. Catecholamines are associated with stress reactions and, interestingly, GI microbiota respond to stress with changes in their efferent and afferent catecholamine responses (reviewed in [98]).

A trial of 36 autistic children found pain, chronic diarrhea, bloating, GI irritability, chronic gastritis, esophagitis, chronic duodenitis, diminished carbohydrate digestive enzymes and reduced pancreatic exocrine secretion in response to secretin challenge [88]. Secretin has not been found to be an effective treatment for autism. In a survey of parents of 500 autistic children, half responded that their children had loose stools or chronic diarrhea, and intolerance for wheat and cow's milk [99]. A number of reports mention improvements in autistic symptoms when reduced gluten and casein diets are implemented and the return of symptoms when these diets are terminated (cited in [100]).

Lucarelli et al. [101] observed an improvement in social skills and the ability to communicate in a trial of 36 autistics who were given diets with diminished

gluten and/or cow's milk, with improvements observed in 5 of 7 objective behavioral scales. Similar findings have been reported by others [102–105]. Following one year on this diet symptoms returned upon termination of the dietary restrictions [104]. Intestinal permeability was found increased to lactose in a number of high functioning autistic children compared to age matched controls, with no increased permeability to mannitol, which was interpreted to mean a diminution in the tight junctions of gut epithelium [106] and the subsequent release of incomplete gluten and casein digestive products. Autistic patents reportedly manifest significantly higher levels of IgA for casein, gluten, lactalbumin and β-lactoglobulin [101, 107].

These observations give rise to the Leaky Gut Hypothesis of Autism, which states that various digestion products can enter the blood through leaky tight junctions in the gut and interact with the immune and central nervous systems in ways that facilitate the onset of autism. Gut peptidases release short chain peptides called exorphins that have structural similarity to endorphins. Gliadomorphins and casomorphins are stable examples of these peptides that are known to induce psychosis [108]. β-Casomorphin-7 is elevated in the urine of autistic patients [104], and when in infused into the blood stream of rats has been shown to activate the transcription of the gene *c-Fos* in the brain [109]. However, dietary restrictions do not cure autism.

While controversial, elevated short chain fatty acids (SCFA) have been associated with autism [110], and both central and peripheral administration of propionic acid (PPA) to rats induces ASD-like impairments that include aberrant motor movements, stereotyped repetition, EEG changes, cognitive deficits, perseveration and social impairment, as well as increased oxidative stress, glutathione depletion, neuro-inflammation, altered lipid profiles and more [111]. SCFA are digestive products derived from fiber and protein. The most common SCFA include propionic PPA and butyric acid (BA) [112]. BA and PPA are metabolized in the liver via the portal circulation, however areas of the distal colon are outside of the portal circulatory bed, and the systemic effects of BA and PPA are believed to be significantly underestimated [110, 112]. SCFA, including PPA, activate G protein coupled neural, effect neurotransmitter synthesis and release, and mediate such diverse events in the nervous system as Ca^{++} gating, mitochondrial function, lipid metabolism, immune function, gene expression and the role of tight junctions [110]. SCFA are believed to modify the activity of TH, and there are 3 ways in which the SCFA BA modifies TH activity: (1) modulation of transcription via chromaffin remodeling, (2) activation of various transcription mediators, and (3) by interfering with TH mRNA [113–116]. Subsequent work by Nankova, et al. [117] have shown that PPA elevates TH mRNA levels and that SCFA increase TH and subsequent catecholamine synthesis.

This dietary model allows for the elevation of cortical and striatal dopamine activity via elevated TH synthesis and activation, and which has been invoked as a potential mechanism for the actions of risperidone [118, 119], one of the two drugs approved for the treatment of the irritability associated with autism. It is worth noting in this context that PPA is structurally similar to valproic acid (VA), and has similar effects to VA, which is a treatment known to induce autistic symptoms, and used as a model for this purpose [120–122]. As cited above, VA appears to induce ASD- like symptoms by stimulating TH transcription in a manner similar to butyrate [86].

It is worth noting that relative to the participation of gastrointestinal events which may underlie autism, recent developments in the study of the human biome and investigations into GI function have revealed that the gut is the source of a number of neurotransmitters and neurotrophic factors, thus opening a previously

understudied source of pharmacologic agents which may regulate CNS function. *E. coli* and Clostridium sp. have been shown to elevate free GI catecholamines and dopamine increases colonic water absorption [123]. GI microbiota produce catecholamines and recognize them in the environment [124–126]. Epinephrine and norepinephrine are implicated in the virulence, ability to adhere, and chemotaxic properties of luminal bacteria [127].

In the work discussed above, it is important to note that PPA activates peroxisome proliferator-activated receptor gamma (PPAR-γ), and that this orphan receptor has been shown to have independent effects on the mediation of catecholamine and opioid pathways by SCFA [128], and that, as discussed below, PPAR is considered to be a "master regulator" of lipid homeostasis both centrally and peripherally. This later finding plays into the growing literature of lipid metabolism dysregulation in autism. PPAR also has immunologic functions that have been found to be related to metabolic and neurologic pathologies [129].

5. Dopamine underlies autistic symptoms in the gut and the CNS

Any comprehensive approach to the treatment of autism must accommodate many different organ systems, certainly the gut and the CNS. As discussed above, the neurotransmitter function of dopamine is well known, including its modulatory effects on motor function, mood, emotion, irritability, reward, and other systems which are affected by autism. However, there also exists in the mesentery a paracrine dopaminergic system that regulates the secretion of bicarbonate [130], the secretion of digestive enzymes by the exocrine pancreas [131], and which controls sodium transport in the lower intestine [132]. Dopamine also has documented effects on gut motility and mucosal blood flow [133–135]. As early as 1994 elevated blood levels of levels of dopamine have been associated with autism [136]. It is known that ASD is associated with elevated levels of dopamine in the tracts linking the amygdala and prefrontal cortex in children with ASD [137, 138].

What is less known is that approximately 42–46% of the dopamine in the body is produced in the gut. Eisenhower and colleagues at NIH [139] studied 8 patients undergoing elective abdominal surgery and 47 patients who underwent cardiac catheterization. Tissue samples from the stomach and duodenum were obtained and compared, as were arteriovenous concentration differences and rates of renal clearance of dopamine and its metabolites in conditions of different sympathetic nervous backgrounds for dopamine not converted to norepinephrine. They found considerable dopamine synthesis in the stomach, pancreas, and duodenum, with renal elimination of dopamine and its metabolites. Dopamine has a naturetic function in the kidney; however, there was significant overflow of dopamine into the renal venous circulation that allows for systemic effects. As expected, cells in the stomach, pancreas, and duodenum stained positive for TH. The authors could not account for the amount of dopamine added to the mesenteric venous circulation, as it cannot be explained by sympathetic activity or diet, and their results were consistent with findings in swine [140].

In keeping with the concepts presented herein, it is relevant that in the liver, bile salt production and release are also under the control of dopamine [141, 142]. Bile salts are known to occur not only in the periphery, but in the CNS as well, where they appear to contribute to neurologic decline and blood brain barrier permeability [143, 144]. Bile acids are the predominant steroid in the brain, with levels that are 10x greater than those found in the blood indicating local synthesis, and with higher titers than that of pregnanolone, which was once considered the predominant neurosteroid [145].

In the brain, it has been observed that chenoxydecholic acid or deoxycholic acid induce the phosphorylation of occludin and increase the permeability of tight junctions via an Rac-1 dependent mechanism [146], making it conceivable that tight junctions in the gut are similarly effected by bile salts under the control of dopamine. It is well known that bile salts upregulate the orphan X receptors Farnesoid X Receptor (FXR), Liver X Receptor (LXR), Retinoid X Receptor (RXR) and PPAR. These nuclear receptors regulate the metabolism and homeostasis of glucose and lipids in numerous ways, including the transcription of the genes that regulate energy metabolism. Beyond the role of lipids in cell membranes and myelin sheaths, there is a growing body of literature to support the concept that lipids play a crucial signaling and regulatory role in cognition and other CNS events. This would appear to be significant as the brain comprised fundamentally of lipid and has the highest rate of glucose utilization in the body.

While it is commonly stated that autism occurs more frequently in males, at a rate of 4 boys for each girl [147], it is less commonly known that in severe autism this ratio increases to 11 to 1 [148]. Numerous sexual dimorphisms in the brain have been described (reviewed in [149]), such as brain size, hemispheric communications, differential gene expression, and more. It is worth noting that there is a growing body of literature implicating dopamine modulation of behavior as part of these sexual dimorphisms. One mechanism which may underlie the sexual dimorphism seen in the expression of ASD may relate to *SRY*, the sex-determining region on the Y chromosome, which is responsible for many male traits, including the differentiation of bipotential embryonic gonads to become testes. *SRY* is an intronless gene that co-localizes with dopaminergic neurons in the hypothalamus, frontal and temporal cortex, striatum, ventral tegmental area (VTA), locus coeruleus and substantia nigral. In humans, *SRY* expression is found in a population of TH positive neurons in the VTA, which is the origin of the dopaminergic cell bodies of the mesocorticolimbic dopamine system which is widely implicated in the drug and natural reward circuitry of the brain. It is important in cognition, motivation, orgasm, drug addiction, intense emotions relating to love, and several psychiatric disorders. SRY has been found to regulate the transcription of TH via the AP-1 binding site on the TH promotor. The synthesis of MAO-A, an enzyme which inactivates DA, and which has polymorphisms associated with the severity of ASD, is also mediated by *SRY* in a manner that elevates extracellular dopamine. Thus, *SRY* appears to be expressed in regions of the brain, and have pharmacologic activity on dopaminergic function, in a manner that is consistent with the preponderance of ASD in males that is pathognomic for this syndrome (reviewed in [149]).

Consistent with the increased prevalence of ASD in males, work in a mouse model has shown that a 16p11.2 gene deletion, which is associated with autism, affects the striatal reward system. While both sexes had 50% reductions in mRNA associated with ERK1, an important signaling kinase, in males there was an increase in ERK1 activation at baseline and in response to sugar in a manner associated with reduced striatal plasticity not shown in females. These changes were associated with an overexpression of dopamine D2 receptors in the striatum [150].

A mechanism by which sleep disturbances associated with ASD may be mediated involves the striatum, an area known to coordinate reward, learning and cognitive behaviors [151, 152] as well as to modulate circadian locomotor and retinal responses [153, 154]. This locus has been shown to be responsible for the maintenance of normal circadian rhythm functionality, and this system appears to be controlled by dopamine. Activation of D_2 receptors has been found to regulate clock genes in the striatum, controlling circadian events. Hood et al. [155] found that depletion of striatal dopamine by various methods, including the use of

AMPT, blunts normal circadian functions and that daily dopaminergic activation is required to maintain normal circadian rhythmicity.

In the context of a dopamine mediated model of autism, it is interesting to note that bile acids under catecholamine control inhibit the $GABA_A$ receptor [156, 157] in a manner that that diminished GABA related inhibitory post synaptic potentials. Inhibition was observed to occur in a stereospecific receptor-ligated, ion channel dependent manner, independent of lipophilicity, and consistent with the behavior of other known GABA receptor blockers. Interestingly, the inhibitory potencies of various bile salts corresponded best with their binding constants with albumin.

Taken together there is evidence for a dopaminergic system which might underlay and unite the symptoms of autism which manifest as central nervous system changes in mood, attention, cognition, socialization, etc., and those seen in the gut as changes in secretory, digestive and excretory functions.

6. The role of energy metabolism

ASD and energy metabolism are associated in several ways beyond the gut with glucose and lipid metabolism affected. Key among them is the role of bile salts under the control of catecholamines. Bile regulates FXR, LXR, and PPAR which are involved in the regulation of glucose metabolism, insulin sensitivity, lipid signaling and homeostasis. As a class, these ligand-inducible receptors are upregulated in the presence of their ligand, such as bile salts, and after binding they migrate to the nucleus where they exert genomic and epigenomic effects upon transcription and translation of the genes that mediate glucose and lipid utilization (for reviews see: [158–165]).

Diabetes and metabolic syndrome are recognized comorbidities of ASD [154]. Catecholamines regulate bile acid release and the FXR upregulates the synthesis and secretion of bile salts from the gall bladder by stimulating the bile salt efflux pump in order to provide bile to solubilize fat soluble nutrients and vitamins from the gut and may have a similarly stimulate FXR in the brain. In mice, FXR deficiency leads to insulin resistance and reduced glucose tolerance [163, 166, 167], and the finding of FXR in pancreatic islet cells that affects insulin release allows for a regulatory role of local bile acid concentrations in insulin release and glucose tolerance [168, 169]. In the CNS, it is commonly known that the influence glucose receptors in the hypothalamus, carotid bodies, and other sites summate to mediate the central nervous control of glucose metabolism. Eating, satiety and similar energy mediated events in the brain are known to be modulated by the sympathetic nervous system, predominantly by dopaminergic systems [170]. Sympathetic afferents from the hypothalamus and other central site, under the control of various agents such as catecholamines and leptin are known to regulate glucose synthesis, insulin sensitivity and similar events (reviewed in [171–174]). Severing the autonomic projections to the islets of Langerhans resulted in a 75–90% impairment in the ability to regulate serum glucose in response to insulin induced hypoglycemia [175]. Although the effects of catecholamines on lipid homeostasis have been defined in the gut, these mechanism can also serve as a model in brain tissue, since this organ contains 25% of the body's cholesterol but only 2% of its mass [176], and most of the lipid synthesis and metabolism in the brain occurs *de novo* within the CNS.

There exists a growing body of work that lipid metabolism underlies cognitive function. Accumulating evidence supports the idea that HDL and the mechanisms that regulate lipid metabolism also influence neurodegenerative diseases including autism, amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and others [177]. Just as HDL have a demonstrably cardio-protective role, they

also appear to have a neuro-protective role. HDL are made throughout the body and serve to remove excess cholesterol from peripheral tissues for excretion in the bile and for steroidogenesis. In a study of 139 centenarians it was found that plasma HDL correlated with mental acuity in age [178]. This was confirmed in another study of 159 centenarians [179], again in a longitudinal population study in Amsterdam [180], and supported by the finding that low HDL was associated with intellectual impairment in age [181–183]. Effectors like cholesteryl ester transfer protein (CETP), which increase HDL are similarly associated with durable cognitive function in later age [179, 184, 185].

Bile salts can be released inappropriately via a "leaky gut" syndrome that ASD or they can be made locally in the brain under the control of catecholamines. Their synthesis and biologic functions have been described in a variety of non-gastric tissues, including the brain. As reviewed by Quinn and DE Marrow [186], bile acids and their salts are now viewed as steroid hormones, and not merely as detergents that solubilize lipids. Consistent with their role as the predominant brain steroid [145], in the rat that the primary bile acid chenoxydecholic acid composed 95% of brains bile acid. Further, the most abundant oxysterols found in the CNS are the C_{22} and C_{26} intermediates of bile acid synthesis.

One of the agents that regulates HDL homeostasis is the LXR, which is upregulated in the presence of the bile salts that solubilize and accompany plasma and tissue lipids. LXR is a cholesterol sensing and regulating molecule and cholesterol functionality is necessary for heathy cell membrane function, which is crucial to synaptic function. LXR has been demonstrated to improve cognitive performance in animal models of Alzheimer's disease presumably via the induction of HDL [cited: [177]].

Once believed to be the master regulator of glucose and lipid metabolism, PPAR-γ is associated with the maturation and development of adipocytes, the deposition of lipids, glucose metabolism, insulin sensitivity and other related events [187]. PPAR-γ has been shown to be mediated by bile salts and dopamine via phospholipase C in a calcium dependent manner, with elevations in dopamine resulting in increased PPAR-γ in cardiac myocytes [188]. PPAR-α is abundantly expressed in skeletal muscle, liver and brain [189, 190], and is associated with dyslipidemia, a condition often seen in autistic patients [190–193]. PPAR-α has been associated in the literature with central dopaminergic function as it appears to influence the activity of antipsychotic agents known to interact with dopaminergic neurologic systems [194, 195]. It has also been implicated in reduced GABAergic interneuron firing in pyramidal neurons resulting in cortical excitation [196–199]. D'Agastino et al. [200] have shown that central nervous system reduction in this "Master Regulator of Lipid Homeostasis" is associated with autistic like behaviors that include; repetitive and perseverative behaviors, loss of cognitive flexibility and reduced spatial information processing. They documented PPAR-α deprivation resulted in resistance to central glutamate stimulation via NMDA receptors, reduced GABAergic interneurons in the frontal cortex and hippocampus with dystrophic neurons in these structures, and increased gamma waves with decreased theta wave frequency.

Historically, there is a well-defined relationship between stress, catecholamines, and plasma lipids (reviewed in [25]). Stress, which is characterized by elevated levels of circulating catecholamines, is associated with increased plasma lipids, reduced glycemic control, diminished insulin secretion and insulin insensitivity, all of which can be associated with ASD. This is consistent with the aggressive fighting responses associated with catecholamines significantly elevating NGF in sympathetic ganglia and in the absence of ACTH or corticoids [201]. Various central mechanisms have been implicated in these events, including, the ventromedial

nucleus of the hypothalamus and hippocampal efferents to the hypothalamus. These central nervous system events can be translated into hyperlipidemia in three ways: via adrenal epinephrine release, via elevated pancreatic glucagon secretion, and via the regulation of hepatic glycolysis and gluconeogenesis. All three of these pathways are regulated by the sympathetic nervous system.

These findings fit with an emerging metabolic model of autism in which CNS control of energy metabolism and the autonomic nervous system as an integrating modality that senses and regulates those changes in the periphery and modifies these effects centrally. Integration of reward, satiety, insulin release and sensitivity, related endocrine events, as well as circadian clock mechanisms and similar systems which are impaired in autism appear to be mediated largely in the hypothalamus and brain stem via various nutrient sensing mechanisms which reticulate throughout the CNS to the cortex, basal ganglia, pyramids and so forth. (for a review see [202]). Cholesterol, LXR, PPARs and other agents which are known to regulate energy metabolism in the periphery appear to do so in the CNS as well and these mechanisms map well to the deficiencies seen in autism. It is particularly noteworthy that many of the events mediated by the nuclear receptors LXR and PPAR are cell and ligand specific, and that changes in cholesterol metabolism can have profound changes on membranes and their functions. That these changes can vary as a function of cell type provides a mechanism by which metabolic impairments in discrete brain regions may occur in ways that compromise specific nuclei and tracts.

Taken together, there appears to be linkage between catecholamine metabolism both centrally and peripherally, the regulation of energy homeostasis, and central nervous system function in a variety of pathologic states. There is a growing body of evidence to indicate a relationship between central and peripheral nervous system regulation of glucose and energy homeostasis, and abnormal cognitive function, as exemplified in autism.

7. Nerve growth factors

Nerve growth factor (NGF) in the brain is stimulated by catecholamine synthesis [203] and regulates the morphology of the catecholamine synapse. Neurotropic NGF is required for catecholaminergic neuron survival and differentiation. It is released into the synapse with catecholamines and it determines the synaptic architecture with elevated levels of NGF resulting in elevated levels of TH, catecholamine synthesis and synaptic neurotransmission [204–206] since NGF concentrations have a direct effect on the budding and arborization of catecholamine dendrites [207, 208] as well as the density of target tissue innervation [209, 210] Similarly, elevated catecholaminergic transmission is associated in a dose dependent manner with brain derived nerve growth factor (BDNF) in a pre-synaptic manner [211]. This is consistent with the finding that the loss of a Brain Derived Nerve Growth Factor (BDNF) allele in a mouse knockout model.prevented the loss of sympathetic islet innervation in an immune based diabetic model [212].

NGF has a hyperplastic, hypertrophic effect on catecholaminergic neurons characterized by elevated TH [213–215] that results from binding to its tyrosine kinase receptor TrkA expressed on the axons of catecholaminergic neurons [216]. In this way pre-synaptic release of neurotransmitters exerts a differentiating effect post-synaptically to mediate catecholamine synaptic architecture, the number of neurons, and innervation density [207, 217].

NGF is known to increase with increased catecholaminergic nerve traffic and with stress [218], and results in the sprouting of new nerve fibers in the stellate ganglion and elsewhere in the sympathetic nervous system [219, 220]. NGF and

BDNF are important mediators of neurologic function in the brain with the ability to mediate short and long term neurologic function in areas associated with ASD like the cortex and hippocampus [221, 222]. NGF has been shown to promote sympathetic neural growth, differentiation and to enhance target innervation [205, 208–210, 223, 224] and NGF is known to be elevated in PTSD [225–227], a disease with a similar constellation of symptoms to autism. NGF leads to sympathetic sprouting and supports dendritic geometry of the newly sprouted nerve terminals for the life of the sympathetic neural substrate [228, 229]. NGF is known to effect memory directly [230], indirectly [220, 231, 232], and through its actions on NE, as well as indirectly via hypothalamically mediated release of cortisol [233].

8. L1-79

LI:79 is D,L α-methyl-para-tyrosine, abbreviated AMPT. It inhibits the activity of TH, which catalyzes the first transformation in catecholamine biosynthesis, i.e., the conversion of tyrosine to dihydroxyphenylalanine (DOPA) which is the rate limiting step in catecholamine synthesis. L α-methyl-para-tyrosine was approved by the FDA in 1979, is marketed under the name Demser®, and is typically called metyrosine and abbreviated AMT.

α-methyl-para-tyrosine is a tyrosine analog that competes competitively for TH and is excreted mostly unchanged in the urine. Demser was approved for presurgical use in the treatment of pheochromocytoma, a catecholamine producing tumor which when manipulated surgically releases pathologic levels of catecholamines into the circulation that can result in serious AE. Demser minimizes this potentially serious complication and can treat pheochromocytoma patients who were not qualified for surgery. It is approved for use in doses between 1 and 4 g/day in divided doses. The doses of L1-79 used in autism clinical trials was 90 mg tid to 400 mg tid of which only 50% is the L-isomer.

While Demser is intended to deplete adrenal medullary catecholamines as fully as possible L1-79 is intended to reduce catecholaminergic tone slightly, a use for which Demser is inappropriate. The published half-life for Demser is 3.53 hours [234], whereas the half-life for L1-79 has been found to be between 10.3–14.3 hours [235]. This is presumed to result from a competitive inhibition between the dextro and levo forms of the molecule for the L-amino acid transport mechanisms in the body resulting in more time on target for the racemate. Since only 50% of L1-79 is the active L-isomer, and as it persists at the receptor for a longer duration, L1-79 is suitable for bid dosing and is much better tolerated at the lower doses used to get a therapeutic effect in ASD.

Adverse events associated with Demser include sedation that typically habituates but might persist at doses >2 g/d, temporary changes in sleep, extrapyramidal signs including tremor at high doses, trismus and parkinsonism at high doses, dose dependent confusion that resolves with dose reduction, dose related diarrhea, and infrequent AE that include crystalluria, nausea and vomiting, and impotence. None of these have been observed in autism except for 2 patients who manifest crystalluria without clinical consequence at the 200 mg tid dose.

It should be noted that D,L α-methyl-para-tyrosine as described herein for the treatment of autism is also used in a polytherapeutic regimen for the treatment of patients with late stage cancer (SM-88) under the Tyme Technologies Inc. at doses that are a fraction of the lowest approved dose for Demser, and has been well tolerated.

9. Preliminary clinical observations

In a proof of concept trial in 8 patients of both sexes between the ages of 2.75 to 24 years of age and without Rett or Fragile X syndrome. Doses began at doses of 90 mg tid and were escalated to 200 mg tid for most patients with two patients receiving a brief course at 400 mg tid, which was not found to increase efficacy. All doses were well tolerated. Patients were washed out of their legacy medications and 6 patients were maintained on L1-79 alone. Two patients were restarted on one of their legacy medications at lower than their pre-study dose. L1-79 in this study had a therapeutic effect on the core symptoms of autism as defined by the ABC-C (**Figure 1**), the CPRS (**Figure 2**), ADOS (**Figure 3**), and the CGI (**Figure 4**). This includes improvements in socialization, communication, repetitive movements, sleep disturbances, and other symptoms of ASD. Interestingly, the Autism Diagnostic Observation Schedule 2 (ADOS), which is the "gold standard" for quantifying the lifetime severity of ASD was profoundly influenced by L1-79 treatment. In the 6 patients in whom the ADOS was measured a mean decrease of 30% was observed with one patient experiencing a reduction of 47% (**Figure 3**) which took him below the threshold for a diagnosis of autism following 10 weeks of treatment, although

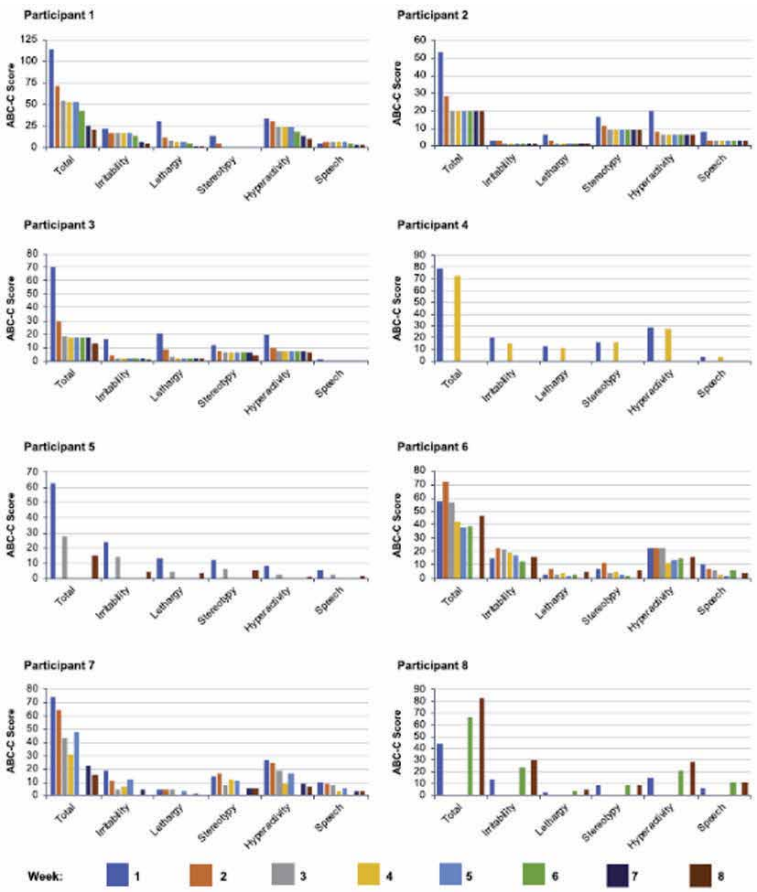

Figure 1.
Proof of concept study: Aberrant behavior checklist-community (ABC-C) scores. Domain scores for each participant during weeks 1 to 8. Because of participant-specific factors, ABC-C scores were not recorded at all visits for all participants.

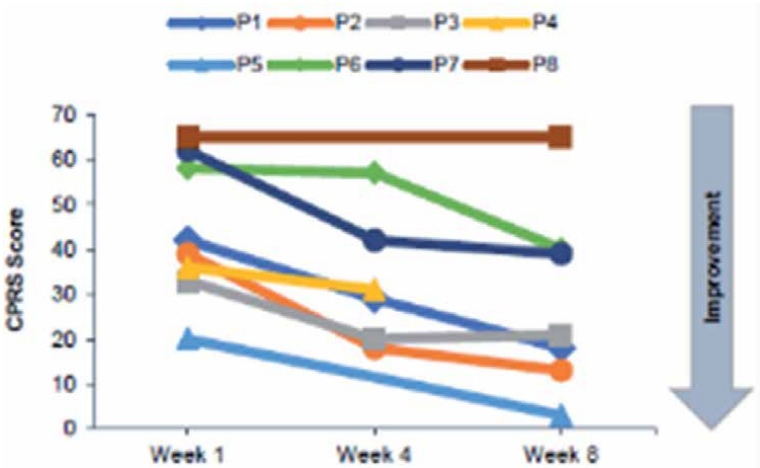

Figure 2.
Proof of concept study: Connor parent rating scale (CPRS) at 4-week intervals for 8 participants. Some participants did not have all assessments.

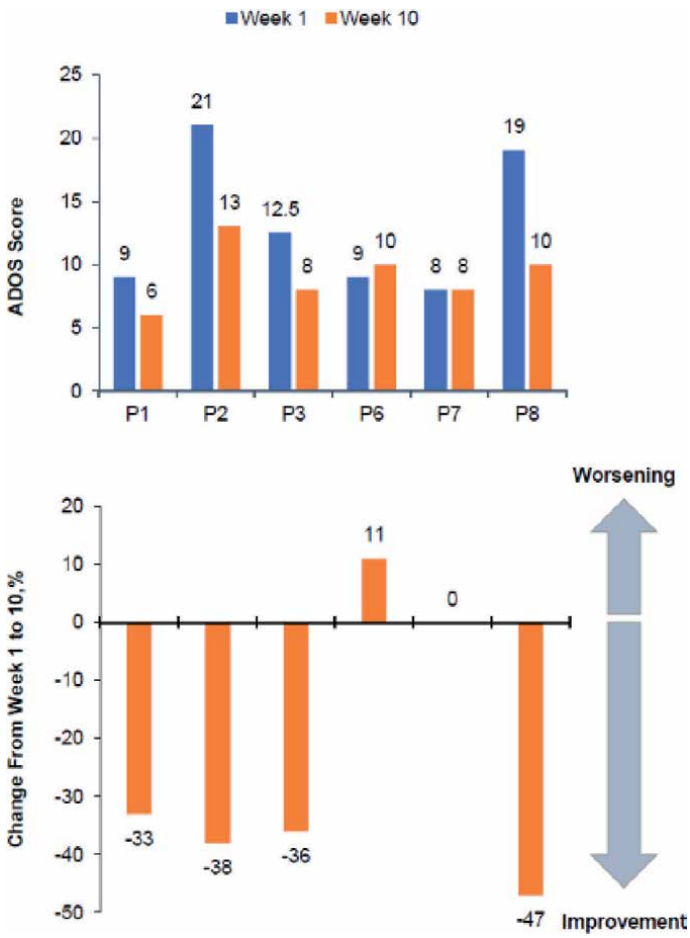

Figure 3.
Proof of concept study: Autism diagnostic observation schedule (ADOS) scores for the 6 participants tested at baseline and week 10.

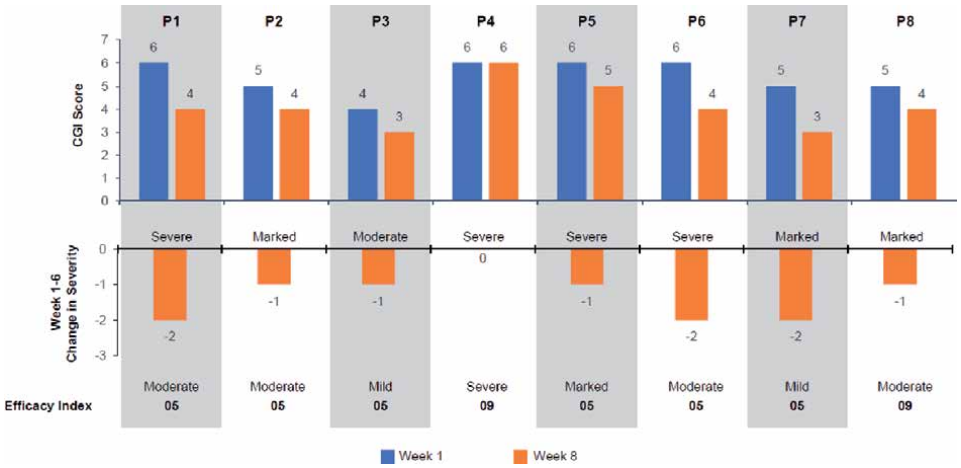

Figure 4.
Proof of concept study: Clinical global impressions (CGI) scores for 8 participants. Top panel shows absolute scores at week 1 and week 8. Bottom panel shows the change from week 1 to week 8. The qualitative interpretation at week 1 appears above each participant change in score, and the interpretation at week 8 is shown below each bar. Each participants efficacy index at week at appears at the bottom.

he was still on the spectrum [236]. As can be seen in the ABC-C scores, these effects were observed rapidly.

A follow-up randomized, double blind, placebo-controlled 28-day study of 39 patients has been completed and the results are currently in preparation for publication [237]. While 28 days was too short to demonstrate much of an effect, per the FDA the existing toxicology did not permit a longer treatment duration at that time. Participants were male patients between the ages of 13 and 21 years of age with a diagnosis of ASD based on DSM-5 criteria and confirmed by Autistic Diagnosis Interview-Revised (ADI-R), ADOS and expert clinical opinion, stable on no more than one concomitant medication with no planned changes in psychosocial interventions during the study and sufficiently tolerant and capable of complying with the requirements for this study. Results from this brief study can be seen for the CGI (**Figure 5**), Social Response Scale-2 (SRS) social motivation T scores (**Figure 6**), ADOS (**Figure 7**), Vinland Adaptive Behavior Scale-II (VABS) socialization standard score (**Figure 8**), and the SRS DSM-SCI T scores (**Figure 9**).

Anecdotally, numerous salutary behaviors were observed in these studies. Two teenage boys hugged and kissed their parents for the first time. One teenager with a history of self mutilating behavior stopped hurting himself. Subjective aspects of socialization such as empathy, effective communication, emotional expression, better sleep patterns, and engagement with peers were reported by parents and teachers and a school bus driver who were unaware of the trials.

The proof of concept study was conducted under the assumption that, per the 505(b)(2) guidelines, any stereoisomer of a drug is considered to be the same drug, and therefore the use of L1-79 was an unapproved use of an approved agent (Demser). In subsequent discussions with the FDA this was disallowed. The FDA required that the proof of concept study be discontinued and approved a follow-on pilot study that was limited to 28 days based upon the toxicology then in existence. It is noteworthy that when study drug was discontinued after the 28-day pilot study patients regressed to baseline within one week whereas the proof of concept patients who had been on study drug for as long as 6 months maintained some residual benefit following the discontinuation of medication.

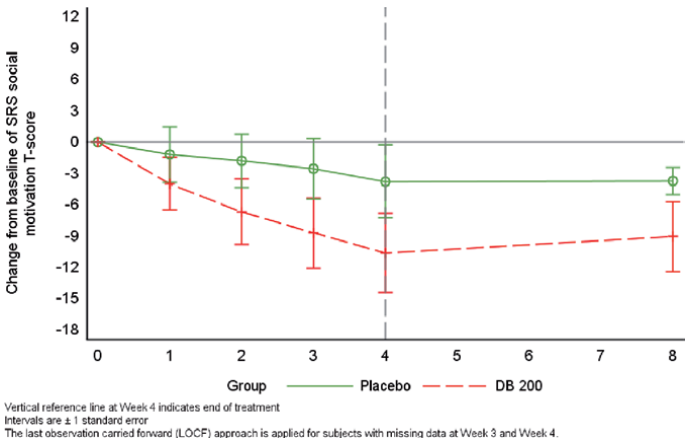

Figure 5.
Pilot study: Change in clinical global impression-severity (CGI-S) over time and individual patient responses from baseline to week 4. Upper panel is the change over time by study week for L1-79 200 mg and placebo. The lower panel is individual patient responses for L1-79 200 mg and placebo.

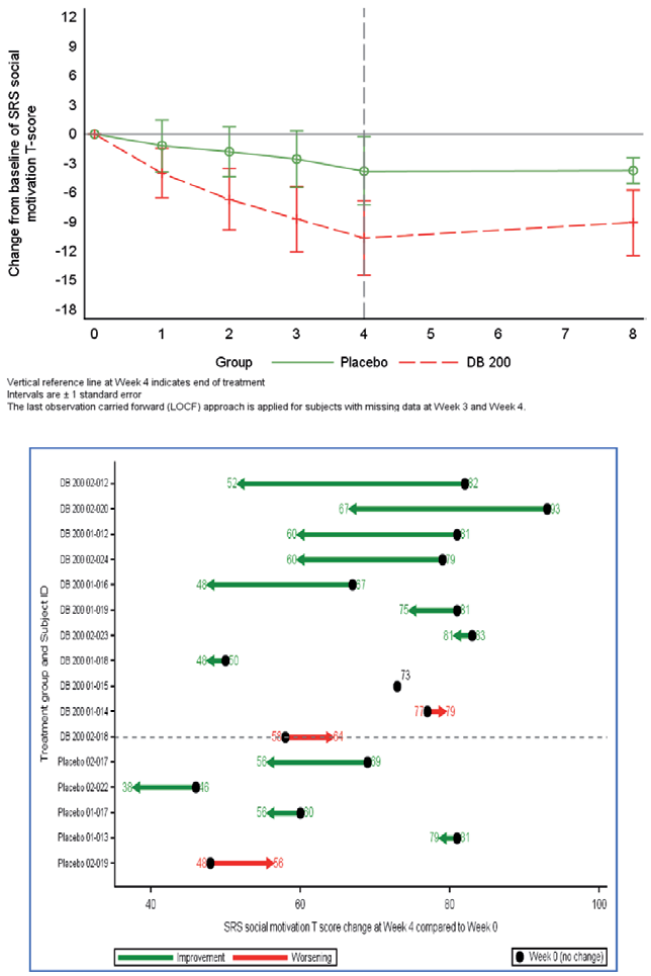

Figure 6.
Change from baseline over time and individual patient responses in the social responsiveness Scale-2 (SRS-2) social motivation T-score at week 4. Upper panel is change from baseline over time for L1-79 200 mg and placebo. Lower panel is individual changes for L1-79 200 mg and placebo.

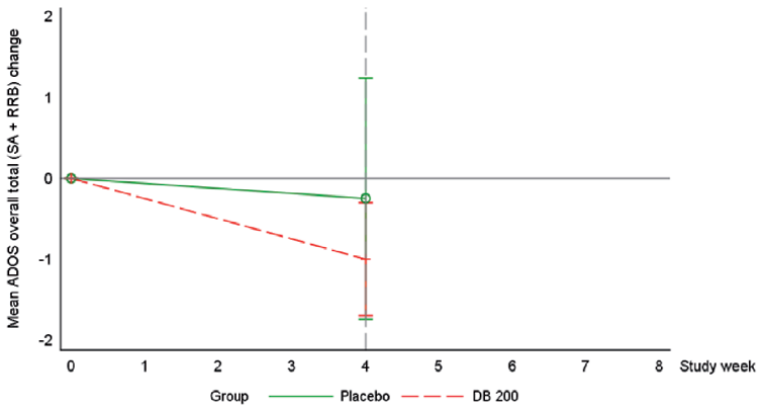

Figure 7.
Pilot study: ADOS score changes for 200 mg dose and placebo after 28 days of treatment. Negative scores represent improvement.

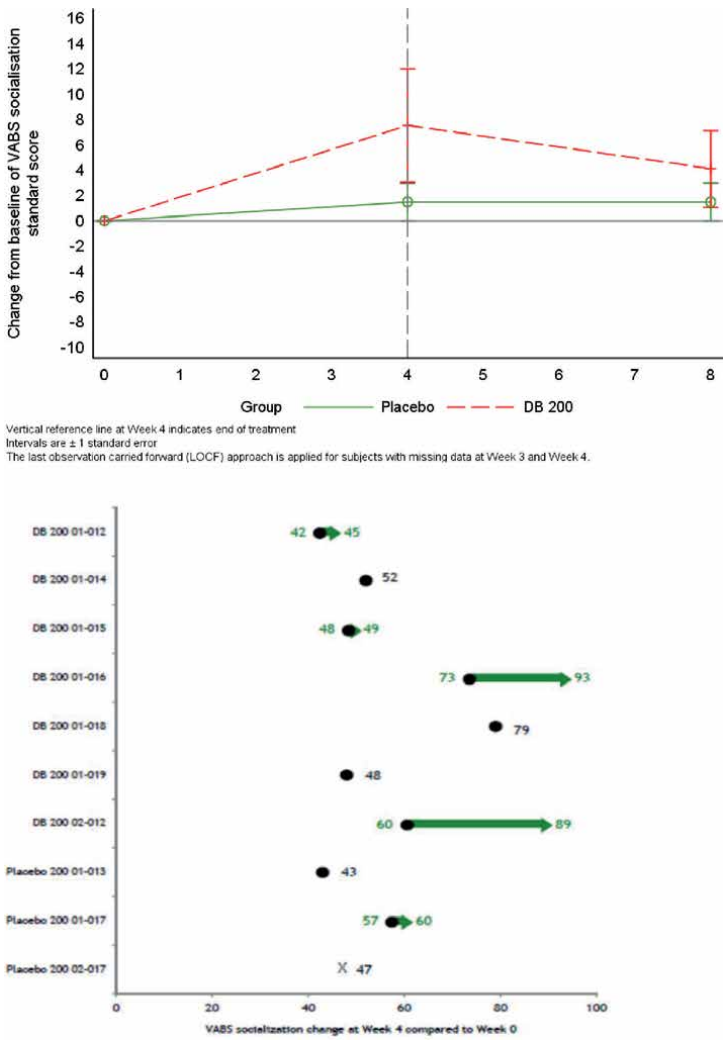

Figure 8.
Upper panel: Change from baseline over time and individual patient responses Vineland adaptive behavior-II (VABS) socialization standard score at week 4 for L1-79 200 mg and placebo. Lower panel individual changes for L1-79 200 mg and placebo.

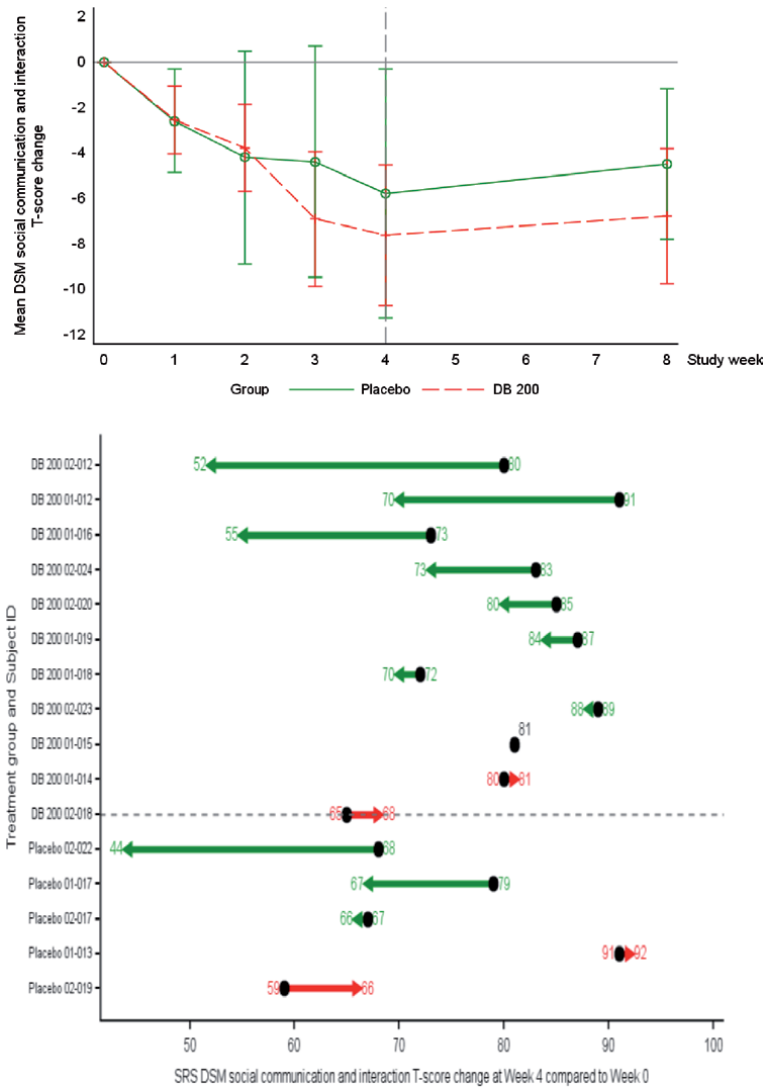

Figure 9.
Pilot study: Upper panel is the change from baseline over time in the social responses scale – 2 DSM-SCI for week 4 L1-79 200 mg and placebo. Lower panel individual changes for L1-79 200 mg and placebo.

10. Mechanism of action

We hypothesize that the mechanism begins with some process that stimulates massive levels of sympathetic neural traffic that gives rise to high levels of catecholamine synthesis and release which is associated with high levels of both BDNF and NGF [238]. NGF receptors are found on sympathetic nerves [239] and are responsible for maintaining catecholaminergic synaptic architecture due to their control on the budding and arborization of catecholamine dendrites [207, 208] and density of innervation [209, 210].

Elevated levels of synaptic nerve growth factors associated with catecholamine release may result from a variety of factors including genetics, cognitive or biological stress such as exposure to pesticides, fever in utero, complications of pregnancy, or other causes. This catecholamine elevation results in elevated levels of nerve traffic due to growth factor induced budding and arborization of catecholamine

nerve terminals and collaterals. As these growth factors are required to support the dendritic architecture of the neurons over their life, this elevated level of NGF & BDNF become chronic, resulting in an enhanced level of synaptic morphology and a consequent elevation of catecholamine release from these new hypertrophic synapses. This change in the level of catecholaminergic tone and the elevated release of catecholamines creates a persistent imbalance in the CNS and between the sympathetic and parasympathetic arms of the autonomic nervous system resulting in an overstimulation of some tracts and depletion in others. This imbalance caused by the growth factors centrally and peripherally results in both neurologic and metabolic pathology.

Because this involves catecholaminergic mechanisms in the brain, gut, mesentery, and elsewhere in the body, changes in emotional expression, speech, cognition, memory, circadian rhythms, gut function, energy metabolism, and the entire panoply of autism related symptoms can potentially be ascribed to aberrant catecholaminergic function. It is worth noting that NGF exerts presynaptic functionality with both pre- and post-synaptic effects with both short- and long-term effects on catecholaminergic neurotransmission [216]. Thus, the effects of L1-79 are not mimicked by receptor blocking agents which only reduce post-synaptic depolarization but do nothing to address the underlying abnormality of excessive catecholaminergic collaterals and a hypertrophic dendritic architecture induced and maintained by growth factors.

Since NGF is known to stimulate TH [215, 240–242] and L1-79 inhibits TH, and given both the short and long term effects of NGF exposure on sympathetic substrates, L1-79 is likely to have a therapeutic effect in the short and intermediate term of treatment of autism and may even have a disease modifying effect in the long run if the hypertrophic synaptic architecture regresses to a more homeostatic morphology. That is, if the underlying pathology of ASD is due in whole or part due to elevated catecholaminergic tone due to the release of growth factors associated with catecholamine release, then by reducing catecholamine synthesis, storage and release along with the associated release of NGF and BDNF a reduction of symptoms is likely to result. If, over a longer period, the reduction of NGF and BDNF enables a restoration of normal synaptic morphology then a persistent reduction of ASD symptoms may be possible, even in the absence of treatment.

11. Conclusions

L1-79 inhibits the rate limiting step in the synthesis of catecholamine, including dopamine and norepinephrine. Unlike the L-isomer of AMPT (Demser), L1-79 has a better kinetic profile for the use of L1-79 as a treatment for ASD. It's presynaptic mechanism of action likely results in a diminution of both catecholamines and related growth factors which we hypothesize will reduce symptoms of ASD in a manner not possible with receptor blockers, and which with long term use may reduce the hypertrophic architecture of catecholamine synapses in ASD back to a homeostatic morphology. An exaggerated catecholaminergic mechanism underlying ASD and its associated comorbidities can explain a variety of potential influences on the disease including the effects of bile, orphan receptors, lipids, glucose and other factors.

Preliminary results observed following the administration of L1-79 to autistic juveniles and adolescents has resulted in consistent improvement in the core symptoms of in two early studies [214, 215] not seen with previous agents.

L1-79 appears to be an effective therapy for the treatment of autism in children empirically with a novel mechanism of action that is supported by the scientific literature.

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Conflict of interest

Dr. Rothman is the managing director of Yamo Pharmaceuticals.

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Chapter 7

Exposure to Xenobiotics and Gene-Environment Interactions in Autism Spectrum Disorder: A Systematic Review

João Xavier Santos, Célia Rasga and Astrid Moura Vicente

Abstract

Heritability estimates indicate that genetic susceptibility does not fully explain Autism Spectrum Disorder (ASD) risk variance, and that environmental factors may play a role in this disease. To explore the impact of the environment in ASD etiology, we performed a systematic review of the literature on xenobiotics implicated in the disease, and their interactions with gene variants. We compiled 72 studies reporting associations between ASD and xenobiotic exposure, including air pollutants, persistent and non-persistent organic pollutants, heavy metals, pesticides, pharmaceutical drugs and nutrients. Additionally, 9 studies reported that interactions between some of these chemicals (eg. NO_2, particulate matter, manganese, folic acid and vitamin D) and genetic risk factors (eg. variants in the *CYP2R1*, *GSTM1*, *GSTP1*, *MET*, *MTHFR* and *VDR* genes) modulate ASD risk. The chemicals highlighted in this review induce neuropathological mechanisms previously implicated in ASD, including oxidative stress and hypoxia, dysregulation of signaling pathways and endocrine disruption. Exposure to xenobiotics may be harmful during critical windows of neurodevelopment, particularly for individuals with variants in genes involved in xenobiotic metabolization or in widespread signaling pathways. We emphasize the importance of leveraging multilevel data collections and integrative approaches grounded on artificial intelligence to address gene–environment interactions and understand ASD etiology, towards prevention and treatment strategies.

Keywords: autism spectrum disorder, xenobiotic exposure, early-life exposure, genetic risk factors, gene-environment interactions, exposome

1. Introduction

Many neuropsychiatric disorders are thought to have a multifactorial etiology, with interactions between genetic susceptibility and environmental factors likely contributing to their onset and progression [1]. ASD has a particularly complex genetic architecture, with implicated genes accumulating thanks to more accessible and less costly high-throughput genotyping and sequencing technologies. Between 15 to 25% of ASD cases occur in the context of clinically defined monogenic syndromes and chromosomal rearrangements [2], and therefore have a genetic

diagnosis. However, most patients still do not have a clearly identified genetic cause. Genome-wide association studies (GWAS), carried out in large cohorts using SNP arrays, did not find consistently associated ASD genes [3], but showed that individuals with ASD carry a significantly higher burden of *de novo* Copy Number Variants (CNVs) than expected [4, 5]. More recently, exome and genome sequencing studies have been detecting a growing number of loss-of-function Single Nucleotide Variants (SNVs) in patients [5, 6]. Some of these SNVs are rare *de novo* genetic variants with high penetrance, but most have low to moderate effects, indicating that a multiplicity of common, low effect variants are discrete contributors to ASD risk variance. These CNVs and SNVs map to dozens of different candidate genes, which frequently cluster in neurobiological pathways (*e.g.* synaptic processes, behavior regulation, cognition and neuronal signaling) as well as in chromatin modification and gene expression regulation processes [4–7], providing evidence for the biological mechanisms disrupted in the disorder.

Recent ASD heritability estimates vary between 64 and 85% [8, 9], and incomplete concordance rates between monozygotic twins are reported [10, 11]. These observations suggest that ASD, and its hallmark clinical heterogeneity, is not solely determined by genetics, and that environmental factors may contribute to its risk. Due to the extreme vulnerability of the developing brain to environmental stressors [12], the impact of environmental factors in this neurodevelopmental pathology is of particular concern. In this context, the environment comprises all non-genetic factors that can influence the onset or progression of the disease. Generally, environmental factors include xenobiotics, *i.e.* any natural or synthetic foreign agent that enters the organism through ingestion, inhalation, dermal absorption, injection or by placental transfer, and also other external factors like medical events or lifestyle, psychosocial and cultural variables [13, 14].

From conception to death, individuals are to some degree shaped by an ever-changing environment. However, its impact in health and disease through the life course is still mostly unexplored. Given the early onset of ASD, environmental exposure during the prenatal period to the second year of life is of particular relevance, while at later stages it may still modulate disease progression and possibly treatment efficacy [13, 15]. In this review we focus specifically on the role of xenobiotics in ASD, and on the impact of interactions between genetic variants and xenobiotic exposure. Literature reporting xenobiotic exposure in ASD is already extensive. We expect this systematic review may guide and encourage further studies to elucidate the impact of gene–environment interactions in ASD.

2. Methods

We systematically reviewed studies in two categories: (a) studies reporting xenobiotic exposure implicated in ASD; (b) studies reporting interactions between the previously defined xenobiotics and any genetic factor. We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) standard checklist [16]. Systematic reviews of the literature were performed successively for categories (a) and (b).

2.1 Information sources and search strategy

PubMed and EBSCO were queried from inception to November 2020, for records published in peer-reviewed English-language journals.

For records in category (a) PubMed and EBSCO were interrogated using updated and dropped clinical terms (“autis*”; “asperger” and “pervasive

developmental disorder") in combination with the terms "environment*", or "xenobiotic", or "toxin" or with terms for xenobiotics' names ("antidepressants"; "air pollutants"; "bisphenol A"; "folic acid"; "metal"; "PBDE"; "PCB"; "pesticide"; "PFC"; "phthalate"; "vitamin D"). Regarding category (b) the query was done using the same clinical terms in combination with "gene–environment" term and with terms for xenobiotics names identified in previous search.

2.2 Screening and eligibility criteria

All identified records were imported to the Mendeley reference manager. PRISMA flowcharts for (a) and (b) categories are shown in **Figure 1**. For record screening, the following exclusion criteria were applied: 1) review articles and letters to editor; 2) articles where the participants' diagnosis of ASD was not confirmed according to criteria from *The Diagnostic and Statistical Manual of Mental Disorders III, IV* or 5 editions or from the *International Classification of Diseases 9* or *10* editions; 3) articles not related to exposure to xenobiotics (category (a)) or not related to gene–environment interactions (category (b)); 4) articles focusing only on animal models, because despite the existence of several robust animal models that provide insight into the biological mechanisms and therapeutics for the disorder, these are unable to fully comprise the behavioral spectrum; 5) articles reporting associations between vaccination or thimerosal exposure and ASD (category (a)), because a role for vaccination and exposure to thimerosal preservative has been discredited [17].

After screening, for category (a) eligible articles were included in the final results if they reported statistically significant associations between xenobiotic exposure and ASD risk. Prenatal to early postnatal (*i.e.* preconception to the second

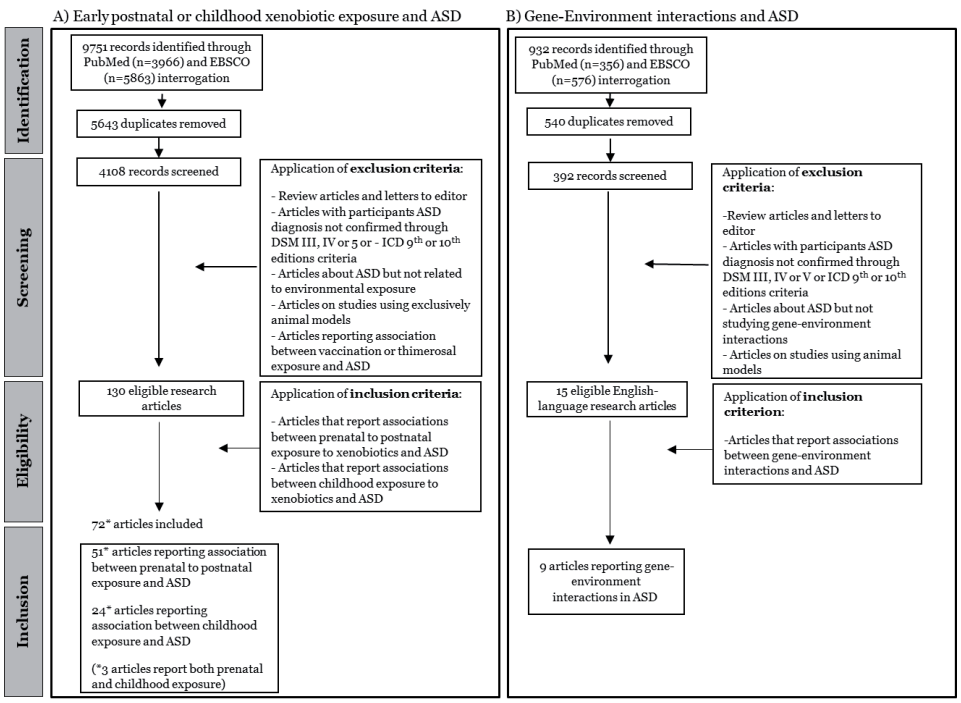

Figure 1.
PRISMA flowcharts for (1A) the identification of articles reporting associations between xenobiotic exposure and ASD; (1B) the identification of articles reporting associations between gene–environment interactions and ASD.

year of life) and later childhood exposure were considered separately. For category (b) eligible articles were included if they implicated gene–environment interactions in ASD risk, as long as the environmental component was the exposure to any of the xenobiotics' identified in category (a).

3. Results

3.1 Xenobiotic exposure associated with ASD

Figure 1A shows the flowchart for the identification of relevant publications. After removing duplicates, a total of 4108 unique records were screened using the defined exclusion criteria, resulting in 130 eligible research papers. Application of the inclusion criterion (*i.e.* to report an association between exposure and ASD) resulted in 72 articles selected to the final list of publications [18–89], shown in **Table S1**. From the 72 articles included, 51 (70.8%) reported prenatal to early postnatal exposure (up to 2 years of age), while 24 (33.3%) reported later childhood (from 2 years) to early adulthood exposure (**Table S1**). Three records reported association of both prenatal/early postnatal and childhood exposure with ASD [20, 28, 81].

The identified xenobiotics were categorized in seven major groups: Air Pollutants, Toxic Heavy Metals, Non-Persistent Organic Pollutants (non-POPs), Persistent Organic Pollutants (POPs), Pesticides, Pharmacological Drugs and Nutritional Factors (**Figure 2**). POPs include bisphenol A and phthalates, while non-POPs include polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and perfluorinated compounds (PFCs). The first five groups comprise ubiquitous toxins present in air, daily use products and the food chain, while exposure to the last two groups occurs through ingestion.

Historical proof-of-concept evidence for a role of xenobiotic exposure in ASD comes from three studies, which reported for the first time a very high prevalence of the disorder among subjects prenatally exposed to teratogens. Specifically, these studies reported an ASD prevalence of 4% among individuals exposed to thalidomide [63], of 8.8% in subjects exposed to valproic acid [59] and of 21.4% in individuals exposed to misoprostol [64] (**Table S1**).

Evidence supporting an association with ASD is stronger for exposure to air pollutants and pesticides, as all studies examining these toxins report an increased risk for the disorder (**Table S1**). Usually, these studies gather air quality or pesticide application data for large geographical areas and, by applying geocoding methods, investigate how exposure patterns relate to ASD prevalence. Each of these studies includes, at least, one hundred cases, with larger ones examining exposure in thousands of subjects [18, 22, 23, 29, 30, 52]. Environmental agencies are instrumental for collection of airborne pollutant and pesticide data in large populations from geographically defined areas, enabling valuable geocoding approaches. Because heavy metals can circulate in the air, large population geocoding studies, involving hundreds of subjects, are also applied to assess exposure to these chemicals [31, 32, 34]. Some studies quantifying exposure to heavy metals, as well as those that analyze POPs, non-POPs or vitamin D, need to resort to biological matrices. Because this data is so labor intensive to collect, most evidence comes from small datasets of less than one hundred subjects. For instance, this review identified 4 studies assessing heavy metals in biological matrices like hair, nails and teeth, all carried out in small numbers of subjects [33, 35, 36, 38]. Regarding POPs and non-POPs, evidence for an association with ASD is still limited, as fewer reports addressed these chemicals (**Table S1**). Two studies provide evidence for an increased risk of ASD from prenatal exposure to PCBs [43, 44] while, in other two, PFCs prenatal exposure was found to decrease ASD

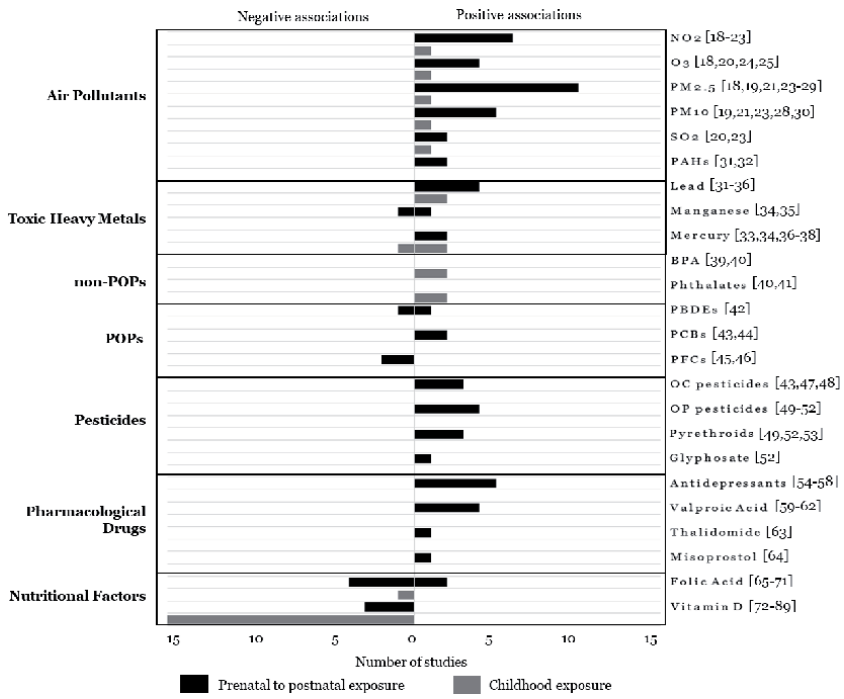

Figure 2.
Number of studies reporting negative and positive associations between exposure to xenobiotics and ASD, including prenatal to early postnatal and childhood to early adulthood exposures. NO_2 – Nitrogen dioxide; O_3 – Ozone; $PM_{2.5}$ – Particulate matter with a diameter less than 2.5 μm; PM_{10} – Particulate matter with a diameter between 2.5 and 10 μm; SO_2 – Sulfur dioxide; PAHs – Polycyclic aromatic hydrocarbons; BPA – Bisphenol a; PBDEs – Polybrominated diphenyl ethers; PCBs – Polychlorinated biphenyls; PFCs – Perfluorinated compounds; OC pesticides – Organochlorine pesticides; OP pesticides – Organophosphate pesticides.

risk [45, 46]. Concerning PBDEs, the only study reporting associations with the disorder observed a decreased risk due to exposure to BDE-153 and BDE-100 congeners, but an increased risk, only in girls, due to exposure to BDE-47 [42]. For bisphenol A and phthalates, two small size studies for each chemical report an increased risk of ASD associated with childhood exposure [39–41]. All studies on antidepressants report an increased risk of ASD (**Table S1**) and, as these usually resort to medical records to assess exposure, include thousands of subjects. A decreased risk of the pathology due to folic acid supplementation is observed by assessing medical records from large samples [68, 69]. However, two recent small size reports, which measured folic acid levels in maternal serum, show an increased risk of ASD associated with prenatal folic acid intake at very high concentrations [70, 71]. In case–control datasets a decreased risk for the disorder is associated with higher prenatal and childhood blood concentrations of 25-hydroxyvitamin D, the main circulating form of this nutrient, with mean serum concentrations values ranging from 9.9 ng/ml to 28.5 ng/ml in cases and 15.0 ng/ml to 40.1 ng/ml in controls [72–79, 83, 85–88]. Most of these studies comprise less than one hundred subjects, however 3 studies examining dried blood spots [84, 86] or medical records [81] were carried out in hundreds or thousands of subjects.

3.2 Gene-environment interactions associated with ASD

Figure 1B shows a flowchart for the identification of relevant publications. The query revealed 392 unique records, of which 15 remained after application of

exclusion criteria. Nine research articles reported gene–environment interactions in ASD (**Table 1**). The environmental component of these interactions included air pollutants (PM_{10}, NO_2 and O_3), PCBs, manganese and nutritional factors

Study	Genetic factor	Xenobiotic	N_{cases}|$N_{controls}$	Main conclusion
Schmidt et al 2012 [65]	677 C > T genotype in *MTHFR*	Folic acid	272|154	Daily prenatal maternal folic acid intake >600 μg was associated with a reduced ASD risk when the mother, the child or both had the low-activity 677 C > T variant.
Volk et al 2014 [21]	rs1858830 CC genotype in *MET*	NO_2 and PM_{10}	251|156	Carriers of the CC genotype with higher prenatal exposure to NO_2 or to PM_{10} were at increased risk of ASD when compared to subjects with CG or GG genotypes and lower exposure.
Rahbar et al 2015 [90]; and Rahbar et al 2018 [91]	Ile/Ile genotype of *GSTP1*	Manganese	100|100 [90]; 163|163 [91]	Among carriers of Ile/Ile *GSTP1* genotype, those with blood manganese concentrations >12 μg/L had higher risk of ASD.
Schmidt et al 2015 [92]	rs10741657 AA genotype in *CYP2R1*	Vitamin D	384|234	AA genotype associated with a decreased ASD risk when maternal vitamin D intake was <400 IU.
Coşkun et al 2016 [93]	rs2228570 TT genotype in *VDR*	Vitamin D	237|243	Trend for an association of the TT genotype with elevated circulating 25(OH) D levels in children with ASD.
Kim et al 2017 [94]	Copy number duplications burden	O_3	158|147	Higher burden of CNVs, namely duplications, and O_3 exposure increases ASD risk.
Mandic-Maravic et al 2019 [95]	*GSTM1*-null genotype	Any medication	113|114	Maternal use of medication during pregnancy associated with high ASD risk in offspring with a *GSTM1*-null genotype.
Bach et al. 2020 [96]	*GSTM1*-null genotype	PCB-153	169|169	Positive association between PCB-153 levels and ASD risk among carriers of *GSTM1*-null genotype, when adjusting for eating yogurt and fish, and paternal age at birth.

25(OH)D – 25-hydroxyvitamin D; CYP2R1 – cytochrome p450 2r1; GSTM1 – *glutathione S-transferase Mu 1; glutathione S-transferase Pi 1; IU – International Units;* MET – *MET proto-oncogene, receptor tyrosine kinase;* MTHFR – *methylenetetrahydrofolate reductase;* VDR – *vitamin D receptor.*

Table 1.
Gene–environment interactions reported in ASD. Listed are gene-environment interactions pairs associated with ASD as identified by the systematic literature review using PRISMA guidelines.

(folic acid and vitamin D), while the genetic component was a specific genotype or, in one study, the overall burden of copy number duplications (**Table 1**).

Most of the genes assessed in these studies are involved in the metabolism of xenobiotics. *GSTM1* and *GSTP1* encode glutathione S-transferases (GSTs), which catalyze the conjugation of substrates with reduced glutathione, easing their clearance from the organism. *VDR* encodes a nuclear receptor of vitamin D, whereas *CYP2R1* encodes a hydroxylase responsible for the conversion of this nutrient to its main circulating form (25(OH)D). *MTHFR* encodes a rate-limiting enzyme involved in folic acid metabolism. Contrary to the other genes, the *MET* gene is not directly involved in the metabolism of xenobiotics, but encodes a pleotropic tyrosine kinase involved in brain development through the MET signaling pathway [97].

4. Discussion

4.1 Exposure to xenobiotics and ASD

4.1.1 Air pollutants, heavy metals, POPs and non-POPs, and pesticides

Five attributes that are transversal to many of the xenobiotics reviewed in this study, including air pollutants, toxic heavy metals, POPs, non-POPs and pesticides, likely account for the increased risk of ASD associated with their exposure: 1) ubiquitous exposure; 2) bioaccumulation potential; 3) neurotoxicity; 4) endocrine-disrupting potential and 5) ability to cross physiological barriers.

Exposure to these toxins is ubiquitous, since they are present in the environment, in everyday household and industrial products, and in food. For airborne toxins, this ubiquity is exacerbated by transboundary flows of pollutants, a phenomenon in which toxins circulate long distances and deposit on land and water bodies far from their sources [98]. POPs exhibit high lipid solubility and low hydrophilicity, and are resistant to environmental degradation through chemical or biological processes, increasing their risk of bioaccumulation in human adipose tissue, the ecosystem and in the food chain [99]. Some air pollutants (*e.g.* PAHs), pesticides (*e.g.* OCs) and heavy metals (*e.g.* lead and organic mercury) are also persistent (resistant to degradation) and bioaccumulative chemicals [99]. For instance, methylmercury (MeHg), one of the main sources of organic mercury, can easily cross the blood–brain barrier and the placenta, and bioaccumulates in the brain, potentially leading to mercury poisoning [100]. Conversely, non-POPs like bisphenol A and phthalates are quickly excreted through feces and urine [101], but their presence in everyday products (*e.g.* water bottles and canned food, cosmetics and personal care products, and toys) and industrial activities is extremely widespread, rendering exposure to these chemicals continuous and universal.

Most of these toxins have well established neurotoxic properties [102]. Many, including bisphenol A, phthalates, pesticides, PAHs, PCBs, PBDEs and lead, are also endocrine-disrupting chemicals (EDCs), defined as any "*exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations*" [103]. While EDCs were initially strictly defined as mimics of estrogens, androgens and thyroid hormones, acting as both agonists or antagonists to hormone receptors, it is now accepted that they act through much broader mechanisms [104]. EDCs interact with neurotransmitter receptors and transcriptional co-activators [104], and have been implicated in dysregulation of trafficking and signaling pathways [105], as well as of epigenetic mechanisms [106]. Early exposure to these toxins, which have overlapping neurotoxic and endocrine-disrupting properties, can therefore lead to

neurodevelopmental complications, with some coining the term neural-disrupting chemicals [107]. Experimental studies in humans and rodents have shown that most of these toxins cross both placental and blood–brain barriers [108, 109], enabling their neurodevelopmental toxicity. The endocrine effects of these toxins may also contribute to the male bias observed in ASD diagnoses. A recent systematic literature review of studies published from 1970 to 2016, concluded that many EDCs exhibit gender-specific effects, and that the male brain seems to be more vulnerable to neurotoxicity [110]. Corroborating this hypothesis, some of the studies identified in this review report gender-specific associations [20, 34, 42, 51].

Given the awareness regarding the hazardous health effects of exposure to these toxins, restrictive policies or bans on their use are often legislated. These include bans on the agricultural application of harmful pesticides [111], the widespread production of bisphenol A-free baby bottles [112] and regulations on PCBs, PBDEs and PFCs production [113]. However, such legislations are not always fully effective. For instance, despite bans, exposure to POPs is still ubiquitous because of their resistance to degradation [113]. Restrictions on bisphenol A use led to replacement by analogues (bisphenol F and bisphenol S) for which harmful effects are also reported [114], and are therefore regrettable substitutions. The transgenerational effects of these toxins are also important, as they can affect not only the exposed individual, but also subsequent generations, through epigenetic mechanisms [99, 104]. Most of the identified chemicals have been persistently used since the 1950s, leading to a growing environmental burden and accumulation of insults over several generations. Consequently, some authors speculate that these delayed effects may account in part for the steady prevalence increase in ASD reported in the last decades [115].

4.1.2 Pharmacological drugs

The increased prevalence of ASD among subjects prenatally exposed to three pharmaceutical drugs (thalidomide, valproic acid and misoprostol) provided the first strong evidence for the involvement of environmental risk factors in ASD. Thalidomide is an immunomodulatory drug, widely prescribed to alleviate morning sickness in pregnant women during the 50s, while misoprostol is a prostaglandin analogue used as an abortion inductor and valproic acid is prescribed for epilepsy and bipolar disorder. These drugs are teratogens (*i.e.* agents that alter the growth or structure of the developing embryo or fetus, causing birth defects), and likely induce brain damage leading to behavioral and cognitive deficits [116]. Nowadays, thalidomide is no longer used during gestation, while the intake of misoprostol and valproic acid by pregnant women is contraindicated.

We also identified 5 research articles associating maternal antidepressant intake during pregnancy with ASD risk, particularly for Selective Serotonin Reuptake Inhibitors (SSRIs). SSRIs act by increasing the extracellular levels of serotonin and are known to cross the placenta [117] and to be secreted through breast milk at low levels [118]. Increased serotonin levels have repeatedly been found in blood samples from ASD subjects [119]. While individual research studies report associations between prenatal exposure to antidepressants and ASD, a recent meta-analysis [120] underpins the inconsistency of overall findings. Thus, a clinical balance between the risks of untreated maternal depression and unclear neurodevelopmental risks of antidepressant exposure for the offspring is warranted.

4.1.3 Nutritional factors

The most encouraging results for protective factors for ASD come from studies examining disease risk and nutrient sufficiency. Overall, there is significant evidence

that folic acid and vitamin D supplementation during pregnancy and childhood are prophylactic for neurodevelopmental disorders.

Folic acid promotes the closure of the neural tube, reducing the risk of early neurodevelopmental problems: periconceptional folic acid intake prevents up to 70% of neural tube defects, with national health agencies recommending that women of childbearing age take 0.4 to 1 mg folic acid daily prior and during gestation [121]. However, while the natural folate is initially metabolized in the gut, folic acid is mainly metabolized in the liver, where the activity of dihydrofolate reductase (DHFR), the enzyme that converts folic acid to its biologically active form tetrahydrofolate, is reduced [122]. Thus, sustained high folic acid supplementation may eventually become noxious due to the accumulation of unmetabolized folic acid [122]. In agreement, two studies have observed a higher risk of ASD when mothers consume extremely high levels of this nutrient during pregnancy [70, 71].

Vitamin D plays a fundamental role in calcium and phosphorus metabolism, and is therefore crucial for various biological processes, among which the maintenance of brain homeostasis. Animal studies have also shown that the vitamin D receptor (VDR) is expressed in the brain since early in development [123]. Despite the growing number of studies reporting insufficiency of vitamin D in children with ASD, ambiguous cut-off levels for vitamin D insufficiency render difficult comparisons between studies [124].

4.2 Gene-environment interactions in ASD

The identification of consistent environmental risk factors for ASD is very relevant in view of the failure of genetics to fully explain the disease etiology and the clinical spectrum. However, integrating the emergent data on environmental risk factors for ASD with established genetic findings has been challenging. In this systematic review we identified 9 studies reporting specific gene–environment interaction pairs in ASD.

Because most of the identified genes cluster in biotransformation processes, their dysregulation may result in a deficient metabolism of xenobiotics, inducing pathological mechanisms that contribute to ASD onset. *GSTM1* and *GSTP1* are expressed in the brain, where they degrade multiple toxins [108]. Brain-expression of *MTHFR* and *VDR*, which are involved in metabolism of folic acid and vitamin D respectively, supports the importance of these nutrients for brain function. The mechanisms through which variants in the *MET* gene and air pollutants interact are unclear, but the role of the MET protein as a key signaling molecule during neurodevelopment [97] suggests that the genetic component of gene–environment interactions pairs goes beyond xenobiotics-responding proteins. In fact, *MET* is a known strong candidate gene for ASD [97], as are *GSTM1*, *MTHFR* and *VDR*, albeit with less supportive evidence.

While relatively scarce, the identified studies already offer valuable insights supporting the potential for preventive strategies based on environmental predictors for subjects carrying a genetic susceptibility variant. For example, controlling exposure to high levels of NO_2 or PM_{10} of carriers of the *MET* gene rs1858830 CC genotype could potentially lower their risk for ASD [21]. For subjects carrying a low-activity variant in *MTHFR* gene, which encodes for methylenetetrahydrofolate reductase, the risk of ASD might be mitigated by an adequate daily intake of folic acid during pregnancy [65]. In these 9 studies the environmental component of the gene–environment interaction pairs includes air pollutants (NO_2, PM_{10} and O_3), a PCB congener, manganese and nutrients (folic acid and vitamin D), opening many possibilities for prevention. While exposure to some factors (eg. outdoor air pollution) may be difficult to control, changes in nutrients intake are easier

to implement, and are particularly important for ASD-subjects carrying known specific variants in genes like *DHFR* [125], *MTHFR* [126] and *VDR* [127].

4.3 Strategies to assess early environmental exposure in ASD: the exposome

In 2005, Wild introduced the term "*exposome*" for a concept that complements the genome, and defined it as *"life-course exposures (including lifestyle factors), from the prenatal period onwards"* [128]. Unlike the genome, the exposome is highly dynamic, and is so diverse that no single technique will be able to completely quantify it. The large number of non-genetic risk factors associated with ASD reflects this, as such factors include not only environmental exposure to xenobiotics, but also psychosocial and lifestyle parameters. The 9 studies identified in this review tended to focus on a single or on a few environmental factors, employing the measuring method best suited for each factor. However, the simultaneous consideration of a set of exposures may much better describe the impact of the environment in individuals, and there are already a number of such studies under development for ASD.

To assess environmental exposure in ASD, the prospective cohort study MARBLES [129] recruits pregnant women who already have a biological child with the disorder, and are therefore at higher risk of a second child with ASD. The MARBLES study collects longitudinal information from the children, up to 36 months old, including environmental exposure, genetic and clinical data. This design allows the assessment of pre and early post-natal exposure to risk factors that may contribute to ASD risk. Because participants are recruited before or during pregnancy, monitoring of gestation and early childhood offers a chance to accurately measure exposures, allowing for the identification of early biomarkers.

Other studies with similar designs apply spectrometric methods to quantify the levels of toxins or their metabolites in biological matrices, usually through the collection of blood [42–45], urine [39, 41, 51] or hair [33, 38] samples. However, prospective designs are not always possible, and cross-sectional studies do not allow assessment of past exposures. Retrospective studies are a viable alternative, benefiting from new methods that allow assessment of previous exposure [14]. For instance, vanguard studies are now using naturally shed deciduous teeth [35] to retrospectively quantify exposure to xenobiotics in ASD subjects. During odontogenesis, deciduous teeth store signatures of exposure to chemicals, from the second trimester *in utero* until their replacement by permanent teeth [35]. The neonatal line, which is formed at birth, marks a histological feature that differentiates pre- and postnatally formed tooth layers. Consequently, teeth can be used to capture both the dose and timing of past exposures.

Another promising matrix takes advantage of archived dried blood spots collected through population-wide newborn-screenings for metabolic and congenital diseases. Chemicals relevant for ASD have been successfully detected in archived blood spots, including bisphenol A, PFCs, lead, mercury, PBDEs and PCBs [130, 131]. When correctly collected and stored, analytes remain stable in neonatal spots for years.

Other retrospective studies employ geo-referencing methods to collect information regarding exposure to air pollutants, pesticides and some heavy metals [18, 19, 24, 27, 32, 34, 49, 52]. These studies leverage indoor and outdoor air quality data, usage of agricultural pesticides or the location of environmentally-significant sites (*e.g.* landfill sites and high-intensity traffic roads) and apply geographic information systems techniques to infer potential associations with ASD risk. Early-life exposure questionnaires can also be used as a tool to assess past exposures and events [50, 66]. Finally, medical and prescription records and registries may be consulted when studying pharmaceutical drugs or supplement intake [54, 56].

Overall, a comprehensive analysis of the exposome must address a multiplicity of factors that includes not only exposure to chemicals in variable settings and situations, but also medical procedures, events and lifestyle, psychosocial and cultural variables.

4.4 Shift towards integrative strategies that address gene-environment interactions in ASD

All research studies identified in this review that report gene–environment interactions in ASD, published up to November 2020, examined specific xenobiotics (**Table 1**). Knowledge regarding interactions between genetics and the environment is vast outside of ASD context, and might be the basis to define what specific interactions to analyze. Leveraging from public, manually curated, literature-based resources, such as the *Comparative Toxicogenomics Database* [132] and the *Toxin and Toxin-Target Database* [133], that compile gene–environment interactions data, is fundamental. Genomic information, including SNV and CNV data, is also available from large international consortiums that aimed at detecting variants in ASD patients, such as the Autism Genome Project [4], the Simons Simplex Collection [134] and the Autism Sequencing Consortium [135]. For subjects for whom genetic data is already available or is currently being generated, an effort to collect exposure data might be very rewarding.

Given the emerging evidence highlighted by this literature review, there is a clear need to shift from studies that separately address the role of genetics and the environment towards multidisciplinary strategies that explore both components as interacting risk factors. Such strategies will inform about the mechanisms through which environmental exposure interacts with genetic background, contributing to ASD onset. Models must further consider ASD phenotypic and genetic heterogeneity. To fully understand the etiology of this very complex disorder, genetic, environmental exposure, epigenetic and clinical data needs to be collected simultaneously for the same group of individuals. It is possible that different gene–gene and gene–environment interactions are associated with distinct clinical subgroups of individuals with ASD and, consequently, phenotypic stratification may also be incorporated into study design. Conceiving such designs is challenging, especially given the large population datasets that are needed to achieve statistical power for the discovery of small-effect variables associated with the disorder [136, 137]. Artificial Intelligence (AI) methods, including data mining and machine learning algorithms, will be crucial to overcome the challenge of integrating substantial amounts of data, allowing the detection of environmental exposure patterns contributing to ASD onset.

4.5 Biological mechanisms underlying gene-environment interactions in ASD

Understanding the biological mechanisms underlying gene–environment interactions that contribute to ASD is fundamental to distinguish between causal and non-causal exposures identified through association studies. While knowledge on this is still limited, given the diversity of risk factors it is likely that multiple mechanisms converge in ASD etiology.

Genetic mutations rendering some individuals more susceptible to certain xenobiotics is the simplest gene–environment interaction mechanism. For instance, a gene functional polymorphism that inhibits the enzymatic degradation of a given toxin may lead to its detrimental accumulation in the organism. Many xenobiotic-responding enzymes, like cytochrome P450 enzymes and GSTs, are expressed in the brain, suggesting the occurrence of metabolic processes that inactivate toxins locally [108].

Epigenetics, a gene expression regulatory process that involves heritable and reversible biochemical modifications of DNA or histones, independent of the DNA sequence, acts at the interface between genes and the environment. These processes include DNA methylation, histone methylation and acetylation events, and post-transcriptional regulation by non-coding RNAs, which are known to be involved in brain development [138]. Environmental factors can modulate genetics through epigenetic mechanisms and xenobiotics implicated ASD are known to alter epigenetic patterns. For instance, valproic acid inhibits histone deacetylases up-regulating the expression of various genes [139]. 5-MethylTHF, a metabolite of folic acid produced by MTHFR enzymatic activity, is a donor of the carbon group used to methylate DNA [140]. Consequently, *MTHFR* gene polymorphisms that result in a diminished activity of the enzyme (i.e. *MTHFR* 677C > T polymorphism) might affect methyl donation and lead to impaired epigenetic regulation [141]. Epigenetic effects of air pollutants [142], BPA [143] and PCBs [144] have also been described.

Neuropathological mechanisms that putatively lead to ASD, such as oxidative stress, neuro-inflammation, hypoxic damage, abnormal signaling pathways and endocrine disruption, can be induced by exposure to xenobiotics. Reduced brain levels of glutathione, the major endogenous cellular antioxidant responsible for the detoxification of xenobiotics, and other oxidative stress biomarkers have been observed in ASD subjects [145]. Evidence for increased levels of neuroinflammation biomarkers in ASD, including brain levels of pro-inflammatory cytokines and microglia activation, which may be stimulated by allergens such as pesticides, has been reported [146]. Proxies for fetal and newborn hypoxia, indicating a deprivation of oxygen supply, have been reported in neonates that later develop ASD [26] and may be elicited by early-life events. Xenobiotics also interact directly with intracellular neurotransmitter pathways [108] leading to signaling impairments. For example, acetylcholinesterase, the enzyme that catalyzes the acetylcholine neurotransmitter breakdown, is the primary target of inhibition by organophosphate pesticides [147] Most of the identified xenobiotics are endocrine disruptors and a role for hormonal imbalances in the disorder is plausible, particularly given the male skewness in ASD diagnoses. Atypical steroidogenic activity, namely increased androgen [148] and estrogen [149] levels in the amniotic fluid, has been reported in affected males. Gender-specific effects of environmental toxins [110] and consequent hormonal imbalances may also be implicated in the female protective effect, a hypothesis proposed to explain the ASD male bias.

A novel area of interest in ASD is the role of gut-brain axis, which refers to biochemical signaling connections between the gastrointestinal tract and the central nervous system. Dysbiosis of the gut microbiome likely accounts for a high comorbidity of gastrointestinal symptoms in ASD patients [150]. While the liver is the predominant site of xenobiotic metabolism, the gastrointestinal tract is the first line of defense against ingested compounds, and is rich in both host and microbial enzymes. As the gut microbiota metabolize hundreds of dietary, pharmaceutical and industrial chemicals, dysbiosis could lead to impairments in the gut-brain axis resulting in neurological insults.

5. Conclusion

This review highlights the accumulating evidence for a role of exposure to xenobiotics in ASD risk, and reinforces the need of developing strategies that consider genetics and the environment as interacting components in ASD etiology. This is

further supported by the still limited but promising results originating from studies that explore gene–environment interactions.

However, the current knowledge is likely just the tip of the iceberg. Given the enormous progress in high throughput methodologies for analysis of biomolecules (genomics, transcriptomics, proteomics, metabolomics), together with the development of comprehensive surveys on environmental exposure and advances in artificial intelligence methods for the integrative analysis of large amounts of data, the field is ripe for new discoveries. The expectation is that knowledge of the exposome of individuals can be integrated with their genomes to define patterns of interactions that cause their particular configuration of behaviors in the autism spectrum. There are however many challenges ahead, particularly concerning the collection of such extensive information from patients in sufficient numbers for integrative analysis.

Because environmental exposure is amenable to adjustment or avoidance, the most important clinical outcome of better understanding gene–environment interactions in ASD is the potential for mitigating risk by controlling exposure of individuals with a genetic vulnerability. This line of research thus opens novel and important perspectives to future prevention and personalized interventions for ASD.

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Conflicts of interest

The authors declare no conflict of interest.

Appendix

Xenobiotic	Study	N_{cases}/ $N_{controls}$	Time of exposure	Exposure assessment	Risk
NO_2	Becerra et al. 2013	7421/ 72253	Prenatal	Geocoding/air quality	Increased
	Volk et al. 2013	279/ 245	Prenatal to postnatal	Geocoding/air quality	Increased
	Jung et al. 2013	342/ 48731	Prenatal to childhood	Geocoding/air quality	Increased
	Volk et al. 2014	251/156	Prenatal	Geocoding/air quality	Increased
	Raz et al. 2017	2098/ 54191	Postnatal (9 m)	Geocoding/air quality	Increased
	Ritz et al. 2018	15387/ 68139	Postnatal (9 m)	Geocoding/air quality	Increased

Xenobiotic	Study	N_{cases}/ $N_{controls}$	Time of exposure	Exposure assessment	Risk
O_3	Becerra et al. 2013	5839/ 55757	Prenatal	Geocoding/air quality	Increased
	Jung et al. 2013	342/ 48731	Prenatal to childhood	Geocoding/air quality	Increased
	Kaufman et al. 2019	428/ 6420	Postnatal	Geocoding/air quality	Increased
	McGuinn et al. 2020	674/855	Prenatal 3rdtrimester	Geocoding/air quality	Increased
$PM_{2.5}$	Becerra et al. 2013	5839/ 55757	Prenatal	Geocoding/air quality	Increased
	Volk et al. 2013	279/245	Prenatal to postnatal	Geocoding/air quality	Increased
	Volk et al. 2014	251/156	Prenatal	Geocoding/air quality	Increased
	Raz et al. 2015	245/ 1522	Prenatal	Geocoding/air quality	Increased
	Talbott et al. 2015	217/226	Prenatal	Geocoding/air quality	Increased
	Chen et al. 2018	124/ 1240	Postnatal to childhood	Geocoding/air quality	Increased
	Ritz et al. 2018	15387/ 68139	Postnatal (9 m)	Geocoding/air quality	Increased
	Kaufman et al. 2019	428/ 6420	Prenatal to Postnatal	Geocoding/air quality	Increased
	Jo et al. 2019	2471/ 243949	Prenatal 1sttrimester	Geocoding/air quality	Increased
	McGuinn et al. 2020	674/855	Postnatal (1st year)	Geocoding/air quality	Increased
PM_{10}	Volk et al. 2013	279/245	Prenatal to postnatal	Geocoding/air quality	Increased
	Volk et al. 2014	251/156	Prenatal	Geocoding/air quality	Increased
	Kalkbrenner et al. 2015	979/ 14666	Prenatal 3rdtrimester	Geocoding/air quality	Increased
	Chen et al. 2018	124/ 1240	Postnatal to childhood	Geocoding/air quality	Increased
	Ritz et al. 2018	15387/ 68139	Postnatal (9 m)	Geocoding/air quality	Increased
SO_2	Jung et al. 2013	342/ 48731	Prenatal to childhood	Geocoding/air quality	Increased
	Ritz et al. 2018	15387/ 68139	Postnatal (9 m)	Geocoding/air quality	Increased
PAHs	von Ehrenstein et al. 2014	104/ 53181	Prenatal	Geocoding/air quality	Increased
	Talbott et al. 2015 (2)	215/ 4856	Prenatal	Geocoding/air quality	Increased

Xenobiotic	Study	N_{cases}/ $N_{controls}$	Time of exposure	Exposure assessment	Risk
Lead	Priya and Geetha 2011	45/50	Childhood (4-12y)	Hair and nails	Increased
	Roberts et al. 2013	325/ 22101	Perinatal (at birth)	Geocoding/air quality	Increased
	von Ehrenstein et al. 2014	348/ 78373	Prenatal	Geocoding/air quality	Increased
	Talbott et al. 2015 (2)	215/ 4856	Prenatal	Geocoding/air quality	Increased
	Arora et al. 2017	22/54	Postnatal (15w)	Deciduous teeth	Increased
	El-Ansary et al. 2017	35/30	Childhood (3-12y)	Red blood cells	Increased
Manganese	Roberts et al. 2013	325/ 22101	Perinatal (at birth)	Geocoding/air quality	Increased
	Arora et al. 2017	22/54	Postnatal (15w)	Deciduous teeth	Decreased
Mercury	Windham et al. 2006	284/657	Perinatal (at birth)	Geocoding/air quality	Increased
	Obrenovich et al. 2011	26/39	Childhood (up to 6y)	Hair	Decreased
	Roberts et al. 2013	325/ 22101	Perinatal (at birth)	Geocoding/air quality	Increased
	Priya and Geetha 2011	45/50	Childhood (4-12y)	Hair and nailS	Increased
	El-Ansary et al. 2017	35/30	Childhood (3-12y)	Red blood cells	Increased
BPA	Stein et al. 2015	46/52	Childhood (10.1 ± 3.7y)	Urine	Increased
	Kardas et al. 2016	48/41	Childhood (7.5 ± 2.9y)	Serum	Increased
Phthalates	Testa et al. 2012	48/45	Childhood (11.0 ± 5y)	Urine	Increased
	Kardas et al. 2016	48/41	Childhood (7.5 ± 2.9y)	Serum	Increased
PBDEs	Lyall et al. 2017 (1)	545/418	Prenatal 2ndtrimester	Maternal serum	Increased Decreased
PCBs	Cheslack-Postava et al. 2013	75/75	Prenatal (early pregnancy)	Maternal serum	Increased
	Lyall et al. 2017 (2)	545/418	Prenatal (2^{nd} trimester)	Maternal serum	Increased
PFCs	Lyall et al. 2018	553/443	Prenatal 2^{nd}trimester	Maternal serum	Decreased
	Long et al. 2019	75/135	Prenatal	Amniotic fluid	Decreased
OC pesticides	Roberts et al. 2007	465/ 6975	Prenatal 1sttrimester	Geocoding/ pesticides data	Increased
	Cheslack-Postava et al. 2013	75/75	Prenatal 1^{st}trimester	Maternal serum	Increased
	Brown et al. 2018	778/778	Prenatal 1^{st} or 2nd trimesters	Maternal serum	Increased

Xenobiotic	Study	N_{cases}/ $N_{controls}$	Time of exposure	Exposure assessment	Risk
OP pesticides	Shelton et al. 2014	486/ 315	Prenatal	Geocoding/ pesticides data	Increased
	Schmidt et al. 2017	296/ 220	Prenatal	Survey	Increased
	Philippat et al. 2018	46/102	Prenatal	Maternal urine	Increased
	von Ehrenstein et al. 2019	2961/ 35370	Prenatal to postnatal	Geocoding/ pesticides data	Increased
Pyrethroids	Shelton et al. 2014	486/315	Prenatal	Geocoding/ pesticides data	Increased
	Hicks et al. 2017	159/298	Prenatal	Geocoding/ pesticides data	Increased
	von Ehrenstein et al. 2019	2961/ 35370	Prenatal to postnatal	Geocoding/ pesticides data	Increased
Glyphosate	von Ehrenstein et al. 2019	2961/ 35370	Prenatal to postnatal	Geocoding/ pesticides data	Increased
Antide pressants	Croen et al. 2011	298/ 1507	Prenatal	Medical records	Increased
	Rai et al. 2013	4429/ 43277	Prenatal	Medical records	Increased
	Gidaya et al. 2014	5215/ 52150	Prenatal	Medical records	Increased
	Harrington et al. 2015	421/ 464	Prenatal	interview and medical records	Increased
	Rai et al. 2017	5378/ 249232	Prenatal	Interview and medical records	Increased
Valproic Acid	Moore et al. 2000	52	Prenatal	Survey	Increased
	Bromley et al. 2008	10/622	Prenatal	Interview and medical records	Increased
	Bromley et al. 2013	12/509	Prenatal	Interview and medical records	Increased
	Christensen et al. 2013	5437/ 630178	Prenatal	Medical records	Increased
Thalidomide	Stromland et al. 1994	100	Prenatal 1sttrimester	Medical records	Increased
Misoprostol	Bandim et al. 2003	23	Prenatal 1sttrimester	Interview	Increased
Folic Acid	Schmidt et al. 2012	429/ 278	Prenatal (early pregnancy)	Interview	Decreased
	Surén et al. 2013	270/ 84906	Prenatal (early pregnancy)	Survey	Decreased
	Al-Farsi et al. 2013	40/40	Childhood (3-5y)	Serum	Decreased
	Nilsen et al. 2013	234/89602	Prenatal	Medical records	Decreased
	Levine et al. 2018	572/ 44728	Prenatal	Medical records	Decreased
	Raghavan et al. 2018	86/1171	Postnatal (2-3d)	Maternal plasma	Increased
	Egorova et al. 2020	100/100	Prenatal	Maternal serum	Increased

Xenobiotic	Study	N_{cases}/ $N_{controls}$	Time of exposure	Exposure assessment	Risk
Vitamin D	Meguid et al. 2010	70/42	Childhood (5.3 ± 2.8y)	Serum	Decreased
	Tostes et al. 2012	24/24	Childhood (7.4 ± 2.7y)	Serum	Decreased
	Mostafa and AL-Ayadhi 2012	50/30	Childhood (8.2 ± 2.4y)	Serum	Decreased
	Neumeyer et al. 2013	18/19	Childhood (10.6 ± 0.4y)	Serum	Decreased
	Gong et al. 2014	48/48	Childhood (3.7 ± 1.2y)	Serum	Decreased
	Bener et al. 2014	254/254	Childhood (5.5 ± 1.6y)	Serum	Decreased
	Kocovska et al. 2014	40/40	Early adulthood (18.9 ± 2.9y)	Serum	Decreased
	Fernell et al. 2015	58/58	Neonatal	Dried Blood Spots	Decreased
	Magnusson et al. 2016	9882/ 499757	Prenatal to childhood	Medical records	Decreased
	Bener et al. 2017	308/ 308	Childhood (5.4 ± 1.7y)	Serum	Decreased
	El-Ansary et al. 2018	28/27	Childhood (7.0 ± 2.3y)	Plasma	Decreased
	Guo et al. 2018	332/197	Childhood (4.9 ± 1.5y)	Serum	Decreased
	Wu et al. 2018	310/ 1240	Neonatal	Dried Blood Spots	Decreased
	Arastoo et al. 2019	31/31	Childhood	Serum	Decreased
	Lee et al. 2019	1399/ 1607	Neonatal	Dried blood spots	Decreased
	Alzghoul et al. 2019	83/106	Childhood	Serum	Decreased
	Sengenc et al. 2020	100/100	Childhood	Serum	Decreased
	Petruzzelli et al. 2020	54/36	Childhood	Serum	Decreased

BPA – bisphenol A; d – days old; m – months old; NO_2 – nitrogen dioxide; O_3 – ozone; OC pesticides – organochlorine pesticides; OP pesticides – organophosphate pesticides; PAHs – polycyclic aromatic hydrocarbons; PBDEs – polybrominated diphenyl ethers; PCBs – polychlorinated biphenyls; PFCs – perfluorinated compounds; $PM_{2.5}$ – particulate matter with a diameter less than 2.5 μm; PM_{10} – particulate matter with a diameter between 2.5 and 10 μm; SO_2 – sulfur dioxide; w – weeks old; y – years old.

Table S1.
Studies reporting xenobiotic exposure associated with ASD, identified through systematic literature review. For each study the numbers of ASD cases (N_{cases}) and controls ($N_{controls}$), the timing of exposure (specific time-points of prenatal, postnatal or childhood periods are shown when stated by the referenced authors), the exposure assessment method, and the direction of association are listed (increased or decreased risk by exposure).

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Section 3

Intervention

Chapter 8

An Observationally and Psychoanalytically Informed Parent-Toddler Intervention for Young Children at Risk of ASD: An Audited Case Series and Convergences with Organicist Approaches

Maria Rhode and Kate Grayson

Abstract

In this paper we describe, with illustrative vignettes, an observationally and psychoanalytically informed parent-toddler intervention for young children at risk of ASD. The intervention was offered to children between 18 and 24 months who fell in the High Risk category of the Checklist for Autism in Toddlers (CHAT), which carries an 83% chance of a diagnosis at the age of 3 ½. In the absence of pathways for children under 2, this preliminary case series comprised 8 children from a heterogeneous clinical population. A significantly lower proportion of treated children were later diagnosed than the CHAT would predict ($p = 0.03$, Fisher's Exact Test), suggesting that this intervention merits further investigation with larger numbers of children and additional instruments. Scores on two routine outcome monitoring measures (the Goal Based Measure and the PIR-GAS) improved both in children who were later diagnosed and in those who were not. We consider these findings in relation to recent non-psychoanalytic research papers (including an RCT on a parent-mediated intervention) that demonstrate the prime importance of parent-toddler interactions, and we suggest that supporting parental confidence is essential to improvement. We discuss emerging convergences between psychoanalytic and organicist approaches, and the possible place of this intervention in conjunction with others.

Keywords: autism, CHAT, early intervention, parent-toddler work, parental confidence, psychoanalytic-organicist convergences, shared emotional experience, 'therapeutic' observation, toddlers at risk of ASD

1. Introduction

In this chapter, we describe an observationally and psychoanalytically informed, non-intensive parent-toddler intervention for children at risk of ASD; we discuss a

preliminary audited case series, and we point out convergences with interventions conducted by organicist workers. An autism diagnosis is usually impressively stable [1, 2], but recent randomized controlled trials suggest that the trajectory of infants and young children may be more modifiable than has long been thought (see French and Kennedy 2017 [3] for a systematic review). As Ozonoff (2013) [4] has written, "By demonstrating that there is solid science behind hope, we can add fuel to the urgency for very early diagnosis and intensive treatment of ASD."

Kanner (1971) [5] emphasized how widely the later fate of his original cohort of children varied, although they had earlier been so similar as to warrant the idea of an identifiable syndrome. Of 11 children, two (and a third to some extent) were employed as adults and respected by the community; most of the others were permanently institutionalized. Kanner was clear that autism stemmed from inborn difficulties in relating, but he also described the "wisdom" of a foster couple who helped the child to channel his obsessions in socially productive ways. He stressed however how little was known, and that no one intervention worked for all children.

Much more recently, Fein et al. (2013) [6] identified a group of so-called "optimal responders" whose performance on various measures came to be indistinguishable from that of controls in spite of a previous reliable autism diagnosis. Again, no shared characteristics could be identified. Moulton et al. (2016) [2] reported that 9% of 200 diagnosed two year olds were "optimal responders" at 4, while some 83% retained the diagnosis. Ozonoff et al. (2015) [1] have similarly found the stability of a diagnosis to be 82% at 24 months (93% at 18 months), while Lord et al. (2012) [7] have identified differing trajectories within a group of at-risk children who were repeatedly assessed between 18 and 36 months. These findings point up both the usual impressive stability of the diagnosis and the fact that a surprising degree of improvement may sometimes be possible: Even suboptimal improvement can make an incalculable difference to the lives of the children and their families [4].

At the same time, the traditional dichotomy between biological and interactional approaches to autism is beginning to narrow. Singletary [8] has proposed an integrated model of autism that brings together findings on brain structure and function, on the effects of hormones and stress, and on social and emotional interaction in attempting to trace how autistic behaviors may be established and perpetuated; he links these formulations with those derived from psychodynamic treatment approaches. Like the overwhelming majority of contemporary workers from all disciplines, Singletary subscribes to Kanner's view that autism stems from congenital factors, but adds that atypical experiences arising from these may lead the child to construct unrealistic pictures of significant adults (see also [9]). For example, the child's atypical sensory endowment may lead it to experience the shared world as a frightening place and to misattribute this to its carers.

Conversely, intensive early intervention of various kinds, including the Early Start Denver Model [10, 11] and pivotal response therapy [12], leads to demonstrable brain changes that can be demonstrated at 2-year follow-up [13]. After a parent-delivered intervention, infant siblings of diagnosed children, who therefore had a heightened risk of ASD, showed measures related to brain systems concerned with social attention that came closer to those in controls [14]. Equally, levels of oxytocin, the "bonding" hormone, which are significantly lower in young children with autism, normalize following 20 minutes of satisfying social interaction with caregivers, but quickly fall off again [15]. These findings illustrate on a biological level that at least one characteristic atypicality in ASD can temporarily be corrected through satisfying experiences of relatedness. They also suggest that ongoing, repeated input will be necessary for consolidation.

In a particularly interesting study, Wan et al. (2013) [16] compared infant siblings of children diagnosed with ASD to a control cohort. The AOSI (a screen for behavioral precursors of autism) was administered at 6–10 months and again at 12–15 months; films of infants playing with their mothers were rated on the Manchester Assessment of Infant-Caregiver Interaction (MACI). Parental non-directiveness and sensitive responsiveness were lower in the high-risk infants at both ages: the authors suggested that the parents might have adopted a more directive style as a consequence of atypical interactions with their older, diagnosed child (a suggestion that agrees with our clinical experience). Another, compatible, explanation was that the parents were responding to the at–risk babies' lower levels of vitality and engagement.

Most interestingly in relation to the possibility of early intervention, an ASD diagnosis at 3 years was not predicted by the siblings' characteristically autistic behaviors as shown on the AOSI (though other studies had suggested that AOSI scores were stable over time). What did predict a diagnosis at 3 years was the at-risk siblings' interactional style with their caregivers as assessed on the MACI, more particularly the caregivers' scores for directiveness and sensitivity (see also [17]). At 12 months, though not earlier, dyadic mutuality, infant positive affect and infant attentiveness to the caregiver predicted an ASD diagnosis at 3 years. It seemed that features suggestive of autism, as assessed on the AOSI, might be modified by helpful infant-caregiver relationship patterns. Importantly, however, most of this group of at-risk siblings, who were not given a diagnosis in spite of difficulties picked up by the AOSI, did show problems when compared to the low-risk siblings: the authors called them the "other concerns" group. The findings were conceptualized on the basis of a transactional model, in which problems in the infants contributed to their carers' sub-optimal interactional style (see also [18]); this in turn reduced the infants' opportunities for social learning. The authors concluded [16] that "intervention efforts to optimize social functioning may need to start early in infancy before interaction patterns become embedded in emerging social atypicality."

In a case series of 8 at-risk infant siblings [19], Green reported definite improvements on a number of measures in comparison with controls after the parents had been offered video interaction feedback, while Bradshaw et al. (2015) [20] and French & Kennedy (2017) [3] have reviewed RCTs of early intervention for infants and young children at risk of ASD. Until 2015, the Early Start Denver Model [10, 11], which provides intensive input over 2 years, was the only intervention after which social communication was significantly better than for Treatment As Usual, though interventions of fewer than 2 hours per week did achieve some improvements. However, Green et al. [21] in 2017 published the follow-up of an RCT of low-intensity video-feedback intervention to promote positive parenting (Modified iBASIS VIPP), conducted with 9–14 month-old at-risk siblings and their parents and first reported in 2015 [22]. Statistically significant improvements were obtained in 'autism prodromal symptoms' over the course of the follow-up, while at the end of the intervention itself the improvements had merely been suggestive (a sleeper effect implying that more rewarding interaction patterns had been internalized). Parental directiveness also decreased significantly, alongside increases in attentiveness to the parent and initiation of interaction by the child. However, no difference could be seen in the rate at which children were given a diagnosis.

2. The present intervention in context

The present Child & Adolescent Mental Health (CAMHS) intervention was informed by work in France, where Houzel [23] had developed outreach provision

for infants and toddlers suffering from various serious problems. Families were offered a modified version of infant observation (originally introduced by Bick [24] as a training module for child psychotherapists and soon adopted in other mental health trainings). Trainee observers learn to position themselves so as to be receptive to whatever is happening without presenting themselves as experts. Fortuitously, mothers who were distressed by the lack of adult company often felt supported by the presence of an interested, non-judgmental person who was there to learn rather than to instruct. Reports began to be published of "participant", or "therapeutic", infant observations where observers took a more active role but where the main emphasis was still on their sensitive, receptive function (for an overview, see [25]). "Therapeutic" observation now forms part of the clinical repertoire of child psychotherapists in many different settings, and has become part of child psychiatry services in a number of French regions; an increasing number of publications report encouraging outcomes of single case studies [26–29].

Houzel stresses that many new mothers can doubt their own competence compared to professionals: the observer's receptive stance can go a long way towards supporting mothers' confidence and self-respect. This emphasis on the observer's sensitive receptivity converges with the later research findings already mentioned [16, 21] on the central role of parental sensitivity and non-directiveness: the observer's modeling of these qualities may support the parents in developing them.

The present case series was originally framed as a pilot research project [1] to investigate the practicality of offering weekly outreach participant observation with parent support for a year to families with toddlers who had been screened with the Checklist for Autism in Toddlers (CHAT) for the risk of a later ASD diagnosis. The CHAT [30–32] is designed for use in primary care and is administered twice, at a week's interval, when the child is between 18 and 24 months. It includes parent reports and direct observation, and addresses the child's capacity for symbolic play and joint attention. Based on standardization on some 16,000 toddlers [31], children who fail in all areas fall in the High Risk category, with an 83% likelihood of an ASD diagnosis at 3 ½. All parents in the pilot gave written informed consent for publication[2]. The two children who could be recruited were later assessed for ASD by clinicians (a child psychiatrist and a multidisciplinary specialist team respectively) who were independent of and blind to the intervention; in the first case, when the child was 3 ½ and the observation had been completed, and, in the second, when the child was just over 2 and had had 4 months of a 1-year observation. The first child was not given an autism diagnosis, while the second was.

This pilot proved impractical due to problems with recruitment and with the geographical matching of observers and families. Fortuitously and over a long period, appropriately-aged at-risk toddlers were referred to the clinic, where one of us (MR) offered participant observation to them and their mothers (and fathers where possible). The only instrument now used was the CHAT at baseline, administered independently of the clinician except in 2 cases (where the issue of bias is not relevant as these children subsequently received a diagnosis). Parents welcomed the CHAT within a clinical context, as they were all concerned about the possibility of autism: it was emphasized that this was a screen, not a diagnosis. Parents knew that their child was part of a case series that would be audited, and subsequently gave consent for the publication of anonymised data. The later diagnostic assessment (by a psychiatrist, a pediatrician, or an interdisciplinary team) was independent of the clinical intervention. In 3 of the 4 cases who did not receive a diagnosis, the

[1] Partners: Prof. Maria Rhode, Dr. David Simpson, Prof. Judith Trowell, Dr. Martin Bellman, Dr. Elizabeth Nevrkla. Observers: Agathe Gretton and Kate Stratton; Supervisor: Margaret Rustin.

[2] Ethical approval granted by the Camden & Islington LREC (Rec Reference Number 05/Q0511/122).

assessors were blind to the treatment; the fourth child was not assessed, as he was obviously not autistic. In all, 2 appropriately-aged children completed the pilot project, while the intervention was delivered at the clinic to a further 6; in each group, the same proportion (50%) received a diagnosis.

The intervention was distinct from child psychotherapy[3] (though 2 children made the transition to psychotherapy after a year, when the parents had become concerned about emotional issues). Clinicians inevitably saw the process through an emotional lens, but comments were not insight-based and did not address the parents' past unless they raised such issues themselves. Any problems between parents and clinicians were dealt with on a realistic basis in the present rather than in terms of past relationships. The main aims initially were to try to engage the child, to make links between family members, to draw the parents' attention to capacities of the child that they might not have noticed, and to think together about what seemed to trigger the child's engagement or to work against it. This was in fact not unlike the aim of VIPP, though no video was involved and the clinician pointed out events to the parents in real time rather than retrospectively. The observational focus, and the emphasis on supporting the parents in observing their child and thinking about him, are features shared with "Watch, Wait and Wonder" [33], though the clinician was more active: toddlers at risk of ASD generally need help to be able to engage, and prolonged "waiting" could be counterproductive. Sharing the emotional experience of all members of the family is central to this approach: this follows naturally from the clinician's receptive attitude. Many parents particularly valued the opportunity to process their own feelings about their child's possible autism and about the many assessments and interventions being offered.

In general terms, the clinician aimed to

- Help the child to engage (for instance, by mirroring their actions or affects, a strategy used in many different autism interventions)
- Respond to parents' anxieties and concerns, and provide a place to process their experience
- Describe the child's actions, and consider possible meanings, so as to encourage communication between parents and child
- Remind the parents of their importance to the child, and foster their sense of competence
- Validate and support the parents' own observational capacities
- Validate satisfying interactions and reflect on the possible meaning of difficulties; accept negative feelings

Some parents responded skeptically to the idea that their child's behavior might be meaningful or communicative: parents of toddlers with autistic features have had to endure their profoundly invalidating lack of response. Some say that they do not exist for their child except perhaps as a source of food. Observation may convince the clinician of just how essential the parents are for the child; but any worker who has experienced the impact of a toddler who completely ignores them is well placed to empathize with the parents' experience. This means that parents

[3] Manual in preparation.

and child may both wish for contact, but that mistiming and the expectation of not being responded to can block this.

For example, an 18-month-old girl (not part of the case series as she was at Medium Risk only) unusually tried to make eye contact with her parents, who happened not to be looking at her at that moment. She turned away and remained impossible to engage for the rest of the hour. The worker shared her observations with the parents, who then realized that the child's behavior was a meaningful example of (unrealistic) disappointment rather than yet another instance of lack of interest or incapacity to respond. They began to hope that there could be a point to paying careful attention to the details of their child's behavior. Over time, this can lead to a virtuous circle of mutual encouragement instead of the vicious circle of discouragement between parents and child [25] in which repeated experiences of invalidation lead the parents to expect nothing else and not to notice the often faint indications that the child might be more open to contact. Again, clinicians are familiar with the experience of suddenly realizing that a child has just done something subtly different that has nearly gone unnoticed. This has potentially far-reaching consequences: what does not get noticed cannot get built on.

In some cases (including that of the little girl in this vignette), it may take a long time for parents to risk believing that their child's behavior could be meaningful. These particular parents told the clinician repeatedly that she must be mad to suggest such a thing. Over time, however, they began to take turns to notice and report what their child had done between sessions, though they might add that this did not mean anything. At length, both parents began to risk being hopeful at the same time, to share pleasure at their little girl's development and to encourage each other when there was a temporary plateau.

We will conclude this section with some further examples of how the clinician might approach specific issues.

- We have already stated that a central aim is to strengthen the links between parents and child. Sometimes the clinician will engage the child first and then point out to the parents what the child has been able to do, or else comment in a way that emphasizes the child's meaningful approach to the parent. For instance, a little boy of 20 months was described as being preoccupied with moving toy cars back and forth repetitiously. He drove a car repeatedly up the arm of the sofa his mother was sitting on, and immediately let it fall to the ground. The therapist commented, "Oh dear, falling down!" (said with a falling vocal inflection). "The car went to see mummy, and then it fell down!" This mother was surprised and delighted to think that her child's play might not be merely repetitious and meangingless, and soon began to engage with him by saying "hello" to the car; while the little boy regularly made eye contact with the therapist whenever he repeated this play, which was the first instance of social referencing that anyone had seen from him. A vital implication of this interaction was that the mother mattered to the child and that he wanted to connect with her.

- Other parents may be actively engaged with their toddler: sometimes directively, but often scaffolding his or her activity very sensitively. The clinician may spend considerable time as a benign witness, sometimes putting into words what is happening but often without a clear role. (Again, the implicit message is that it is the parents who matter). The clinician might comment: "yes, I see,"or "Mummy saw [what you did]" when the child engages in social referencing. When there has been an instance of satisfying communication between mother and toddler, the therapist might say, "Mummy understood

what you wanted/what you were showing her"; or, if the parent has reported an example of progress, "Mummy was very happy when you pointed to the picture/showed her what you wanted/liked playing with the other children." In an intervention that is going well, mother and child may end up playing together for long stretches while the therapist shares and validates the mother's pleasure. In psychoanalytic terms, this could be conceptualized as what Stern calls the "good grandmother transference" [34].

- Imitation is central in establishing contact with toddlers at risk of autism. The clinician may mirror the child's actions and gestures, sometimes in a different mode, as in the first example, where the therapists's falling vocal inflection mirrored the falling of the toy car (see Stern, 1985 [35], on cross-modal attunement). The little boy in that example often banged on the radiator to make a sound: the therapist similarly banged on a metal rubbish bin, and this turned into a "conversation" that could be varied by introducing different rhythms.

- Where a child's actions become repetitive and meaningless, the therapist will need to intervene. This may be by removing a toy car whose wheels the child is spinning, while explaining that it is stopping the therapist and child from being together. The therapist may also introduce a more meaningful context, for instance by placing a doll in the car or by using another car to approach the first and pretending to speak to it.

For example, the same little boy already mentioned, like many children with autistic features, was preoccupied with opening and closing doors, and on one occasion hunched himself over the dolls' house, repetitiously opening and shutting its door in such a way as to exclude the adults. After commenting that she could not see what he was doing, the therapist approached the dolls' house with a toy animal, who popped his head out of different windows, saying "hello" to the boy as though teasing him by appearing in a different place each time. He smiled, returned to contact and produced a stream of lively babbling.

The therapist may model ways of overcoming negative patterns of interaction, or remind the parents of times when they had themselves been able to do this. For example, when a child climbed onto the therapist's lap and repeatedly tugged at her hair, she said that she could see that he was cross, but hair-pulling was not allowed as it hurt her, and she removed his hand from her hair while maintaining eye contact and keeping him on her lap. He focused on looking at her and began to babble, which she mirrored. Later in that hour, he pinched his mother, and she too said that she could see that he was cross; she picked him up and rocked him, and he settled down. In a later session he persistently pinched and strangled his mother in a way that was very difficult to tolerate, and she became increasingly upset. The therapist reminded the mother of how well she had previously managed by rocking him when he had pinched her: the mother tried picking him up and rocking him, and again this was successful in helping him to settle.

3. The case series

3.1 Children's characteristics and later diagnostic status

The children were a heterogeneous group, recruited largely by word of mouth in the absence of pathways for this age [36]. While most research studies we have mentioned concern infants whose older siblings have an autism diagnosis, this was

true of only 2 of our 8 toddlers. Factors well-known to be associated with autism - extreme prematurity; a metabolic abnormality; and a congenital condition together with a neonatal infection – each with a 20% risk of autism – were present in 3 of the children; the other 5 children showed autistic features without these associated factors. Of the 2 children with older diagnosed siblings, one received a diagnosis while the other did not.

Table 1 summarizes the information on the 8 children in the High Risk category of the CHAT with regard to gender, to their later diagnostic status, and to whether or not they had regressed, were born prematurely, or were the younger sibling of a child diagnosed with autism. The small number of children means that no associations can be identified between any of these factors and a subsequent diagnosis.

Table 2 concerns age at referral, at the beginning of treatment[4] and at diagnostic assessment, as well as prematurity status and the presence of an older diagnosed sibling. Of 8 children in the High Risk category, 4 (or 50%) received an autism diagnosis at a range of ages. According to the CHAT, this figure might have been expected to be 83% ($p = 0.033$, Fisher Exact Test [37]).

3.2 CAMHS routine outcome monitoring measures

These were routinely collected in line with clinical practice, but are not available for the child who was seen before they were introduced at the clinic or for the two children seen in their homes. The two measures collected, as shown in **Table 3**, were the PIR-GAS, (where the clinician rates the parent–child relationship) and the Goal Based Measure, where the parent rates how far the child has progressed towards 3 desired goals on a scale of 0 to 10. (Child G moved away before any measures except GAS-1 could be obtained). As is usual, the parents appeared to rate progress more highly than the clinician; the big jump in the GAS score for Child A coincided with his beginning to call his parents Mummy and Daddy, which made an enormous difference to their relationship with him. It will be seen that all parents judged their child to have improved on the agreed goals, whether or not they later attracted a diagnosis.

3.3 A heterogeneous group: autistic features, developmental achievements, and subsequent diagnostic status

Table 4 shows some of the children's developmental achievements. Reliable patterns would not be expected with so few children, though some tendencies were unexpected. The children's characteristics at the beginning of the intervention did not predict their diagnosis [16]. All but 2 of the children had sleeping problems. Perhaps unsurprisingly, only 1 made eye contact, and was subsequently not diagnosed; on the other hand, the one child who initially showed social referencing later was.

In the course of the intervention, all but 1 of the children developed turn taking and reciprocity: the one who did not received a diagnosis. All (4) of the children who were later undiagnosed developed play, whether in response to an adult, initiated by themselves, or symbolic; of the diagnosed children, 3 played in response to an adult, but only 1 initiated play themselves or played symbolically. This underlines the importance of the adult taking the initiative where necessary.

Table 5 concerns the use of words and of two-word and three-word sentences, as well as of capacities such as playing peek-a-boo games, which clinically is often

[4] Exclusion criteria included neonatal atypical brain structure; epilepsy; and child protection concerns or serious mental illness in the family.

		Diagnosis		
		No	**Yes**	**'PENDING'**
CHAT 1	**HIGH**	3	4	1
CHAT 2 (one week later)	**HIGH**	3	4	1
	F	1		
Gender	**M**	2	4	1
	N	3	3	
Regressed?	**Y**		1	1
	N	2	3	1
Sibling?	**Y**	1	1	
	N	2	4	1
Premature?	**Y**	1		

Table 1.
Some characteristics of the 8 children.

Sibling?	**Prem?**		**Mean**	**N**	**Minimum**	**Maximum**	**Range**
No	No	Referral age in months	18.60	5	13	23	10
		began Rx age m months	21.20	5	16	24	8
		Age at assessment in months	30.60	5	22	42	20
	Yes	Referral age in months	24.00	1	24	24	0
		began Rx age m months	28.00	1	28	28	0
		Age at assessment in months	55.00	1	55	55	0
Yes	NO	Referral age in months	15.00	2	12	18	6
		began Rx age m months	19.50	2	15	24	9
		Age at assessment in months	34.50	2	27	42	15

Table 2.
Age of children at referral, beginning of treatment and diagnostic assessment.

a promising sign, and engaging in joint attention and following and producing a point, all of which are targeted by the CHAT. Participating in triadic situations is also encouraging clinically, as is the display of a sense of humor. Again, the small number of children rules out meaningful distinctions between those with and those without a subsequent diagnosis. However, there appear to be some trends that are at least suggestive in respect of initiating play (**Table 4**), producing symbolic play (**Table 4**), producing sentences of 2 words or more (**Table 5**), showing the capacity for humor (**Table 5**), and (not surprisingly as this is a component of the CHAT) producing or following a point (**Table 5**).

CHILD	Goal-based measure T1	Goal-based measure T2	GAS-1	GAS-2
A	4;6;4	6;7;6	21	53
B	2;1;2	7;5;8	50	50
C				
D	2;1;0	3;5;2	32	40
E	1;1;2	2;6;8	55	58
F				
G			21	
H	1;1;0	2;8;4	32	44

Table 3.
Children's scores on routine CAMHS outcome measures (Goal-Based Measure and PIR-GAS).

		Diagnosis		
		No	Yes	('Pending')
Sleeping problems	Y (mild)	1		
	Y	3	3	
	N		1	1
Eye Contact (initial)	Y	1		
	N	3	4	1
Social Referencing (initial)	Y		1	
	N	4	3	1
Turn taking	Y (variable)		2	
	Y	4	1	1
	N		1	
Reciprocity	Y (variable)		1	
	Y	4	2	1
	N		1	
Play: responds	Y (variable)		1	
	Y	4	3	1
Play: initiates	Y (variable)		1	
	Y	4	1	1
	N		2	
Play: symbolic	Y (fleeting)		1	
	Y	4	1	1
	N		2	

Table 4.
Developmental achievements.

Of the 4 children who were diagnosed, 1 was assessed at just under 27 months and one at 23 months. In both cases, the diagnoses relied on the presence of typically autistic behaviors, even though the children were well under 3 (see [16]), and did not involve observation of the child playing with the mother (or even with a clinician as in the ADOS). This point seems important in view of the studies by Wan et al. [16] and by Moulton et al. [2] and we will return to it in the discussion.

		Diagnosis		
		No	Yes	('Pending')
Words: produces	Y	4	3	1
	proto-words		1	
Sentences	Y	3		
	N		4	1
	3-w	1		
Peek-a-boo: moves Mother's hands	Y	3	3	1
	N	1	1	
Peek-a-boo by child	Y	4	4	1
	N			
Joint attention	Y (fleeting)		1	
	Y	4	3	1
Humor	Y++	1		
	Y	3	1	1
	N		3	
Follows a point	Y (fleeting)		1	
	Y	4	2	1
	N		1	
Uses a point	Y (fleeting)		1	
	Y	4		1
	N		3	
Participates in triadic situations	Y	4	2	1
	Y (fleeting)	0	1	0

Table 5.
Further developmental capacities.

The trajectory of the child whose diagnosis is described as "pending" was particularly interesting. At 31 months, he received a diagnosis based on his withdrawn and sensory-seeking behavior after a pediatric appointment in which his mother described his difficulties while he remained withdrawn. At 34 months, his atypical behaviors were confirmed by professionals in a different country (with a high prevailing standard of expertise in autism); but they thought that a diagnosis would be premature in view of his high degree of reciprocal engagement and mutual enjoyment during play with his mother. Both of these positions seemed to us to be understandable in view of this child's behavior in the clinical context. He came to be highly engaged with his mother and responsive to her; showed evidence of Theory of Mind in everyday interactions with his parents; engaged in triadic situations; produced words and two-word sentences, and imitated animal sounds on request; followed and produced a point; and sustained humorous 'proto-conversations' with the therapist as well as with his parents. However, if his mother and therapist spoke together and he was receiving no adult attention, his gaze went blank and he reverted to spinning the wheels on a truck that he had previously been playing with appropriately. It was not until some months after the original diagnosis that he became able to remain present and engaged even when adults did not focus on him for a brief time.

Finally, a possibly suggestive trend concerns the presence or absence of "associated factors". As we have stated, these were having an older diagnosed sibling; extreme prematurity; a metabolic abnormality; and a congenital condition and neonatal infection (each with a 20% risk of autism). In each of the diagnosed and undiagnosed groups, one child was a younger sibling. Otherwise, the undiagnosed group contained 3 instances of the "other factors", while none were present in the diagnosed group. It is conceivable that autistic behaviors may be more persistent where they are not associated with such other risk factors.

4. Discussion

Like iVIPP-Auti, this intervention involves the parents in identifying interactions that promote or inhibit the toddlers' engagement. In addition, the therapist models receptivity, and aims to empower the parents and support their capacity to observe. Perhaps the most significant distinguishing characteristic of this psychoanalytically-informed kind of therapeutic observation is that the main focus of the clinician's thinking concerns the possible meaning of what is taking place – indeed, the belief that the child's behavior is meaningful – even though the meaning is not necessarily articulated (see Britton, [38]). It remains mysterious how receptive attention promotes development (see [39] for a psychoanalytic perspective) or grows the social brain (to think biologically). In any case, findings such as those of Wan et al. [16] attest that it does so, as does the association, repeatedly documented from Ainsworth et al. [40] onwards, between maternal sensitivity and secure attachment (see [41]).

The parents involved in this pilot all valued the opportunity to focus on their child and to discuss their own feelings about the process the family was going through. To some extent they were a self-selected group, as they were largely referred by word of mouth in the absence of established pathways. The intervention does not suit all families: One couple, for instance, felt unable to take time off work to attend, and instead wanted very intensive input for their son. Matching interventions to families is an important issue for future exploration. So is the issue of even earlier intervention, concerning which promising case reports exist [42, 43] and for which neurological markers at 6 months could serve as a baseline [44, 45].

The trajectory of one particular High Risk toddler illustrates the degree to which improvement can be mediated by the parents. Initially this boy made no contact with the parents, screamed uninterruptedly to the point of making himself sick, and often had to be taken out of the room. The parents' lives were seriously restricted by his fear of other children and by his other major difficulties. After some 5 meetings, the therapist went on sick leave for 3 months: She returned to find that the child had begun to speak. Clearly, this was not the consequence of any direct input from her; but the parents had felt listened to and were able to maintain a different mind-set while interacting with their child. This boy remains somewhat delayed, but is doing well with support at a mainstream school. He enjoys a wide range of activities and friendships, though his behavior could have justified an autism diagnosis for some 18 months after work began: an example of how much change can take place between 2 and 4 years, a period during which a diagnosis is usually stable [1, 2].

The case of the child whose diagnosis is "pending" (p. 11) illustrates how important it is that a diagnosis should take account of how the child plays with the mother, not just the presence of autistic features which, as Wan et al. have shown, do not predict a diagnosis at the age of 3 [16]. Two of our children who were given

a diagnosis received it very early (at 23 months and before 27 months respectively), on the basis of a checklist of symptoms. These families did not continue with the intervention: in one case, because they moved away and, in the other, because the diagnosis gave them access to excellent local services.

All 8 of the present children, whether or not they received a diagnosis, improved considerably in terms of pleasurable engagement with their parents and other markers of relatedness, as summarized earlier (the fact that this is a preliminary audit rather than a research study means that unfortunately there is no control group to compare them with in this respect). However, unlike "optimal responders", all showed residual difficulties to a greater or lesser degree; some of these appeared to be emotional, and, with 2 children, were subsequently addressed in psychotherapy. This links with Wan et al.'s [16] description of their "Other Concerns" group: High-Risk siblings who showed early autistic features on the AOSI but whose parents demonstrated high receptivity and low directiveness, and who, at 3 years of age, did not receive a diagnosis, but still had problems compared to low-risk siblings.

Despite all the recent research demonstrating that autistic features in early childhood are far from immutable and can be ameliorated through parent-mediated interventions, professionals (as well as parents) still often think of "having autism" as though it were something concrete and fixed. This can understandably make them reluctant to intervene early for fear of prematurely labelling a child. Many parents tell a painful story of being advised that their child "will grow out of it", which can leave them feeling unheard and invalidated. If the findings of studies such as those of Dawson [10, 11] and Green [21] were fully taken on board, professionals might feel more able to act early and with a realistic degree of hopefulness.

A diagnosis is often needed to access essential services, but it should be emphasized to parents that children are heterogeneous in respect of their trajectory [7, 46] and that, with intervention, there may be considerable scope for review between the ages of 2 and 4. Such uncertainty can be difficult for parents – and professionals – to sustain [47], but doing so may be central to being able to remain receptive and non-directive – the factors that predict a diagnosis at 3 years in the way that early autistic features do not [16]. The present intervention could help parents to manage the wait for a definite diagnosis as well as to foster receptivity, non-directiveness and the capacity to trust their own feelings and observations. The intervention is low-key, and could potentially be delivered by well-supported mental health workers at a far lower intensity than an effective intervention such as ESDM, as it is in France; it could also work well in conjunction with iVIPP-Auti. As the American Academy of Pediatrics has stated [48], listening to parents and early screening are both essential.

5. Conclusion

The rate of diagnosis of the toddlers in this case series, unlike the rate in many studies of other interventions, was markedly lower than might be expected on the basis of the CHAT ($p = 0.033$), suggesting that a larger study is warranted. The results illustrate the heterogeneity of a clinical sample and the changes that can take place in very young children with autistic behaviors, as documented in recent research. The key features of the present intervention are parental involvement; the clinician's sharing of the family's emotional experience, privileging of meaning, and support of the parents; and the promotion of receptive behavior.

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Conflict of interest

The authors declare there is no conflict of interest.

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Autism spectrum disorder (ASD) is a complex condition that has extreme heterogeneity, which makes it extremely challenging from a diagnostic and etiological point of view. To add to the complexity, ASD typically has co-morbidity and overlap with other conditions outlined in this book, including epilepsy, attention-deficit/hyperactivity disorder (ADHD), and others. This book also examines monocyte cytokine profiles and catecholamines in ASD, genetic studies of autism, treatments, and controversial issues.

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