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Thesis title:

PIRAMiD: Predicting Early Onset Autism through Maternal Immune Activation and
Proteomic Discovery

Thesis submitted to National University of Ireland, Cork, in fulfilment of the requirements
for the degree of Doctor of Philosophy

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, at either University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

The Murray, O'Keeffe and English lab groups completed all laboratory work. Detailed author contributions can be found included in each experimental chapter.



05/04/2023

Dr Michael Carter

Date:

Abstract/Premise of this Thesis

Autism spectrum disorder (ASD) is a heterogeneous developmental disorder arising early in life. ASD is composed of a wide variety of clinical characteristics, neuropsychological impairments and complex phenotypes. The classical triad of ASD symptoms includes disrupted social function, atypical verbal and non-verbal communication skills, and restricted interests with repetitive behaviours. These core symptoms often coexist with other psychiatric and neurological comorbidities. Attention Deficit Hyperactivity Disorder (ADHD), epilepsy, migraine, and anxiety are much commoner in children with ASD (1, 2). Children and adults with ASD often encounter difficulties with emotional and behavioural problems (EBPs) such as emotional reactivity, aggression, and depression (3, 4). Up to 50% of those affected can have intellectual disability (ID) and limited verbal communication (5, 6, 7). Social, emotional and behavioural deficits in children with ASD are also important modifiers of outcome and are linked to elevated stress, mental and physical health problems, and lower overall family and caregiver well-being (8, 9). We know that early intervention can be effective, and may be parent or therapist delivered (10). Pharmacological treatment of ASD can be successful insofar as it is useful for symptomatic management of some ASD comorbidities such as ADHD, and depression.

Although genetic susceptibilities are increasingly recognised, the mechanism of disease development in ASD remains unknown. We are aware of both common and rare genetic risk factors with more than four hundred diverse high confidence genes now linked to ASD (<https://www.sfari.org/resource/sfari-gene/>). Singly, these genetic factors each convey only a modest increase in ASD risk (~1%), however collectively they can contribute to a far greater risk (11, 12). Both *de novo* and inherited genetic defects are recognised but ASD risk in progeny does not follow a clear pattern of inheritance (13, 14). Estimates of heritability of ASD in twin pairs vary widely between 50 – 90% (15, 16). The apparent male preponderance in ASD persists with a clear bias towards males. Rates of ASD among males exceed those of females by three or fourfold hinting at a possible sex differential genetic foundation (17, 18). Up to 20% of individuals with ASD may possess copy number variants (CNV) and *de novo* loss of function single nucleotide variants (SNV) that are individually rare but in combination, increase an individual's overall ASD risk (11). While newer methods of genetic analysis (such as whole genome sequencing) are uncovering new candidate genes with regularity (19), the heterogeneity of the clinical and phenotypic groups within ASD strongly suggest that in those with a genetic predisposition, environmental factors may act in concert to bring about a

multisystem dysfunction leading to ASD. Despite recent advances in gene analysis, we are yet to discover a single gene determinant that can account for more than a small percent of ASD cases. The current ASD literature suggests that mutations occurring in genes involved in synapse formation, cell adhesion molecule production (Cadherin), scaffolding proteins (SHANK proteins), ion channels (sodium, calcium, and potassium channels), and signaling molecules can disrupt regulatory or coding regions and affect synapse formation, plasticity and synaptic transmission (20). All this suggests that we cannot explain many cases of ASD by genetic factors alone, or at least we cannot explain them using our current understanding of ASD genetics or our current techniques of genetic analysis.

The flawed picture of ASD genetics has led some to investigate the role of environmental exposures in the aetiology of ASD. Researchers have identified many environmental risks in ASD. Advanced parental age, foetal environmental exposures, perinatal and obstetric events, maternal medication use, smoking and alcohol use, psychosocial hardship, nutrition and toxic exposures have all been implicated as risks in the pathogenesis of ASD (21, 22). While authors attribute between 17 - 41% of ASD risk to non-genetic or environmental exposures, the exact balance between genetic and environmental determinants and their roles in aetiology remains disputed (11, 22, 23).

Multiple mechanisms have been proposed through which each of these exposures may exert an influence on genetic and epigenetic risk in ASD, but there are only a handful that are likely to effect abnormal neurodevelopment. Animal models of inflammation and maternal immune activation are particularly well characterised, and have successfully modelled ASD type behaviours and social difficulties in mice, rats and non-human primates (24, 25, 26).

Maternal immune activation (MIA) is defined as an increase in measured levels of inflammatory markers in mothers during pregnancy. Through this process, a cytokine cascade transmits to the foetus, resulting in adverse neurodevelopmental phenotypes and even remodelling of the immature foetal brain. Many studies have profiled cytokine, chemokine, immune cell and inflammatory signatures in ASD affected individuals (27, 28, 29, 30, 31, 32). Only a much smaller number have characterised cytokine profiles in expectant mothers who progressed to birth children who develop ASD (33, 34). The few previous studies, which have examined gestational serum, have indicated mid-gestational upregulation in specific pro-inflammatory cytokines or indeed down-regulation in anti-inflammatory cytokines.

Metabolomic analysis refers to the systematic identification and quantitation of all metabolites in a given biological sample (35). This collection of metabolites, known as the metabolome, is thought to directly reflect the biochemical activity of the studied system at a specific point in time (36). The metabolome has become an area of interest, as some inborn errors of metabolism (IEM) are clearly linked to ASD phenotypes. Phenylketonuria (PKU) and Smith-Lemli-Opitz syndrome (SLOS) are disorders of amino acid and cholesterol metabolism respectively. Untreated PKU is associated with strongly autistic phenotypes (37), while SLOS is phenotypically heterogeneous, but autism remains a common feature in these children (38).

Similarly, proteomics is defined as the study of the complete protein profile in a given tissue, cell or biological sample (39). Proteomic studies of human sera have so far noted altered levels of proteins involved in inflammation or immune system regulation, including acute phase reactants and interleukins (40). Abnormalities of the complement system have also been found in ASD and other psychopathologies such as schizophrenia (41, 42). Recent works demonstrate that the complement pathway can affect synaptic remodelling and has roles in neurodevelopmental processes (43).

The initial focus of ASD research on genomics has largely failed to result in the much-hoped-for silver bullet of ASD aetiology, i.e. a common genetic cause. Instead, the genetic landscape has proven to be exceedingly complex and interdependent on a multitude of factors, including environmental exposures and other modifiers of genetic risk. Research examining the aetiology of ASD has shifted focus from genetics to a multimodal approach. In recent years, funding has become available for a far wider variety of ASD aligned research topics, beyond those with a focus on genetics. Opportunities now exist to adopt a multifaceted approach to ASD aetiology, shifting the focus from a narrow genetic base, to a broader multimodal approach to examine other potential mechanistic players. While this adds further complexity to what is already a complicated picture, the strived for parsimonious answer is simply never likely to materialise. Newer fields and modalities such as proteomics, metabolomics and machine learning will help to further refine and untangle the complicated web of ASD, and this variety of granular detail is what is likely to result in a practicable biomarker or effective therapy in the future. In this thesis using a multimodal approach (ELISA, metabolome and proteome analysis) we aim to explore further the role of MIA and alterations of the proteome and metabolome in the pathophysiology of ASD. We hope that our findings may ultimately help to identify a potential gestational biomarker of ASD, which will improve access to early diagnosis and treatment. We also aim to characterise co-morbid

emotional and behavioural problems, which arise early in children with ASD and are pervasive throughout all spheres of life. Early recognition and intervention with these co-morbidities can improve treatment outcomes, patient, and family wellbeing.

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I thank my parents, Jim and Kathleen, they got me here, and I would not be where I am today, without their love and guidance. To my little brothers Dan and Tony, you helped shape the man I am today, and I continue to learn from you. And to my extended family, too many to mention, thank you all. To my mother in law, Fidelma, who has been unerring in her support and care for Gail, myself, and the kids over the past ten years. Thank you all for being caring, loving, supportive presences in my life.

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Finally, I dedicate this thesis to the memory of my beloved grandmother, Margaret. Her life, was well lived. Her influence can be seen and felt through the families of her children, and now her children’s children. Her legacy, one of love, respect, resilience and dignity. Her unwavering love, kindness, and wisdom have been a constant source of inspiration and strength throughout my life. Her memory and influence will continue to guide me and my children in the decades ahead. I offer this thesis as a tribute to her life.

Publications and Presentations

Peer Reviewed Publications:

1. *Maternal Immune Activation and Interleukin 17A in the Pathogenesis of Autistic Spectrum Disorder, and Why It Matters in the COVID-19 Era.*

M Carter, S Casey, GW O’Keeffe, L Gibson, L Gallagher and DM Murray (2022)
Front. Psychiatry; doi:10.3389/fpsyt.2022.823096

*This article was among the top 10 most viewed articles published in the Autism section of Frontier of Psychiatry in 2022

2. *Mid-gestation cytokine profiles in mothers of children affected by autism spectrum disorder: a case–control study.*

M Carter, S Casey et al. Scientific Reports November 2021; doi: 10.1038/s41598-021-01662-z

3. *Maternal mid-gestation cytokine dysregulation in mothers of children with autism spectrum disorder.* Journal of Autism and Developmental Disorders.

S Casey, **M Carter** et al. Sept 2021; doi: org/10.1007/s10803-021-05271-7

Publications pending submission/in review:

4. *Dysregulated Steroid and Sulfur Metabolism, Glycolysis, and Cell Adhesion molecular pathways in cord blood preceding ASD diagnosis.*

D O’Boyle #, A Noone #, K Dowling #, **M Carter**, C Scaife, D Ryan, BH Bech, TB Henriksen, L Gallagher, C Mooney, A Khashan, DM Murray, JA English.

5. *Characterising the temporal evolution of emotional and behavioural problems (EBP) in an Irish cohort of Autism Spectrum Disorder (ASD) affected children in early childhood.*

M Carter, L Gibson, V Livingstone, D Murray.

Published Abstracts and Posters:

6. *Characterising the temporal evolution of emotional and behavioural problems (EBP) in an Irish cohort of Autism Spectrum Disorder (ASD) affected children in early*

childhood. *Developmental Medicine & Child Neurology* 2022, 64 (Suppl. 1), 14–8;
doi.org/10.1111/dmcn.15123,

M Carter, L Gibson, D Murray.

***Top three posters presented at BPNA 2022 conference**

7. *Maternal mid-gestation cytokine dysregulation in mothers of children with autism spectrum disorder*. Poster Presentations. *Developmental Medicine & Child Neurology*. 2020; 62(S1):15-75; <https://doi.org/10.1111/dmcn.14411>;
S Casey, **M Carter** et al.

Oral Presentations:

8. *Mid-gestation cytokine profiles in mothers of children affected by autism spectrum disorder: a case-control study*. *Developmental Medicine & Child Neurology* 2022 Mac Keith Press, 64 (Suppl. 1), 5–13 DOI: 10.1111/dmcn.151225
M Carter

Chapter 1:

An Introduction to the PiRAMiD study

A Description of the Cohort

The PiRAMiD cohort as a continuation of the BASELINE birth cohort study

Participants in the PiRAMiD study were drawn from a nested cohort within the BASELINE birth cohort study. The BASELINE Birth Cohort Study (Babies after SCOPE: Evaluating the Longitudinal Impact on Neurological and Nutritional Endpoints) (www.baselinestudy.net) was based in Cork, Ireland and in total; recruitment ran for just over three years, from August 2008 to October 2011. The SCOPE Ireland pregnancy cohort (www.scopestudy.net) formed the basis of recruitment of infants to BASELINE [n = 1537] and an additional 600 infants were recruited after delivery providing a total sample of 2137. The research team performed assessments on day of life 2 and at 2, 6, 12, 24 and 60 months of age. Team researchers performed specific developmental assessments at 24 months (using the Ages and Stages parental questionnaire, and the Child Behaviour Checklist (CBCL)) and at 60 months (using the Kaufman Brief Intelligence Test – 2 (KBIT-2) and the CBCL). Blood and DNA samples were bio-banked at 15 and 20 weeks' gestation, at birth, and at 24 and 60 months. Participants in PiRAMiD were selected based on CBCL scores at 5 years indicating high ASD risk or subsequent ASD diagnosis at a point after the 5 year BASELINE follow up. Participants with underlying genetic or developmental conditions (e.g. PTEN mutations) were excluded from analysis. Please see Figure 1 for graphical representation of recruitment process. Due to the dual recruitment streams in BASELINE, approximately 20% of participants had no stored midgestation serum samples or cord blood. This led to some variability in participant numbers between experiments (e.g. mid-gestation cytokine analysis versus proteomics and metabolomics in the older child cohort).

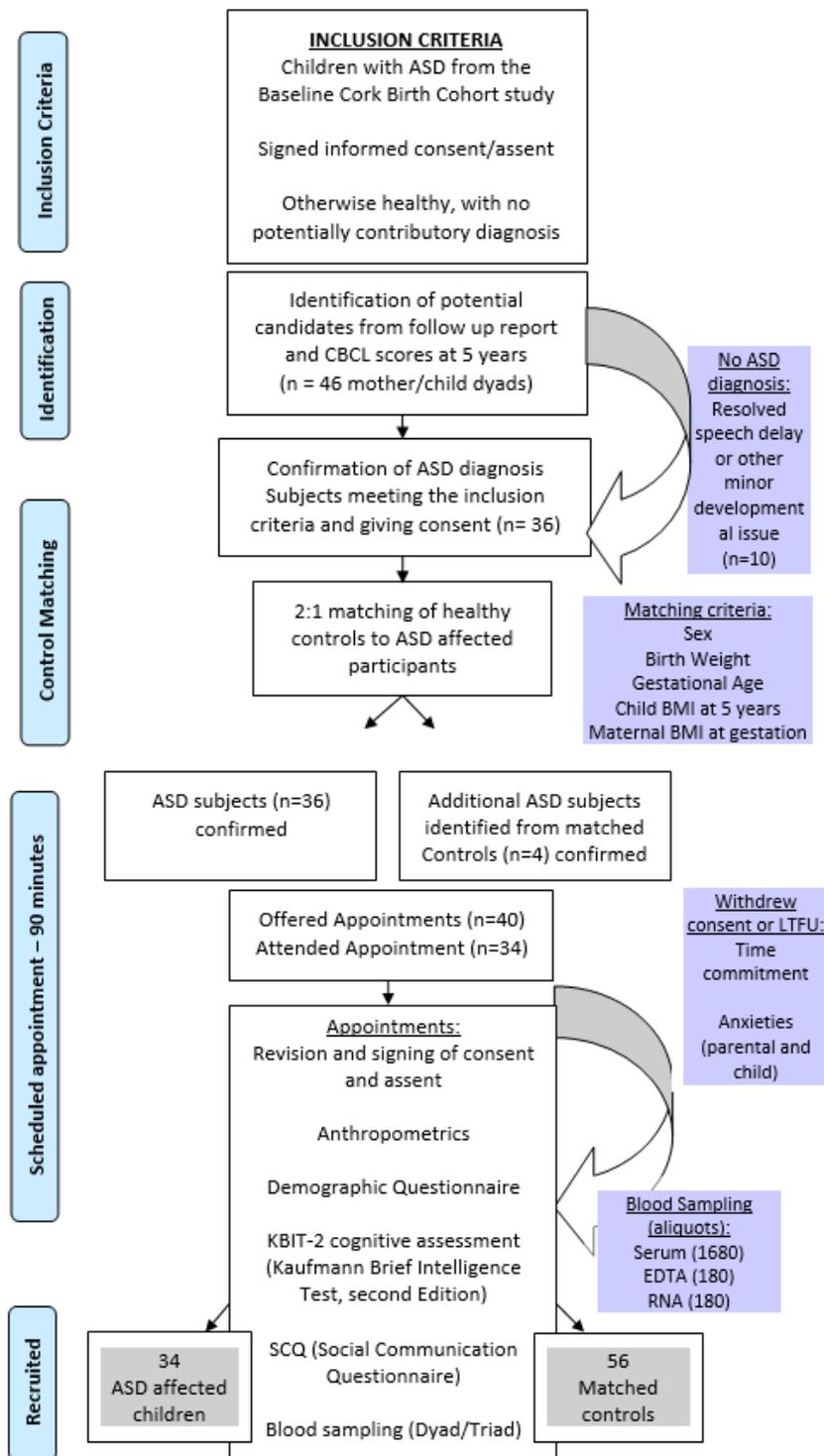


Figure 1: Prisma diagram of the recruitment and follow up process in PiRAMiD

An Introduction to Autism Aetiology, Diagnostics, and Management

Autism as a Clinically Distinct Disorder

We derive the Irish word for Autism, *Uathachas*, from the term *Uath*, which itself means self or singular. It is something of a peculiarity then that the diagnostic concept of Autism as a distinct or singular clinical entity is a relatively modern one. It is merely in the past 100 years or so that autism, and indeed child psychiatry as a whole, have become the subject of serious academic scholarship. A leading figure in the development of early nomenclatures and classifications of Autism was Leo Kanner (1894 – 1981), a Ukrainian post World War I immigrant to the United States. Kanner, an ex-cardiologist and self-taught practitioner of child psychiatry, would later help establish the first dedicated child psychiatry centre in the United States, at Johns Hopkins Hospital, Maryland. He would go on to publish his own text on child psychiatry in 1935, simply titled “Child Psychiatry”. Always an advocate for child health, the development of his ideas led to new diagnostic approaches to children with developmental disorders and psychopathology. In a later work, in which he published his behavioural observations of eleven children with atypical development, he perceived that all the children shared specific core features (44). These features, a lack of communicative use of language, preservation of sameness, restricted interest in activities, and stereotypical and repetitive patterns of behaviour, would go on to define the Autism we know today. Initially, he termed this distinct disorder Infantile Autism. “Autism” had previously been coined by Bleuler in 1908 to describe the introspective symptoms typically seen in adults with schizophrenia (45). Kanner argued that “Infantile Autism” was a unique disorder and not a precursor to Schizophrenia, as had previously been believed. Autism was first included in the third edition of Diagnostic and Statistical Manual of Mental Disorders (DSM) in 1980. Over time, the issues of diagnosis and classification of childhood-onset disorders such as autism came to be addressed in mainstream, official diagnostic systems such as the aforementioned American Psychiatric Association’s DSM (now in its 5th edition DSM-5, see Table 1) (5), and the World Health Organization’s International Classification of Disease, currently in its 11th edition (ICD-11) (46). Both definitions now reflect the wide heterogeneity inherent within the ASD diagnosis, offering an ASD umbrella, which can be qualified with a variety of additional modifiers and clinical specifications (including a measure of severity). Care must be taken however to ensure that on a systems level, the approach to diagnosis is consistent. Subtle differences between the classification criteria exist and may affect how ASD diagnoses are concluded, particularly in the setting of ASD and co-occurring intellectual disability.

Inconsistent or interchangeable use of DSM-5 or ICD-11 may have knock on effects on reported prevalence and the characterisation of ASD cohort between regions.

Table 1: DSM-5 Criteria for Autism Spectrum Disorder (5)

Domains	Criteria: Deficits	Examples
Persistent deficits in social communication and social interaction across multiple contexts, as manifested by the following, currently or by history; must have all 3 symptoms in this domain	Social-emotional reciprocity	Abnormal social approach and failure of normal back-and-forth conversation; reduced sharing of interests, emotions, or affect; failure to initiate or respond to social interactions
	Nonverbal communicative behaviours used for social interaction	Poorly integrated verbal and nonverbal communication; abnormalities in eye contact and body language or deficits in understanding and use of gestures; total lack of facial expressions and nonverbal communication
	Developing, maintaining, and understanding relationships	Difficulties adjusting behaviour to suit various social contexts; difficulties in sharing imaginative play or in making friends; absence of interest in peers
Restricted, repetitive patterns of behaviour, interests, or activities, as manifested by at least 2 of the following, currently or by history; must have 2 of the 4 symptoms	Stereotyped or repetitive motor movements, use of objects, or speech	Simple motor stereotypies, lining up toys or flipping objects, echolalia, idiosyncratic phrases
	Insistence on sameness, inflexible adherence to routines, or ritualized patterns or verbal nonverbal behaviour	Extreme distress at small changes, difficulties with transitions, rigid thinking patterns, greeting rituals, need to take same route or eat food every day
	Highly restricted, fixated interests that are abnormal in intensity or focus	Strong attachment to or preoccupation with unusual objects, excessively circumscribed or perseverative interest

	Hyper- or hypo-reactivity to sensory input or unusual interests in sensory aspects of the environment	Apparent indifference to pain/temperature, adverse response to specific sounds or textures, excessive smelling or touching of objects, visual fascination with lights
<p><i>Symptoms must be present in the early developmental period (but may not manifest until later when social demands exceed the child's limited capacities or be masked by learned strategies). Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning. These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay.</i></p>		

Prevalence and Epidemiology of Autism Spectrum Disorder

Collections of epidemiological data regarding ASD began in the early 1960s (47, 48). Prevalence, which represents the proportion of subjects within a specific population affected by a condition at that specific time, is one of the most commonly reported metrics of ASD epidemiology. In order to report prevalence accurately, case definition (what constitutes a case of ASD) and case identification (how these cases are identified), should be predetermined and consistent throughout the timeframe of the study. This has not always been easy, as the definition of an ASD case has changed frequently over the years. Initial iterations of ASD's definition (such as those of Kanner), were narrowly focused and reflected more the severer phenotypes characterised by severe language difficulties and cognitive impairment. In recent decades, less severe forms of ASD such as "high functioning autism" and Asperger's disorder received more recognition (49). The most recent interpretations of ASD within the DSM-V (see Table 1 and Figure 2) and ICD-11, adopt a much broader spectrum model of ASD to better reflect the heterogeneity of clinical phenotypes, disability and care requirements within the ASD umbrella (5, 46).

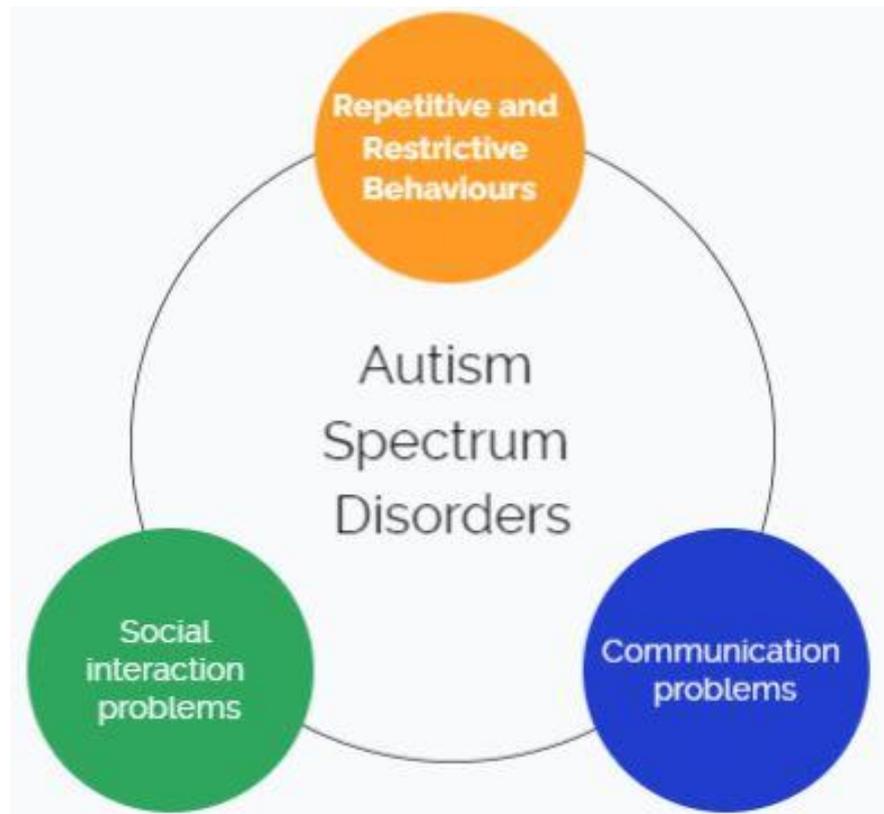


Figure 2: Schema of the neuropsychiatric features of ASD.

ASD is characterised by impaired social interaction (maladaptive responses in social settings, lack of interest in peers), communication difficulties (verbal and non-verbal including body language) and restricted or repetitive behaviours such as ritualistic movements, or rigid manner.

The prevalence of ASD has been progressively increasing for decades, and is likely more common than previously believed (50, 51, 52). The condition was once considered rare, affecting less than 0.05% of the population (53, 54). Yet, the reported prevalence is generally on the rise (52), ranging from 0.39% to 2.6% in different jurisdictions (17, 55, 56, 57). Recent figures from the United States indicate a prevalence of 2.4% among American children. This represents a remarkable shift from a prevalence of approximately 1 in 1,000 in the 1960s (58), to 1 in 44 today in the United States (59). Increasing prevalence may in part, be explained by changes in reporting practices, increased recognition of ASD symptoms, broadening of the ASD diagnosis (5), and improved accessibility to services (51, 52). We expect that if the ASD case definition is broadened or case ascertainment improves that reported prevalence will also improve. The source of data used is also important; data acquisition based on referral data into an ASD service is prone to inaccuracy. Changes in public awareness, changes in clinical awareness, and the availability of services locally can all affect the referral rates to a specific ASD service. Another methodological reason behind

apparent increases in prevalence is diagnostic substitution. Diagnostic substitution occurs when we categorise children with multiple diagnoses differently over time, or when children receive a diagnosis today that may differ to one given in the past, due to altered referral or diagnostic practices (51, 60). This is particularly true of girls who may previously have been diagnosed with Generalised Anxiety Disorder (GAD), Obsessive-Compulsive Disorder (OCD) or an Eating Disorder (ED), when in reality they suffered from ASD or Asperger Syndrome (61).

While methodological issues plague the epidemiology of ASD, so do more social or human issues such as health inequality. Many studies have demonstrated lower rates of ASD in marginalised groups (based on socioeconomics or race) (50, 59, 62). Minority race often correlates with lower socioeconomic status (SES) and poorer health awareness. Access to healthcare is an issue in areas with poor health literacy and service provision. Areas with well-developed referral pathways and access to diagnostics and assessment consistently report higher prevalence of ASD versus areas that are resource poor (59, 63). No studies to date have identified an underlying biological reason for this disparity. It remains likely that in marginalised groups and resource poor settings that ASD is under-diagnosed. Variability in health service use and health literacy may contribute to this effect (63, 64).

In general, we accept that the overall prevalence of ASD currently is approximately 1.0 – 1.5% (51, 65, 66). Our recent understanding of ASD prevalence suggests that the current and enduring increase in ASD prevalence is likely attributable to methodological factors, issues of access to health, and health inequality. While there exists little evidence to suggest some underlying biological determinant, one must not eliminate this possibility altogether. Serendipitously, the methodological failings that we see are also an excellent opportunity to tailor targeted interventions aimed at reducing these inequalities, easing early access and intervention, improving service provision, and enabling service user engagement.

The Varied Aetiologies Underpinning Autism Spectrum Disorder:

In this section, we will explore some of the established theories and aetiologies of ASD. Generally, we recognise that genetic risk are responsible for a large fraction of overall ASD risk but also that environmental risk factors, while less important, play a significant role. In the following paragraphs, we will look first at pathological and radiological findings in ASD, and then outline genetic risk, environmental factors, and the interplay between genes and environment, which constitutes our most robust understanding of ASD pathophysiology thus far.

The Genetics of ASD

The first indicator of the importance of genetics in ASD is the high concordance of ASD between twins and its high heritability in family groups. Estimates of heritability of ASD in twin pairs vary widely between 50 – 90% (15, 16), and others suggest that ASD is among the most heritable of all medical conditions (67). A recent meta-analysis of twin studies reported that the heritability estimates ranged from 64% to 93% (68). Sex linked difference are also recognised with an apparent male preponderance in ASD. Rates of ASD among males exceed those of females by three or fourfold (17, 18), while recurrence rates among siblings are found to be higher in siblings of female probands (69, 70). This may suggest some underlying female protective effect or indeed an increased male vulnerability as a potential explanation.

In terms of categorising risk genes in ASD (<https://gene.sfari.org/database/human-gene/>), we can consider:

- i. Rare single gene variants (disruptions/mutations, and sub-microscopic deletions/duplications directly linked to ASD) account for only a small percentage of all ASD cases.
- ii. Syndromic genes (those implicated in syndromes in which a significant population develop symptoms of autism (examples: Angelman Syndrome, Fragile X Syndrome). These account for <10% of all autism cases (1).
- iii. Association/Common genes variants (Small risk conferring common polymorphisms identified from genetic association studies in idiopathic ASD). These account for the majority of idiopathic autism cases.

Overall, most risk for ASD stems from common variants and association genes, each with an individually small effect, acting additively across the genome to create a greater effect. The use of Genome Wide Association Studies (GWAS), has allowed the investigation of common

variants across the human genome using an association study design, which allows analyses of both cases and controls as well as parental transmission to offspring. This approach has yielded incredible results in identifying underlying risk in a multitude of psychopathologies (71, 72). While initial GWAS in autism were underpowered, the yield of these studies has improved as the population sizes studied has increased (73, 74). A small but significant subset of Rare and de novo ASD gene variants (those not inherited from parents, and usually not shared with unaffected siblings) confer substantial risk, singly, but account for only a small fraction of autism overall. Variants such as these are unmasked with increasing regularity owing to advances in whole exome sequencing (WES) and higher resolution genomic analysis (75). The yield for finding these high-risk rare variants is greatest in ASD cohorts with associated developmental delay, intellectual delay, and systemic anomalies or in those with comorbid seizures (76, 77). The biobanking of genetic samples and phenotypic characterisation of individuals with ASD and their families has played a major role in the availability of well-characterized subjects for WES research (such as the Simons Simplex Collection). WES studies using these curated data sets have confirmed that advanced parental age is a significant risk factor for the aggregation of de novo mutation rates (78). While also clarifying whether particular biological pathways underlie potential causal mechanisms in ASD (for example, through loss of function mutations). One study found that among individuals with ASD, there was a two times greater incidence of homozygous loss of function mutations versus neurotypical controls. These homozygous loss-of-function mutations effectively result in knock out of specific (potentially critical) genes, of which genes expressed in the brain were overrepresented (79).

Copy number variants (CNV) are another mechanism through which ASD risk is conferred. CNVs arise when specific segments of the genome differ between individuals with each individual expressing a variable number of copies of the segment in question. Segments can be short or long, and some encompass whole genes or multiple genes. Copies of the gene segment arise through duplications and deletions and lead to structural differences between individuals' chromosomes (see Figure 3). This excess or diminution of genetic material occurs with increased frequency in individuals with ASD compared to neurotypical controls. The 16p11.2 microdeletion/microduplication syndrome, which confers susceptibility to ASD, is a well characterised example of this phenomenon (80). Now, It is estimated that approximately 15% of people with autism a rare mutation or CNV that is contributing to their clinical presentation (81).

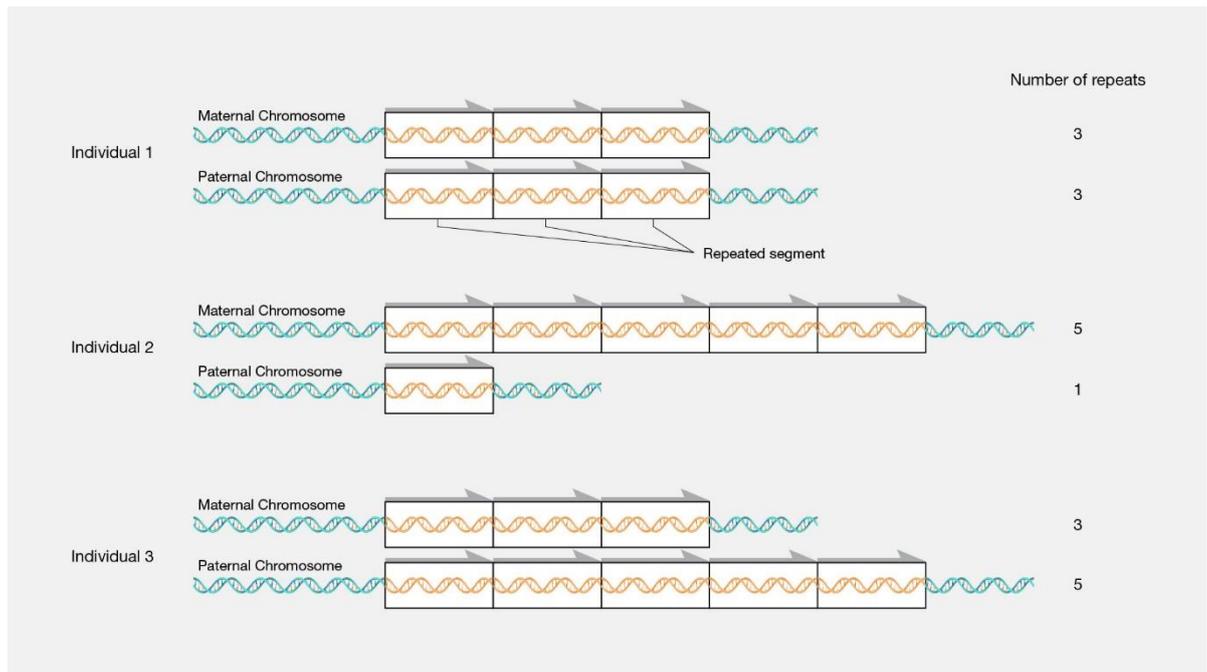


Figure 3: Copy number variation (CNV):

Refers to a circumstance in which the number of copies of a specific segment of DNA varies among different individuals' genomes. The individual variants may be short or long (thousands of bases). These structural differences may have come about through duplications or deletions and can affect long stretches of DNA. Such regions may or may not contain a gene or multiple genes.

Image courtesy of the National Human Genome Research Institute at <https://www.genome.gov/genetics-glossary>

In recent times, much effort has gone into functional modelling of single gene (syndromic) versions of autism and rare or de novo mutations. What we have found is that the genes and mutations contributing to ASD represent diverse biological pathways, including synaptic function, chromatin modification, transcriptional regulation (13, 82) and even innate immune processes (83). Many of these genes are co-associated with a shared risk for a range of neurodevelopmental disorders and psychopathology (84). Our sum knowledge of ASD genetics demonstrates that this highly heterogeneous array of genes and mutations can converge to bring about a phenotypically similar clinical presentation; while on the other hand, highly specific genetic lesions can be associated with widely variable clinical findings. We have witnessed a dizzying array of advances in ASD genetics over the past decade, and it is clear that there is a major and definite genetic basis to ASD aetiology. The question however remains, how exactly do these genetic findings bring about the clinical phenotype? Is it that mutations encoding for specific neurobiological functions converge to cause ASD as a distinct clinical entity, or do a variety of biological pathways and various ASD related genes work in concert to bring about a more general risk of a spectrum of heterogeneous

developmental and psychopathological conditions (of which ASD is one)? We will later explore how underlying genetic risks and environmental factors (G x E) may interact to bring about phenotypic ASD outcomes.

Neurobiology of ASD

Keeping to the general theme of ASD as an entity typified by heterogeneity, findings at the neuropathological level also, unsurprisingly, demonstrate similar heterogeneity. Initial approaches to post-mortem study of autistic brains has suffered to that end, with subjects displaying a wide spectrum of symptoms, comorbidities and a variety of genetic risks. It follows that there is a likelihood that a variety of brain areas may therefore be affected with so many variables at play. So far, the commonest findings at post-mortem or from structural MRI studies are:

- i. Alterations in brain volume (megalecephaly (85) and microcephaly (86)),
- ii. Purkinje neuronal loss in the cerebellum (87),
- iii. Activation of the Innate immune system (microglia) (88)
- iv. Focal developmental malformations (cortical, hippocampal or cerebellar) (89).

Generally, in children with autism, the head circumference at birth is typically normal or near normal (see Figure 4). However, by approximately two years, the head circumference becomes abnormally enlarged (90). By the age of 3 or 4 years, MRI studies find that brain volumes are increased (by up to 10%) versus typical controls (91). While there are reports of increases in the volume of both grey and white matter, a 2020 meta-analysis of structural MRI findings in ASD found that ASD was typified by increased grey matter volume in the fronto-temporal regions (92). Megalecephaly can frequently co-occur with underlying syndromic ASD. Genetics syndromes with a strong ASD phenotype associated with increased brain or head size include Cowden's disease or Bannayan-Riley-Ruvalcaba syndrome (Phosphate and tensin homolog deleted in chromosome 10 gene (PTEN)), the neurocutaneous syndrome, neurofibromatosis type 1 (neurofibromatosis-1 gene (NF1)) and ASD syndromes arising through mutations in the chromodomain helicase DNA binding protein 8 gene (CHD8)(93). In microcephaly, again this may be syndromic such as in Cohen, Rett, or Angelman syndrome. Mutations in numerous high confidence ASD candidate genes such as DYRK1A (dual specificity tyrosine-(Y)-phosphorylation-regulated kinase 1-A) can also lead to ASD microcephaly phenotypes (94).

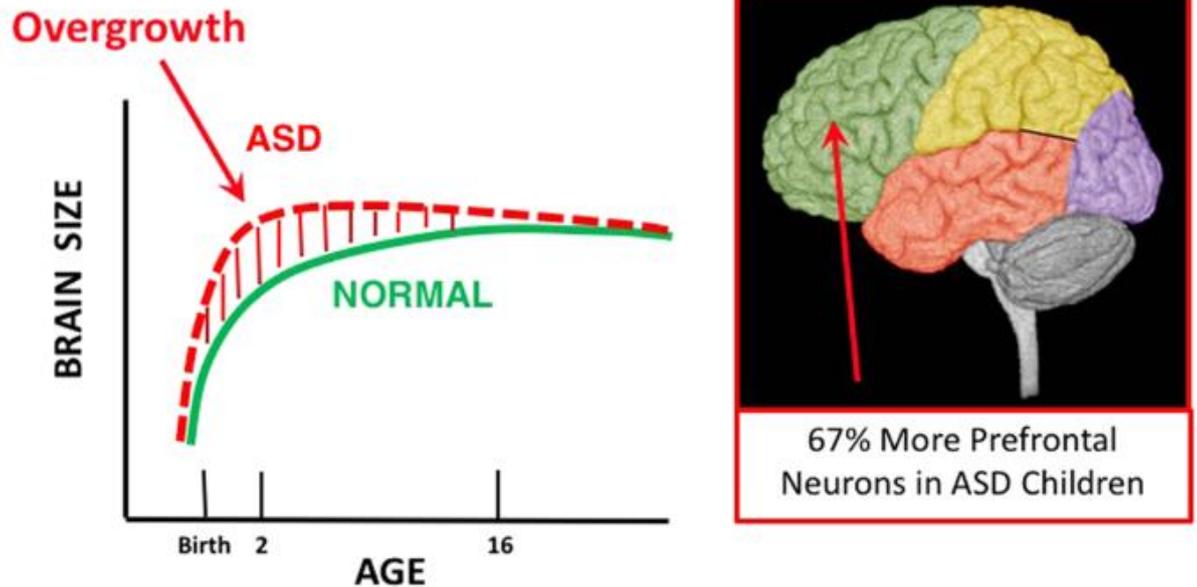


Figure 4: Illustration of early brain growth in ASD.

Brain overgrowth in infancy occurs in many ASD toddlers and is due to an overabundance of cortical neurons. In humans, cortical neuron generation occurs only in prenatal life suggesting that ASD begins in the womb. The overabundance of cortical neurons (up to two thirds more according to some authors (90)) is theorised to lead to disrupted neural network development and function, and ASD symptoms (95). (Figure courtesy of E. Courchesne from *The ASD Living Biology: from cell proliferation to clinical phenotype. Molecular psychiatry. 2019*) (95)

Histopathologic changes in the cerebellum have been observed frequently in post-mortem studies of ASD brains (87). Loss of cerebellar Purkinje cells (mainly in the lateral cerebellar hemispheres) has been a consistent finding. Interestingly, Purkinje cell density has been found to correlate with the use of social eye contact in patients with autism (96). This may suggest that regional Purkinje cell cerebellar pathology may contribute to specific features of the ASD phenotype.

Numerous studies have identified elevated levels of cytokines, chemokines, mRNA immune signatures and other markers of innate immunity in the examination of post-mortem brain and CSF samples (97, 98). Microglia are the resident macrophages of the central nervous system. They possess varied morphologies and functions and under normal physiological conditions, they exist mainly, in a resting (ramified) state and constantly monitor their microenvironment, surveying neuronal and synaptic activity (99). They play a crucial role in synaptic pruning during postnatal brain development, a process crucial for the promotion of

synapse formation and the regulation of neuronal activity and synaptic plasticity (100). Linking this with causes of megalencephalic ASD, we suspect that brain overgrowth may be due to failure of this synaptic pruning or excessive dendritic arborisation. When triggered by brain injury, infection or neuro-inflammation, microglia activate and proliferate. Activated microglia assume an amoeboid shape, migrating to sites of inflammation and secreting cytokines, chemokines and reactive oxygen species. These inflammatory molecules can interrupt synaptic plasticity and may lead to learning deficits associated with autism. These findings in ASD are useful in establishing that a pathological process is ongoing, but these findings alone are insufficient to implicate a specific aetiology and a multifactorial or even individualised collection of aetiologies may be at play.

Some groups have observed altered cortical thickness in ASD cohorts (both thickened and thinned) particularly in the prefrontal cortex, abnormalities in which may underlie deficits in social functioning, attention, and impulsivity (101). We commonly find malformations of cortical development (MCDs) such as focal cortical dysplasia (FCD) during histopathological evaluation of post-mortem autism tissue that may be visually appreciable on MRI (102). Tuberous sclerosis (TS) is a syndrome characterised by drug resistant epilepsy, developmental delay and autism in approximately 50% of sufferers. TS is driven by the development of cortical tubers, which are abnormally formed cerebral gyri with bulky, disorganised and dysplastic structure. TS tubers are formed by mutations in either of the tuberous sclerosis complex genes (TSC 1 or TSC 2). When we examine brain tissue samples from individuals with TSC mutations, we find disorganisation of the normal cortical layering pattern, and neurones of abnormal size, quantity and location. These resultant abnormal neurones can promote abnormal cellular growth in the cortex.

Environmental Factors:

There is growing interest in the role of environmental risk in ASD, with observational evidence for associations between prenatal and perinatal factors that lead to increased ASD risk. A systematic review from 2017, which comprehensively covered the literature in this sphere, found that factors such as advanced parental age, and birth trauma or asphyxia-related birth complications were likely contributors to ASD risk (103). Other authors have indicated elevated risk associated with preterm birth, maternal obesity, gestational diabetes, short inter-pregnancy interval, and the use of specific medications (valproic acid) (103, 104, 105). While explorations of the role of nutritional factors and chemical exposures have shown mixed results (104, 105). A large Danish cohort study was emphatic in finding no ASD risk associated with MMR vaccination (106). The mechanisms through which the

environment may influence ASD risk are complex and include genetic, epigenetic, inflammatory and immunological interactions (103). As changes in brain morphology are seen soon after birth, it is likely that any environmental risk factor must begin pre-natally.

The role of epigenetics:

Epigenetics refers to molecular modifications of DNA that can regulate gene activity, are independent of DNA sequence, and remain stable through mitosis. The most widely recognised epigenetics effects are DNA methylation, histone modifications (including acetylation and deacetylation), and non-coding RNAs (107). Epigenetic changes can arbitrate access to specific genomic loci, altering transcription, as well as influence the stability of mRNAs. When these effects occur in the crucial fetal and early childhood window, they can attribute long-term epigenetic modification and impart a greater impact on neurological development (108).

We recognise that epigenetic effects cause a number of genetic conditions, which share an autistic phenotype. Rett syndrome, a condition characterised by developmental regression, stereotypies, severe intellectual disability and autism, is caused by mutation in the MECP2 gene (resulting in a critical loss of function) which encodes the DNA methylation protein MeCP2, inhibiting this protein's transcription, and producing the Rett phenotype (109). Another X-linked disorder, Fragile X syndrome, is similarly caused by loss of function mutations in the critical FMR1 gene. The FMR1 gene becomes hypermethylated leading to failed production of the FMR1 gene product, fragile X messenger ribonucleoprotein (FMRP), which itself has a central role in gene expression and potentially regulates translation of hundreds of mRNAs. Many of these mRNAs are involved in the development of synaptic connections, the absence of which can truncate neuroplasticity (110). More broadly, evidence of dysregulated DNA methylation in ASD exists on multiple levels: genetic mutations in genes encoding epigenetic machinery, abnormally methylated loci, and genome-wide correlations of both hyper- and hypo-methylation (111). With compelling evidence to support a role for maternal immune activation in the pathogenesis of ASD, researchers hypothesise that epigenetic effects may be one of the mechanisms through which MIA can alter neurodevelopment (112). Gestational exposure to maternal inflammation may lead not only to significant ASD risk in progeny, but also to potentially far-reaching risk of psychopathology in the child's later life (83, 113).

Immunological findings in Autism Spectrum Disorder:

Under normal conditions, we consider the brain to be an immunologically privileged organ. The blood brain barrier limiting the movement of most peripheral immune cells (and pathogens) into the central nervous system. The brain is therefore heavily dependent on its own resident microglia (monocyte lineage) for immune protection and surveillance (114). Upon activation, microglia become roving phagocytic cells scavenging damaged tissue, metabolic waste products and pathogens. As previously mentioned, they also play a critical role in synaptic pruning during early childhood development (100). There is burgeoning evidence that disturbances of typical inflammatory and immune response may be significant contributing factors behind the pathophysiology of many psychiatric disorders (115, 116, 117). Alterations to the local homeostasis maintained by microglia can disrupt normal prenatal microglia development and maturation leading to microglial epigenetics alterations, and affecting the developing fetal brain and increasing ASD risk (118).

There also exists a large body of evidence, which suggests aberrant activation of the immune system in ASD (119, 120). Researchers have documented alterations of immune expression repeatedly in ASD affected children and adults as well as animal models with ASD-like phenotypes (27, 121, 122). A prenatal inflammatory challenge using lipopolysaccharide (LPS) in mice previously resulted in ASD-like behavioural alterations (122). A prenatal viral challenge in rhesus monkeys resulted in ASD-like phenotypes such as repetitive behaviours and decreased sociability (123). Large epidemiological studies have shown that immune disorders and mid-trimester viral illnesses, which lead to a pro-inflammatory state in mothers during pregnancy, are associated with increased ASD, schizophrenia and bipolar disorder risk in offspring (113, 124, 125, 126). In the following paragraphs, we will explore some of the role of infection, inflammation and antenatal exposures in ASD aetiology.

Intrapartum viral and infectious exposures:

The idea that congenital or early life infection can lead to ASD is not a new one, and evidence to support this theory has been building for decades. In 1977, Chess noted ASD rates of 8 – 13% in offspring of pregnant US mothers who were infected by the 1964/65 Rubella outbreak (125). In recent decades, large epidemiological studies indicate that conditions such as maternal autoimmune disorders and mid-trimester viral infections that trigger gestational pro-inflammatory states (i.e. Maternal Immune Activation(MIA)), are linked with elevated ASD, schizophrenia and bipolar disorder risk in offspring (113, 124, 125, 126). Not only Rubella (125), but other “TORCH” infections (Toxoplasmosis, Other, Rubella, Cytomegalovirus (CMV), and Herpes) have been implicated in cases of ASD arising from

intrauterine infection (127). There are also reports of “reversible” ASD phenotypes in children who acquired HSV encephalitis in early childhood, who later fully recovered (128). Children affected by ASD arising from intrapartum or congenital infection tend to suffer from other sequelae of those infections (such as deafness, or microcephaly in congenital CMV). These features are not typical of the large majority of “idiopathic” ASD cases that we see. Overall, congenital infections themselves seem to contribute to only a small percentage of the overall prevalence of ASD.

The evidence for maternal infection and an association with ASD is somewhat conflicting. In a large Danish cohort, maternal influenza infection and prolonged periods of gestational fever bestowed a twofold and threefold risk of infantile ASD respectively (129), while a meta-analysis found that ASD risk was increased following maternal bacterial infections and negative life events occurring during the third trimester (124). Conversely, another group (in a much smaller cohort) failed to reproduce the influenza findings but did find a twofold risk association between maternal fever and infantile ASD, which ameliorated somewhat with use of antipyretics (130). Overall, the data suggests that there is a significant risk of ASD and other neurodevelopmental disorders bestowed upon infants exposed to in utero inflammation (131). Maternal pro-inflammatory states brought about by underlying inflammatory disorders, infection or metabolic stress, are likely to arise through a variety of underlying pathological mechanisms (immunological, infectious, oxidative stress, neuroendocrine mechanisms) (112). Maternal immune activation represents one of the best-characterised models of such gestational inflammation with a growing body of evidence linking MIA to ASD risk.

Allo- and Autoimmunity:

To a degree, the immunological privilege afforded to the brain is also one enjoyed by the developing foetus. In a typical pregnancy, the fetal graft and placenta do not generate the expected host allo-immune response against the fetal alloantigens. This is due to a combination of factors, chief among which is the HLA-G complex (132). This human leukocyte antigen, through action on NK cells, and within the decidua, creates the immune tolerance needed to allow the fetus to develop unhindered (132). Some authors link maternal polymorphisms of HLA-G to ASD phenotypes in offspring, suggesting a potential aberrancy of the gestational inflammatory balance (133). While the placenta typically does a good job of protecting the fetus from maternal immunoglobulins (Ig), occasionally there are exceptions (IgG autoantibodies in maternal Lupus or Graves’ disease), which can lead to negative effects on the fetus (134, 135). There is some evidence from small primate models

that Ig harvested from human mothers of children with ASD can impart an ASD phenotype on offspring from pregnant monkeys injected with the pooled human Ig (136, 137). Several groups have identified brain antigens that cross-react with maternal IgG and may contribute to neurodevelopmental impairments in children (138, 139, 140). Based on these maternal Ig findings some authors have even suggested a specific ASD subtype, maternal autoantibody-related ASD (MAR-ASD).

The placental interface:

The placenta plays a critical role here in protecting the fetus from viral and bacterial infection, and in maintaining an environment conducive to normal development (114). A host of proinflammatory and anti-inflammatory cytokines are normal constituents of the placenta interface. These cytokines play an important role in maintaining this complex homeostasis, but aberrations in this balance can imperil the fetus, and deleteriously alter fetal development (see Figure 5 below) (141, 142). A well-characterised environmental factor known to affect this homeostasis, alter early fetal brain development and increase ASD risk is maternal inflammation in pregnancy, commonly called maternal immune activation (MIA). Animal studies demonstrate convincingly that maternal inflammation is conducted to the fetus through a variety of direct and indirect (stimulation of the fetuses own immune response) mechanisms at the levels of the placental interface. Maternal cytokines can passively transfer to the fetal compartment, or placental inflammation can trigger the fetal cytokine response (143). In severe cases, MIA induced placental inflammation can lead to fetal hypoxemia and fetal demise (144). The inflammation arising in MIA is not necessarily infection specific and recently a range of conditions associated with proinflammatory states in pregnancy (obesity, autoimmune disorders, psychosocial stress and pre-eclampsia) have also been associated with increased ASD risk in children (145, 146). Toll like receptors (TLRs) which we will discuss in more detail later, are over-expressed in mothers who are obese. The specific TLR involved, TLR4, is expressed during bacterial infections and correlates with increased IL-6 expression in the placenta (147). This all suggests that gestational maternal immune activation (MIA) plays a role in the pathogenesis of ASD in exposed offspring. It is also likely that while MIA arises through a variety of mechanisms, in some instances, these mechanisms may converge into a common end effector pathway.

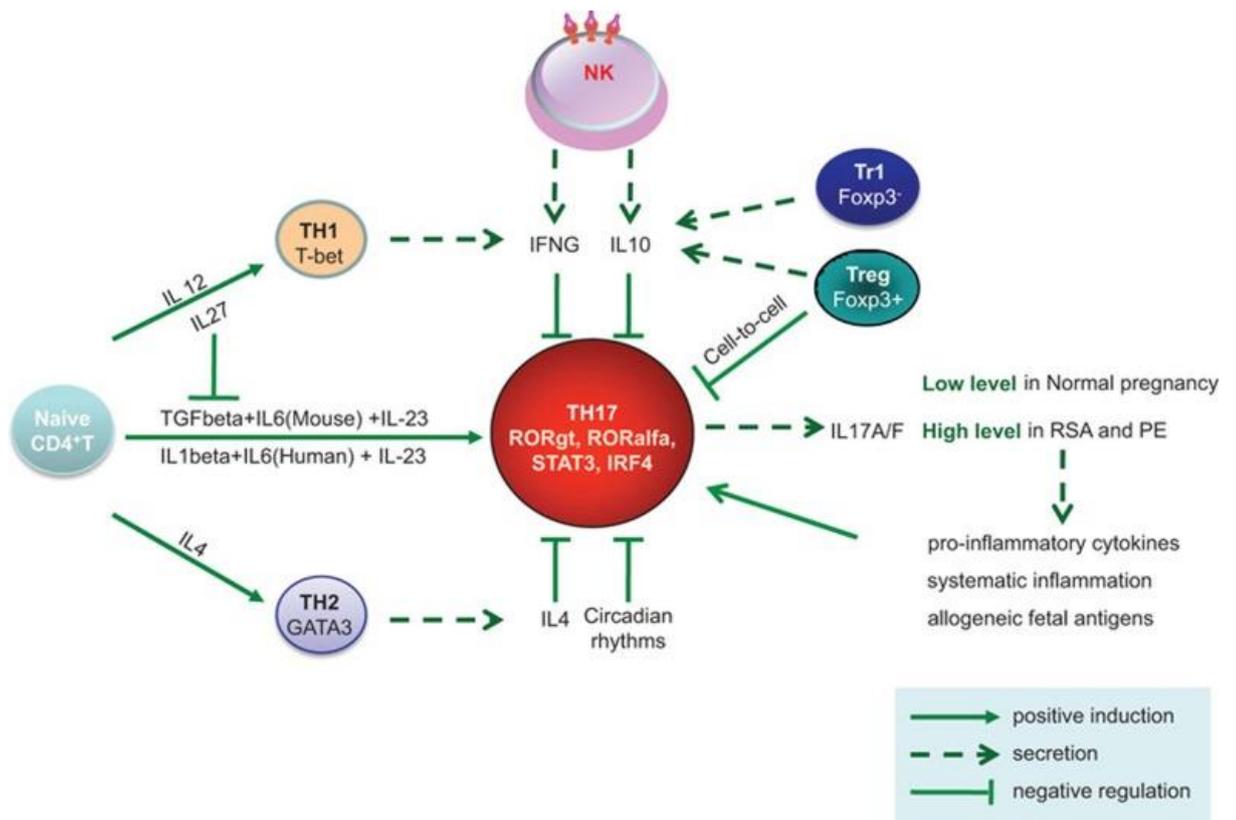


Figure 5: Key regulatory pathways of TH17 cells during pregnancy.

Naive CD4⁺ T helper cell develop into different T helper subsets, including TH1, TH2, TH17 (T helper 1, 2, 17 cells), Treg and Tr1 cells (T regulatory and type 1 T regulatory cells), under different cytokine environments. TH17 cells, characterized by the production of IL-17, have an important role in pregnancy and have been found to be upregulated in RSA (recurrent spontaneous abortion) and pre-eclampsia (PE) patients. A complex immune network at the placental interface orchestrates regulation of TH17 cells and IL-17. Decidua Natural Killer (NK) cells, resident in the placenta decidua can inhibit inflammatory TH17 cells through interferon gamma and IL-10 secretion. While, interferon gamma from TH1, IL-4 from TH2, and IL-10 from Treg and Tr1 cells can also inhibit TH17 cells. IL-27 counters the polarization of naive CD4⁺ T cells, but has little or no effect on mature TH17 cells.

[\(Adapted from Fu B, Tian Z, Wei H. TH17 cells in human recurrent pregnancy loss and pre-eclampsia. 2014\)](#)

Maternal Immune Activation:

Maternal immune activation (MIA) is defined as an increase in measured levels of inflammatory markers in mothers during pregnancy, and more specifically refers to a triggering of the maternal immune system by infectious or infectious-like stimuli resulting in an increase in measurable inflammatory markers (148, 149). Numerous epidemiological studies have linked gestational infections with elevated risk of ASD in offspring (33, 125, 126), while animal models of MIA have simulated gestational infection resulting in MIA-induced

neural and behavioural abnormalities analogous to those seen in ASD (24, 150, 151). In mouse models, MIA can lead to overproduction of neurones as well as illicit macroscopic structural changes in the developing brain including increased cortical thickness, focal cortical dysplasia and increased brain volume (24, 152, 153), findings remarkable similar to those seen in children with ASD (see Neurobiology of ASD).

Many studies have also sought to identify blood-based biomarkers of ASD in affected adolescents and adults (27, 28, 29, 30, 31, 32, 120, 154) and have reported alterations of molecules involved in iron transport (155), inflammation (28, 156), brain development (157), hormonal regulation and metabolism (158). A much smaller number of studies have characterised cytokine profiles in expectant mothers who progressed to give birth to children who develop ASD (33, 34). The few previous studies, which have examined gestational serum, have indicated mid-gestational upregulation in specific pro-inflammatory cytokines or indeed down-regulation in anti-inflammatory cytokines. None to date has identified and validated any reliable mechanistic biomarkers.

Cytokines as key players in ASD risk:

Cytokines are cell-signalling molecules, are the chief regulators of inflammation and immune processes, and are ubiquitous throughout all bodily tissues. They stimulate other cytokines to proliferate, and can even self-stimulate through positive feedback loops. While there is a dizzying variety of cytokines, many will share similar functions, known as redundancy (159). Many cytokines demonstrate pleiotropism whereby they have multiple roles or functions, and can be sneakily versatile, with unknown or unexpected interaction and effects (160). This versatility can make deciphering cytokine function difficult in that each cytokine may have a completely different function, depending on the source cell, the intended target, or the specific phase of the immune response during which the cytokine is elaborated. In this way, many cytokines can fulfil both anti-inflammatory and pro-inflammatory roles depending on the prevailing conditions at a given time (159).

Evidence from animal models indicates that cytokines play a key role in acting out the effects of MIA on the developing fetal brain (24, 152). Maternal production of proinflammatory cytokines, leads to increased placental, amniotic and fetal brain cytokine levels, with resultant altered fetal brain development or behavioural outcomes (24, 161). In humans, along with complement and chemokines, cytokines are expressed at the level of the synapse (112). Acting within the CNS, cytokines are involved in several pathophysiological functions, including synaptic transmission and neuronal excitability (162). Other human based studies

indicate that aberrations of the immune system may play a role in ASD (34, 120, 163). Some propose that alterations in cytokine expression could facilitate the classification of ASD subtypes as well as work as biomarkers of response to treatment (27, 34, 121). In the diagnosis and management of ASD, earlier is better, and identification of reliable biomarkers during pregnancy may allow for targeted behavioural interventions from early infancy. This could also aid the development of targeted pharmacological strategies which have already shown promise in animal models (24), and analogues of which are currently in use in routine medicine practice (164, 165).

Animal-based studies.

Although there are very few human studies that have examined the molecular links between MIA and ASD, many animal-based studies have addressed the question of MIA and the association of perturbation of cytokine expression and parallel behavioural changes in offspring. MIA has been replicated in a variety of small animal models: mouse, rat and simian phenotypes of ASD have been created through intrauterine inflammatory exposure (123, 142, 161). Early murine models showed behavioural phenotypes and aberrant brain morphology in offspring born to influenza-infected dams (166). Later models used immune-stimulants, viromimetic polyinosinic:polycytidylic acid (polyI:C) and the bacteriomimetic lipopolysaccharide (LPS) to model ASD phenotypic offspring and to examine cytokine aberrations during pregnancy and their role in deviant fetal neuro development (167, 168). While rat and simian models have provided valuable insights into the effects inflammation can have on social and communicative behaviour in progeny (123, 142). Interestingly, remedial steps have been possible with improvements in and resolution of some ASD traits following blockade of specific inflammatory pathways (IL-6 and IL-17A) (24). This work suggested that these two cytokines in particular are significantly involved in the neuronal dysfunction brought about through MIA (24, 142, 161, 169). MIA-mouse models of ASD have shown increased IL-17A levels in maternal blood, the postnatal brains of offspring and in placental mRNA levels of the IL-17A (170). This suggests upregulation of IL-17A activity at the feto-maternal interface. In 2016, Choi et al. demonstrated persuasively that simulated MIA in murine models leads to elevation in maternal IL-6, leading to downstream activation of maternal Th17 cells. Maternal Th17 cells produce IL-17A that is hypothesised to cross to the foetus via the placenta leading to increased expression of IL-17AR in the foetal brain, contributing to cortical malformations and behavioural abnormalities (24, 160). In effect, the cytokines that are normally expressed in the fetal brain (via microglia etc.), and that have key functions in normal development become altered. This leads to disruption of the optimal

cytokine balance triggering long lasting alterations to synapse function, neural connectivity and developmental processes (25).

The most recent studies seem to indicate a stacking of risk with multiple fetal exposures, timing of exposure also being important (100, 171). Experiments in animal models of multiple antenatal exposures indicate that multiple environmental exposures (such as MIA and gestational diabetes) may amplify the MIA response and result in more severe or alternate behavioural outcomes (172).

Is there a common convergent pathway for MIA?

Findings from animal models suggest that Toll-Like Receptors (TLR) are important mediators of inflammation in MIA (88, 160). As a family, these receptors may represent a pathway upon which varied sources of maternal inflammation converge, bestowing risk upon offspring. TLR 3 receptors, for instance, are stimulated by polyI:C or viral infection to produce a potent immune response characterised by the production of inflammatory cytokines such as IL-1b, IL-6 and interferons (173). TLRs are expressed on many peripheral tissues and CNS based immune and parenchymal cells including T-cells, microglia, neurones and the placenta. Activation of placental and immune TLRs leads to cytokine production leading to placental innate immune activation and modulation of the gestational inflammatory milieu (112). A variety of human medical conditions suggest links with TLRs. TLR4 receptors which are the mediators of inflammation in bacterial infections also become activated by stress, depression, lupus (SLE), psoriasis and pre-eclampsia in women during pregnancy (174, 175, 176, 177).

TLRs are crucial activators and potentiators of innate immune pathways, and it may be through elaboration of cytokines from downstream effectors that TLRs create a convergent quality and can mediate effects on the fetal brain (160).

Human studies.

Quite a large number of human based studies have examined immune and cytokine aberrations in individuals (adults and children) affected by ASD, and found altered expression. For the purposes of this thesis, I examined the literature and selected eight cytokines that were commonly found to be altered according to existing evidence. In Chapter 2, I outline each of the analytes, Tumour Necrosis Factor – α (TNF α), Interleukin 1 beta (IL-1 β), Interleukin 4 (IL-4), Interleukin 6 (IL-6), Interferon γ (IFN γ), Interleukin 8 (IL-8),

Interleukin 17 (IL-17), and Granulocyte-macrophage colony-stimulating factor (GM-CSF), examine their function and highlight their purported role in ASD (178, 179, 180).

While the cytokine profiles of ASD affected individuals have been well characterised, very few studies have investigated the relationship between mid-gestation cytokine levels and ASD risk in offspring. To our knowledge, only three human studies have examined maternal serum (33, 34, 181), and one more has examined amniotic fluid cytokine profiles in mothers of ASD affected children (182). The findings from these studies effectively provide all of our current understanding of gestational cytokine profiles in the setting of ASD.

Previous literature on gestational samples analysis in ASD.

Working from the same laboratory and using similar methods, Goines et al. (2011) and more recently, Jones et al. (2017) both demonstrated elevated mid-gestational cytokine levels between groups of ASD affected children versus controls or children without ASD. Goines et al. demonstrated elevated levels of mid-gestation (15–19 weeks' gestation) IFN γ , IL-4 and IL-5 with an associated 50% increased ASD risk. While Jones et al. showed elevated levels of mid-gestation GM-CSF, IL-6, IFN γ and IL-1 α in the ASD affected group versus children with developmental delay, but not ASD. In the Goines study, ASD cases were matched with neurotypical controls based solely on child characteristics (sex, birth month and year), something which the authors acknowledge in their limitations. Neither study had access to comprehensive maternal health information during the pregnancy (including intrapartum infections). Nor did they have a record of relevant maternal medical history, all, information important to the interpretation of their findings. Irwin et al. (2018) demonstrated alterations in IL-4, MCP-1 and IL-10 levels in 28-week gestation serum of mothers who birthed ASD affected children (181). Specifically, IL-4 (usually anti-inflammatory or involved in allergic type inflammation (119)) was increased and associated with higher ASD symptomology (as measured by the Social Communication Questionnaire (SCQ)) in offspring. Higher concentrations of IL-10 (anti-inflammatory) were associated with fewer ASD symptoms in offspring (measured by the Social Responsiveness Scale (SRS)), and finally, elevated MCP-1 was associated with fewer ASD symptoms (as measured by the SCQ). No controls were used in this analysis, instead a large cohort of ASD affected individuals were enrolled, and the 28-week gestation cytokine concentrations were correlated with ASD symptomology at 7 years of age. This is novel in two senses, none has previously assessed the cytokine profile in the third trimester, and none has correlated cytokine findings with severity of ASD symptomology in this way. As with previous authors, they had no access to relevant maternal pre-conceptual medical history or gestational infections data.

Finally, Abdallah et al. (2013) examined amniotic fluid samples and found elevated levels of IL-4, IL-10, TNF α , and TNF β . In a preliminary study (2012), they also identified elevations in MMP-9 in ASD cases relative to controls (183). Advanced sample age is again an issue with the oldest samples in this analysis being 29 years old, the youngest 10 years old. The samples were stored at -20°C according to local guidance (184). Both the storage conditions and the samples ages are likely to have contributed to significant cytokine degradation (185)

Linking immunity, MIA, and genetics in ASD:

Bioinformatics analysis of large CNV studies suggest strongly that innate immune processes are implicated in ASD risk (83), this may indicate that immune dysfunction in ASD may be genetically driven or influenced. MIA downregulates expression of susceptibility genes known to be highly penetrant in ASD and heavily involved in neurogenesis, cell signalling, synaptogenesis and axonal guidance in the early stages of fetal development (83, 95). When compared with curated ASD associated gene sets (e.g. via the SFARI Gene database (<http://gene.sfari.org/>), MIA downregulated genes were substantially enriched. The strongest enrichment of MIA downregulated genes was observed in the ASD gene categories with the highest likelihood of a link to ASD i.e. SFARI “High Confidence” or “Syndromic” ASD gene sets. This suggests that MIA may bestow increased ASD risk through downregulating the expression of the same genes that are highly penetrant in ASD during the early stages of fetal development. Loss of function mutations in TSC1 and TSC2 (tuberous sclerosis complex 1 and 2) genes are linked to syndromic ASD, and these genes are critical upstream regulators of the mTor (mammalian target of Rapamycin) pathway. mTor has important functions in innate immunity and metabolism in particular (186, 187, 188).

MIA also has downstream effects, in some cases influencing the transcriptome rather the genes themselves. FMR1 and CHD8 are both highly penetrant genes for ASD, yet MIA does not seem to influence expression of these genes directly. Rather, it wields an influence on downstream gene targets such as FMRP (fragile X syndrome protein complex). This raises the possibility that MIA may act as an environmental factor disrupting crucial early developmental genomic pathways through influence on downstream gene targets (83). This might suggest that MIA could act both in a direct (genetic) and indirect fashion (epigenetic/regulatory) with the end effects converging on similar pathways.

Preclinical studies are investigating the epigenetic effects of cytokines and inflammation in humans. A number of pro-inflammatory cytokines are known to influence histone modifications through methylation and acetylation pathways (112). While MIA can alter

offspring epigenetics through other modifiers such as oxidative stress and circulating folate levels (112), and is shown to alter methylation patterns in other psychopathology (189). In children exposed to MIA, epigenetic modifications have been found in the placenta, cord blood and even peripheral blood samples taken in later childhood, which suggests that these changes are enduring and long-lived (190). Again, taking this information altogether, our understanding of epigenetic effects in MIA and ASD suggests that prenatal exposure to inflammatory factors is associated with altered epigenetics in progeny, and resultant aberrations of neurodevelopment which may be long lasting.

A possible role for intrapartum sex hormones in ASD aetiology:

Steroid hormone alterations in the fetal environment have been implicated in elevated ASD risk (191). The male brain theory of ASD is a theory of cognition that claims autism can be described as an extreme variant of the male phenotype (192). There is some evidence to support this as testosterone in fetal development has been shown to affect vocabulary, eye gaze, restriction of interests and increased attention to detail (193). Functional imaging studies have indicated a structural effect of testosterone on the fetal brain with a resultant effect on the reward system and behaviours akin to those seen in autism (194). Genetic mutations (single nucleotide polymorphisms) in specific sex steroid synthesis genes were associated with ASD (with retained cognitive ability), for example, CYP19A1 (195). A study of amniotic fluid in a large birth cohort study indicated elevated levels of progesterone, 17 α -hydroxyprogesterone, androstenedione, and testosterone in an ASD affected group versus controls (191). Polycystic Ovarian Syndrome (PCOS) is a disorder in women, which can cause infertility and leads to elevated circulating testosterone levels. PCOS can affect between 5 – 10% of women of childbearing age (196). A Swedish study found that children born to mothers with PCOS had elevated ASD risk (197). This factor was strengthened if the mother was hirsute (suggesting higher circulating levels of testosterone) (198). Androstenedione is an endogenous androgen steroid hormone that is a precursor of testosterone, as well as other androgens and oestrogens. Serum androstenedione levels have been found to be significantly higher independent studies in pre-pubertal children with ASD (199), and adults with ASD (200). Another group profiled amniotic fluid samples from the Danish birth cohort and found that in children with confirmed ASD that levels of progesterone, 17 α -hydroxyprogesterone, androstenedione and testosterone are all significantly elevated versus matched controls (191). These exposures in the antenatal period may be important epigenetic fetal programming effectors and may interact with other important pathophysiological factors contributing to autism.

Where do the metabolome and the proteome reside in the span of autism aetiology?

We currently know of no specific, sensitive or early biomarkers that can reliably detect ASD, and are dependent of the clinical onset of physical symptoms to make a diagnosis. Newer “Omics” disciplines allow granular investigation of ASD and can draw from a wide variety of biological samples or tissues, perhaps setting the stage for new biomarkers. The premise of Omics research originates from a broad vision of the system in question, taking in the whole, rather than a fraction. This complements the precision of the classical genetic or biochemical approaches based on single or few target molecules. A major strength of the omics approach is its ability to produce a complete profile of the biological milieu (transcripts/proteins/metabolites) to obtain a holistic description of the contemporaneous biology within a sample.

Metabolomics:

Metabolites are small molecule end-products of cellular metabolic pathways, and metabolomics is the comprehensive identification and quantification of these products in a tissue or fluid (35). Metabolomics can offer an interesting insight in to the individual biochemistry of each person with ASD at a given point in time (36). A metabolomic profile can complement the clinical/behavioural profile of the ASD phenotype and can intimate an underlying molecular phenotype. The biochemical outputs of metabolomic analysis represent the interplay of genetic, epigenetic, proteomic and environmental factors and may offer individualised insights into a subject’s specific ASD pattern. In a sense, the metabolome can provide an individualised, time-locked description of each person’s unique biochemical signature. We can employ a variety of experimental methods including ultra-performance liquid and gas chromatography, and machine learning techniques to explore the metabolome in high fidelity, and to observe change over time. With these technologies, there exists a unique and novel opportunity to discover sensitive, specific and personalised biomarkers which may potentially allow for earlier diagnosis of ASD, before symptoms become apparent (108). Metabolomics could aid the detection of patterns suggestive of maternal immune activation or mitochondrial dysfunction during pregnancy, potentially allowing antenatal intervention. While detection of biochemical patterns in young children may allow early diagnosis and behavioural interventions which are proven to be effective (10).

Proteomics:

The development of the field of proteomics is providing powerful tools to investigate the complex biological systems of disease. Study of the proteome can scope the identification of biomarkers for clinical diagnosis, the monitoring of disease states, the study of pathogenic molecular mechanisms, and potentially even the choice of appropriate therapeutic interventions. In combination with other –omics platforms i.e. transcriptomics, proteomics may improve current gene modelling by profiling molecular phenotypes at targeted areas of the genome that are transcriptionally active. The detection of proteomic biomarkers may contribute to the advancement of ASD diagnostics and clinical management, and may uncover novel biomarkers that could improve the outcomes of individualized interventions. In a similar vein to the large bio-repositories established for ASD genetics, it is imperative that researchers globally have access to big data outputs obtained from metabolomic and proteomic studies of ASD subject's biological specimens. Studies to date have generally been small with a variety of samples tested (brain, saliva, serum, plasma and urine). Generally, we produce data from proteomic techniques by using one or two-dimensional gel electrophoresis (1/2DE) and liquid-chromatography mass spectrometry (LCMS) to analyse biological samples. Crucially in recent years, advances in computational power and techniques has allowed bioinformatics and machine learning platforms be used for a deeper understanding of the proteomic outputs (often extremely large datasets). This big data analysis allows new interpretations and can help determine relevant signals and patterns through automated techniques. To date, proteomic research (utilising a variety of tissues and bio fluids) has highlighted aberrations of synaptic vesicle regulation, myelination, energy metabolism (201), and altered expression of proteins associated with inflammation and regulation of the immune system (154, 201). Altered expression of proteins involved in lipid metabolism (154, 202, 203, 204), and the complement system (41) in ASD affected cohorts versus controls have been identified. Proteomics is clearly a promising yet evolving field, and studies to date have generally suffered from being small, and methodologically heterogeneous. Blood based analysis may be the most promising substrate given its ease of availability and storage, but questions remain over aspects of its suitability given the likely lack of brain specific proteins therein (blood-brain barrier impermeability). With larger populations, the use of big data technologies, correlation with clinical characteristics and the integration of a multi-modal omics platforms and approaches, proteomics and metabolomics can offer a real personalised insight into the granular detail of functional pathways and biomarkers of ASD.

This may allow for earlier ASD detection, intervention and even novel or personalised therapeutic strategies (204).

Important clinical, phenotypic and intervention aspects in Autism

Autism Diagnosis

As we have previously discussed in Table 1 and Figure 2, the autism diagnosis arises from the assessment of behaviours. The presentation is heterogeneous with varied behaviours, and often-significant phenotypic differences between affected individuals. In spite of this heterogeneity, the foundation of diagnosis is relatively concrete, encompassing the core clinical features of social and communications difficulties, and restricted or repetitive behaviours (Figure 2), features that are readily identified by experienced clinical practitioners using validated and established assessment tools. The latest diagnostic criteria in the DSM-V and ICS-11 improve on previous iterations in a numbers of ways:

- i. Use of the consolidated umbrella term ASD, incorporating Asperger's and PDD-NOS (pervasive developmental disorder – not otherwise specified), entities which were previously poorly defined.
- ii. The unification of social and communication criteria to a social-communication factor, reducing the number of broad categories from three to two.
- iii. The incorporation of sensory issues within the restricted and repetitive behaviours factor reflects the clinical experience of their ubiquity within ASD phenotypes.
- iv. Onset within the first 3 years of life, previously a requirement in DSM-IV, is no longer a condition. This modification accounts for the heterogeneity of age at diagnosis. We recognise that ASD symptoms can be well-masked (known as camouflaging) by children right up in to mid to late childhood (especially in girls) (205). Other ASD symptoms may not become apparent until the child encounters difficulties later in life, when external social demands exceed the child's limited capacity to deal with them (205).

The gold standard for ASD diagnosis encompasses two core assessments, the structured interview and both structured and unstructured observation of behaviour. The interview is conducted with the parents and involves a detailed developmental history, relevant family history, age at first concern, symptoms of concern, co-morbid issues etc. Preferably, the observations of child behaviour and social interactions should occur in home and in a peer group setting (typically school). Instruments commonly used to complete these assessments are the Autism Diagnostic Observation Schedule 2nd Edition (ADOS-2)(206) and

the Diagnostic Interview for Social and Communication Disorders (DISCO)(207). Typically, these instruments are used in an MDT setting with trained clinical personnel undertaking the assessments. While recent evidence suggests that the ASD diagnosis can be strongly fixed from as young as 14 months of age (208), there is a growing acknowledgement that ASD symptomology can fluctuate over time (209). Whether this is a biological effect in some cases of ASD or a treatment effect secondary to early intervention is yet to be determined. Finally, all children at the point of ASD diagnosis should undergo a comprehensive medical examination(210). Co-morbidity medical conditions are very common in ASD, as well as ASD arising in the syndromic context. Neurological and physical examination thus are important (head size, neurocutaneous syndromes) and may guide further investigations (brain imaging, genetics) especially in the context of co-existing intellectual difficulties or abnormal examination findings.

Autism Screening

ASD as a medical condition is an extremely important one, with a huge individual and societal cost (56). The prevalence (50), and possibly the incidence (211) of ASD has been increasing for decades. Yet as diagnostics and interventions have evolved over this time, access to services is often a critical issue for those affected by ASD and their caregivers. Many ASD services, especially those serving marginalised areas remain chronically underfunded (212), and some populations that are most at risk have the longest wait for a diagnosis or multi-disciplinary therapy (213).

Most recognised indicators of ASD in very early life are based on abnormal developmental attainment or recognition of subtle physical and behavioural signs (eye contact, proto-declarative pointing, responsive to name, social gestures and imitative play). There is evidence to suggest that symptoms of ASD may be apparent in infants as young as 6 months, and we know that the diagnosis is reliably stable from around 14 months (208, 210). Both these observations raise the issue of ASD screening in early childhood (214, 215).

Since 2015, the American Academy of Paediatrics (AAP) has recommended universal population based ASD screening in early childhood (216). This recommendation has remained, in spite of some reservations expressed by the United States Preventive Services Task Force (USPSTF) about the lack of data about the potential harms or benefits of such a universal screening scheme. Certainly from the parent's perspective, there is some evidence that early interaction with developmental services and increased knowledge of

child development outweighed any short term emotional distress triggered by ASD screening (even in false positive cases) (217). Regardless, screening may be particularly effective in higher risk groups such as those with ASD affected siblings or specific perinatal exposures (prematurity or neonatal infection). Given the strong evidence for the role of intrapartum infections in ASD risk, the ongoing COVID-19 pandemic perhaps represents an opportunity to follow closely, an important cohort of children whose mothers contracted COVID-19 while pregnant during the current pandemic (218).

In spite of some initial reservations about universal screening, it is clear that positive change can be brought about through increasing public and professional awareness, and early engagement with services. This should lead to downward revisions of the age at which ASD symptoms are recognised, at which ASD is diagnosed and when interventions are implemented, all leading to better outcomes.

Comorbid Psychiatric, Medical, Emotional and Behavioural Problems

Children and adolescents with Autism Spectrum Disorders (ASD) frequently suffer from co-existing clinical disorders (in up to 70% of autistic individuals) (210). In younger age groups, fine motor problems, epilepsy, restrictive eating habits and sleep problems are extremely common (219, 220, 221, 222). While in older paediatric cohorts, anxiety, tics, obsessive-compulsive disorder (OCD), attention deficit disorder (ADD), cognitive difficulties, dyspraxia, eating disorders and depression are all more prevalent (210, 223, 224, 225).

It is reported that children with ASD have an increased prevalence of both internalizing (emotional) problems, such as social difficulties, anxiety, depression, and withdrawal symptoms; and externalising (behavioural) problems such as attention problems, hyperactivity, and conduct disorders (226, 227). Researchers using the Child Behavior Checklist (CBCL) (a caregiver administered questionnaire that characterises internalising and externalising traits in children at different age groups) report that scores on the Social, Thought, and Attention problems scales were more than two standard deviations higher in a cohort of children with autism compared to a neuro-typical sample (228). A CBCL ASD profile, consisting of high scores on the Withdrawn, Social problems and Thought problems scales has also been previously described (229, 230). Children with ASD often exhibit maladaptive behaviours, defined as co-occurring, internalising and externalising behavioural problems that negatively affect everyday activities (231). These behaviours are sometimes more distressing to caregivers than the core autistic symptoms themselves

(232). EBPs can restrict remedial interventions, and negatively affect long-term outcomes of ASD affected children.

While other authors have highlighted the occurrence of emotional and behavioural problems (EBPs) in various ASD groups and at various time points, consensus on how and when EBP present and evolve over time is lacking (2, 3). Later in this thesis, we aim to illustrate the temporal evolution of EBPs in our ASD cohort using serial CBCL scoring, highlighting how EBPs development in early childhood (2 years) and how these maladaptive problems evolve over the important early childhood window. Early identification of these EBPs may help to identify children with subtle features of ASD (who have not been identified yet), with specific EBP syndromes (which can hamper education and socialisation), allowing effective and timely engagement with services or interventions.

Interventions and therapy

No two children with ASD are alike and we should tailor interventions as much as practicable to best suit the needs of each individual. Behavioural and speech therapy based approaches have shown the best results so far, with pharmacological treatment most useful in the management of comorbid conditions (seizures, depression, ADHD). The aim of treatment should be to maximise the individual's independence, to create a nurturing educational environment geared towards optimisation of their relative strengths, to manage treatable comorbidities, and to improve social and communicative abilities. We should also consider the family unit, with practical, financial, social and respite supports available as required and on an ongoing basis (233).

Early intervention should be prioritised, as young children with ASD can become socially isolated (even from their parents) as their ASD progresses. This can bring about impaired educational opportunities owing to the development of core ASD features and confounding comorbidities. Interventions in these early years may take advantage of optimal brain plasticity, which may confer additional benefit to interventions versus those instituted later in life (234, 235). There is emerging evidence that “prodromal” social and communication interventions can be effective when administered even before core ASD symptoms have developed (10).

The cornerstone of early interventions are based on early intensive behavioural intervention models, themselves originally derived from the core components of applied behavioural analysis (ABA) (cognition, language, sensorimotor and adaptive behaviours) (236). Newer interpretations such as the Early Start Denver Model (ESDM) give added

emphasis to parental involvement and improve upon developmental and relationship aspects, fostering a more holistic approach. This is a high intensity model delivered by therapists for 15 hours per week, with concurrent parental training. Children engaged with ESDM have demonstrated greater improvements in autism symptoms, intelligence, language, and adaptive and social behaviours versus standard community intervention groups (237).

Low intensity, parent administered approaches have also shown positive results, such as improved joint attention and joint engagement (238). Parents are tutored to have improved awareness of, and to become more attuned to, their child's communication style and signals. The aim is to facilitate joint engagement, practice positive parenting techniques and to improve social and communication skills in the child (238). These techniques can help frustration and low confidence levels experienced by parents who can experience great difficulty responding to their child's needs (237, 239). The Picture Exchange Communication System (PECS) can be used in nonverbal children with demonstrable improvements in functional communication (240), while targeted behavioural interventions (cognitive behavioural therapy (CBT)) can be effective in reducing anxiety and aggression in persons of all ages with ASD (241, 242).

Conclusion:

In summary, autism has proven to be a more complex entity than previously believed. ASD prevalence is increasing due to a mixture of biological and methodological factors. It is an extremely important disorder with a vast financial health burden for governments and a vast psychological one for family units and affected loved ones. Recent progress in our understanding of ASD aetiology is moving our scope beyond a purely genetic underlying cause. Environmental risks play an important role, and the variety of environmental risks implicated in ASD is broad. Already these risks have helped to illustrate how gene-environment interplay can affect immunity and fetal development, as well as offering potential for new therapeutic targets and interventions. Already the avenues of inquiry through which we may identify a reliable biomarker are expanding. The marriage of established techniques (ELISA, genomic sequencing), newer methodologies (proteomics, metabolomics) and technological solutions (machine learning, artificial intelligence) may present novel signatures or biomarkers of ASD which may be useful in identifying ASD antenatally or in early infancy thereby allowing early intervention.

In this thesis I aim, first, to investigate the role of maternal immune activation in autism aetiology using a variety of these techniques, with the expressed aim of identifying possible early onset or antenatal biomarkers of autism. Second, I aim to elaborate on the natural history of emotional and behavioural problems arising in autistic children and to assess their impact on children and their families.

Aims and Objectives:

The general aim of this thesis is,

1. To investigate, in a well-characterised cohort of ASD affected children, the potential contributory role of maternal immune activation and early protein or metabolic dysregulation in ASD aetiology; with the aim of identifying a potential biomarkers which may aid in the early identification of, and timely management of, the disorder.

A secondary aim of this work is,

2. To characterise the clinical features of this ASD cohort with a focus on the temporal development of emotional and behavioural comorbidities and the timing of their onset in this group.

To that end, I will achieve these aims via the following objectives:

- a. Examination of mid-gestation cytokine profiles in mothers of children with an ASD diagnosis at two mid-gestation time points (15 and 20 weeks) across two sites as part of a large multi-centre pregnancy study with the aim of identifying a potential gestational ASD biomarker.
- b. Validation of candidate maternal cytokines (including an ultrasensitive assay of interleukin 17) at a single specific mid-gestational time-point (20-weeks' gestation) in a carefully characterised expanded birth cohort.
- c. Discovery analysis of proteomic and metabolomic signatures in cord blood plasma and late childhood serum samples from the ASD cohort versus matched controls
- d. Characterise clinically the development of emotional and behavioural problems (EBPs) in our ASD cohort with an emphasis on the evolution of EBPs over time and the timing of onset.
- e. Development a maternal and child genetic biobank/repository of bio-samples taken from mother-child dyads enrolled in the study. Children included with ASD had either an early (<5 years old) or late (>5 years old) ASD diagnosis and samples were stored for interval RNA, genetic and HLA-G analysis.

f.

Thesis layout and chapter summary

The thesis contains seven chapters in total. In Table 2 (below), I have outlined the individual manuscripts created for each chapter. In addition to those noted and numbered (papers I – V), I have also written an introductory (Chapter 1) and discussion chapter (Chapter 7).

Table 2: Snapshot of thesis chapters and papers

Chapter	Paper	Question	Hypothesis	Methods	Key Findings
1	0	Introduction chapter and general review of the autism literature	Broad review regarding key facets of autism aetiology as well as important historical and clinical features.	Literature review	ASD aetiology is not fully understood. Novel tools are providing exciting opportunities to investigate the interplay of genes and the environment
2	I	Literature review, published article	The COVID-19 pandemic presents an ideal opportunity to investigate the effects of maternal infection and inflammation on foetal and early childhood development	Literature review	We outline literature concerning the role of maternal inflammation in ASD and review the emerging pathology of COVID-19 and its potential impact on child development.

3	II	Examination of mid-gestation cytokine dysregulation in mothers of children with ASD in two geographically remote cohorts	Mid-gestation cytokine dysregulation is linked with abnormal foetal development and childhood developmental disorders including ASD	Using multiplex ELISA technology, we examined mid-gestation cytokine concentrations in two distinct birth cohort groups (in Ireland & New Zealand). We compared the levels of a range of cytokines in mothers of ASD affected children versus controls.	Altered expression of the pro-inflammatory cytokine, interleukin-17 (IL-17), between groups. The ASD affected group displayed significantly higher levels of IL-17.
4	III	A more focused examination of cytokine dysregulation during pregnancy in mothers of children with ASD	Mid-gestation cytokine dysregulation is linked with abnormal foetal development and childhood developmental disorders including ASD	Using multiplex and ultrasensitive ELISA (specifically for IL-17), we examined mid-gestation cytokines in an expanded Irish ASD cohort. We compared cytokine levels in mothers of ASD affected children versus controls.	Altered expression of mid-gestation interleukin-4 levels (an anti-inflammatory cytokine). The levels of which were reduced in the ASD cohort versus matched controls
		Can an -omics approach highlight longitudinally persistent altered expression of proteins or	Given the heterogeneity within ASD symptomology and phenotypes, we cannot account for the origin of ASD by a single	Using a combination of proteomic (mass spectrometry), metabolomic (ultra-performance liquid	Our findings suggest in utero dysregulation of glycolysis, sulphur metabolism, mitochondrial and androstenedione networks, with dysregulated serum GAPDH,

5	IV (TBP)	metabolic molecules in children with ASD?	genetic mutation or environmental exposure. Combined methods capturing an array of metabolomic and proteomic signatures may be predictive of ASD	chromatography), and machine learning techniques we compared the protein and metabolome milieu of archived cord blood and late childhood samples in ASD affected children versus matched controls.	SELENBP1, and BLVRB persisting into late childhood in ASD
6	V (TBP)	At what age do emotional and behavioural problems (EBPs) arise in ASD cohorts, how do they change over time, and how are they characterised?	EBPs are important modifiers of outcome in children with ASD. They are, however, under-recognised by both parents and clinicians. Improved and earlier identification of EBPs may improve clinical outcomes in ASD affected groups.	We followed a population of children with proven ASD from the BASELINE birth cohort from birth until late childhood. We measured EBPs using the Child Behavioural Checklist (CBCL) at 2 years and 5 years, We compared scoring between the ASD group & matched controls.	EBPs are important modifiers of outcomes in children with ASD that develop in early childhood. Early intervention can improve outcomes with children with EBPs so early assessment for EBPs in children with ASD is warranted.
7	0	Discussion chapter		Summary and discussion of findings	Summary of findings, contextualisation of findings in literature, strengths & limitations of the thesis, future directions of research.
TBP = To be published at a later date					

Chapter 2

Paper 1: Literature and Narrative Review Article

Maternal Immune Activation and Interleukin 17A in the Pathogenesis of Autistic Spectrum Disorder; and why it matters in the COVID-19 era.

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Abstract:

Autism spectrum disorder (ASD) is the commonest neurodevelopmental disability. It is a highly complex disorder with an increasing prevalence and an unclear aetiology. Consensus indicates that ASD arises as a genetically modulated and environmentally influenced condition. Although pathogenic rare genetic variants are detected in around 20% of cases of ASD, no single factor is responsible for the vast majority of ASD cases or that explains their characteristic clinical heterogeneity. However, a growing body of evidence suggests that ASD susceptibility involves an interplay between genetic factors and environmental exposures. One such environmental exposure which has received significant attention in this regard is maternal immune activation (MIA) resulting from bacterial or viral infection during pregnancy. Reproducible rodent models of ASD are well established whereby induction of MIA in pregnant dams, leads to offspring displaying neuroanatomical, functional and behavioural changes analogous to those seen in ASD. Blockade of specific inflammatory cytokines such as interleukin-17A during gestation remediates many of these observed behavioural effects, suggesting a causative or contributory role. Here we review the growing body of animal and human-based evidence indicating that interleukin-17A may mediate the observed effects of MIA on neurodevelopmental outcomes in the offspring. This is particularly important given the current COVID-19 pandemic as SARS-CoV-2 infection during pregnancy is a potent stimulator of the maternal immune response, however the long-term effects of maternal SARS-CoV-2 infection on neurodevelopmental outcomes is unclear. This underscores the importance of monitoring neurodevelopmental outcomes in children exposed to SARS-CoV-2-induced MIA during gestation.

Introduction:

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterised by a spectrum of deficits in social interactions and communication combined with stereotypical and repetitive behaviours. Up to 50% of those affected can have intellectual disability (ID) and limited verbal communication (1-3). In recent decades, the prevalence of ASD has consistently increased from approximately 1 in 1,000 in the 1960s (4), to 1 in 44 today in the United States (5). Increasing prevalence may in part, be explained by changes in reporting practices, increased recognition of ASD symptoms, broadening of the ASD diagnosis (1), and improved accessibility to services (6, 7). A significant ratio of 4:1 from male to female still exists with markedly differing prevalence rates between the sexes, 1/38 in males and 1/151 among females (8). Although genetic susceptibilities are recognised, the mechanism of disease development is unknown and does not follow a clear pattern of inheritance (9, 10). This suggests possible mediation by additional unknown biological or environmental factors (11). Both common and rare genetic risk factors have been identified with more than four hundred diverse genes now linked to ASD. Singly, these genetic factors each convey only a modest increase in ASD risk (~1%), however collectively they can contribute to a far greater risk (12, 13). Up to 20% of individuals with ASD may possess copy number variants (CNV) and de novo loss of function single nucleotide variants (SNV) that are individually rare but in combination, increase an individual's ASD risk (12). While newer methods of genetic analysis (such as whole genome sequencing) are uncovering new candidate genes with regularity (14), the heterogeneity of the clinical and phenotypic groups within ASD strongly suggest that in those with a genetic predisposition, environmental factors may act in concert to bring about a multisystem dysfunction leading to ASD. A well-characterised environmental factor known to impact early fetal brain development and increase ASD risk is maternal inflammation during pregnancy, which is commonly called maternal immune activation (MIA). Numerous epidemiological studies have linked gestational infections with elevated risk of ASD in offspring (15-17), and animal models of MIA have simulated gestational infection resulting in MIA-induced neural and behavioural abnormalities analogous to those seen in ASD (18-20).

Focused early intervention in young children with ASD has been shown to result in normalized patterns of brain activity, and is associated with improved functional outcomes and reduced morbidity (237, 243). Most children affected by ASD can have a reliable and stable ASD diagnosis from as early as 14 months of age (208), yet in spite of this, the average age of ASD diagnosis is closer to 5 years (244, 245). Numerous studies sought to identify

blood-based biomarkers of ASD in affected adolescents and adults (120, 154) and have reported alterations of molecules involved in iron transport (155), inflammation (28, 156), brain development (157), and metabolism (158). None to date has identified and validated reliable mechanistic biomarkers with the ability to improve ASD detection in the crucial early developmental period. Multiple descriptive ASD biomarkers such as characteristic MRI brain findings, abnormalities of gaze preference on eye tracking or characteristic EEG findings in infants with ASD; show promise in terms of aiding earlier ASD detection. However, none is directly involved in the pathogenesis of ASD and arises of the condition rather than contributes to it. The infant brain doubles in volume over the first year coinciding with maximal neuroplasticity and synaptogenesis. Recognition of an early mechanistic biomarker gives us the best chance of implementing strategies during this critical early childhood window allowing ASD diagnosis and intervention at the earliest possible stage.

Here we highlight recent research in this area, both from pre-clinical animal studies and epidemiological human studies, along with a proposed mechanistic pathway, that we can encourage other research groups with access to suitable maternal-child cohorts to examine this question. We encourage researchers to look at the prospective study of children born during the COVID-19 era, when their gestations may have been complicated by mild or even asymptomatic SARS-CoV-2 infection. Otherwise, the long-term effect, if any, of COVID-19 on the fetal brain could remain unknown for years to come.

Inflammation, viral infection and ASD: What are the implications of the COVID-19 pandemic?

There is growing scientific evidence that aberrant immune activation occurs in ASD (119, 120) based on studies of autistic children and young adults (27, 121). As early as 1971, Stella Chess reported ASD cases associated with the 1964 Rubella outbreak in the United States (246), and in a 1977 follow up study, Chess et al. quoted ASD prevalence rates of 8 to 13% in children of mothers who were infected during that outbreak (125). Large epidemiological studies indicate that conditions such as maternal autoimmune disorders and mid-trimester viral infections that trigger gestational pro-inflammatory states (i.e. MIA), are linked with elevated ASD, schizophrenia and bipolar disorder risk in offspring (113, 124, 125, 126). More recently, a range of conditions associated with proinflammatory states in pregnancy such as obesity, psychosocial stress and pre-eclampsia were associated with increased ASD risk in children (145, 146). Thus, gestational maternal immune activation (MIA) appears to play a role in the pathogenesis of the ASD phenotype in exposed offspring.

Maternal Immune Activation and neurodevelopmental outcomes.

We define MIA as a triggering of the maternal immune system by infectious or infectious-like stimuli resulting in an increase in measurable inflammatory markers during pregnancy (148, 149). MIA has been most commonly simulated in preclinical rodent, murine and nonhuman primate (rhesus macaque) animal models by Poly (I:C) (polyinosinic-polycytidylic acid) or LPS (lipopolysaccharide) injection which respectively model viral and bacterial infection (24, 26, 123). Poly (I:C) is a synthetic analogue of double stranded RNA, mimics the effects of viral infection (168). The triggered immune response results in offspring with behavioural, immunological, and neurological abnormalities that approximate to autistic symptoms observed in humans, notably, impaired sociability and repetitive behaviours (24, 142, 247). Offspring born to poly (I:C) treated dams have consistently, across all exposure categories (administration of varying doses of poly (I:C) and at varying gestations), shown impairment of social interaction, this is manifest as reduced communication in ultrasonic vocalisations which are usually triggered by separation from the dam in the first two postnatal weeks. Marble burying, a well-recognised behavioural paradigm to measure repetitive behaviours in rodents, again is consistently increased in murine offspring following poly (I:C) treatment (248). These offspring have proven useful in pre-clinical etiological studies as well as identification of therapeutic targets.

Cytokine dysregulation may play a causative role in observed neuronal dysfunction in pre-clinical models of MIA (142, 151, 169). In a recent study, Choi et al convincingly demonstrated that simulated MIA in murine models leads to elevation in maternal IL-6, which in turn activates maternal Th17 cells. These maternal Th17 cells produce IL-17, which is thought to cross the placenta triggering increased expression of IL-17AR in the fetal brain and leading to cortical malformations and behavioural abnormalities (24, 160). These malformations parallel abnormalities found in brain development in children, adolescents and adults with ASD (249, 250). Poly (I:C) treatment also leads to raised IL-17A mRNA levels in placental tissue of these mice (24). Through inhibition of IL-6 and IL-17A signalling with antibody blockade of the IL-17A cytokine, Choi et al also determined that a sustained increase in IL-17A expression seemed to be pathogenic in ASD, as IL-17A blockade prevented the development of ASD-like phenotypes (24). Specific behaviours in mice which model core diagnostic features of ASD (including repetitive burying and increased neonatal ultrasonic vocalisations (USV)) were normalized in the previously MIA-exposed offspring (251, 252).

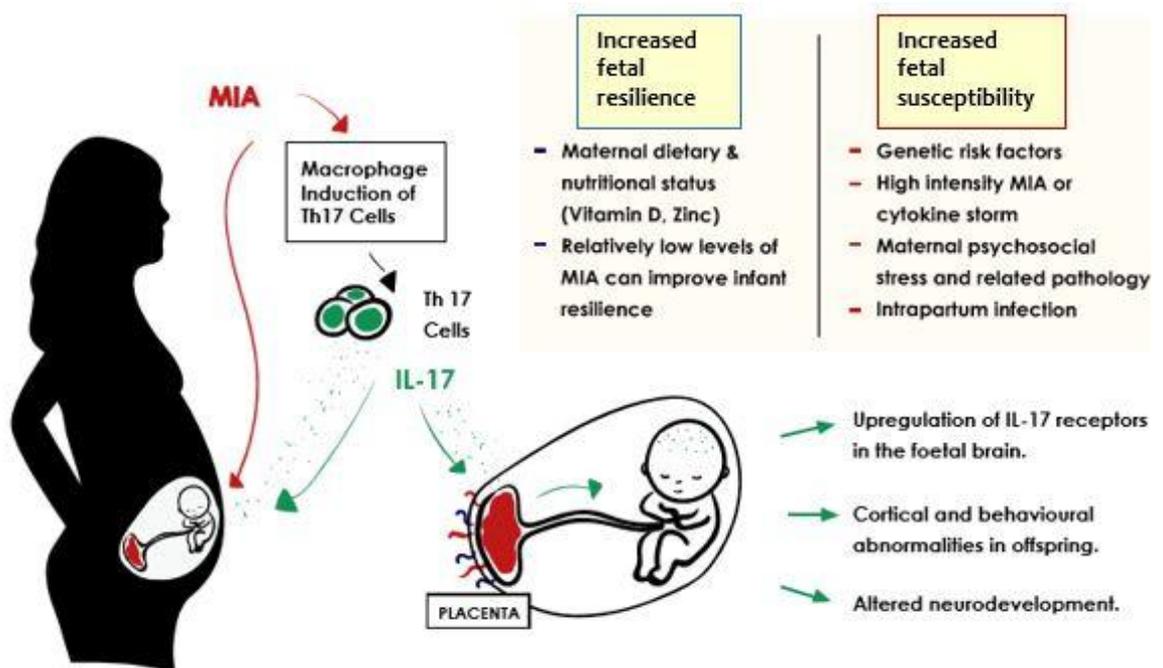


Figure 6: Potential outcomes in the inflammation-exposed fetus in the context of MIA related IL-17 induction

Improved fetal resilience is associated with lower intensity of maternal immune activation. ASD risk after prenatal exposure to maternal fever has been found to increase in a dose dependent manner (129, 253) and similar effects were identified in animal models of MIA (254). A balanced maternal diet seems to contribute to improved fetal resilience also (167, 255, 256). Exposure to relatively higher grades of immune activation via high intensity MIA (146), intrapartum infection (257, 258) and genetic

risk factors lead to reduced fetal resilience, and increased likelihood of unfavourable developmental outcomes.

Alterations in cytokine expression in human studies:

While many studies have examined the cytokine profiles of individuals with ASD, only a very limited number of studies to date have examined mid-gestation cytokine levels in mothers of children who subsequently develop ASD. Three studies retrospectively analysed maternal blood sampled during pregnancy. A 2017 study by Jones et al, reported elevated mid-gestation cytokines and chemokines in mothers of children with ASD associated with ID, and particularly early onset ASD (as defined by the authors as early or sustained delays in language or social skills, and excluding those showing clear skill regression) (34).

Dysregulation was noted in a number of cytokines including interleukins IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, and IL-17A between 15 and 19 weeks' gestation. An earlier study noted elevations in mid-gestation serum IL-4, IL-5 and IFN-gamma levels in mothers of ASD affected children (33). While, more recently, Irwin et al (2018) demonstrated alterations in IL-4, MCP-1 and IL-10 levels in 28-week gestation serum of mothers who birthed ASD affected children (181). Other authors have examined amniotic fluid at mid-gestation and found elevated levels of IL-4, IL-10, TNF- α and TNF- β in ASD patients versus controls (182). Yet, amniotic fluid cytokine concentrations are more indicative of the fetal immune state rather than the maternal state (259, 260). In Table 3, we outline a number of the cytokines most frequently found to be dysregulated in the serum or cerebrospinal fluid of ASD affected individuals, and gestational serum and amniotic fluid samples from mothers of ASD affected children.

Table 3: Cytokine dysregulation in ASD affected individuals and in gestational serum and amniotic fluid samples of mothers with ASD affected offspring

Cytokine	Category	Altered in blood/CSF of ASD individual	Altered in gestational blood	Altered in amniotic fluid	Cytokine characteristics Relevance to ASD
TNF α	Pro-inflammatory	(156, 261, 262, 263)	(34)	(182)	Apoptosis of infected cells. Elevated in the CSF and blood of ASD affected individuals (156, 261, 262).
IL-1 β	Pro-inflammatory	(156, 261, 264, 265)	(34)		A potent pro-inflammatory cytokine involved in both acute and chronic inflammation. Correlated with ASD symptom severity (121).
IL-6	Pro-inflammatory	(156, 261, 263, 264, 265, 266, 267)	(34)		Induces production of acute phase proteins and stimulates B-cell antibody production (268). Pleiotropic (affects hematologic, hepatic, endocrine and metabolic function). Thought to impact synapse formation and neuronal migration (269). Potentially mediates IL-17 linked ASD risk in pregnancy (24, 142)
IFN γ	Pro-inflammatory	(120, 156, 266)	(33, 34)		Interfaces between innate and adaptive immune response. Secreted by NK cells, and promotes NK killing. Activates macrophages, which produce IL-12 and -23, stimulating Th1 and Th17 cell respectively. Inhibits Th2 cells. Versatile, with a role in defence against intracellular

				pathogens, tumour surveillance, autoimmunity, allergy and the protection of the amniotic space during pregnancy (270).	
IL-17	Pro-inflammatory, Chemotactic	(27, 29, 156, 263, 267, 271)	(34)	Derived from Th17 cells, a subset of CD4 cells. Potentiates the innate PMN response throughout inflammation. Postulated to trigger alterations in the blood brain barrier and lead to cortical dysplasia (142).	
IL-4	Pro-/Anti-inflammatory, Allergy	(265)	(33, 34, 181)	(182)	A Th2 derived cytokine, often linked with asthma and allergic type inflammation (119). Dual role: pro/anti-inflammatory properties. Crucially important in mitigating inflammation during pregnancy (primarily through suppression of Th1 T-cells and associated cytokines (IL-2 and IFN γ)).
GM-CSF	Growth factor	(272)	(34)	A colony-stimulating factor. Produced by stromal cells, it targets bone marrow, and precursor cells, mediating haematopoiesis.	
IL-8	Chemotactic	(30, 264, 266)	(34)	Produced by fibroblasts, neutrophils and macrophages. Chemo-attractant for phagocytes at site of inflammation.	

Note: The numbers in parentheses indicate the relevant references

A growing body of evidence supports a role in ASD pathogenesis for Th17 cells and their product cytokine, IL-17A (See Figure 6 (271, 273)). The IL17A gene itself has been identified by a small genome-wide CNV study to amplify copy number variants (CNVs) in ASD affected cohorts (274). Elevated levels of IL-17A have been reported in the blood of ASD affected individuals, and these correlate positively with severity of ASD behavioural symptoms (27, 34, 271). Yet, others have found high concentrations of IL-17A in individuals affected by obesity or high BMI (275), both of which are more likely in ASD groups (276). This is a potential confounder for any retrospective cohort based study designs.

STRING analysis (Figure 7 (277)) indicates that IL-17A has proven or predicted interactions with IL-2, IL-6, IL-10, IL-13, IL-17F, IL-17RA, IL-17RC, CTLA4, STAT3 and STAT6. Each of these proteins have been previously reported to have altered expression in children with ASD, as outlined below. Of these, the most persistently described, and hence, potential key player is IL-17A, along with its receptor IL17RA and receptor complex, IL17RC.

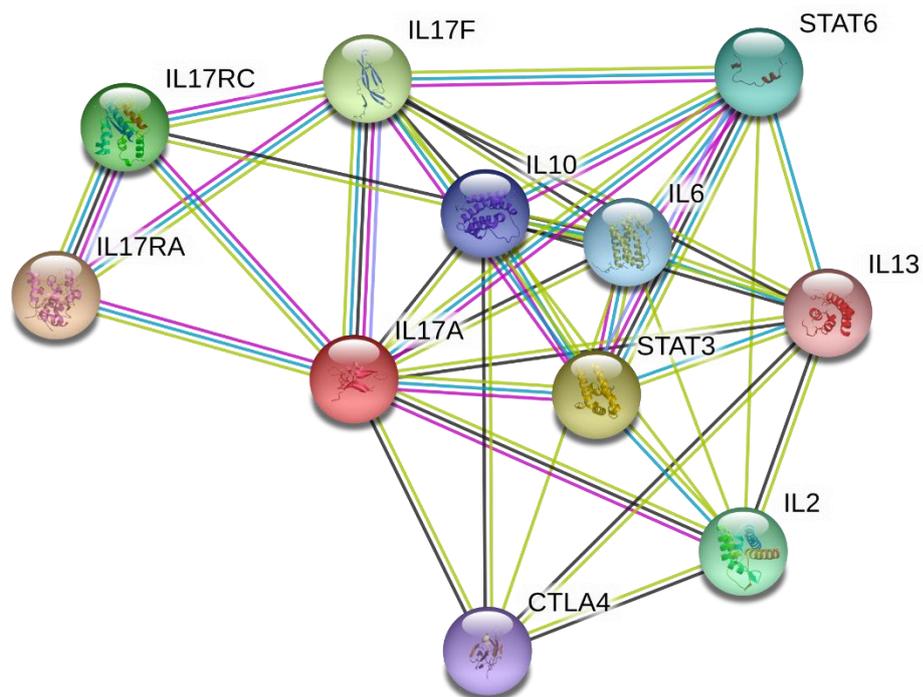


Figure 7: STRING diagram illustrating the known and predicted protein IL-17A interactions

Network nodes represent proteins - each node represents all the proteins produced by a single, protein-coding gene locus. Edges (lines) represent protein-protein associations that are specific and meaningful, i.e. proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding each other. Blue connecting lines indicate that protein interaction information

was derived from curated databases, pink indicates the interaction was experimentally determined, yellow indicates the interaction was determined via text mining, black indicates protein co-expression, and lilac indicates protein homology. Analysis was performed on 28 July 2021 via the string-db.org domain.

IL-17A associated pro-inflammatory mediators in ASD:

Upregulation of pro-inflammatory pathways has been persistently associated with ASD. IL-6 is a versatile cytokine, with multiple functions throughout the body. It plays roles in immunity, inflammation, hematopoiesis and oncogenesis. IL-6 works to promote pro-inflammatory Th17 cells (IL-17 producers) and to downregulate anti-inflammatory Treg cells (regulatory T-Helper cells) (278, 279). Th17 cells produce cytokines that cross the placental barrier (151). This transplacental effect has been well characterised with IL-6, which was shown to alter offspring behaviour and brain development (161, 280).

Like IL-17A, IL-17F is also produced by Th17 cells (281). IL-17F is reported to be involved in the regulation of proinflammatory gene expression and responses (282). IL-17RA and IL-17RC are both members of the IL-17 receptor family. In order for IL-17A (or indeed IL-17F) to have biological effects on tissues, IL-17RA must be present (281). IL-17RA is expressed in immune cells, and some children affected by ASD appear to possess higher levels of this receptor compared to neuro-typical controls (283). IL-17RA blockade may reduce monocyte associated oxidative stress which may improve neuro-inflammation associated with ASD (283). IL-17RC is also essential for the formation of the IL-17 receptor complex (284). IL-17RC levels in neutrophils are raised in children with ASD compared to neuro-typical controls. In fact, expression of this receptor (mRNA and protein) was completely absent in a cohort of neuro-typical children. The presence of both IL-17A receptor subunits in ASD patients may magnify the effects of IL-17A resulting in an autistic phenotype (285).

The transcription factor STAT3 (signal transducer and activator of transcription 3) is a key player in the development of T helper cells and regulates the expression of the T helper cell specific transcriptional regulator – retinoic acid receptor related orphan receptor γ -t (ROR γ t) via IL-6 (286, 287). IL-6 is a potent driver of ROR γ t activity. ROR γ t is exclusively found in lymphoid cells such as Th17 cells (CD 4 helper cells), and is required for differentiation of Tregs to Th17 cells (287). STAT3 proteins occur at elevated levels in the peripheral blood mononuclear cells (PBMCs) of children affected by ASD (288). Inhibition of STAT3 mitigates MIA associated behavioural and immunological abnormalities seen in animal models (169), while ROR γ t KO models reverse outcomes in MIA exposed mouse pups (24).

Lastly, IL-13 is a cytokine derived from T cells, which has both inflammatory and anti-inflammatory properties. IL-13 inhibits the production of other inflammatory cytokines (IL-1 α , IL-1 β , IL-6) through its effects on inflammatory macrophages (289). IL-13 is recognised as a key driver in allergic and inflammatory airway disease, where its effects are potentiated by IL-17 (290). Raised IL-13 has been noted in the plasma and PMBCs of children affected by ASD (156, 291), particularly those with comorbid asthma (although IL-13 is known to be skewed in those with co-morbid atopic conditions) (292).

IL-17A associated anti-inflammatory mediators in ASD:

Another member of the STAT family, STAT6, suppresses the IL-17A inflammatory response. In certain conditions, STAT6 signalling attenuates IL-17A producing T-cells, reducing their production of IL-17A (293). IL-4 mediated inhibition of Th17 cells and IL-17A production is STAT6 dependent (294). In human studies, children with ASD reportedly have reduced levels of STAT6-expressing CD45 cells (CD45⁺STAT6⁺) in their PBMC profile compared to neuro-typical controls (295). STAT6, as part of the IL-4 signalling cascade can enhance the expression of anti-inflammatory mediators. This pathway is critical for acceptance of the fetal graft, through reduction of Th17 cells and increase of both IL-4 and Tregs in the fetal environment (114, 296).

In addition to downregulation of the STAT6 mediated pathways, downregulation of other anti-inflammatory cytokines is also reported in autism. Anti-inflammatory cytokine IL-10 acts as a “master” immuno-regulator (297) and IL-10 concentrations are significantly lower in ASD children compared with neuro-typical controls (298, 299). Cytotoxic T-lymphocyte antigen 4 (CTLA4) is a glycoprotein located on T cells (300) and is induced following T cell activation. This anti-inflammatory molecule is expressed at lower levels in the peripheral blood mononuclear cells (PBMCs) of children with ASD (301). Reductions in the levels of these anti-inflammatory and regulatory proteins may lead those with ASD to acquire a more pro-inflammatory state.

Linking immunity and genetics in ASD.

Bioinformatics analysis of large CNV studies suggest strongly that innate immune processes are implicated in ASD risk (83), this may indicate that immune dysfunction in ASD may be genetically driven or influenced. MIA downregulates expression of susceptibility genes known to be highly penetrant in ASD and heavily involved in neurogenesis, cell signalling, synaptogenesis and axonal guidance in the early stages of fetal development (83, 95). When compared with curated ASD associated gene sets (e.g. via the SFARI Gene database (<http://gene.sfari.org/>), MIA downregulated genes were substantially enriched. The strongest enrichment of MIA downregulated genes was observed in the ASD gene categories with the highest likelihood of a link to ASD i.e. SFARI “High Confidence” or “Syndromic” ASD gene sets. This suggests that MIA may bestow increased ASD risk through downregulating the expression of the same genes that are highly penetrant in ASD during the early stages of fetal development.

Loss of function mutations in TSC1 and TSC2 genes are linked to syndromic ASD, and these genes are critical upstream regulators of the mTor pathway. mTor has important functions in innate immunity and metabolism in particular (186, 187, 188).

MIA also has downstream effects, in some cases influencing the transcriptome rather the genes themselves. FMR1 and CHD8 are both highly penetrant genes for ASD, yet MIA does not seem to influence expression of these genes directly. Rather, it wields an influence on downstream gene targets such as FMRP (fragile X syndrome protein complex). This raises the possibility that MIA may act as an environmental factor disrupting crucial early developmental genomic pathways through influence on downstream gene targets (83). This might suggest that MIA could act both in a direct (genetic) and indirect fashion (epigenetic/regulatory) with the end effects converging on similar pathways.

As previously, mentioned, normal pregnancy is associated with suppression of immunity, allowing the fetus to develop inside the mother’s innate immune system. HLA-G antigen recognition controls the placental immune response and allows acceptance of the fetal graft. HLA-G interacts with the CD8 cell surface antigen found on most cytotoxic T-lymphocytes that mediate efficient cell-cell interactions within the immune system (302). Higher rates of HLA-G mutations have been found in mothers of children with ASD (133). The Th17 pathway in particular has been identified as a likely effector of inflammatory changes on the developing fetal brain, with downstream effects on behaviour and cognitive

development (142, 303). We hypothesize that the physiological changes in maternal immunity during pregnancy are dysregulated in some mothers of children with ASD.

Genome-wide association studies (GWAS); comparing cases to controls, represent the gold standard for identifying common genetic risk variants for multifactorial disorders like ASD. Until now, GWAS have been limited largely by their small sample sizes. Extremely large samples are needed to find robustly associated risk variants, increasingly; researchers are examining larger and larger GWAS numbers by accessing population-based cohorts. A recent study linked four immune related genes with ASD traits seen in the general population such as rigidity and attention to detail. This raises the possibility that ASD candidate genes may be associated with ASD symptomology, even in the general population. This may allow gene studies on a population basis rather than specifically in ASD cohort, greatly increasing power in these studies (304).

Many of the inflammatory proteins reported to have altered expression in ASD are linked to pro-inflammatory Th-17 cells, their product IL-17A, and the IL-17 receptors and receptor complexes. It appears that IL-6 activation (regulated by STAT3 and STAT6 via ROR γ t activity) of IL-17 expression, and subsequent upregulation of IL-17 receptors and receptor complexes may have a key role in the pathogenesis of ASD. The majority of linked molecules identified above are pro-inflammatory and found in higher quantities in those with ASD, with a corresponding downregulation of anti-inflammatory proteins. Whether this dysregulation of IL-17 is an inherent or acquired state is unclear.

Circulating T cell and IL-17A levels are altered in a subset of children with ASD. MIA (including IL-17A) seems to play a role in altering important developmental pathways through direct interaction with ASD susceptibility genes, and indirectly, through interaction with their gene products. Circulating levels of IL-17A are dysregulated during pregnancy in mothers of children who develop ASD and ID (34, 271, 274). Murine models support a causative role for IL-17A in the pathogenesis of ASD. We conclude from the existing evidence that IL-17A dysregulation in the mother or developing infant could play a causal role in the development of at least some subsets of ASD and may be the link between environmental exposure and genetic susceptibility. Understanding the role of IL-17A and its associated targets on neurodevelopmental outcomes is now becoming increasingly important.

What is the relevance of the ongoing COVID-19 pandemic to MIA-induced ASD risk?

Coronavirus disease 2019 (COVID-19), a disease caused by the novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a pandemic, affecting every corner of the globe. Although, the disease (COVID-19) affects primarily the respiratory systems of those affected, it has been found to affect and damage other organs, including the kidneys (305), liver (306), brain (307, 308) and heart (309, 310). Worldwide reported cases and COVID-19 related mortality are most likely an underestimate due to variability of public health capacities between countries, but as of August 2021, there have been almost 200 million confirmed cases of COVID-19, and over 4.2 million deaths reported to the WHO (311).

Our current knowledge of COVID-19 is based only on our limited experience with SARS CoV-2 since December 2019 and analogously, through our experience of other coronaviruses (SARS CoV and MERS, Middle East Respiratory Syndrome). The long-term consequences of *in-utero* SARS-CoV-2 exposure and/or congenital infection are almost entirely unknown. There is clear evidence that prenatal exposure to viral infections increases the risk of adverse developmental, neurological and psychiatric outcomes in later childhood and adult life (25, 26, 113). In this next section, we discuss the implications of the COVID-19 pandemic in the context of MIA-induced alterations in neurodevelopmental outcomes.

COVID-19 and cytokine storm:

Preclinical work shows that MIA, which stimulates interleukin-17A release from Th17 cells, can establish sustained fetal-placental inflammatory responses. This inflammatory milieu can persist into childhood and affect the development of the young “primed” brain. Remarkably, in murine models, social difficulties in MIA-exposed offspring are remediable through a variety of mechanisms including IL-17 blockade (24, 142). Cytokine storm is a general term applied to maladaptive cytokine release in responses to infection and other stimuli (312). In the context of sepsis, cytokine storm is considered one of the major causes of acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS) and multi-organ failure (313, 314). In COVID-19, cytokine storm seems to play a role in disease aggravation and correlates positively with severity of disease (315). IL-17A target IL-6 and C-reactive protein (CRP) specifically, have been shown to correlate positively with increased mortality (316). Elevated numbers of Th17 cells have been isolated in the blood of individuals with fatal COVID-19 infection (317), while many authors have demonstrated significantly elevated levels of IL-17A in those with both mild and severe COVID-19 (318, 319, 320). Coronavirus infection results in macrophage, and dendritic cell activation and IL-6

release (321). This instigates an amplification cascade (JAK – STAT1/3 pathway) that results in cis signalling (binding of cell membrane bound IL-6 receptors) in lymphocytes with downregulation of Tregs and increased differentiation of TH17 cells; as well as trans-signalling (binding of soluble IL-6 receptor) effects on many other cell types (endothelial cells). This widespread immune activation and cytokine production contributes to the pathophysiology of severe COVID-19 (322). Indeed, some authors have specifically suggested therapies intended to target both Th17 cells and IL-17A in COVID-19 disease (323, 324). We have already outlined how Th17 specific (T-helper 17 cell) pathways are initiated via activated macrophages that produce IL-6 and IL-1 β . As outlined, IL-6 in particular, is a potent potentiator and trigger for IL-17A release (312, 323, 325). IL-17A therefore, may be a key player in the COVID-19 cytokine storm.

Coronavirus (SARS-CoV-2) neurotropism and neurological effects:

Coronaviruses have a demonstrated specific neuro-tropism that allows them access to, and to proliferate in, the host's CNS (326, 327). Cell entry seems to occur through the angiotensin-converting enzyme – 2 (ACE-2) and transmembrane protease serine 2 (TMP S2) receptors, both of which are widely expressed in the placenta and at the feto-maternal interface. While trans-placental infection of the fetus is, yet to be proven conclusively, vertical transmission is certainly plausible and may lead directly to inflammatory processes in the fetal brain, in addition to indirect effects via the host/ maternal immune response. The neurological sequelae of COVID-19 are wide-ranging and relatively common. The majority of neurological presentations so far have fallen into 5 categories, (i) Encephalopathy (including delirium and impaired consciousness), (ii) Inflammatory CNS disorders (including encephalitis and Acute Disseminating Encephalomyelitis (ADEM)), (iii) Cerebrovascular accident/stroke, (iv) PNS disorders (including Guillain-Barré Syndrome and cranial nerve palsies, (v) "Miscellaneous" central neurological disorders (such as raised intracranial pressure, seizures, and myelitis) (328). Hyposmia/Anosmia and hypogeusia (329) are recognised as 2 important hallmarks of acute SARS-CoV-2 infection, while more severe neurological complications have included cerebrovascular accidents (CVA), encephalitis, encephalopathy and neuropsychiatric disorders (308, 330). Protein-protein network analysis for Guillain-Barre syndrome (GBS) and COVID-19 revealed that the combined gene set showed an increased connectivity as compared to COVID-19 or GBS alone, this was particularly true of genes related to Th17 cell differentiation. Transcriptome analysis of peripheral blood mononuclear cells (PBMC) from patients with COVID-19 and GBS demonstrated the activation of interleukin-17 signalling in both conditions (331). Viral RNA

has been isolated in clinical CSF samples in those with COVID-19 and neurological symptoms (332), and post-mortem examination of brain tissue has identified both viral RNA and neutrophilic infiltrates suggestive of aberrant immune response (333).

Recent pluripotent stem cell derived organoid models have been used to model SARS-CoV-2 infection in a wide range of tissues including gut, lung, liver, kidney and brain (307, 334). These models demonstrate the virus' ability to infiltrate and proliferate in a variety of different cell/tissue types. Within the brain, the areas with the highest avidity for SARS-CoV-2 are the choroid plexus and the hippocampus (307). This is an interesting finding, as the choroid plexuses themselves represent the interface between cerebrospinal fluid (CSF) and blood compartments (in a similar fashion to the blood-brain barrier). They are located in each of the four ventricles, and are intimately related with immediately adjacent CSF, capillary blood supply and neural tissue. ACE-2 receptors also appear to be highly expressed in the choroid plexus (335). In this sense, they provide a comprehensive roadmap upon which SARS-CoV-2 can potentially travel. The neurological features on COVID-19 infection are diverse and wide-ranging. Most studies to date have focused on symptomology in adult patients, but novel models of SARS-CoV-2 infection in a variety of human and animal tissues is casting new light on the mechanisms underlying COVID's infectivity and its ill-effects. There appears to be a variety of mechanisms underlying COVID's pathogenicity, not limited to direct viral effects on tissue, but also collateral effects via immune and thrombotic processes (336). Although there is little research on the effects of COVID on fetuses in early pregnancy, the same processes of direct viral effects and secondary immune and inflammatory effects are likely to be at play.

Maternal COVID-19 infection and perinatal exposure:

Pregnant women are not thought to be more susceptible to contracting coronavirus than the general population (337), but given alterations in the pregnant immune state (114), they may be more susceptible to more severe infection (338, 339). Studies from previous pandemics, H1N1 influenza (2009), SARS (2003) and MERS (2012), suggest the possibility of significant maternal and neonatal morbidity and mortality (340, 341). Indeed, both MERS and SARS resulted in maternal death in a significant number of cases, but the specific risk factors for a fatal outcome during pregnancy are not clear. Our experience with these previous coronaviruses indicates higher risk of adverse outcomes for the fetus and infant including fetal growth restriction (FGR), and preterm delivery, both of which have previously been linked to increased ASD incidence (342) as well as NICU admission, spontaneous abortion and perinatal death. As with other Coronaviruses, maternal SARS-CoV-2 infection has been

associated with negative perinatal outcomes. Preterm delivery, fetal distress, stillbirth and perinatal death have been widely reported (339, 343, 344, 345). Figures from China show that while up to 3% of pregnant women infected with COVID-19 required admission to intensive care (346, 347), a UK study showed 1% of pregnant women admitted with SARS CoV-2 required ECMO (Extra-corporeal membrane oxygenation) and 10% Intensive Care Unit (ICU) management (348).

Caesarean section (CS) has been implicated as a risk factor for the development of ASD in offspring. The mechanisms underlying this are unclear, yet the risk of ASD is increased by approximately 33% in both elective and emergency Caesarean section procedures (349). In a systematic review of perinatal and maternal outcomes during the pandemic, Caesarean section rates were reported at extremely high levels, up to 90% in some centres (range from approximately 50 – 90%) (350). For comparison in work published in 2020, Turner et al noted an all-cause national Caesarean section rate in Ireland of approximately 26% (351). These higher rates were observed in most centres despite recommendations from the Royal College of Obstetrics and Gynaecology (RCOG) and the International Federation of Gynecology and Obstetrics (IFGO) against decisions for CS being influenced by maternal SARS CoV-2 status.

More specifically to neonatal outcomes, the WHO quotes worldwide preterm delivery rates of approximately 10% (352). Two large review studies reported preterm delivery rates of 20 – 25% in SARS CoV-2 affected pregnancies (353, 354). Women with SARS CoV-2 seemed to be more likely to endure a preterm delivery (354). The majority of these deliveries were iatrogenic, but in some reviews, up to half were attributable to either fetal or maternal compromise (355).

Maternal and neonatal ICU admission rates were also higher in the SARS CoV-2 affected cohorts. Maternal ICU admission and mechanical ventilation rates were high versus age matched non-pregnant women (354). While rates of stillbirth and neonatal death appear similar to uninfected fetuses, NICU admission rates were notably higher in COVID affected pregnancies (348), commonly as a precautionary step in the care of the neonate. Neonatal morbidity was higher in the SARS CoV-2 affected groups and was associated with preterm delivery in mothers with more severe COVID-19 primary infection. Hypoxaemia and respiratory difficulties in mothers had knock on effects of reduced placenta perfusion, pre-placental hypoxemia, fetal distress, and preterm delivery (356).

Given our knowledge of the potential developmental effects of Th17 activation in pregnancy, children in utero during this pandemic may have significant inflammatory exposures if maternal infection occurs. There remain unanswered questions about the impact that asymptomatic and mild maternal infection has on the fetal brain in early pregnancy. Prospective follow up studies will need to follow infants whose mothers were infected as well as health unaffected controls. There is enormous potential to leverage archived serological samples from pregnancy and neonatal cohorts to study the relationships (or associations) between markers of maternal inflammation and later neurodevelopmental outcomes in offspring born during the pandemic. While in general, the likelihood of intrauterine maternal-fetal transmission of coronaviruses is low—there have been no documented cases of vertical transmission occurring with either SARS or MERS. There are current reports of possible vertical transmission of SARS-CoV-2 in several cases of third trimester maternal infection (357, 358, 359). Little to no information exists about children exposed in the 1st and 2nd trimesters yet. While generally placental seeding does not seem common, some cases have reported strong evidence of placental infection with the demonstration of high viral load and immuno-histological evidence of SARS-CoV-2 in placental tissue (357). Currently, we can only surmise what the true effect (if any) of gestational COVID-19 on the incidence of ASD will be, but already some have concerns that the incidence may increase (360, 361). No studies have yet been reported on neurodevelopmental outcomes, as the oldest offspring are still in early childhood. Still, the evidence we have outlined within this review from MIA studies examining IL-17A and its pathway members provides a strong basis to build upon our current hypothesis and ask the question; could COVID-19 induced MIA act via IL-17A signalling to increase the risk of ASD-like phenotypes in vulnerable offspring?

Discussion: improving outcomes for ASD affected individuals and families:

We believe that in spite of the tragedy of the COVID-19 emergency, we are presented with a serendipitous opportunity to progress scientific knowledge regarding prenatal exposures and ASD risk. During the COVID-19 pandemic, we have witnessed a novel infection, affect an immunologically naïve population within an extremely well defined period of exposure. COVID-19 is now a notifiable illness, and has been characterised and monitored more than any illness in history. Many countries have developed stringent mandatory testing protocols, and track and trace programmes. Within all this, exists an opportunity to study the longitudinal effects of this infection on offspring of those affected by gestational COVID. Further investigation of mid-gestational cytokine profiles (IL-17A in particular) and their

potential for genetic interplay could be a crucial cog in the development of actionable and cost-effective improvements in the current models of ASD care. Identification of pathways of immune dysregulation during pregnancy could lead to the identification of a risk marker of ASD that could be characterised in broader ASD cohorts. This would facilitate the identification of a predictive marker of ASD allowing earlier dedicated ASD screening in at risk children. Coupled with these potential biochemical markers, known early clinical signs of ASD exist. Crystallisation of the ASD diagnosis can be as early as 14 months old according to some authors, and there are clinically detectable signs of ASD from a younger age still (208, 362, 363). The first children born of this pandemic are now reaching their toddler years, and they may represent a group with increased risk of ASD or other developmental conditions. Taken together, a postulated early biochemical marker and established early clinical markers could allow targeted early ASD screening, which would lead to earlier intervention, and improved outcomes. Therapies instituted in this age group have the potential to significantly improve clinical outcomes in ASD affected children. The timing of therapy is important with the most dramatic symptomatic and developmental improvements in those detected at an earlier age of diagnosis (364, 365).

We believe that it is the obligation of the scientific community to glean what benefit we can from this pandemic. In spite of social distancing measures, systematic national “lockdowns” and working from home, there has been unprecedented scientific collaboration to try to counter the scourge of COVID. This has led to some outstanding success, not least in the development of two highly effective mRNA vaccines. In order to facilitate international research, the development of an international gestational COVID-19 consortium and registry would be an important step in coordinating research activities and aims. Isolation of relevant clinical bio-samples and prospective identification of patients will have already begun in some centres, and should be facilitated by the public health infrastructures that have been built up around the pandemic. Multidisciplinary collaborative follow up programmes should be established to identify, assess and treat children with potential negative post-COVID outcomes.

List of abbreviations:

ACE-2	Angiotensin-converting enzyme – 2
ADHD	Attention Deficit Hyperactivity Disorder
ARDS	Acute respiratory distress syndrome
ASD	Autism Spectrum Disorder
CS	Caesarean section
CD8 cell	Cluster of Differentiation 8, Cytotoxic T-lymphocytes
CHD8	Chromodomain helicase DNA binding protein 8 gene
CNV	Copy Number Variant
COVID-19	Corona Virus Disease-2019
FMR1	Fragile X mental retardation 1 gene
GWAS	Genome-wide association study
HLA-G gene	Human Leukocyte Antigen G coding gene
ID	Intellectual Disability
IL	Interleukin
IL17A gene	Interleukin 17A gene
LPS	Lipopolysaccharide
MERS	Middle Eastern Respiratory Syndrome
MIA	Maternal Immune Activation
mTor	Mammalian Target of Rapamycin
Poly (I:C)	Polyinosinic: polycytidylic acid
PNS	Peripheral nervous system
ROR γ t	Retinoid-related orphan receptor gamma t
SARS-CoV-2	Severe Acute Respiratory Syndrome – Coronavirus 2
SNV	Single Nucleotide Variant

Th17	T helper 17 cell
TSC1; TSC 2	Tuberous sclerosis complex 1 / 2

Chapter 3

Paper 2: Maternal Mid-gestation Cytokine Dysregulation in Mothers of Children with Autism Spectrum Disorder

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MATERNAL MID-GESTATION CYTOKINE DYSREGULATION IN MOTHERS OF CHILDREN WITH AUTISM SPECTRUM DISORDER.

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Abstract

Autism spectrum disorder (ASD) is a developmental disorder characterised by deficits in social interactions and communication, with stereotypical and repetitive behaviours. Recent evidence suggests that maternal immune dysregulation may predispose offspring to ASD. Independent samples t-tests revealed downregulation of IL-17A concentrations in cases, when compared to controls, at both 15w ($p = 0.02$), and 20w ($p = 0.02$), which persisted at 20w following adjustment for confounding variables. This adds to the growing body of evidence that maternal immune regulation may play a role in foetal neurodevelopment.

Keywords: IL-17A, Autism Spectrum Disorder, Cytokine, Inflammation, Maternal Immune Activation

Declarations of interest: none

Introduction

ASD is an intricate continuum of neurodevelopmental disorders all of which have an onset in early childhood. These disorders are characterised by impairments in social interaction and communication, and the presence of restricted, ritualistic or repetitive interests, behaviours and activities (6, 7). To meet the diagnostic criteria, symptoms must have been present during the early developmental period, and must cause significant functional impairments (social or occupational) of varying severities(366). It reportedly affects approximately 1.5% of the population in the developed world (367). Although deficits can be present from infancy, diagnosis is often delayed. Classic Autism is typically formally diagnosed at an average of 5.6 years (standard deviation (SD) 4.1), and Asperger's at an average of 9.9 years (standard deviation (SD) 5.3) (368). An early, accessible biomarker which could aid early detection and intervention (369) would be a significant step forward in the care of these children.

There is growing evidence that disturbance of inflammatory and immune responses may be a significant contributing factor behind the pathophysiology of many psychiatric disorders (115, 116, 119, 370). Alterations of immune cell expression have been documented repeatedly in ASD affected children and adults as well as animals with an ASD-like phenotype (27, 121, 122), and maternal viral or bacterial infections have been found to be significantly associated with ASD in offspring (371). Maternal immune activation (MIA) is believed to disrupt the delicate processes underlying neuronal development, increasing the risk of disordered neurodevelopment (279, 372).

MIA may typically be modelled in animals using lipopolysaccharide (LPS), Polyinosinic:polycytidylic acid (Poly(I:C)), or valproic acid. MIA in rodents results in a wide array of enduring ASD-like behavioural alterations in offspring. Neurodevelopment of the rodent brain is said to be equivalent to that noted in human mid-gestational neurodevelopment between gestational days 10 – 20 (373). Inflammatory insults during this time have resulted in reduced social approach and reciprocal social behaviour, increased repetitive and stereotypical behaviours,

typically measured using a marble burying test, abnormal prepulse inhibition and ultrasonic vocalisations, impaired learning and memory, measured using a variety of maze tests, and reduced novel object recognition (26, 374). Few large models of MIA induced ASD exist, though non-human primate models are more common than others are, and extend findings in rodent models. A mid-gestation viral challenge in the rhesus monkey may manifest as repetitive behaviours, decreased affiliative vocalisations, inappropriate social interactions with novel animals, and impaired social attention (375, 376).

Human epidemiological studies have shown that immune disorders and mid-trimester viral illnesses, which lead to a pro-inflammatory state in mothers during pregnancy, are associated with increased ASD, schizophrenia and bipolar disorder risk in offspring (113, 124, 125, 126). In 1977, Chess noted ASD rates of 8 – 13% in offspring of United States (US) mothers who were infected in the 1964 Rubella outbreak (125). More recently, Maher *et al.* linked preeclampsia to increased ASD risk (377).

Midgestation in particular appears to be an important neurodevelopmental period. Some of the key processes occurring during this period include the development of the hippocampus, cortical plate, the longitudinal fissure, sulci and gyri, cerebellum, superior and inferior colliculi, primary visual, motor and sensory cortices, the cerebrospinal tract, as well as spinal cord myelination, as well as neurogenesis. The brain also significantly increases in size between gestational weeks 13 and 21 (378, 379, 380, 381). Insults during this time have been found to result in neurodevelopmental and psychiatric disorders in both humans and animals (382, 383, 384).

Very few clinical studies have examined the cytokine profiles of mothers who go on to have a child with ASD. A retrospective 2017 study reported elevated levels of several circulating cytokines and chemokines in mid-gestational mothers who progressed to bear a child affected by ASD. This study was able to examine children with an early diagnosis of ASD, with and without intellectual disability. These included granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1 α , IL-6, interferon- γ (IFN- γ), IL-8 and monocyte chemoattractant protein-1 (MCP-1) (385). An

earlier study performed by Goines *et al.* showed dysregulation in a number of serum cytokines including IFN- γ , IL-4, IL-5 and IL-10 at a single time point between 15 and 19 weeks' gestation (386). Elevated MCP-1 has also been observed in amniotic fluid samples of ASD infants (387). Brown *et al.* identified increased levels of the inflammatory marker C-reactive protein (CRP) in prospectively collected maternal serum samples during early pregnancy (388). In recent times more conditions with a pro-inflammatory milieu, such as obesity, psychosocial stress and pre-eclampsia have also been reported to increase the risk of ASD (145, 377),(146). Thus, MIA and cytokine dysregulation during pregnancy seems to play a role in the pathogenesis of the ASD phenotype.

In the present study, we wished to examine the mid-gestational cytokine profiles in mothers of children with a subsequent ASD diagnosis examined at two mid-gestation time points (15 and 20 weeks) across two sites of a large multi-centre pregnancy study with the aim of identifying a gestational ASD biomarker which may aid in the timely treatment and management of the disorder.

Methods

Study Population

Maternal-child dyads were recruited to the population-based SCOPE study (www.scopestudy.net). This study used a cohort from the two SCOPE centres from which paediatric follow-up was completed. These sites were Cork, Ireland (Cork ECM5 (10) 05/02/08) and Auckland, New Zealand (SCOPE-NZ) (AKX/02/00/364). In Cork, children had detailed follow-up from birth to 5 years through the Cork BASELINE Birth Cohort Study. All children who scored below the “cut-off value” in the Ages and Stages Questionnaire (suggestive of abnormal development) were referred for paediatric assessment. Those with suspected ASD at 2 or 5 years were referred to early intervention services for full ASD assessment. Further follow-up was completed after Early Intervention Services (EIS) assessment to confirm diagnosis of ASD. Diagnosis was considered to be confirmed if made by a professional (EIS or child psychiatrist). In Auckland, telephone follow-up using standardised questionnaires was carried out as part of the Children of SCOPE study at 6 years and ASD diagnosis was by parent report. Cases from both sites were enrolled to the cohort.

Inclusion criteria for enrolment were:

- Biobanked maternal antenatal serum samples
- Developmental follow-up completed for the child at 5 or 6 years of age (site dependant)
- Cases had a diagnosis of ASD according to the local selection criteria
- Controls had no underlying medical or developmental conditions

SCOPE-IRELAND and the Cork BASELINE Birth Cohort study was carried out with local ethical approval from the Cork Research Ethics Committee (Cork ECM5 (10) 05/02/08). Full written informed consent was obtained in all cases. SCOPE-NZ and the Children of SCOPE study was carried out with ethical approval gained from local ethics committees (New Zealand Health and Disability Ethics Committees (AKX/02/00/364 and NTX/10/10/106) and all women provided written informed consent. A patient recruitment flowchart is outlined in Figure 1.

Demographic Variables

Demographic and relevant clinical data regarding the participants is presented in Table 3. 'Age, maternal' represents maternal age in years at the time they were approached to participate in the study whilst pregnant. 'Birthweight, g' is the infant's birthweight in grams. 'SEI' stands for Socioeconomic Index and this variable was calculated using the New Zealand Socioeconomic Index guide. The same index was used across both locations, and Cork participants were scored based on the same criteria as their New Zealand counterparts (389). Perceived Stress Scores (PSS) are based on the 10 question PSS questionnaire (390). An individual's scores on the PSS can range from zero to 40 with higher scores indicating higher perceived stress. Low stress scores range from 0-13, moderate stress scores range from 14-26, and high stress scores range from 27-40. Gestational age at delivery is presented in weeks, and APGAR (appearance, pulse, grimace, activity and respiration) scores are presented as being < 7 or ≥ 7 at both 1 and 5 minutes of age. Household income represents the combined household income and is quoted in New Zealand Dollars (\$) and Euros (€). Body Mass index (BMI) is categorised using the World Health Organisation (WHO) criteria and is measured in kilograms per metre squared. Underweight and normal BMI categories are considered together as are overweight and obese categories. Folate intake was categorised as yes or no for: (i) any supplemental folate in the preconceptional period and (ii) at the 15-week visit.

Biofluid Collection

Serum samples were obtained from mothers recruited to the SCOPE-NZ and SCOPE-Cork studies at both 15 and 20 weeks gestation within the greater Auckland area, New Zealand and Cork University Maternity Hospital, Cork, Ireland. Biobank specimens were archived at -80°C until required. Maternal mid-pregnancy specimens from 15 and 20 weeks were retrieved from the multi-centre SCOPE study sites with ongoing paediatric follow-up. Identical protocols for collection, processing and storage of samples were followed at both sites.

Venepuncture was performed by SCOPE study specific research midwives at each of the sites in accordance with best practice guidance (SCOPE Consortium standard operating procedures (SOP)). Maternal specimens were collected in serum separator tubes (Becton-Dickinson Franklin Lakes, New Jersey) and immediately placed on ice and transported to the laboratory. Before proceeding to centrifugation, serum samples were stored at 4°C for 30 minutes from time of collection to allow clot formation. Presence of the clot was confirmed visually, and samples were then centrifuged at $2400\times g$ for 10 minutes at 4°C . Serum samples were transferred into ice-cold 5mL sterile polypropylene tubes (VWR, Radnor, Pennsylvania) via sterile Pasteur pipettes. The samples were centrifuged again at $3000\times g$ for 10 minutes at 4°C . Sera were then aliquoted to red capped, barcode-labelled cryovials (VWR) in volumes of 250 microlitres. Aliquots were logged in the SCOPE database (MedSciNet), and stored at -80°C within four hours of collection(391). For transport of NZ serum samples to Cork, Ireland: the maternal specimens were packed on dry ice and shipped directly to the SCOPE Ireland biobank repository, where they were stored at -80°C until their use in cytokine and chemokine profiling.

Cytokine Analysis

Serologic concentrations (pg/ml) of eight cytokines, chemokines and proinflammatory proteins were investigated at 15- and 20-weeks gestation using the Mesoscale Discovery V-plex cytokine, chemokine and proinflammatory electrochemiluminescent assays (Meso Scale Diagnostics, Rockville, Maryland). Cytokines were chosen for further examination based on evidence of dysregulated expression in preclinical models (392, 393, 394, 395) and autistic patients (370, 396, 397, 398).

IL-16 and IL-17A were examined using the V-plex multiplex Cytokine Panel 1 kit (KD15050D). Eotaxin and MCP-1 were examined using the V-plex multiplex Chemokine Panel 1 kit (K15047D). IFN- γ , IL-1 β , IL-6 and IL-8 were examined using the V-plex multiplex Proinflammatory Panel 1 kit (K15049D). All standards and samples were run in duplicate.

All plates were prepared according to manufacturer's instructions and analysed on the Meso QuickPlex SQ 120. Results were generated as calculated concentration means on the Mesoscale (MSD) Discovery Workbench 4.0 assay analysis software. Calibration curves used to calculate concentrations of individual cytokines are established by fitting the calibrator signals to a four-parameter logistic model with a $1/Y^2$ weighting. The MSD analysis software determines individual cytokine concentrations from electrochemiluminescent signals via backfitting to the calibration curve. Calculated concentrations are also multiplied by the dilution factor applied to the samples, which in this case, was 4. Samples were excluded if %CV was higher than 25% between duplicates as previously described (399). Lower and upper limits of detection (LLOD and ULOD) as well as interassay coefficients of variation (CVs) for each protein are outlined in Table 4. Limits of detection represent calculated concentrations, which correspond to signals 2.5 standard deviations above/below the blank (zero calibrator).

Table 4: Median LLOD and ULOD for each of the tested cytokines. All units are pg/ml.

Proteins	Median LLOD (pg/ml)	Median ULOD (pg/ml)	Interassay CV (%)
IL-17A	1.60	6560.00	8.63
IFN-γ	0.34	1400.00	10.13
Eotaxin	0.44	1820.00	12.04
MCP-1	0.13	530.00	10.43
IL-16	0.83	3400.00	6.35
IL-1β	0.14	575.00	9.07
IL-6	0.19	765.00	8.76
IL-8	0.15	599.00	9.18

Samples were chosen due to early ASD presentation (formal diagnosis prior to 5 years) and sample availability. Several were excluded from the individual final analyses due to either poor %CV values or concentrations reading below the LLOD for individual cytokines. Of the combined 25 cases and 38 controls, the final sample numbers for cases after all exclusions are outlined in Table 5.

Table 5: Final Sample Numbers for Combined Cork and Auckland Cases and Controls. Derived from the original 25 cases and 38 controls.

Proteins	Cases	Controls	Excluded	Cases	Controls	Excluded	Total Excluded (Below LLOD)	Total Excluded (%CV >25%)
	15w	15w	15w	20w	20w	20w		
IL-17A	20	34	9	20	31	12	10	11
IFN-γ	20	28	15	19	30	14	2	27
Eotaxin	15	23	25	18	16	29	7	47
MCP-1	21	32	10	19	32	12	1	22
IL-16	22	35	6	21	37	5	5	6
IL-1β	14	19	30	14	22	27	25	32
IL-6	20	28	15	20	29	14	1	28
IL-8	22	28	13	19	29	15	0	28

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA) and IBM SPSS Statistics 24/26 (SPSS Statistics, Chicago, IL). All cytokine variables were Log_{10} transformed prior to analysis to achieve normality (400). Independent samples t-tests were used to investigate differences between cases and controls for the cytokine variables. Multiple logistic regression models were used to assess whether cytokine concentrations can predict ASD outcome after adjusting for individual confounding variables. A confounder was defined as a variable that was associated with both case/control status and the cytokine variable under investigation. For comparisons of continuous variables between groups, independent samples t-tests were used when there were two groups and one-way ANOVAs were used when there were more than two groups. Relationships between categorical variables were investigated using the chi-squared test. Statistical significance (2-tailed) was set at $p \leq 0.05$ and all tests were two-sided.

Results

Participant Details

Table 6: Demographic characteristics of participants.

Demographics for combined NZ and IRE cohorts (n=63)			
Variables	Cases (n=25)	Controls (n=38)	p-value
Age (maternal), years	30.4 (5.7)	30.6 (3.6)	0.9
Birthweight, g	3604.0 (666.0)	3439.0 (431.0)	0.2
Sex (Infant)			0.02
Male	23 (92)	25 (66)	
Female	2 (8)	13 (34)	
Mode of Delivery			0.04
Unassisted vaginal	9 (36)	16 (42)	
Assisted vaginal	4 (16)	15 (40)	
Pre-labour LSCS	1 (4)	2 (5)	
Labour LSCS	11 (44)	5 (13)	
Gestational Age at delivery	39.9 (1.5)	40.0 (1.4)	0.9
1-minute Apgar			0.08
<7	2 (8)	0	
≥7	23 (92)	38 (100)	
5-minute Apgar			*
<7	0	0	
≥7	25 (100)	38 (100)	

Ethnicity			1
Caucasian	23 (92)	35 (92)	
Non-Caucasian	2 (8)	3 (8)	
SEI (maternal)	52.6 (16.2)	49.8 (11.7)	0.4
Household Income			0.4
Unknown	2 (8)	2 (5)	
<\$75K (<€64K)	6 (24)	11 (29)	
\$75 – 100K (€64-84K)	10 (40)	8 (21)	
>\$100K (>€85K)	7 (28)	17 (45)	
Smoking status in pregnancy			0.4
No, never smoked	20 (80)	24 (63)	
No, ex-smoker	4 (16)	11 (29)	
Yes, current smoker	1 (4)	3 (8)	
PSS (Perceived stress score)	13.8 (7.3)	14.7 (6.7)	0.6
BMI (WHO categories)			0.2
Underweight/Normal ($\leq 25\text{kg/m}^2$)	14 (56)	27 (71)	
Overweight/Obese ($> 25\text{kg/m}^2$)	11 (44)	11 (29)	
Folate – pre-conceptual			0.6
No	9 (36)	11 (29)	
Yes	16 (64)	27 (71)	
Folate – 15 week visit			0.02
No	3 (12)	15 (40)	

Yes	22 (88)	23 (61)
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Comparison is made between cases and controls across the whole cohort. P-values are calculated using the Pearson Chi square for categorical data, and independent samples t-test where appropriate for continuous variables. Variations in local Caesarean section practices from each site likely give rise to the significant difference in Mode of Delivery rates. Eight of eleven (73%) of the ASD cases delivered by lower segment Caesarean section - “Labour LSCS” were in NZ. “Pre-labour LSCS” was excluded when identifying confounding variables due to small sample numbers (n = 3). There are no significant differences in birth weight, either between cases and controls, or between subjects from each site. Numbers are presented as mean (SD) or n (%).

Of the 2034 mothers recruited to SCOPE-NZ, 1208 agreed to participate in the follow up birth cohort study, Children of SCOPE. 16 NZ children who completed developmental follow-up and had an ASD diagnosis by 6 years were selected for cytokine profiling (compared to 16 controls). While the NZ cohort was originally matched, one case was excluded from analysis due to possible chromosomal abnormality, and its corresponding control was one of only two females remaining in the cohort, so was not excluded, resulting in 15 NZ cases total.

Of the 2183 mothers recruited to Cork’s Baseline birth cohort study, 1537 were recruited from SCOPE Ireland at the 20 weeks visit and an additional 600 children were recruited to the cohort postnatally. In total, 1249 children completed 5-year follow up assessment in the Cork BASELINE Birth Cohort Study. Of these children, 23 had a reported diagnosis of ASD, and 10 had available mid-gestation samples and were selected for cytokine profiling (compared to 22 controls). The study clinical research fellow contacted cases selected from the Cork cohort via telephone in June/July 2019, and all cases were verbally confirmed to have ASD (diagnosed by local EIS or child psychologist). While the Cork cohort was originally matched, numerous samples were excluded, resulting in a lack of matching.

The cohort of ASD cases from NZ and Cork were combined (n=25), and samples from the mothers of these children were analysed alongside those from the mothers of neurotypical controls n=38 (see Figure 8).

Detailed clinical characteristics of participants and mothers from both cohorts are provided in Table 6. As previously stated, several samples from both locations were excluded from the final analysis due to either poor %CV values or concentrations reading below the LLOD for individual cytokines. This resulted in an altered male/female ratio between cases and controls and ultimately an unmatched cohort. Other significant differences between cases and controls included mode of delivery and folate use in early pregnancy (15w).

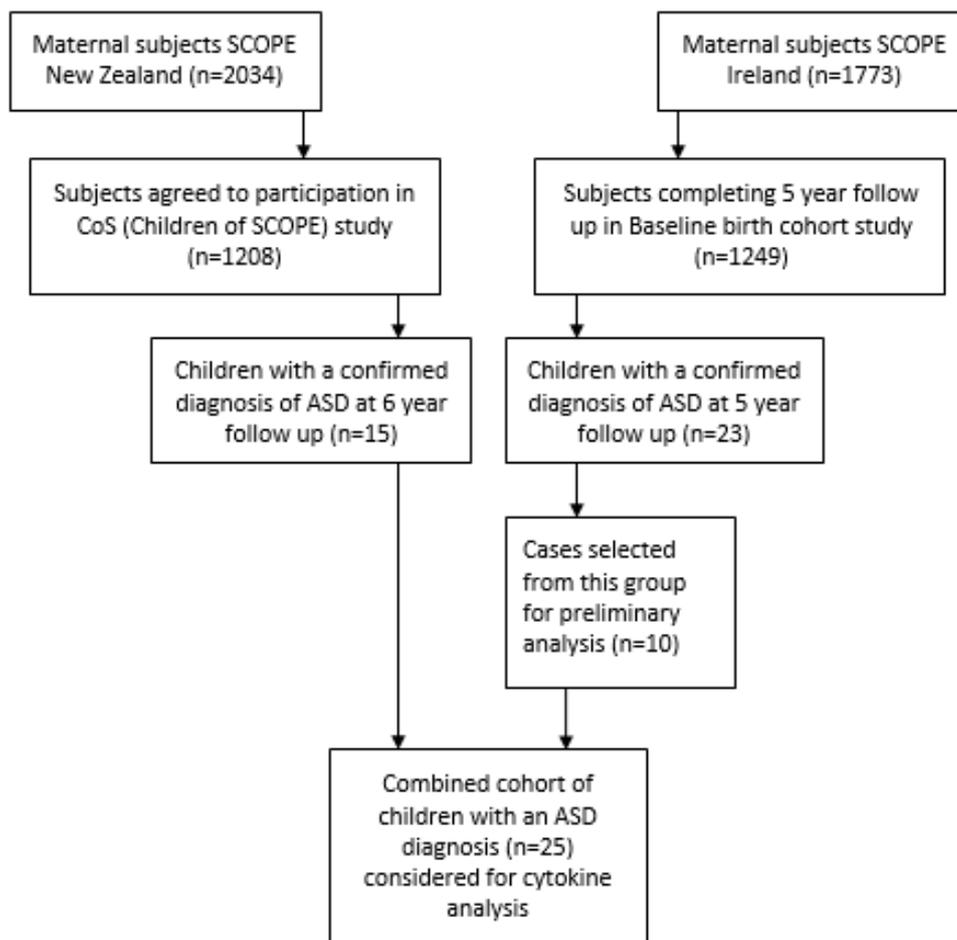


Figure 8: Flow chart outlining participant enrolment and follow up across both sites.

Mid-Gestational Cytokine Analysis

To determine whether there was any difference in inflammatory markers between mothers of ASD and neurotypical children at either 15 or 20 weeks gestation, electrochemiluminescent Mesoscale assays were performed.

Of the original panel of eight cytokines, one was significantly altered - IL-17A. IL-17A was significantly altered at both 15w and 20w in mothers of children who went on to have a child affected by ASD, compared to controls. IL-17A concentrations were significantly different between cases (Mean (M) = -0.22; Standard Deviation (SD) = 0.28) and controls (M = -0.001; SD = 0.35) at 15w ($t(52) = 2.43$; $p = 0.02$), and between cases (M = -0.26; SD = 0.38) and controls (M = -0.002; SD = 0.40) at 20w ($t(49) = 2.32$; $p = 0.02$) (**Figure 9a**). After adjusting for confounding by folate, IL-17A no longer showed a statistically significant association with ASD risk at 15w (adjusted odds ratio [aOR] = 0.17 (95% CI = 0.02 – 1.57); $p = 0.12$). Downregulation at 20w remained, as there were no changes in associations after adjustment for confounding by folate (aOR = 0.14 (95% CI = 0.02 – 0.87); $p = 0.03$).

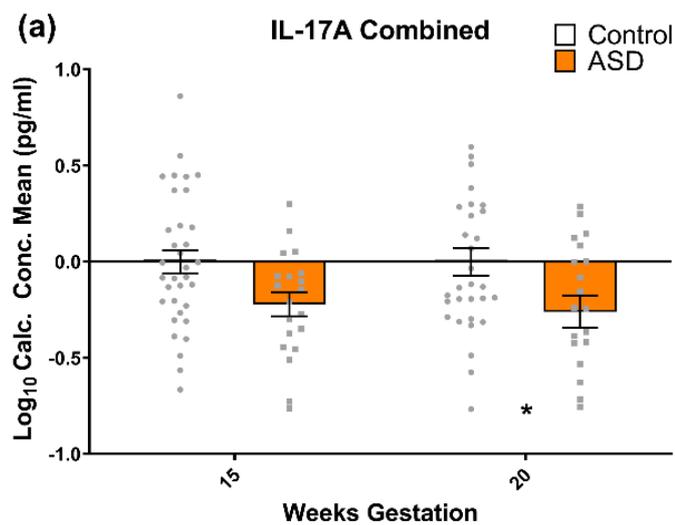


Figure 9 – (a) IL-17A is Downregulated, at 20 Weeks Gestation in Mothers of ASD Children versus Controls.

*This remains after adjusting for confounding variables –Folate intake at 15w. All data are mean ± SEM; independent samples t-tests, analysed on a case vs control basis. * = p < 0.05.*

Expression of IFN- γ , IL-16, Eotaxin, MCP-1, IL-1 β , IL-8 and IL-6 was not significantly different in mothers who went on to have a child with ASD when compared to controls at either time point. Therefore, levels of these cytokines were not associated with increased ASD risk.

IFN- γ was not found to be significantly different between cases (M = 0.26; SD = 0.28) and controls (M = 0.25; SD = 0.31) at 15w (t (46) = 0.19; p = 0.85) or between cases (M = 0.34; SD = 0.31) and controls (M = 0.39; SD = 0.41) at 20w (t (47) = 0.51; p = 0.62) (Figure 10a). IL-16 was not significantly different between cases (M = 2.04; SD = 0.16) and controls (M = 2.01; SD = 0.18) at 15w (t (55) = 0.64; p = 0.52), or between cases (M = 2.01; SD = 0.19) and controls (M = 2.02; SD = 0.20) at 20w (t (56) = 0.12; p = 0.92) (Figure 10b). Sex was found to be a confounder for IL-16 at 15w, though levels remained not significantly associated with development of ASD after adjusting for confounding by sex (aOR = 2.38 (95% CI = 0.63 – 89.61); p = 0.64). Eotaxin was not significantly different between cases (M = 1.50; SD = 0.27) and controls (M = 1.57; SD = 0.31) at 15w (t (36) = 0.73; p = 0.47), or between cases (M = 1.61; SD = 0.31) and controls (M = 1.62; SD = 0.23) at 20w (t (32) = 0.11; p = 0.91) (Figure 10c). MCP-1 was not significantly different between cases (M = 1.87; SD = 0.26) and controls (M = 1.87; SD = 0.23) at 15w (t (51) = 0.10; p = 0.92), or between cases (M = 1.87; SD = 0.29) and controls (M = 1.90; SD = 0.19) at 20w (t (49) = 0.58; p = 0.56) (Figure 10d). IL-8 was not significantly different between cases (M = 0.56; SD = 0.25) and controls (M = 0.57; SD = 0.36) at 15w (t (48) = 0.15; p = 0.88), or between cases (M = 0.54; SD = 0.23) and controls (M = 0.61; SD = 0.28) at 20w (t (46) = 0.89; p = 0.38) (Figure 10e). IL-1 β was not significantly different between cases (M = -1.39; SD = 0.83) and controls (M = -1.03; SD = 0.75) at 15w (t (31) = 1.28; p = 0.21), or between cases (M = -1.37; SD = 0.89) and controls (M = -1.23; SD = 0.65) at 20w (t (34) = 0.54; p = 0.59) (Figure 10f). Mode of delivery was found to be a confounder for IL-1 β at 15w and 20w, though IL-1 β at 15w (aOR = 0.83 (95% CI = 0.28 – 2.45); p = 0.74) and 20w (aOR = 0.76 (95% CI = 0.26 – 2.23); p = 0.61) remained not significantly associated with development of

ASD after adjusting for confounding by mode of delivery. IL-6 was not significantly different between cases (M = -0.44; SD = 0.22) and controls (M = -0.40; SD = 0.24) at 15w (t (46) = 0.54; p = 0.59), or between cases (M = -0.36; SD = 0.27) and controls (M = -0.39; SD = 0.19) at 20w (t (47) = 0.50; p = 0.62) (Figure 10g). Sex was found to be a confounder for IL-6 at 15w, though IL-6 at 15w remained not significantly associated with development of ASD after adjusting for confounding by sex (aOR = 0.30 (95% CI = 0.17 – 5.17); p = 0.41).

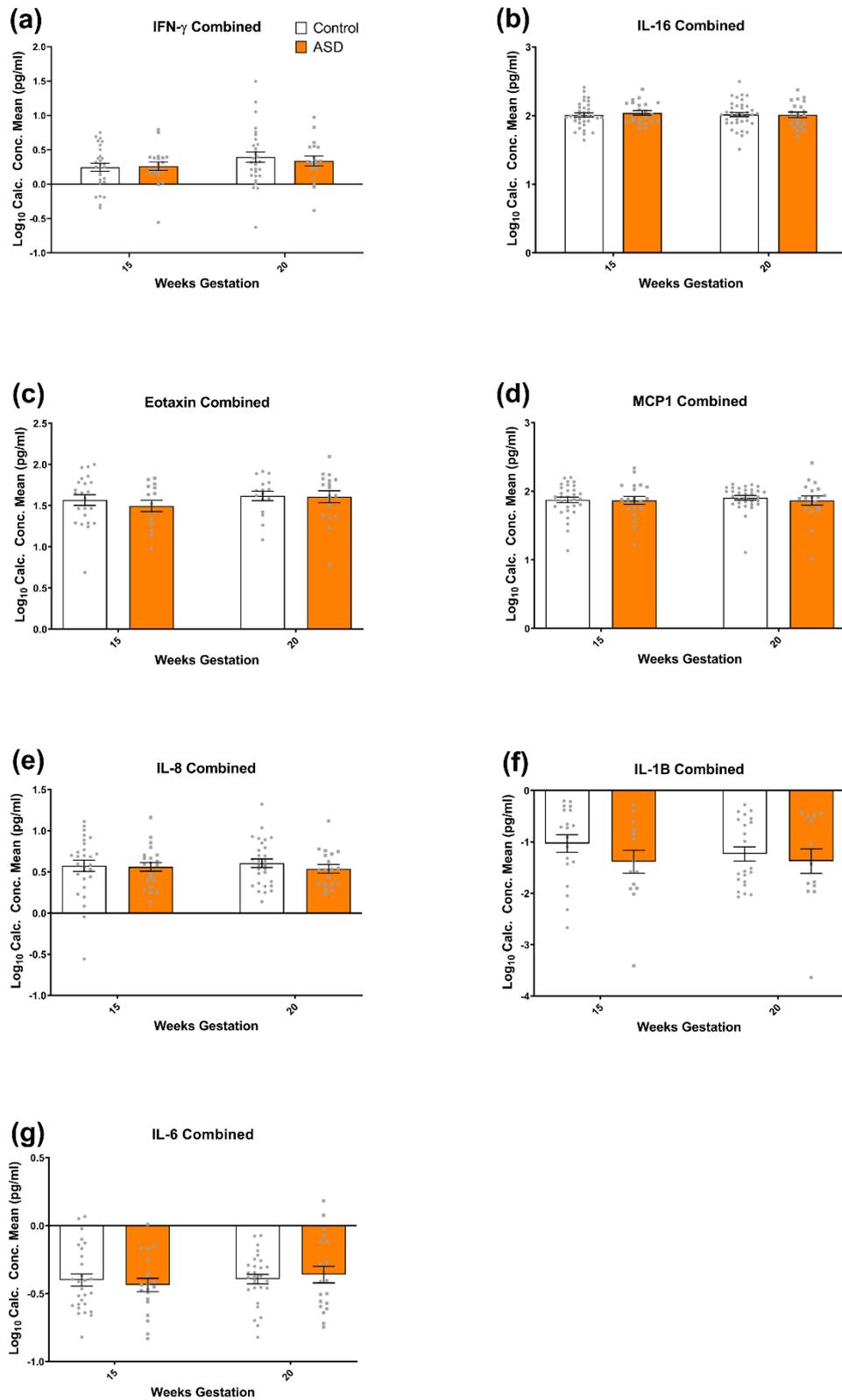


Figure 10 – (a) IFN- γ , (b) IL-16, (c) Eotaxin, (d) MCP1, (e) IL-8, (f) IL-1 β and (g) IL-6 were not Significantly Altered at Either 15 or 20 Weeks Gestation

in Mothers of ASD Children when Compared to Neurotypical Controls. All data are mean \pm SEM; independent samples t-tests, analysed on a case vs control basis.

Maternal health

To examine whether other factors might have altered maternal cytokine profiles we examined maternal health factors and medication use during pregnancy.

None of the participants had any of the following pre-existing inflammatory conditions – inflammatory bowel disease, rheumatoid or inflammatory arthritis, venous thromboembolic disease. No subjects suffered from either psoriasis or ankylosing spondylitis. The commonest reported medical condition was asthma. Several participants from each site had physician diagnosed asthma; 12 suffered from mild asthma and 3 suffered from moderate asthma. The individuals with moderately severe asthma (one case (Cork), two controls (NZ)) were being treated with regular low dose inhaled corticosteroid and long acting beta agonist or Terbutaline combination therapy. The subjects with mild asthma were 7 controls (4 Cork, 3 NZ) and 5 cases (2 Cork, 3 NZ). None of the asthmatics received oral steroid therapy at any point during pregnancy or in the preceding year. Hypothyroidism was also relatively common and occurred in three cases (2 NZ, 1 Cork) and two controls (1 NZ, 1 Cork). Two of the three cases had evidence of hypothyroidism first detected during the 1st trimester and became euthyroid with treatment. Those others with a history of hypothyroidism were treated prior to pregnancy and were euthyroid throughout. Finally, a single case in Cork had coeliac disease (on gluten free diet).

With regard to antepartum infections, between 0 and 15 weeks gestation, upper respiratory tract infections were reported in 12 subjects, 5 (4 NZ, 1 Cork) cases and 7 (5 NZ, 2 Cork) controls. Other infections were also reported in three (all NZ) cases and seven (3 NZ, 4 Cork) controls (5 gastroenteritis, 3 lower urinary tract infections (UTI), 1 case of genital herpes and another vaginal candida, treated with Clotrimazole. At 15-20 weeks, no infections were reported in the NZ group, but two controls from Cork had a UTI and one case had an unspecified infection. None of the participants was taking regular anti-inflammatories and those taking paracetamol or aspirin did so only occasionally or for a specific episode. There was no significant

difference between case and control groups in terms of reported paracetamol/aspirin use.

In summary, there were no significant differences in maternal health, inflammation or medication use between the two groups.

Discussion

In the present report, we have identified IL-17A as a potential cytokine biomarker whose expression is significantly reduced in mid-gestation (20 weeks) in pregnancies resulting in a child with ASD after adjusting for folate intake at 15w. This novel finding adds to the growing body of evidence that *in utero* exposure to MIA and resultant cytokine dysfunction is associated with an increased risk of the subsequent development of ASD.

Interestingly, the potential confounders identified within this study – sex, mode of delivery and maternal folate intake – are widely discussed risk factors for the development of ASD (401, 402, 403, 404). After adjusting for maternal folate intake at midgestation, IL-17A levels at 15w were no longer significantly associated with ASD development in offspring. A high number of case subjects (22) answered ‘yes’ to taking folate supplements during midgestation, while only three answered no. While no data are available on the doses of folate taken here, studies have linked both low and high dose maternal folate intake to DNA hyper/hypomethylation, gamma-aminobutyric acid (GABA), dopamine and serotonin dysfunction, and altered synaptic plasticity, neurogenesis and growth cone development. These events trigger neurodevelopmental disturbances, which may lead to the development of ASD (401, 402, 405). As previously mentioned, the current study had a larger ratio of males to females. It is widely understood that ASD is more commonly diagnosed in males. There are a number of theories on why this is the case. It appears that males may tend to externalise symptoms of the disorder, whereas females typically internalise symptoms, complicating diagnosis for females (406, 407). Mode of delivery was also identified as a confounder. Indeed, over 50% of mothers of ASD cases delivered by C-section which was initiated after the onset of labour. Emergency C-section is typically preceded by either foetal or maternal indications which may themselves be independent risk factors for ASD (408). C-section delivery has been linked to impaired cognitive and behavioural outcomes in both humans and animal models (404, 409, 410). Delivery by C-section has been linked to reductions in endogenous oxytocin (411), and subsequent social deficits in mice. These deficits may be reversed in mice by exogenous oxytocin therapy early during the postnatal period (412).

Although this is one of the few human studies to examine maternal midgestation cytokine dysregulation linked to ASD, there is an abundance of data from animal studies on the cytokine and behavioural changes resulting from MIA. MIA has been replicated in small animal models where induction of MIA through maternal infection leads to an autistic phenotype in offspring, characterised in mice by increased self-grooming, increased marble burying behaviour (repetitive, stereotyped behaviours) and deficits in ultrasonic vocalisations (communication). These alterations may be prevented by inhibition of specific cytokines (IL-6 and IL-17A), which suggests that the cytokines themselves may have a causative role in the resultant neuronal dysfunction (142, 151, 413).

In the murine MIA model of ASD, Poly(I:C) treatment has been found to increase IL-17A levels in maternal blood and the postnatal brain as well as placental messenger RNA (mRNA) levels of the cytokine. To determine whether alterations in IL-17A expression are symptomatic of, or pathogenic in ASD, a recent study inhibited IL-17A signalling in Poly (I:C) treated pregnant mice and reported that ASD-like phenotypes in the offspring were prevented (24). IL-17A and IL-6 appear to work in tandem. Knockout of IL-6 in Poly(I:C) treated dams results in failure to alter IL-17A levels in offspring, which suggests IL-6 acts upstream of IL-17A (393). Poly(I:C) is a synthetic analogue of double stranded RNA which mimics the effects of viral infection when injected into test subjects (168). It is used as a model of MIA extensively in rat, mouse and non-human primate studies. Pups of MIA-exposed dams in Poly(I:C) murine models have demonstrated communication challenges, reduced social approach, increased repetitive behaviours (24) and alterations in development of the cerebral cortex and cerebellum (150, 414).

Accumulating evidence supports a role for T-helper 17 (Th17) cluster of differentiation 4 (CD4) cells and their product cytokine IL-17A in ASD. Th17 cells have previously been implicated in the pathogenesis of a variety of autoimmune and neuroinflammatory disorders (415). Upstream IL-6 is also a key player in differentiation of these Th17 cells (393). Th17/IL-17 mediated immunity has been found to cause severe damage to the brain in response to inflammation-sensitised hypoxia (416). The gene for IL-17A (IL17A) has been identified in a genome-wide

analysis to have enriched/overexpressed copy number variants in ASD cohorts (274). In subsets of children with ASD, IL-17A has been found at elevated levels in the blood (both plasma and serum) and correlated with increased severity of behavioural symptoms (27, 29). Nadeem *et al.* report that children affected by ASD have an increased number of IL-17A receptors in monocytes and that activation via IL-17A increases the child's oxidative inflammation. Blocking the receptor may ameliorate inflammatory effects, which suggests an interesting therapeutic option for both inflammatory and behavioural symptoms (283). Indeed, IL-17A administration in a murine model improves sociability following MIA (417). IL-17A/IL-17A receptor blockade has also been shown to ameliorate the symptoms of other disorders such as atherosclerosis (418), inflammation-sensitised encephalopathy (419) and ankylosing spondylitis (420). IL-6 has been detected at elevated levels in cerebellar tissues of humans affected by ASD in their lifetime. Altered levels of this cytokine have been linked to dysfunctional adhesion and migration of neural cells, as well as imbalanced excitatory and inhibitory functions. This suggests that altered expression of IL-6 may contribute to the autistic phenotype and pathogenesis (421). Levels are also significantly increased in the frontal cortex and plasma of ASD patients (422, 423). Elevated IL-6 in the murine brain also results in an autistic behavioural phenotype, as well as abnormal dendritic morphology and distribution (424). Though we do not observe any notable alterations in IL-6 in this study, perhaps it acts at later time points when the nervous system is more developmentally mature.

The present study has a number of strengths, which increase our confidence in the findings. It involves a multi-centre, multi-national maternal cohort of over 4000 women, with very detailed maternal demography and 1st trimester health and lifestyle data at 15 weeks gestation. Of these women, 39 went on to have a child affected by ASD (~1% ASD rate). The rate of ASD seen in this cohort is similar to that seen across the developed world (~1.5%), so this study is a realistic reflection of ASD incidence. For this reason, we are confident that we have identified the majority of expected cases. Serum samples from both SCOPE study centres were collected, processed and biobanked according to identical protocols to ensure uniformity. Though it appears that our finding IL-17A downregulation goes against the previous

reports regarding induced upregulation of IL17 in animal studies (425), one must consider that this is currently one of the only studies in humans which has examined IL-17A in midgestation, and is therefore a novel finding. There is increasing evidence that IL-17A may cross the placenta from mother to foetus (425), which may, in theory, explain reduced levels in maternal serum and increased levels typically seen in the serum of offspring.

Though the present study has some major strengths, we must also address its limitations. One major shortcoming of the current study is its inability to replicate the findings of similar mid-gestation ASD cytokine studies (385, 386, 387). However, results are conflicting amongst the previous studies. Goines *et al.* reported midgestational elevation of IFN- γ in mothers of children who develop ASD, which contrasts with the current study (386). Jones *et al.* from the same research group detected midgestational downregulation of IL-8 and MCP-1 in mothers of children who develop ASD without intellectual disability. We did not find significant alterations in these cytokines in our cohort (385). Abdallah *et al.* utilised amniotic fluid to profile elevated MCP-1 in mothers of children who developed ASD. While we see very slight downregulation of MCP-1 at 20w, Abdallah *et al.* do not specify weeks gestation at measurement in their study (387). The differences in findings between studies may relate to several factors: assays and measurement of cytokines (Luminex/Millipore – neither used Mesoscale assays), differences in the stage of gestation at measurement, and our small study size compared to other similar studies. The relatively small numbers of ASD cases makes it difficult to draw meaningful conclusions regarding different sub-types of ASD. A number of ASD samples were also lost due to poor quality, reflected by large but inconsistent (across multiplex plates) numbers lost due to poor %CV, further reducing our cohort size, subsequently resulting in a disproportionately large percentage of male cases compared to females. In addition to this, a large number of samples were below the LLOD for many cytokines (up to 25 – Table 2), which suggests the assay used may not have been sensitive enough. This ultimately created an unmatched cohort. The follow-up procedure was different at both sites, with a more detailed follow up at 2 and 5 years available to the Cork BASELINE study. However, the diagnosis of ASD was

similar: parental report (Auckland), parental report of confirmed EIS or psychiatrist diagnosis. In Cork, children were diagnosed relatively early and so may be more on the severe end of the spectrum to that in Auckland. A large percentage of cases were delivered via Caesarean section. This may skew results, as this mode of delivery has previously been associated with increased ASD incidence (426, 427, 428). Larger, longer-term studies, which consider long-term outcomes, will be required with repeated maternal cytokine profiling to attempt to replicate and expand our findings.

Conclusion

To conclude, this study has identified dysfunctional IL-17A expression at 20 weeks gestation in mothers of ASD children. IL-17A may act as a potential early marker of maternal immune dysfunction and if validated would aid screening of high risk infants to support focused early therapeutic intervention in infancy (429). The current study provides a foundation for further investigation of IL-17A in large maternal cohorts. This multicentre study also provides novel insight into the midgestation cytokine profiles in mothers of both neurotypical and ASD offspring and is another piece in the puzzle of this elusive disorder.

Chapter 4

Paper 3: Mid-Gestation Cytokine Profiles in Mothers of Children Affected by Autism Spectrum Disorder: a Case-Control Study

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Title: Mid-Gestation Cytokine Profiles in Mothers of Children Affected by Autism Spectrum Disorder: a Case-Control Study

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Abstract:

Background:

Alterations of gestational cytokine profiles have been reported in mothers of children with Autism Spectrum Disorder (ASD). There is increasing evidence that the intrauterine environment is an important determinant of ASD risk. The aim of this study is to examine the mid-gestational maternal serum cytokine profiles of the mothers of children affected by ASD from a well-characterised birth cohort.

Methods:

A nested sub-cohort within a large mother-child birth cohort were identified based on a confirmed multi-disciplinary diagnosis of ASD before the age 10 years and neuro-typical matched controls (sex, birth gestation and birthweight) in a 2:1 ratio. IFN- γ , IL-1 β , IL-4, IL-6, IL-8, IL-17A, GM-CSF and TNF α were measured in archived maternal 20-week serum using Meso Scale Diagnostics multiplex technology and validation of our IL-17A measurements were sought using an ultrasensitive single analyte assay.

Results:

From a birth cohort of 2137 children, 38 had a confirmed diagnosis of ASD before 10 years. Of these, 25 had available maternal serum at 20 weeks' gestation. In the final analysis, we examined the sera of 25 cases and 50 matched controls. The sex ratio was 4:1 males to females in each group, and the mean age at diagnosis of ASD was 5.09 years (SD 2.13). Concentrations of IL-4 were significantly altered between the ASD-affected group and the matched control group. No other significant differences were found in the concentrations of the other cytokines analysed using either the multiplex or ultra-sensitive assay techniques.

Conclusions:

In a well-characterised prospective cohort of children with ASD, and supplementary to promising evidence from animal experiments and retrospective screening programmes, we confirmed mid-gestational alterations in maternal IL-4 cytokine levels in ASD-affected pregnancies versus matched neuro-typical controls.

Keywords: *Autism · Cytokine · ASD · IL-17A · Maternal immune activation · Early intervention*

Background:

Autism spectrum disorder (ASD) is an intricate continuum of neurodevelopmental disorders, all of which have an onset in early childhood and persist throughout life. These disorders are characterised by core impairments in social communication, and the presence of restricted and repetitive interests and behaviours (5, 6, 7, 65). There exists within this spectrum a broad range of heterogeneity. Clinical phenotypes vary widely, aetiology remains unclear, and many different comorbidities afflict those with ASD. There is clearly a strong genetic component in many cases with heritability estimates of 50 – 90% (15, 16), while the apparent male preponderance with rates exceeding that of females three to fourfold, also hints at a strong genetic foundation (17, 18). Yet, even using newer techniques such as ASD-optimised ultrahigh resolution chromosomal microarray, we only find a single gene determinant in approximately 25% of cases (430, 431). A recent study of monozygotic twins (MZ) (who share 100% similar copies of their genetic material) quoted ASD concurrence rates as low as 59% between MZ siblings (22). We are yet to discover a single gene determinant that can account for more than a small percent of ASD cases. All this suggests that we cannot explain many cases of ASD by genetic factors alone, or at least we cannot explain them using our current understanding of ASD genetics or our current techniques of genetic analysis.

This imperfect picture of ASD genetics has led some to investigate the role of environmental exposures in the aetiology of ASD. Researchers have identified many environmental risks in ASD. Advanced parental age, foetal environmental exposures, perinatal and obstetric events, maternal medication use, smoking and alcohol use, psychosocial hardship, nutrition and toxic exposures have all been implicated as risks in the pathogenesis of ASD (21, 22). Some authors attribute up to 17% of ASD risk to these exposures, yet the exact balance between genetic and environmental determinants and their roles in aetiology remains disputed (22, 23). Multiple mechanisms have been proposed through which each of these exposures may exert an influence, but there are only a handful that are likely to effect abnormal neurodevelopment. Animal models of inflammation and maternal immune activation are particularly well characterised, and have successfully modelled ASD

type behaviours and social difficulties in mice, rats and non-human primates (24, 25, 26).

Maternal immune activation (MIA) is defined as an increase in measured levels of inflammatory markers in mothers during pregnancy, and more specifically refers to a triggering of the maternal immune system by infectious or infectious-like stimuli resulting in an increase in measurable inflammatory markers (148, 149). Through this activation, a cytokine cascade transmits to the foetus, resulting in adverse neurodevelopmental phenotypes and even remodelling or malformations of the developing foetal brain. There have been many studies, which have profiled cytokine signatures in ASD affected individuals (27, 28, 29, 30). A much smaller number of studies have characterised cytokine profiles in expectant mothers who progressed to give birth to children who develop ASD (33, 34). The few previous studies, which have examined gestational serum, have indicated mid-gestational upregulation in specific pro-inflammatory cytokines. These findings arise from retrospective examination of stored serum samples from the wide 15 – 19 week gestation window. None of these studies confirmed a formal psychiatric or multi-disciplinary team diagnosis of ASD, nor did they account for important and relevant underlying maternal inflammatory conditions such as inflammatory bowel disease (432) and rheumatoid arthritis (433). Our aim in this study was to measure candidate cytokines at a single specific mid-gestational time-point (20-weeks' gestation) in a carefully characterised prospectively recruited birth cohort.

Methods:

Study population:

Mother and child dyads were recruited from the Cork BASELINE Birth Cohort Study (Babies after SCOPE: Evaluating the Longitudinal Impact on Neurological and Nutritional Endpoints) (www.baselinestudy.net). In total, recruitment ran for just over three years, from August 2008 to October 2011. The SCOPE Ireland pregnancy cohort (www.scopestudy.net) formed the basis of recruitment of infants to BASELINE [n = 1537] and an additional 600 infants were recruited after delivery providing a total sample of 2137. The research team performed assessments on day of life 2 and at 2, 6, 12, 24 and 60 months of age. Team researchers performed specific developmental assessments at 24 months (using the Ages and Stages parental questionnaire, and the Child Behaviour Checklist) and at 60 months (using the Kaufman Brief Intelligence Test – 2 (KBIT-2) and the Child Behaviour Checklist). Blood and DNA samples were bio-banked at 15 and 20 weeks' gestation, at birth, and at 24 and 60 months. Children with low scores at either time-point were examined further by the study paediatrician (DM) and were referred for early intervention assessment. In this study, archived mid-gestational (20 weeks) serum samples were analysed.

The inclusion criteria for the study were:

- i. Subjects had bio-banked mid-gestational serum samples available,
- ii. All participants had completed 5 year follow up (ideally including developmental assessment),
- iii. Children who were suspected ASD cases had received a confirmed ASD diagnosis according to local practices,
- iv. Those participants with alternate developmental conditions (such as recognised genetic syndromes) were excluded

Clinical Diagnosis:

The majority of children received their ASD diagnosis through the Health Service Executive (HSE) ASD service. The standard tests utilised in this setting are the Autism Diagnostic Observation Schedule (ADOS), and parent report via either the Diagnostic Interview for Social & Communication Disorders (DISCO) or Autism Diagnostic

Interview-Revised (ADI-R) questionnaires. A small number of children received their initial diagnosis through private multidisciplinary teams using the same assessment tools. All of these children later received a confirmatory diagnosis with the HSE ASD service.

Demographic Variables:

We have presented the demographic and relevant clinical data regarding the participants in Table 8. Male sex is indicated as a percentage in each participant group. Infant birthweight is presented in grams. Gestational age is in weeks. Customised birth centile indicates the percentile of the child's birthweight in relation to their gestational age at birth. Centiles were adjusted for mothers' height, weight at 15-week visit, ethnicity, and infant sex. The centiles were calculated using an online research calculator and were based on UK standards <https://www.gestation.net/> (434). Maternal age is presented in years and sub-categorised into three age ranges, 18 – 28, 29 – 39, >40 years. We present maternal BMI in kg/m² and sub-categorise according to WHO criteria, underweight BMI <18.5, normal BMI 18.5 – 24.99, overweight BMI 25 – 29.99 and obese BMI >30 kg/m². We present the Apgar scores (435) at one and five minutes as the proportion from each group with a tally less than seven. In our group there were three categories of marriage status, single, married or de facto (stable relationship akin to marriage) and finally we document smoking status in this pregnancy as (No) non-smoker, (Yes, but stopped) smoked until pregnancy was discovered, and (Still smoking) continues to smoke. The 10-question Perceived Stress Score questionnaire forms the basis for the Perceived Stress Scores (PSS). An individual's scores on the PSS can range from zero to 40 with higher scores indicating higher perceived stress. Low stress scores range from 0-13, moderate stress scores range from 14-26, and high stress scores range from 27-40 (436). Past medical history indicates the relevant past medical history of mothers in the study, and intrapartum infections correspond to reported infections in the first 20 weeks of pregnancy.

Ethical Approval:

Ethical approval for both the SCOPE study (Cork ECM5 (10) 05/02/08) and Cork BASELINE Birth Cohort Study (ECM3 (x) 05/04/19) were provided locally by the Cork

Research Ethics Committee (CREC). We obtained written informed consent from the mothers of each case and control recruited for additional enrolment in the PiRAMiD study (Predicting early onset Autism through Maternal Immune Activation and Proteomic Discovery). Additional ethical approval for the PiRAMiD study was obtained from CREC (ECM 3 (k) 03/12/19).

Bio-fluid collection:

We obtained archived serum samples of mothers recruited to the SCOPE-Cork study at 20 weeks' gestation within Cork University Maternity Hospital, Cork, Ireland. Biobank specimens were archived at - 80°C in the SCOPE (Cork) ISO accredited biobank facility until required. SCOPE study specific research midwives in accordance with best practice guidance (SCOPE Consortium S.O.P.) had performed venepuncture at the 20-week visit. Maternal specimens were collected in serum separator tubes (Becton-Dickinson Franklin Lakes, New Jersey), immediately placed on ice, and transported to the laboratory. Before proceeding to centrifugation, serum samples were stored at 4°C for 30 minutes from time of collection to allow clot formation. Researchers confirmed the presence of the clot visually, and samples were then centrifuged at 2400xg for 10 minutes at 4°C. Serum samples were transferred to ice cold 5mL sterile PP (polypropylene) tubes (VWR, Radnor, Pennsylvania) via sterile Pasteur pipettes. Samples were again centrifuged at 3000xg for 10 minutes at 4°C. Sera were then aliquoted to red capped, barcode-labelled cryovials (VWR) in volumes of 250 microlitres. Aliquots were logged in the SCOPE database (MedSciNet), and stored at -80°C within four hours of initial collection (391).

Cytokine analysis:

We selected our candidate cytokines (IL-1 β , IL-4, IL-6, IL-8, IL-17A, GM-CSF, TNF α , IFN γ) based on previous literature highlighting aberrations in cytokine levels in individuals with ASD (120) versus healthy controls. We also reviewed the literature and focused on a number of publications which have measured mid-gestation (15 – 19 weeks) cytokines previously (33, 34), and on IL-17A in particular. Much of the recent literature espouses IL-17A's potential as a key player in MIA associated neurodevelopmental outcomes (24, 113, 251). In order to quantify IL-17A more precisely, we examined IL-17A as part of a multiplex ELISA (enzyme-linked

immunosorbent assay), and we measured IL-17A levels individually, using a separate ultrasensitive ELISA assay.

MSD Multiplex V-plex assay

We profiled the serologic concentrations (pg/ml) of eight cytokines and proinflammatory proteins at 20 weeks' gestation using the Mesoscale Discovery V-plex cytokine and proinflammatory electro-chemi-luminescent (ECL) assays (Meso Scale Diagnostics, Rockville, Maryland 20850-3173, United States).

We used the V-plex multi-spot Cytokine Panel 1 (human) kit (LOT No: Z0047047) to examine IL-17A and GM-CSF, and we examined IFN- γ , IL-1 β , IL-4, IL-6, IL-8 and TNF α using the V-plex multi-spot Proinflammatory Panel 1 (human) kit (LOT No: Z0047096). We ran all standards in triplicate, but we ran all participant samples in duplicate due to low sample volumes.

MSD S-plex IL-17A ultrasensitive assay

We profiled serologic concentrations (fg/ml) of IL-17A at 20 weeks' gestation using the Mesoscale Discovery S-plex (Lot No: Z00S0003) IL-17A ECL assay (Meso Scale Diagnostics, Rockville, Maryland 20850-3173, United States). We ran all standards and participant samples in triplicate (single analyte kits require a smaller volume of serum for analysis than multiplex kits).

We performed all experiments as per the manufacturer's instructions and analysed the plates on a MESO QuickPlex SQ 120 instrument. Numeric results were generated as "calculated concentration means" on the MSD Discovery Workbench 4.0 assay analysis software. Samples were excluded if the coefficient of variation (%CV) was higher than 25% between duplicates/triplicates as previously described (399). We have outlined the Lower limits of detection (LLOD), lower limits of quantification (LLOQ) and the upper limits of quantification (ULOQ) as well as inter-assay CV (Coefficient of variation) for each cytokine in Table 7 for both the multiplex and ultrasensitive assays.

Table 7: Sensitivity of assays per each analyte examined

	LLOD Median	LLOD Range	LLOQ	ULOQ	Inter- assay CV
Proinflammatory Panel	<i>pg/mL</i>	<i>pg/mL</i>	<i>pg/mL</i>	<i>pg/mL</i>	%
IFNγ	0.37	0.21 – 0.62	1.76	938	8.16
IL-1β	0.05	0.01 – 0.07	0.646	375	7.95
IL-4	0.02	0.01 – 0.03	0.218	158	6.23
IL-6	0.06	0.05 – 0.09	0.633	488	8.62
IL-8	0.07	0.03 – 0.14	0.591	375	7.8
TNFα	0.04	0.01 – 0.13	0.690	248	6.74
Cytokine Panel	<i>pg/mL</i>	<i>pg/mL</i>	<i>pg/mL</i>	<i>pg/mL</i>	%
GMCSF	0.16	0.08 – 0.19	0.842	750	10.78
IL-17A	0.31	0.19 – 0.55	3.19	3650	11.45
Ultrasensitive IL-17A	<i>fg/ml</i>	<i>fg/ml</i>	<i>fg/ml</i>	<i>fg/ml</i>	%
IL-17A	13	N/A	60	140000	8.67

LLOD, LLOQ, ULOQ for each analyte tested using the MSD proinflammatory panel 1, cytokine panel 1, and MSD S-plex Human IL-17A kits. The units of measurement used in the multiplex assays are pg/ml (10^{-12} grams (picograms) per millilitre), while the units in the ultrasensitive assay are fg/ml (10^{-15} grams (femtograms) per millilitre). The quantitative range of the assay lies between the LLOQ and ULOQ.

Inter-assay CV is a measure of the variance between runs of sample replicates on different plates and assesses plate-to-plate consistency – which is satisfactory.

Statistical analysis

We compared the ASD cases (n=25) as a whole with the neuro-typical controls (n=50), All data were analysed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA) and IBM SPSS Statistics 26 (SPSS Statistics, Chicago, IL). ROUT analysis (437) was performed to remove outliers for each analyte (Q = 1%). Data were analysed using Mann-Whitney U-test, as data were non-parametric. Statistical significance (2-tailed) was set at $p < 0.05$.

Results:

Of the initial 2137 recruited, 1249 completed 5-year follow up (see Figure 11) included in the final analysis group were 75 child-mother pairs. Each mother had stored serum from 20 weeks' gestation for analysis and had no significant past medical history of inflammatory disease. The case and control split was one case to two controls (25 cases to 50 controls). We selected neuro-typical, healthy controls from the same BASELINE birth cohort, and we matched controls to cases based on

- i. Infant sex,
- ii. Gestational age at birth,
- iii. Birthweight and
- iv. Maternal BMI at 15-week visit.

We identified 22 children with a confirmed diagnosis of ASD at the 5-year developmental assessments and a further 13 cases were diagnosed between 5 and 10 years. These "later" cases consisted of children who received their formal ASD diagnosis after 5 years of age. These cases were identified on review of the 5-year follow up documentation. Those with expressed parental concern about ASD, developmental assessment suggestive of ASD, or at risk ASD scoring in the Child Behavior Checklist (CBCL) were added to the cases cohort. The clinical research fellow verified these additional later ASD cases via a follow up telephone interview. Following confirmation of each ASD diagnosis, the research fellow invited each candidate and his or her parents to attend a follow on cognitive (KBIT-2) and ASD

symptomology (SCQ) assessment. The cohort and their parents' medical histories were further characterised using a study-specific health questionnaire.

In total, there were 10 case exclusions. Nine (9) cases had no stored serum from mid-gestation. We excluded these cases along with their matched controls. We excluded one further case (and matched controls) due to a genetic diagnosis of Bannayan-Riley-Ruvalcaba syndrome. We have depicted the recruitment stream in Figure 11. Of the 1249 children still enrolled at 5-years, 38 children contacted at 10 years had a confirmed diagnosis of ASD. This gives us a prevalence of 3%, generally, in line with what others have quoted recently (17, 50).

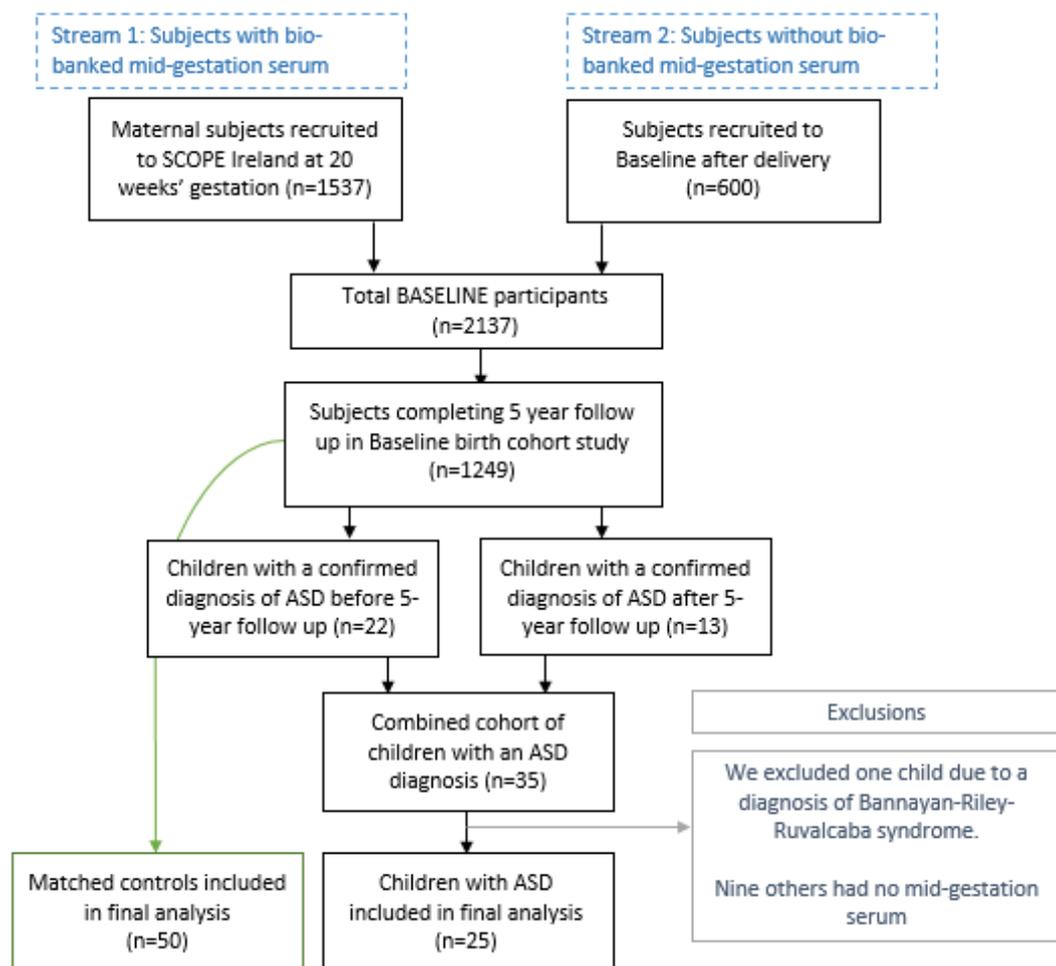


Figure 11: Recruitment numbers flow chart:

Participants in Baseline were drawn from two streams, those recruited at 15-week booking appointment (n=1537) and those recruited in the immediate post-natal

period (600). Twenty-two participants had a known ASD diagnosis at 5-year follow up and a further 13, a diagnosis between 5 and 10 years.

Cohort Characteristics:

In our cohort, the ratio of male to female ASD affected children was 4:1; this is consistent with most consensus of male preponderance in ASD (18, 70). There was no difference between groups in relation to infant birthweight or gestational age at delivery. There were no significant differences between those infants with low (<7) reported 1- and 5-minute Apgar scores. The groups matched closely in terms of maternal age and maternal BMI. All mothers participating in the study were first time mothers. The groups were ethnically homogenous, with all participants bar one of Caucasian European background. The exception was a single control of Australasian descent. With regard to inflammatory conditions and potential modifiers of inflammation, no participants reported use of any anti-inflammatories or steroids during pregnancy. Each group reported approximately equal rates of smoking. Perceived stress scores (PSS) did not significantly differ between groups, though more controls reported moderate to high stress. One mother in the control group reported suffering from Psoriasis, and a mother in the cases group reported having coeliac disease. There was no significant difference between groups in the commonly reported medical conditions of anaemia (diagnosed prior to pregnancy), asthma, depression (none on active treatment) and thyroid disease. Of those with thyroid disease, one participant from each group had hyperthyroidism; the remainder were euthyroid following treatment. In the first 20 weeks of pregnancy, 44% of controls reported at least one infection (most commonly a Respiratory Tract Infection), while only 20% of cases did so, again, this was non-significant. We have detailed the participant demographics in Table 8.

Table 8: Demographic characteristic of study participants.

Variable	Cases (n=25)		Controls (n=50)		p-value
	n or M	(%) or SD	n or M	(%) or SD	
Male sex	20	(80%)	40	(80%)	1
Infant birthweight	3488	SD 532	3496	SD 455	0.80
Gestational age	39.65	SD 1.5	39.78	SD 1.5	0.75
Customised birthweight centile	48.23	SD 26.5	51.90	SD 26.9	0.51
Maternal age	30.76	SD 5.3	31.46	SD 3.9	0.52
18 – 28	8	(32%)	9	(18%)	0.26
29 – 39	17	(68%)	39	(78%)	
>40	0	(0%)	2	(4%)	
Maternal BMI	25.80	SD 4.9	25.23	SD 4.0	0.60
Underweight	1	(4%)	1	(2%)	0.25
Normal	13	(52%)	26	(52%)	
Overweight	7	(28%)	14	(28%)	
Obese	4	(16%)	9	(18%)	
Apgar 1 minute <7	4	(16%)	3	(6%)	0.16
Apgar 5 minute <7	1	(4%)	1	(2%)	0.61
Marital Status					0.87
Single	2	(8%)	3	(6%)	
Married	20	(80%)	39	(78%)	
De facto	3	(12%)	8	(16%)	

Smoked (pregnancy)					0.85
No	20	(80%)	37	(74%)	
Yes, but stopped	2	(8%)	5	(10%)	
Still smoking	3	(12%)	8	(16%)	
PSS (moderate or high)	8	(32%)	24	(48%)	0.24
Past Medical history					
Anaemia	2	(8%)	8	(16%)	0.34
Thyroid disease	4	(16%)	3	(6%)	0.26
Depression	2	(8%)	5	(10%)	0.74
Asthma	4	(16%)	5	(10%)	0.41
Intrapartum infection (<20w)					
Respiratory tract infection (RTI)	3	(12%)	13	(26%)	0.16
Urinary tract infection (UTI)	2	(8%)	7	(14%)	0.45
Gastroenteritis (GE)	0	(0%)	2	(4%)	0.31

In Table 8, we calculated all p-values using the Pearson Chi square for categorical data, and independent samples t-test or Mann-Whitney U-test where appropriate for continuous variables depending on the normality of the distribution. There are no significant differences demonstrated between the groups in any of the variables listed. Cases and controls are well matched with little variance between the key matching variables, infant sex, gestational age and birthweight. Data are presented as either the mean (SD) with continuous variables or n (percentage) with categorical ones.

Cytokine Analysis:

Table 9: Summary table of cytokine analysis results.

	Cases		Controls		p-value	ROUT analysis	
	<i>Median</i>	<i>IQR</i>	<i>Median</i>	<i>IQR</i>		<i><0.05</i>	<i>Case</i>
IFN-γ	2.773	2.164 – 4.403	2.763	1.793 – 4.699	0.99	2	3
IL-1β	0.032	0.011 – 0.049	0.067	0.018 – 0.109	0.09	2	0
IL-4	0.027	0.019 – 0.031	0.053	0.029 – 0.074	0.04	1	0
IL-6	0.444	0.240 – 0.756	0.404	0.252 – 0.556	0.49	0	3
IL-8	5.519	3.895 – 7.001	4.881	3.515 – 5.785	0.10	0	5
TNFα	1.127	0.845 – 1.690	1.114	0.921 – 1.464	0.69	0	2
GM-CSF	0.120	0.081 – 0.279	0.163	0.103 – 0.242	0.38	0	2
IL-17A	0.691	0.487 – 0.985	0.842	0.394 – 1.010	0.85	0	0
IL-17A (U)	3.468	3.364 – 3.585	3.449	3.313 – 3.613	0.80	0	0

We quote all analyte concentrations in pg/mL except for ultrasensitive IL-17A assay (IL-17A (U)) which we quote in fg/mL. p-values are statistically significant at values less than 0.05. Outliers were removed using ROUT analysis on GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA). The final column “ROUT analysis” indicates the number of outliers removed from each group per analyte. We used Mann Whitney U-tests for the calculation of p-values as data were non-parametric.

MSD Multiplex V-plex:

IFN- γ

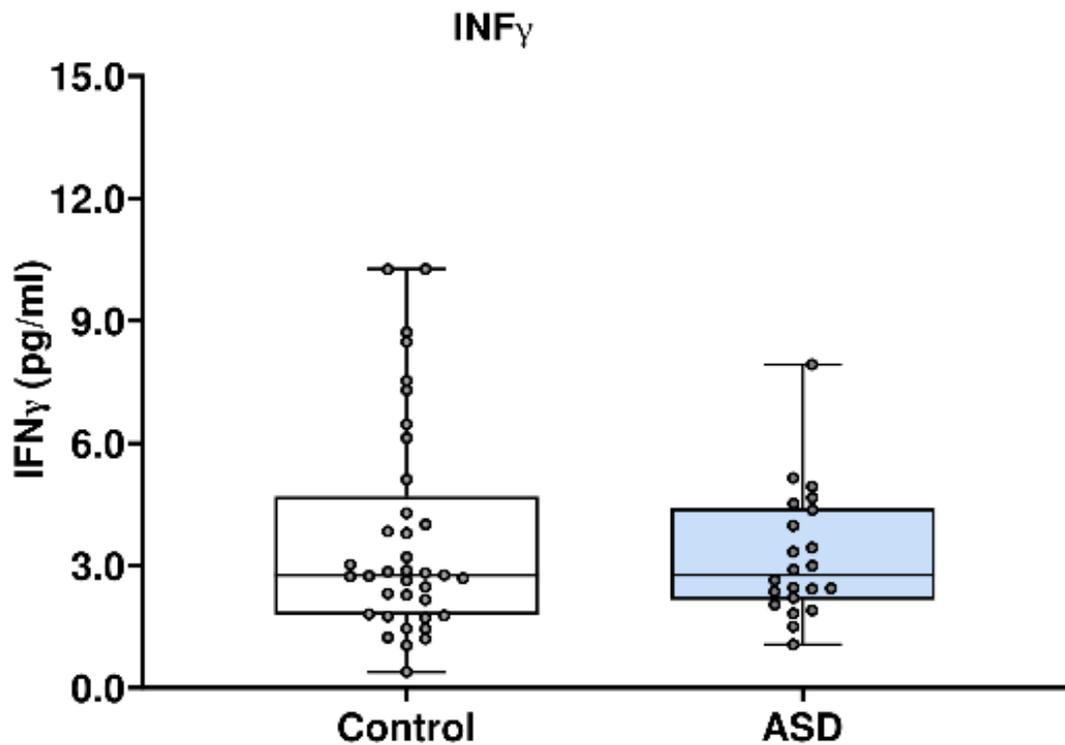


Figure 12: IFN γ concentration in ASD cases versus matched controls

IFN γ was not significantly altered in mothers of ASD affected children (median 2.773) at 20 weeks' gestation compared to neuro-typical controls (median 2.763). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. In total, five outliers were removed (3 controls and 2 cases). Final analysis was performed on $n=22$ cases and $n=37$ controls $p = 0.99$.

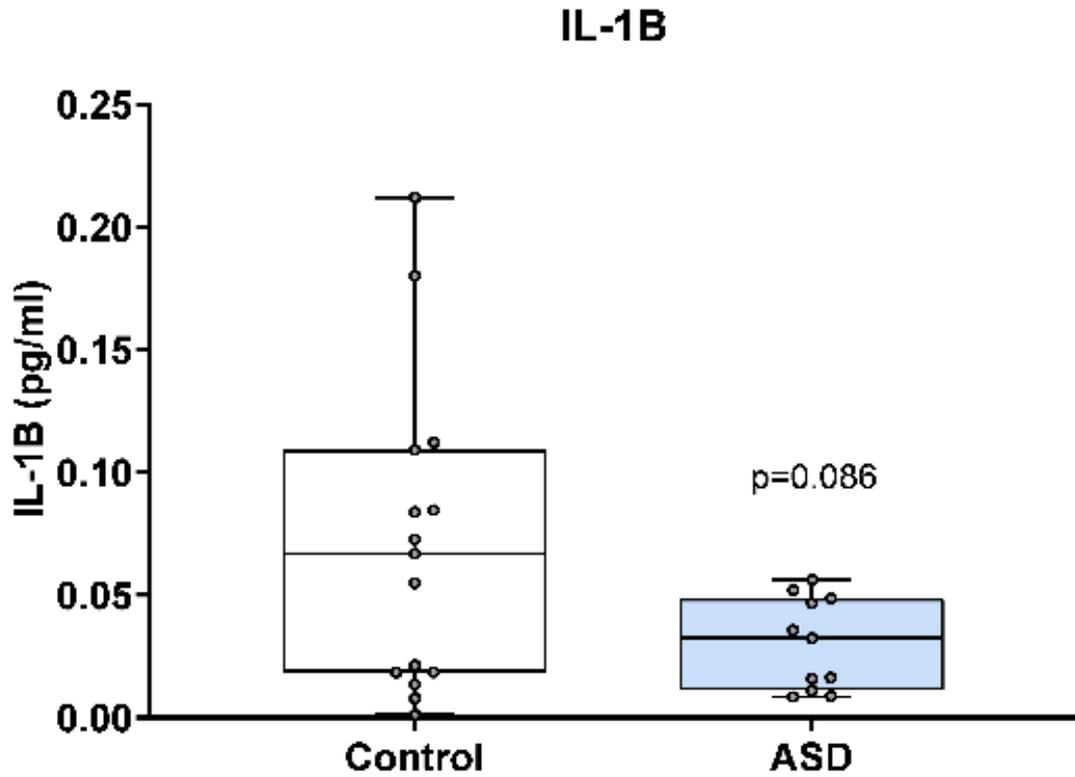


Figure 13: IL-1 β concentration in ASD cases versus matched controls

IL-1 β was not significantly altered in mothers of ASD affected children (median 0.032) at 20 weeks' gestation compared to neuro-typical controls (median 0.067). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. In total, two outliers were removed (2 cases). Final analysis was performed on $n=11$ cases and $n=15$ controls $p = 0.09$.

IL-4

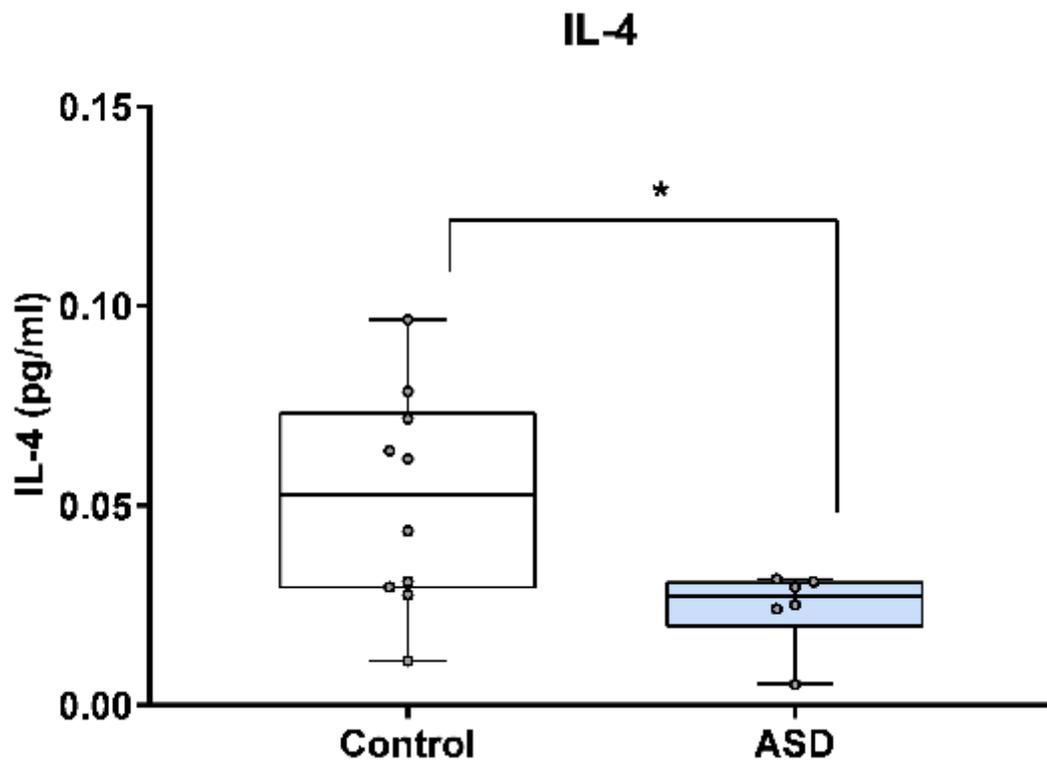


Figure 14: IL-4 concentration in ASD cases versus matched controls

IL-4 was significantly altered in mothers of ASD affected children (median 0.027) at 20 weeks' gestation compared to neuro-typical controls (median 0.053).

ROUT analysis (Q = 1%) was performed to identify and exclude outliers. 1 outlier was removed (1 case). Final analysis was performed on n=6 cases and n=10 controls. *p = 0.04

IL-6

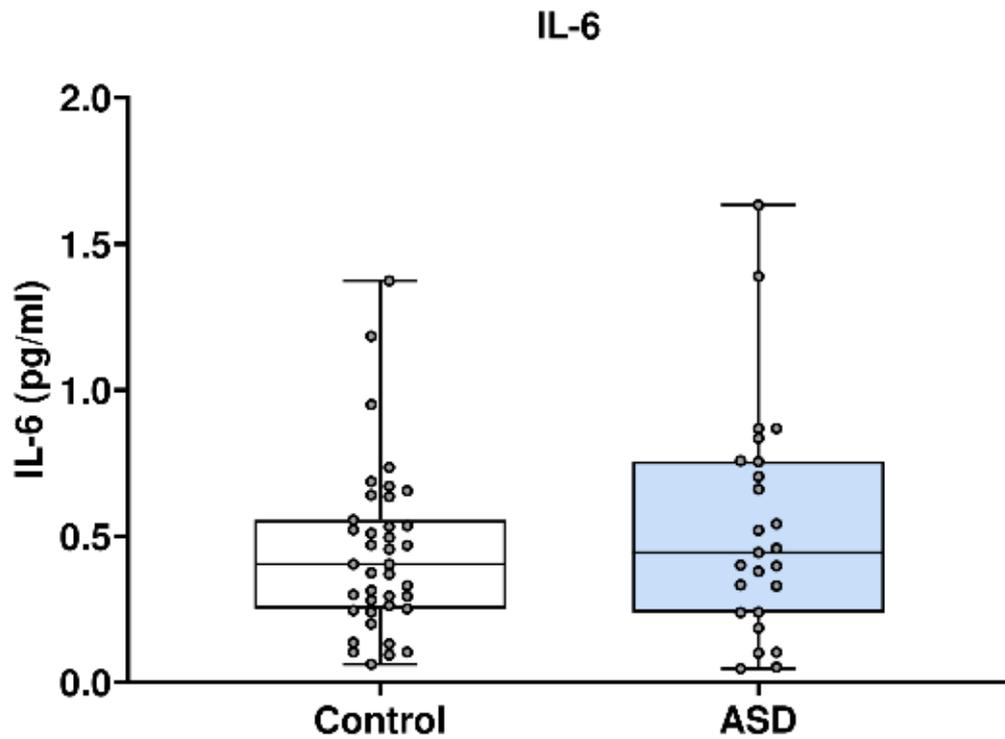


Figure 15: IL-6 concentrations in ASD cases versus matched controls

IL-6 was not significantly altered in mothers of ASD affected children (median 0.444) at 20 weeks' gestation compared to neuro-typical controls (median 0.404). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. 3 outliers were removed (3 controls). Final analysis was performed on $n=25$ cases and $n=39$ controls. $p = 0.49$

IL-8

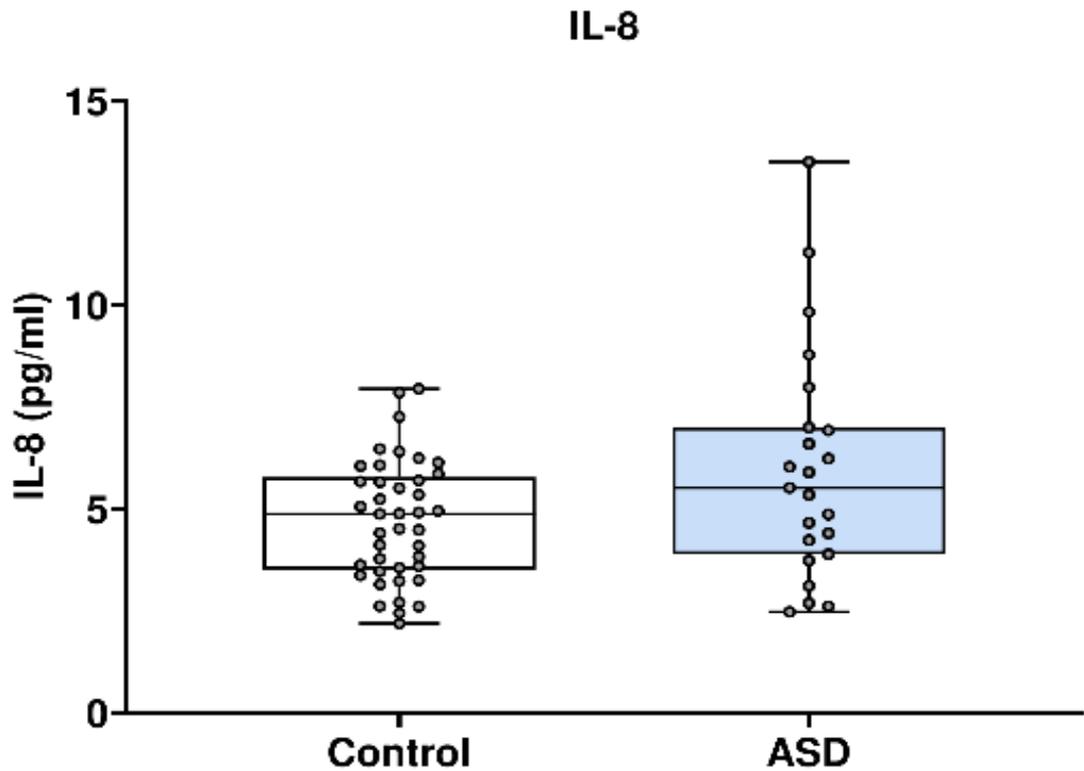


Figure 16: IL-8 concentration in ASD cases versus matched controls

IL-8 was not significantly altered in mothers of ASD affected children (median 5.519) at 20 weeks' gestation compared to neuro-typical controls (median 4.881). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. 5 outliers were removed (5 controls). Final analysis was performed on $n=23$ cases and $n=36$ controls. $p = 0.10$

TNF α

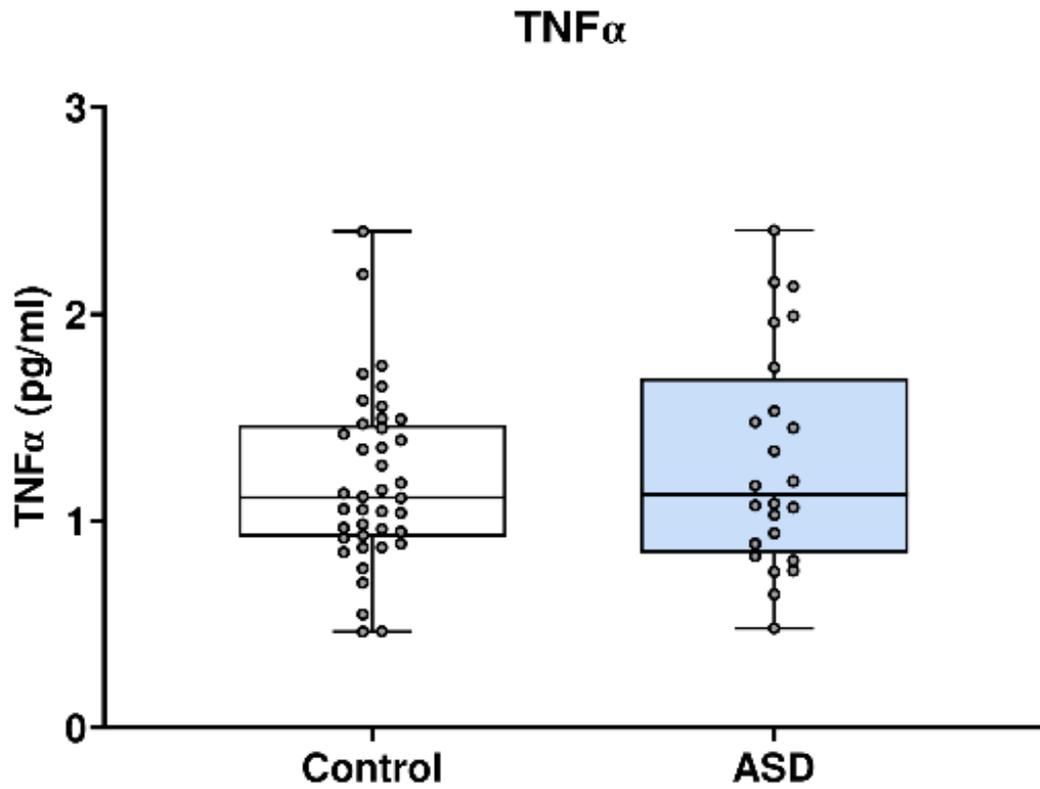


Figure 17: TNF α concentration in ASD cases versus matched controls

TNF α was not significantly altered in mothers of ASD affected children (median 1.127) at 20 weeks' gestation compared to neuro-typical controls (median 1.114). ROUT analysis (Q = 1%) was performed to identify and exclude outliers. 2 outliers were removed (2 controls). Final analysis was performed on n=24 cases and n=40 controls. p = 0.69

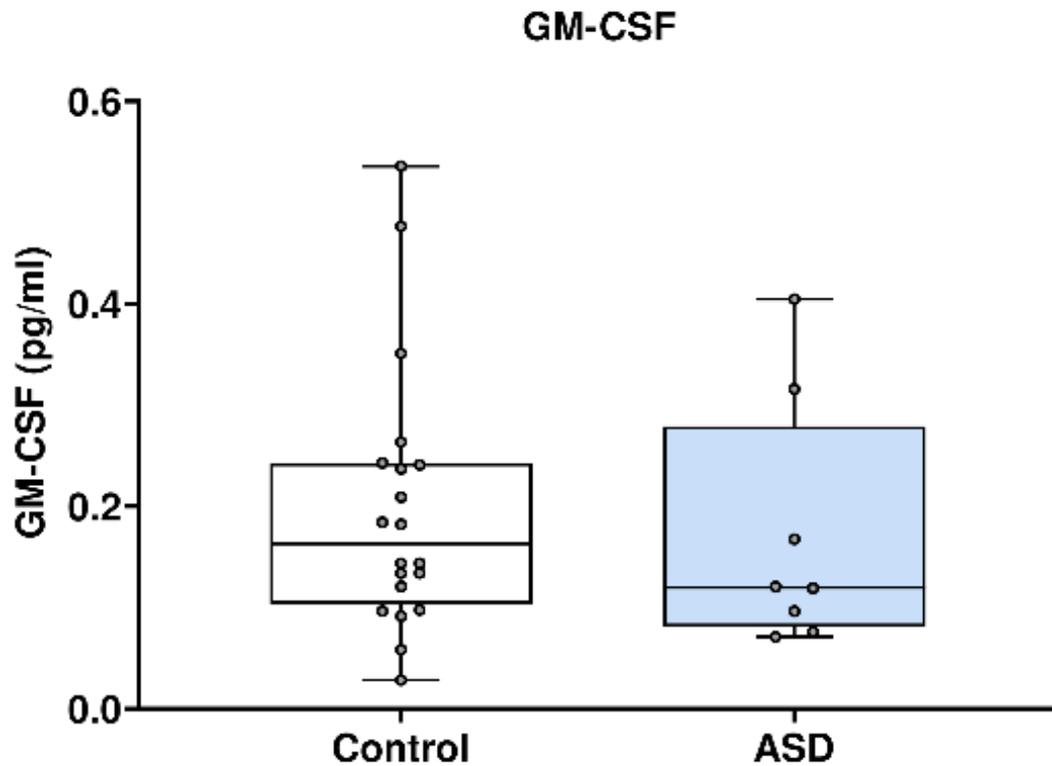


Figure 18: GM-CSF concentration in ASD cases versus matched controls

GM-CSF was not significantly altered in mothers of ASD affected children (median 0.120) at 20 weeks' gestation compared to neuro-typical controls (median 0.163). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. 2 outliers were removed (2 controls). Final analysis was performed on $n=8$ cases and $n=22$ controls. $p = 0.38$

IL-17A

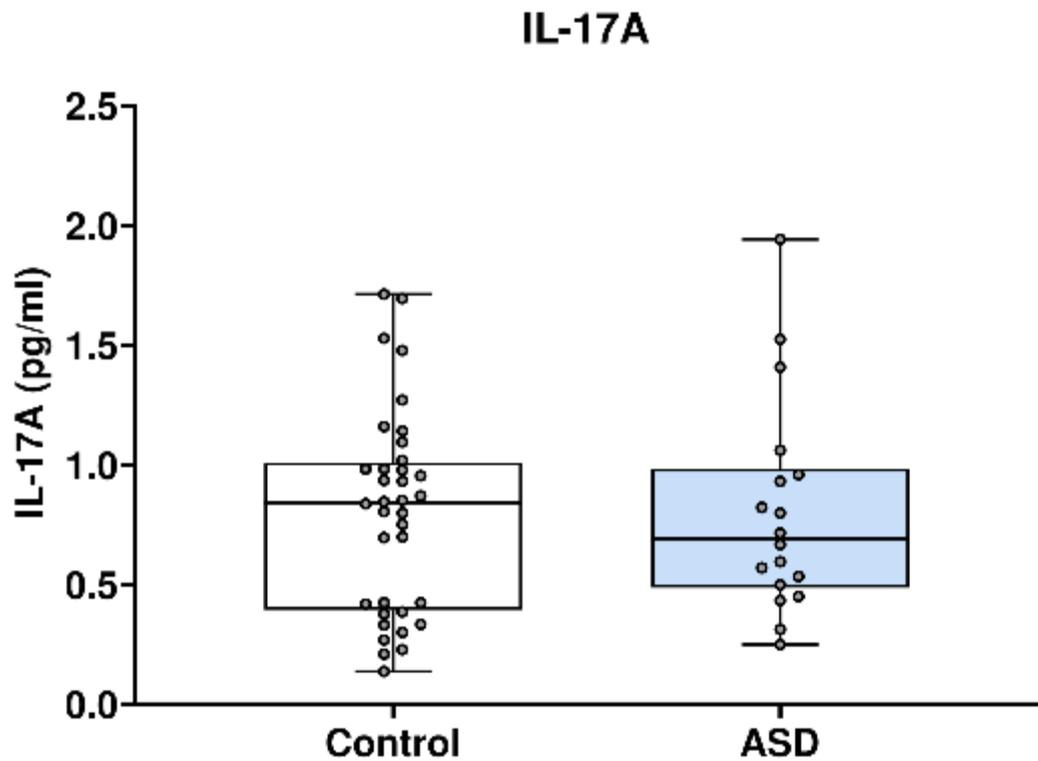


Figure 19: IL-17A concentrations from the Multiplex analysis in ASD cases versus controls

IL-17A was not significantly altered in mothers of ASD affected children (median 0.691) at 20 weeks' gestation compared to neuro-typical controls (median 0.842). *ROUT analysis (Q = 1%) was performed to identify and exclude outliers, none were found. Final analysis was performed on n=18 cases and n=36 controls. p = 0.85*

MSD S-plex ultrasensitive assay:

IL-17A (U)

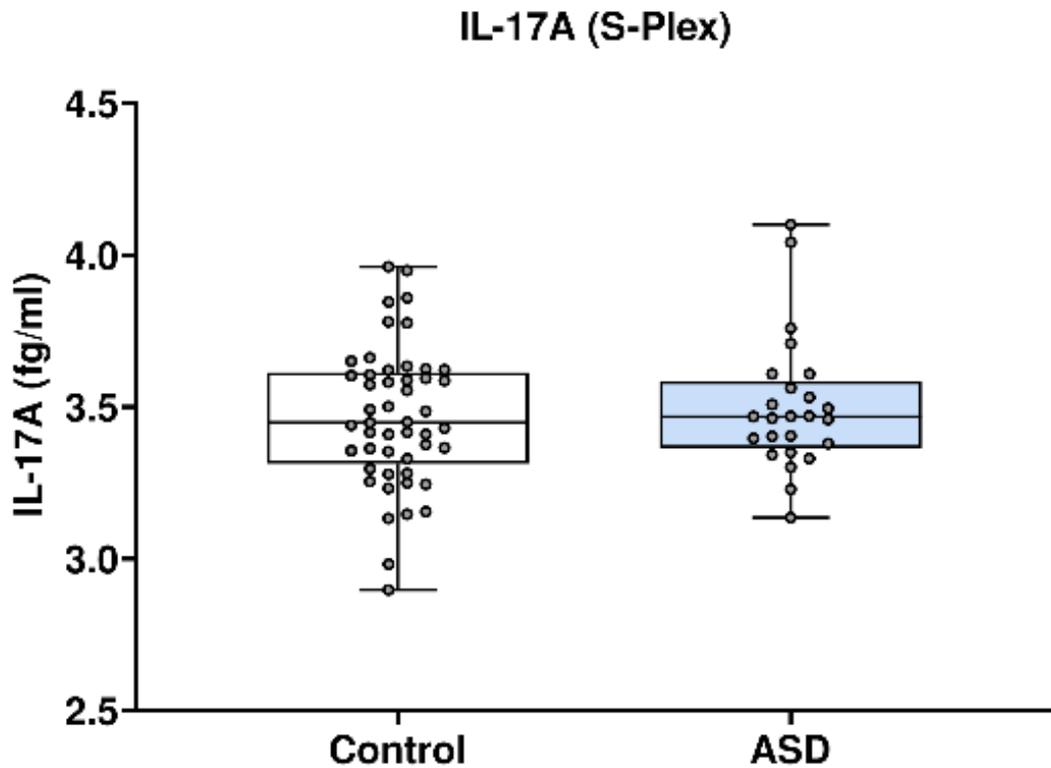


Figure 20: IL-17A (Ultrasensitive) concentrations of IL-17A in ASD cases versus controls

IL-17A was not significantly altered in mothers of ASD affected children (median 3.468) at 20 weeks' gestation compared to neuro-typical controls (median 3.449). ROUT analysis (Q = 1%) was performed to identify and exclude outliers, none were identified. Final analysis was performed on n=25 cases and n=49 controls. $p = 0.80$

Post hoc analysis to examine the effect of storage duration on sample quality:

Our study used samples that had been stored for an extended time (ranging from 9.1 year to 11.8 years) before their analysis (Table 10). Somewhat mitigating this, the majority of samples were collected and stored for a similar duration before use. While degradation is highly likely to have occurred in each sample, we expect that, as all samples were stored under similar conditions, that the degree of cytokine degradation is comparable across all samples. To test this, we correlated sample age with the concentrations of each analyte (Table 11). We found that seven of nine analytes correlated negatively with sample age suggesting some degradation over the period from the most recent to the earliest sampling. Two analytes, IL-6 and GM-CSF, correlated positively but the correlations were “weak” and “negligible” respectively. One analyte, TNF α demonstrated a significant “fair” negative correlation (438) with sample age ρ (*Rho*) -0.308 ($p = 0.01$), which is also reflected by linear regression analysis ($F(1, 63) = 5.037$; $p = 0.028$. $R^2 = 0.074$ (439)). This finding confirms significant and moderate TNF α degradation in the timeframe of our sample acquisition, but no other cytokines were significantly altered in this period. While we undertook steps to reduce cytokine loss from degradation, by avoidance of freeze thaw cycles and remotely monitored ultra-low temperature storage at -80°C (185, 440), it remains likely that sample degradation occurred irrespective of remedial action. However, both controls and ASD samples were stored for similar lengths of time.

Table 10: Sample ages (years)

	Total sample number	Median sample age (IQR)	p - value
<i>Case</i>	25	9.75 (9.48 – 10.71)	0.61
<i>Control</i>	50	10.08 (9.52 – 10.57)	

p-value was calculated using the Mann-Whitney U test as data is not normally distributed

Table 11: Correlation between sample age and analyte concentration

	IFN γ	IL-1 β	IL-4	IL-6	IL-8	TNF α	IL17A	GMCSF	*IL17A
Samples (n)	64	28	17	67	64	66	54	32	74
ρ (Rho)	-	-	-	0.104	-	-	-	0.062	-0.194
	0.103	0.346	0.065		0.137	0.308	0.063		
p	0.42	0.71	0.81	0.41	0.27	0.01	0.66	0.76	0.1

Spearman's Rank Correlation results. We measured correlation using Spearman's rank correlation (Rho) ρ bivariate analysis of sample age and each individual concentration of analyte per sample. Number of samples analysed (n) per analyte. Statistical significance is considered when p value <0.05. *analyte measured using ultra-sensitive MSD assay.

Discussion:

We have shown that the expression of IL-4 in maternal serum is altered significantly between ASD affected and matched control groups at 20 weeks' gestation in a small, but carefully characterised cohort of mothers and children where the child has a diagnosis of ASD by age 10 years.

Previous evidence indicates that aberrations of the immune system may play a role in ASD, (34, 120, 163). Some propose that alterations in cytokine expression could facilitate the classification of ASD subtypes (27, 34, 121) as well as work as biomarkers of response to treatment. In the diagnosis and management of ASD, earlier is better, and identification of reliable biomarkers during pregnancy may allow for targeted behavioural interventions from early infancy. This could also aid the development of targeted pharmacological strategies which have already shown promise in animal models (24), and analogues of which are currently in use in routine medicine practice (164, 165).

IL-4

That we have demonstrated alterations in IL-4 is an interesting finding. In the small number of studies that have examined mid-gestational serum of mothers to ASD affected children, IL-4 is the only cytokine to demonstrate altered expression across all studies (33, 34, 181). Interestingly, while previous authors found levels of IL-4 to be elevated in the ASD affected group versus controls, we have found the opposite. Physiologically, IL-4 is a pleiotropic, generally anti-inflammatory cytokine that functions to suppress the pro-inflammatory milieu. Produced by activated T-cells, NK cells, and mast cell, IL-4 aids the conversion of naïve T helper cells into Th2 cells as well as potentiating the Th2 response (441, 442). IL-4 also has a role in the developmental and maintenance of key regulatory T-cells (Tregs) through STAT6 signalling pathways (443). Tregs are important mediators of inflammation during pregnancy and at the feto-maternal interface (114). We find IL-4 itself at the feto-maternal interface throughout pregnancy (444), indeed in normal pregnancy; levels of IL-4 persist and increase as the pregnancy progresses (445). Low circulating levels of IL-4 during pregnancy have been linked with spontaneous abortions, pre-

eclampsia, intra-uterine growth restriction and pre-term delivery (296, 446, 447). Failure of the usual pregnancy homeostasis (elevated IL-4 levels) may lead to a more pro-inflammatory pregnancy environment with subsequent effects on maternal health, obstetrics outcomes, and child health and development.

Animal-based studies:

Although there are very few human studies that have examined the molecular links between MIA and ASD, many animal-based studies have addressed the question of MIA and the association of elaboration of cytokines and parallel behavioural changes in offspring. MIA has been replicated in a variety of small animal models: mouse, rat and simian phenotypes of ASD have been created through intrauterine inflammatory exposure (123). These models provide valuable insights into the effects inflammation can have on social and communicative behaviour in progeny (123, 142). Remedial steps have been possible with improvements in and resolution of some ASD traits following blockade of specific inflammatory pathways (IL-6 and IL-17A) (24). This work suggested that these two cytokines in particular are significantly involved in the neuronal dysfunction brought about through MIA (24, 142, 151, 169).

In MIA-mouse models of ASD, a commonly used synthetic analogue of double stranded RNA which mimics the effects of viral infection, Polyinosinic:polycytidylic acid or Poly(I:C) (168), is shown to increase IL-17A levels in maternal blood and the postnatal brain of offspring (146). Importantly, there is also an increase in placental mRNA levels of the cytokine, suggesting upregulation of IL-17A activity at the foeto-maternal interface. Determining the role alterations of IL-17A have to play in ASD pathogenicity has become a key question over the past few years. In 2016, Choi *et al* demonstrated persuasively that simulated MIA in murine models leads to elevation in maternal IL-6, leading to downstream activation of maternal Th17 cells. Maternal Th17 cells produce IL-17A that is hypothesised to cross to the foetus via the placenta leading to increased expression of IL-17AR in the foetal brain, contributing to cortical malformations and behavioural abnormalities (24, 160). Conversely, inhibition of IL-17A signalling via IL-17A specific antibodies prevented ASD phenotypes in offspring (24). In support of the synergy between IL-6 and IL-17A, Gene

knockout of IL-6 in Poly(I:C) treated dams results in failure to alter IL-17A levels in offspring, which suggests IL-6 acts upstream of IL-17A (393).

Human studies:

Quite a number of human based studies have examined immune/cytokine aberrations in individuals affected by ASD themselves. In Table 12, for simplicity, we have categorised the cytokines measured in our analysis based on their overall function (178, 179, 180). We also highlight their role in ASD according to the literature

Table 12: Cytokines included in our analysis and their roles and relevance to ASD

Cytokine	Category	Altered in blood/CSF of ASD individual	Altered in gestational blood	Altered in amniotic fluid	Cytokine characteristics Relevance to ASD
TNFα	Pro-inflammatory	(156, 261, 262, 263)	(34)	(182)	Apoptosis of infected cells. Elevated in the CSF and blood of ASD affected individuals (156, 261, 262).
IL-1β	Pro-inflammatory	(156, 261, 264, 265)	(34)	-	A potent pro-inflammatory cytokine involved in both acute and chronic inflammation. Correlated with ASD symptom severity (121).
IL-6	Pro-inflammatory	(156, 261, 263, 264, 265, 266, 267)	(34)	-	Induces production of acute phase proteins and stimulates B-cell antibody production (268). Pleiotropic (affects hematologic, hepatic, endocrine and metabolic function). Thought to impact synapse formation and neuronal migration (269). Potentially mediates IL-17 linked ASD risk in pregnancy (24, 142)

IFNγ	Pro-inflammatory	(120, 156, 266)	(33, 34)	-	Interfaces between innate and adaptive immune response. Secreted by NK cells, and promotes NK killing. Activates macrophages, which produce IL-12 and -23, stimulating Th1 and Th17 cell respectively. Inhibits Th2 cells. Versatile, with a role in defence against intracellular pathogens, tumour surveillance, autoimmunity, allergy and the protection of the amniotic space during pregnancy (270).
IL-17	Pro-inflammatory, Chemotactic	(27, 29, 156, 263, 267, 271)	(34)	-	Derived from Th17 cells, a subset of CD4 cells. Potentiates the innate PMN response throughout inflammation. Postulated to trigger alterations in the blood brain barrier and lead to cortical dysplasia (142).
IL-4	Pro-/Anti-inflammatory, Allergy	(265)	(33, 34, 181)	(182)	A Th2 derived cytokine, often linked with asthma and allergic type inflammation (119). Dual role: pro/anti-inflammatory properties. Crucially important in mitigating inflammation during pregnancy (primarily through suppression of Th1 T-cells and associated cytokines (IL-2 and IFN γ)).
GM-CSF	Growth factor	(272)	(34)	-	A colony-stimulating factor. Produced by stromal cells, it targets bone marrow, and precursor cells, mediating haematopoiesis.

IL-8	Chemotactic	(30, 264, (34) 266)	-	Produced by fibroblasts, neutrophils and macrophages. Chemo-attractant for phagocytes at site of inflammation.
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Note: Reference numbers for supportive scientific literature in parentheses (all are human based studies)

While the cytokine profiles of ASD affected individuals have been well characterised, very few studies have investigated the relationship between mid-gestation cytokine levels and ASD risk in offspring. To our knowledge, only three human studies have examined maternal serum (33, 34, 181), and one more has examined amniotic fluid cytokine profiles in mothers of ASD affected children (182). Below, we have summarised the findings from these studies, which effectively provide all of our current understanding of gestational cytokine profiles in the setting of ASD.

Previous literature on gestational samples analysis in ASD:

Working from the same laboratory and using similar methods, Goines *et al* (2011) and more recently, Jones *et al* (2017) both demonstrated elevated mid-gestational cytokine levels between groups of ASD affected children versus controls or children without ASD. Goines *et al* demonstrated elevated levels of mid-gestation (15 – 19 weeks' gestation) IFN γ , IL-4 and IL-5 with an associated 50% increased ASD risk. While Jones *et al* showed elevated levels of mid-gestation GM-CSF, IL-6, IFN γ and IL-1 α in the ASD affected group versus children with developmental delay, but not ASD. The authors do not mention the age of the samples used in either study, but the samples used were sourced from the same birth cohort in Orange County, California between 2000 and 2003. In both studies, the samples were initially stored at room temperature and later at - 20°C freezer conditions before long-term storage at - 80°C. This initial handling may have contributed to some cytokine degradation. In the Goines study, ASD cases were matched with neuro-typical controls based solely on child characteristics (sex, birth month and year), something which the authors acknowledge in their limitations. Neither study had access to comprehensive maternal health information during the pregnancy (including intrapartum infections). Nor did they have a record of relevant maternal medical history, all, information important to the interpretation of their findings.

Irwin *et al* (2018) demonstrated alterations in IL-4, MCP-1 and IL-10 levels in 28-week gestation serum of mothers who birthed ASD affected children (181). Specifically, IL-4 (usually anti-inflammatory or involved in allergic type inflammation (119)) was increased and associated with higher ASD symptomology (as measured by the Social Communication Questionnaire (SCQ)) in offspring. Higher concentrations of IL-10

(anti-inflammatory) were associated with fewer ASD symptoms in offspring (measured by the Social Responsiveness Scale (SRS)), and finally, elevated MCP-1 was associated with fewer ASD symptoms (as measured by the SCQ). The samples used in this analysis were reported to be at least 5 years old. No controls were used in this analysis, instead a large cohort of ASD affected individuals were enrolled, and the 28-week gestation cytokine concentrations were correlated with ASD symptomology at 7 years of age. This is novel in two senses, none has previously assessed the cytokine profile in the third trimester, and none has correlated cytokine findings with severity of ASD symptomology in this way. As with previous authors, they had no access to relevant maternal pre-conceptual medical history or gestational infections data.

Finally, Abdallah *et al* (2013) examined amniotic fluid samples and found elevated levels of IL-4, IL-10, TNF α , and TNF β . In a preliminary study (2012), they also identified elevations in MMP-9 in ASD cases relative to controls (183). Advanced sample age is again an issue with the oldest samples in this analysis being 29 years old, the youngest 10 years old. The samples were stored at -20°C according to local guidance (184). Both the storage conditions and the samples ages are likely to have contributed to significant cytokine degradation (185).

Limitations:

The samples used in our study fall outside the ideal sample age for accurate analysis of cytokines (185). To our mind, this is the single most important limitation confronting studies of this nature. Unfortunately, the shelf life of archived samples is finite, and even samples in long-term ultra-low temperature storage (-80°C) suffer from degradation of cytokines and chemokines over time (185, 440). Retrospective sample analysis, would present an excellent opportunity to study cytokine aberrations in ASD, if the time to ASD diagnosis was shorter. One UK study found that the average delay between concerns first being noted by parents and the child receiving a diagnosis of an ASD was 4.6 years (SD 4.4 years) (448). ASD services continue to be under-resourced (212) and diagnoses are chronically delayed (449). Under current conditions, our experience of retrospective analysis of archival samples suggests that this style of study design is not well suited to addressing this

question. Even large-scale population based studies would suffer from the same issues of sample fidelity over longer periods.

To ensure future study designs are capable of accurate mid-gestation cytokine analysis, they should be prospective, and concentrate on early ASD case identification or screening. Early identification should be paramount, the diagnostic stability of ASD is reliably fixed from as early as 14 months old (208) so screening and identification within the first 2 - 3 years of life is possible. Cytokines should be analysed contemporaneously, acute phase reactants such as IL-1 β and IL-6 have demonstrated greater than 50% degradation within 3 years even in -80°C freezer conditions (185). IL-4 is stable only for 3 years, while IL-17A, IFN γ , and TNF α , all suffer more than 50% degradation within 4 years at ultra-low temperature storage (185). Basic handling of samples and initial processing requires optimisation to ensure the risk of sample degradation is minimised: (i) Store samples at ultra-low temperatures, (ii) initial processing should be rapid (<1 hour from venepuncture to freezer storage) and (iii) freeze-thaws cycles should be minimised. With robust methods of early screening in place, early confirmatory diagnosis within the first 2/3 years, and analysis of gestational samples within 3 years, it should be feasible to increase the yield and validity of such studies, and greatly reduce cytokine loss through prolonged storage. While this approach would allow for study of children presenting with the earliest signs of ASD, or targeted high-risk groups (ASD affected siblings). It would likely miss those presenting later, including those who are a high-functioning phenotype or of female sex.

Finally, our small sample size is a major limitation, and results should be interpreted with caution. Analysis of IL-4 levels in the groups yielded results on only 16 individuals (6 cases and 10 controls). Attrition of the viable samples was due to a combination of the low absolute concentrations of IL-4 in the samples (likely exacerbated by advanced sample age), concentrations at or below the sensitivity (LLOD) of the MSD multiplex format and high CV values. It is difficult to make inferences about results in samples this size, and larger scale group analysis is warranted.

Strengths:

Although our study has suffered from some of the same limitations as previous studies, our study is strengthened by the quality of our cohort. Each child had a concrete specialist service ASD diagnosis, confirmed by the clinical paediatric fellow. Each child was well characterised clinically and matching was strictly observed. Matching was not only based on child characteristics (Sex, Gestational age, Birthweight), but also on an important maternal characteristic, BMI at 15 weeks' gestation. This enhanced the validity of our results. In addition to detailed child characteristics, we have also included important information regarding the past medical histories, medication or anti-inflammatory use, and pre-existing inflammatory conditions of the mothers included in the study. We present crucial information about infection rates in the first 20 weeks of pregnancy, all of which presents a major confounder to accurate analysis if this information is absent. Our methods were robust, and we identified two key issues of multiplex assay sensitivity and advanced sample age, and remedied the former through utilisation of ultrasensitive single analyte plates.

Conclusion:

In conclusion, in a carefully characterised maternal-child cohort study we did not replicate the findings of similar mid-gestational studies, but did find some evidence of mid-gestational cytokine aberrations (downregulated IL-4) in the mothers of children with ASD. Reduced levels of IL-4 are linked to a pro-inflammatory state during pregnancy and negative obstetric and foetal outcomes. All studies to date have had similar and significant limitations. Future studies should focus on minimising the time between sample acquisition and analysis, use of best practice for initial sample handling, and early identification and characterisation of cases and their mothers. Future analysis should be serial and include investigation of samples taken from early in pregnancy. The first trimester, and particularly 8 - 12 weeks' gestation is a crucial period for organogenesis and differentiation, and analysis from this period will help complete the picture of gestational cytokine fluctuations and their effect on neurodevelopment.

List of abbreviations:

ADI-R: Autism Diagnostic Interview-Revised

ADOS: Autism Diagnostic Observation Schedule

ASD: Autism Spectrum Disorder

BMI: Body Mass Index

CBCL: Childhood Behavioral Checklist

CD 4 cell: Cluster of Differentiation 4 cell

CREC: Cork Research Ethics Committee

CSF: Cerebrospinal Fluid

DISCO: Diagnostic Interview for Social and Communication Disorders

ECL: *Electrochemiluminescence*

ELISA: enzyme-linked immunosorbent assay

GE: Gastroenteritis

GMCSF: Granulocyte-macrophage colony-stimulating factor

HLA-G gene: Human Leukocyte Antigen G coding gene

HSE: Health Service Executive

ID: Intellectual Disability

IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10 and IL-17A: Interleukins 1 alpha, 1 beta, 2, 4, 6, 8, 10, 17A

IL-17AR: Interleukin 17A receptor

IQR: Interquartile Range

ISO: International Organization for Standardization

KBIT-2: Kaufmann Brief Intelligence Test, second edition

LLOD: Lower Limit of Detection

LLOQ: Lower Limit of Quantification

MIA: Maternal Immune Activation

mRNA: messenger Ribonucleic Acid

MSD: Meso Scale Discovery

MZ: Monozygotic

Poly (I:C): Polyinosinic: polycytidylic acid

PMN: Polymorphonuclear cells

PP: Polypropylene

PSS: Perceived Stress Score

ROUT: **Robust regression and **Outlier** removal**

RTI: Respiratory Tract Infection

SCQ: Social Communication Questionnaire

SOP: *Standard Operating Procedure*

SRS: *Social Responsiveness Scale*

STAT6: Signal transducer and activator of transcription 6

Th1, 2 and 17: T helper 1, T helper 2, and T helper 17 cells

TNF α , TNF β : Tumour Necrosis Factor alpha, beta

Tregs: Regulatory T-cells

ULOQ: Upper Limit of Quantification

UTI: Urinary Tract Infection

Declarations:

Ethics approval and consent to participate

The Cork Research Ethic Committee (CREC) approved all research protocols in this study. Each participant gave informed consent. Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall 6 Little Hanover Street, Cork, Ireland.

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Not applicable.

Authors' contributions

MC wrote the manuscript, collated cohort data, designed and performed all experimental work equally with SC, and performed data analysis. SC commented on the manuscript at all stages and performed data analysis, designed and performed experimental work equally with MC. LG commented on the manuscript at all stages and advised regarding characterisation of the cohort and ASD diagnosis. GOK commented on the manuscript revisions and aided in statistical analysis. DM commented on the manuscript, was involved in study design, and was the key supervisor. All authors read and approved the final manuscript

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

Chapter 5

Dysregulation of Steroid and Sulfur Metabolism, Glycolysis, and Cell Adhesion molecular pathways in cord blood preceding Autism diagnosis

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**Dysregulation of Steroid and Sulfur Metabolism, Glycolysis, and Cell Adhesion
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ABSTRACT

While there is considerable evidence implicating maternal immune activation (MIA) and cytokine dysregulation in the pathophysiology of Autism, inflammatory cytokines are unlikely to translate clinically as prognostic biomarkers due to their lack of specificity. Our aim was to identify perinatal molecular dysregulation in umbilical cord blood, which preceded the onset of childhood Autism, and ascertain whether these in-utero biomarker alterations persisted into pre-pubertal childhood. In a cohort of 2,137 mother-infant dyads, we undertook a nested case-control study in the BASELINE Birth Cohort. Proteomics and metabolomics analysis was performed on cord blood plasma from 22 children diagnosed with Autism at or before age 5, and 44 neurotypical controls. In a clinical diagnostic follow-up between the ages of 7 and 10 years (PiRAMiD), 24 children with Autism and 48 neurotypical controls provided blood samples for longitudinal profiling of molecular candidates. In cord blood, Autism outcome was associated with reduced levels of circulating steroid and steroid derivatives, alongside reduced levels of proteins, with roles in sulfur metabolism, glycolysis, and cell adhesion. Dysregulation of GAPDH, SELENBP1, and BLVRB proteins were evident in both cord blood and in serum from pre-pubertal children with Autism. Our findings were further corroborated using machine learning approaches, with AUROC in test sets for top performing models ranging from 0.82 to 0.86 for proteomic and metabolomic prediction models, respectively. Collectively, these data suggest cord blood molecular signatures precede the onset of Autism and have the potential to lead to prognostic biomarkers, while also highlighting potential materno-feto-placental molecular processes, which underpin Autism aetiology, and warrant further investigation.

Keywords: Autism, ASD, cord blood, proteomics, metabolomics, sulfur metabolism, steroid biosynthesis, glycolysis, machine learning.

INTRODUCTION

Autism Spectrum Disorder (ASD) is a childhood onset neurological developmental disorder that affects social communication and behaviour (56). For the remainder of this document we will refer to ASD as Autism, and the names may occasionally be used interchangeably. Autism affects at least 1% of the Irish population, concurrent with rates in the US and the UK (450). It is amongst the top medical and psychiatric conditions in terms of personal, familial, societal, and economic strain (450). Although clinical features of Autism are often evident in early developmental years, the majority of children with Autism are not diagnosed reliably until at least 3 to 4 years old, and in many cases much later (451). Pilot studies demonstrate that targeted intervention in high-risk infants, as young as 6 months old, can improve clinical outcomes (243, 452, 453); yet early intervention services for this cohort are not widely available, or financially supported (454). Robust biological predictors of Autism outcome have the potential to enable early, targeted interventions, elucidate the underlying pathophysiology of Autism, and identify novel therapeutic targets, alleviating symptoms of Autism (455, 456).

The prevalence of Autism has consistently increased over the past two decades, from 1 in 1,000 in the early 1990s, to 1 in 68 presently in the USA (50). Researchers attribute this to increased recognition of Autistic symptoms, improved diagnostics, broadening of the construct over time, and increased environmental influences (52). While genetic variants causative in Autism have been identified, the underlying contributions owing to a large proportion of Autism cases remains unexplained by genomics alone. This makes the clinical and translational utility of genomics indistinct. Additionally, there are substantial differences in Autism prevalence between genders (1/42 males; 1/189 females), which is unexplained by genetic variants suggesting mediation by unknown biological factors (17, 18).

Pharmacological management in Autism generally focuses on symptoms and co-occurring conditions (such as irritability and anxiety respectively), without addressing

the underlying core Autism symptoms (1). To date, risperidone and aripiprazole are the only FDA-approved drug treatments for irritability in Autism. These drugs are antipsychotics with a wide variety of therapeutic indications, none specific to autism (154). In order to develop targeted therapeutic treatments for Autism, and improve diagnosis, classification, and stratification, a better understanding of the molecular pathways underlying autism is required. Greater understanding of Autism pathophysiology may result in the identification of blood biomarkers with practicable therapeutic applications. The emergence of one clear molecular pathway indicative of risk or an Autism specific molecular pathway would be a significant advancement in our understanding of brain development in autism.

Although several studies have explored blood-based biomarkers of Autism in children and adults (154, 455), few have explored cord blood for prognostic markers of Autism, and no studies have had access to longitudinal samples for the exploration of persistent molecular changes from birth into childhood. Numerous studies sought to identify blood-based biomarkers of ASD in affected adolescents and adults (120, 154) and have reported alterations of molecules involved in iron transport (155), inflammation (28, 156), brain development (157), and metabolism (158). There is accumulating evidence that autistic individuals have significant differences in brain development, and this evidence suggests that the neurobiological alterations that occur prenatally or during the first years of life may underlie the neuroanatomical and behavioural aspects of Autism (201, 457). It is therefore plausible that there are differential molecular changes present in cord blood at birth, preceding the diagnosis of Autism. Here, we describe the first integrated proteomic and metabolomics study to profile cord blood from neonates with a later Autism diagnosis. The umbilical cord at birth represents an opportunity to explore the in-utero environment, and capture the early materno-feto-placental molecular mechanisms implicated in neurodevelopmental outcomes. We conducted a nested case-control study recruiting participants from the Cork Baseline Birth Cohort with the aim of undertaking integrated OMICS analysis in cord blood and a follow-up longitudinal profiling study, to identify persistent markers of Autism at birth and in children aged between 7 and 10 years.

MATERIALS AND METHODS

For extended materials and methods, please refer to supplementary information.

Participants

We recruited children from the Cork Birth Cohort Study, BASELINE (Babies after SCOPE: Evaluating the Longitudinal Impact on Neurological and Nutritional Endpoints) (www.baselinestudy.net) (458). The research team performed developmental assessments using the Ages and Stages Questionnaire (ASQ) at 2 years. At 5 years, the children also completed a cognitive assessment (KBIT-2) with a trained research nurse. The parents completed a Child Behaviour Checklist (CBCL) questionnaire at both time-points. Between June 2019 and August 2020, the clinical research fellow contacted each child who had a confirmed or suspected Autism diagnosis at 5 years. Suspected Autism was based on the CBCL findings at 5 year follow up or parental report. The fellow confirmed their diagnosis and invited them to attend a further developmental assessment in later childhood, as part of the PiRAMiD (Predicting early onset autism through maternal immune activation and proteomic discovery) study. In the follow up assessment, children were aged between 7 and 10 years. Children were matched based on sex, birth gestation, birth weight and maternal BMI at 15 weeks' gestation. Neurotypical controls were recruited from the same birth cohort. Two matched controls for each Autism case were initially included, but follow up of all controls was challenging due to safety restrictions and social distancing protocols encountered during the Covid-19 pandemic. Notwithstanding, each case matched with at least one control. Controls also attended a late childhood developmental assessment. Please refer to Figure 21 for participant flow chart.

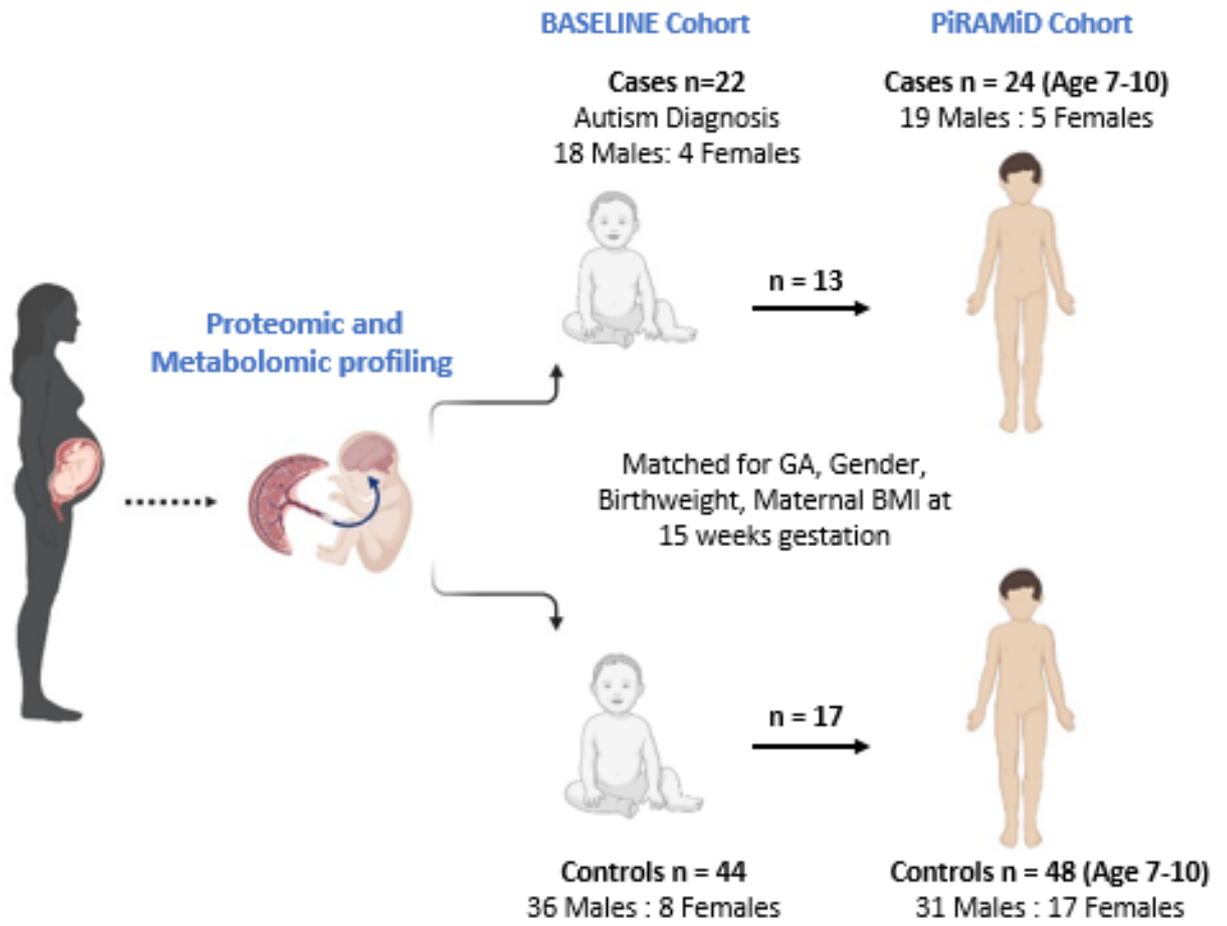


Figure 21: Study design:

A nested case control study of 22 ASD cases and 44 matched controls were selected based on their neurodevelopmental outcomes, and a formal Autism diagnosis at 5-year BASELINE follow-up. We used cord blood plasma collected from these children in the immediate post-natal period for our multi-omics analysis. Longitudinal follow up was at 2 years, and 5 years in BASELINE, and in later childhood (7-10 years) in PiRAMiD. The greater number of children with ASD at this time point is accounted for by recruitment of children who received a formal ASD diagnosis after the 5-year BASELINE assessment. These children were clinically characterised using information gathered at the PiRAMiD follow up appointment as well as extensive prenatal maternal health records from the SCOPE database.

Clinical Diagnosis of Autism

Most children received their ASD diagnosis through the Health Service Executive (HSE) Early Intervention Team (EIT). The standard tests utilised in this setting are the Autism Diagnostic Observation Schedule, Second Edition (ADOS-2), and parent report via, either the Diagnostic Interview for Social & Communication Disorders (DISCO) or Autism Diagnostic Interview-Revised (ADI-R) questionnaires. A small number of children received their initial diagnosis through private multidisciplinary teams (MDTs) using the same assessment tools. All of these children later received a confirmatory diagnosis with the EIT service.

Biospecimens

Cord blood was obtained at birth from children enrolled within BASELINE by researchers involved in the study. Cord blood samples were collected in 4 ml BD EDTA-Vacutainer tubes (Becton Dickinson). Once collected, samples were stored on ice. After centrifugation, the plasma was stored in 200µl aliquots at –80 °C. Children with Autism and controls who attended the later childhood assessments (age range at attendance 7 – 10 years) provided blood for biochemical analysis. Serum was collected in BD Vacutainer serum tubes and following centrifugation, sera were stored in 250µl aliquots (Wilmut) at –80 °C. Samples were stored in – 80°C long-term storage ISO accredited bio-banking facilities. Local standard operating protocols were followed in all handling and storage of human bio-samples.

Proteomics analysis of cord blood

The full list of all reagents and chemicals used for proteomic analysis are detailed in supplementary information.

High-abundance protein depletion of cord blood

To improve the dynamic range for proteomic analysis, 45µl of cord blood plasma from each participant was immunodepleted of the 14 most abundant proteins (Alpha-1-antitrypsin, A1-acid glycoprotein, Serum Albumin, Alpha2-macroglobulin, Apolipoprotein A-I, Apolipoprotein A-II, Complement C3, Fibrinogen alpha/beta/

gamma, Haptoglobin, IgG A, IgG G, IgG M, Transthyretin, and Serotransferrin). This was performed using the Agilent Hu14 Affinity Removal System (MARS) coupled to a High-Performance Liquid Chromatography (HPLC) system (full details in supplementary methods).

Sample preparation and mass spectrometry

Sample preparation for mass spectrometry including protein quantification, protein digestion, and peptide purification was performed as previously described (459) and are further detailed in supplementary methods. All samples were run on a timsTOF Pro mass spectrometer (Bruker Daltonics, Bremen Germany) connected to the Evosep One chromatography system (EvoSep BioSystems, Odense, Denmark). Full methods are detailed in supplementary methods

Data pre-processing, bioinformatics and statistical analysis

Raw data acquired on the timsTOF were analysed in FragPipe (V18.0) using the built-in DIA-Speclib_Quant workflow. Protein group (PG) files generated were subsequently uploaded into Perseus (V2.0.5.0) where the data was grouped by case-control status, filtered for outliers, contaminants, and missing values. Only proteins present in >70% of samples in at least one group and that had a value ≥ 1 were taken forward for quantification. The data was subsequently log₂ transformed, normalised based on median values and analysed for differential expression, using a Student's t-test. The STRING database (<https://string-db.org/>) was employed to explore the converging pathways and coordinated changes in functional, biological, and cellular processes at the level of the proteome. STRING uses protein accession numbers to identify the protein-protein interactions and pathway relationships present. Exports from STRING include KEGG and GO pathway analysis results which were performed on all proteins differentially expressed ($p < 0.05$) within the BASELINE data set.

Proteomic analysis of serum from children

High-abundance protein depletion of serum samples was undertaken whereby 45 μ l of serum collected from children in the PIRAMiD study were immunodepleted using HPLC. Sample preparation and mass spectrometry for proteomic analysis were performed as described above. All data files generated from the serum of our

PiRAMiD cohort underwent analysis in FragPipe (V18.0) with outputs processed in Perseus (V2.0.5.0). Furthermore, candidate biomarkers identified as differentially expressed in cord blood at birth (SELENBP1, GAPDH, BLVRB) were verified at the peptide level in the open-source software tool Skyline (<https://skyline.gs.washington.edu>), for visualisation of MS1 and MS2 fragment data. The library was constructed by searching the QC injections, which were interspersed after every ten injections throughout the MS run. For our peptide targets, mass chromatograms were extracted for +2 and +3 precursor charge states and their associated fragment ions. All parent and fragment level data was visually confirmed, and peak editing was undertaken where necessary, using the peptide Retention Time (RT), dotproduct (idop), mass accuracy (<10ppm), and a confirmed library match, to reliably identify and quantify peptides across the DIA runs (Supplementary methods).

Metabolomics analysis of cord blood

The full list of all reagents and chemicals used for proteomic analysis are detailed in supplementary information. Preparation of cord blood EDTA plasma for metabolomic analysis by LC-MS/MS was carried out according to established protocols for serum/plasma (460). Full details on the sample preparation and mass spectrometry methods in Supplementary information.

Data pre-processing, bioinformatics and statistical analysis

We uploaded MSE data from the metabolic profiling of cord blood samples on to Progenesis QI 2.4 software (Nonlinear Dynamics, Newcastle, U.K.) for peak picking and alignment. Data were normalized to 'All Compounds' in Progenesis QI. The coefficient of variation (CV) was computed for each compound across all QC runs (n=8) to evaluate repeatability of measurement, and compounds with > 30% CV within the QCs were removed. Any compound which had >30% missing values across the entire sample set or >30% in either subgroup (case or control) was removed. 1782 features (429 from negative mode, 1353 from positive) remained which were then log₂ transformed and normalised on the median values. T-tests for significant differences between the cases and controls were then carried out and corrected for false discovery rate (FDR) using the Benjamini-Hochberg procedure. Spearman's

correlation coefficient was used for the assessment of correlation between steroid metabolites. All pre-processing and statistical analysis was conducted using Python (461) and R statistical software (462). Python libraries used include Pandas (463), Numpy (464), Matplotlib (465), Scipy (466), Scikitlearn (467). R packages applied were ggplot2 (468), ggcorrplot (469), Tidyverse (470) and mixOmics (471).

[Targeted steroid analysis in cord blood and serum from children](#)

A targeted LC-MS/MS method using a Waters ACQUITY-Xevo TQMS instrument was developed in house for the quantification of 13 steroids in both cord blood and serum samples from children in the BASELINE and PiRAMiD cohorts respectively. Differential expression of these 13 metabolites between Autism cases and matched controls was assessed using the Mann Whitney U test. ANCOVA was employed to adjust for age of participants at the time of sample collection and potential variations in steroid concentrations due to age ranges. The sample preparation method, the full list of steroids quantified and their acquisition parameters are included in supplementary information.

[Machine Learning \(ML\) for predictive modelling in Autism](#)

ML modelling was performed on the discovery proteomics and metabolomics in cord blood, and the targeted steroid MRM data in cord blood. Compounds that passed QC and missing value filtering were taken forward for machine learning analysis. The metabolite and protein quantifications were standardized to ensure optimum run times for ML analysis. Feature ranking was applied to determine the features with the highest predictive values for the prediction of Autism. Random Forest's Gini index was used to determine the optimum subset of variables for prediction. The top 20, and top 10 features identified in the feature ranking, as well as the features that displayed significant alterations following t-tests were selected for modelling, and comparison of predictive scores between subsets of features. Separate Random Forest and Logistic Regression models were used to assess each subset on their predictive value. Each model was trained using 5-fold cross validation to reduce overfitting. Area Under the Receiver Operator Characteristic (AUROC) curve was used

to compare model performance. ML methods have been undertaken as previously described (472).

RESULTS

Participants

The SCOPE Ireland pregnancy cohort (www.scopestudy.net) formed the basis of initial recruitment of infants to BASELINE [n = 1537] and an additional 600 infants were recruited after delivery providing a total sample of 2137 children who were followed from birth. At 5-year follow up, 22 children had a formal ASD diagnosis. A further 26 children scored in the “at risk/borderline range” for Autism Spectrum Disorder/Pervasive Development Disorder based on the CBCL; expressed parental concern regarding the possibility of Autism; or reports of an Autism associated developmental delay (most commonly speech delay).

The clinical research fellow, between May 2019 and March 2020, contacted all 22 children who had an Autism diagnosis at the 5-year assessment, confirming their Autism diagnosis through parental report. The fellow also verified if an interval diagnosis of Autism had been made in the 26 additional children identified as being at risk of Autism at the earlier 5-year follow up. Of those, 15 children had received an interval Autism diagnosis, meaning the PiRAMiD Autism cohort totalled 37 children. Ultimately, of the children with a confirmed ASD diagnosis (n = 37), 34 (92%) were able to participate. Two families could not attend due to childcare or work difficulties, while one child was excluded due to an underlying genetic syndrome.

We selected healthy, neurotypical controls from the same BASELINE cohort and matched controls with cases for (i) Infant sex, (ii) Gestational age, (iii) Birthweight and (iv) Maternal BMI at 15-week visit. Initial matching was on a 2:1 basis but control recruitment was incomplete due to the constraints of social distancing and COVID-19 safety protocols in 2020. Each case was at least matched with one control, and 56 controls attended the later childhood follow up (PiRAMiD). Of those who attended the later childhood appointments, 25 of 34 (74%) cases and 51 of 56 (91%) controls allowed blood sampling. Participant demographics were assessed for significant differences between groups and are presented in Table 13.

Table 13: Demographics and participant characteristic in the BASELINE cohort for cord blood analysis

Variable	Cases (n=22)		Controls (n=44)		p-value
	n or Median	(%) or IQR	n or Median	(%) or IQR	
Male sex	18	(82%)	36	(82%)	1
Infant birthweight (Kg)	3.54	(3.05 – 3.76)	3.53	(3.25 - 3.7)	0.92
Gestational age	39.8	(38 – 41)	40.2	(38.3 – 41)	0.60
Maternal age	32	(29.5 – 34.3)	31	(29.5 – 32)	0.32
Maternal BMI	25.5	(23.6 – 30)	25.4	(23 – 29)	0.50
Socioeconomic Index	50	(37.5 – 50)	44	(23.3 – 52)	0.39
Alcohol (in pregnancy)					0.13
No	15	(68%)	20	(46%)	
Yes, but stopped	2	(9%)	13	(30%)	
Drank in pregnancy	5	(23%)	11	(24%)	
Smoking (in pregnancy)					0.96
No	18	(82%)	35	(80%)	
Yes, but stopped	2	(9%)	4	(9%)	
Smoked in pregnancy	2	(9%)	5	(11%)	

PSS score at 15 weeks	9.5	(8 – 13.3)	12	(9 - 17)	0.20
Inhaled steroids in pregnancy	1	(4.5%)	4	(9%)	0.51
Paracetamol in pregnancy	6	(27%)	14	(32%)	0.70
Infection during 1st trimester	2	(9%)	4	(9%)	1

Participants and clinical characteristics of the BASELINE Birth Cohort for cord blood analysis. Relevant clinic characteristics for the children with cord blood analysis. The key matching variables were sex, gestational age at birth (in weeks), birthweight, maternal BMI at 15 weeks gestation. Also demonstrated are relevant other variables including maternal age at delivery, SEI recorded at 15 weeks gestation, alcohol and smoking status during pregnancy, paracetamol, inhaled corticosteroid use during pregnancy, and perceived stress scale scores at 15 weeks. Finally, we document the rates of documented infections (Urinary Tract infections (UTI) and upper respiratory tract infections (URTI)) in the first trimester in each group. We present data as numbers and percentages (n %) or medians and interquartile range (IQR). P-values were calculated using Mann-Whitney-U Test for non-parametric continuous data, and using Pearson Chi Square for categorical data. P-values are significant at <0.05.

Table 14: PiRAMiD characteristics

Variable	Cases (n=24)		Controls (n=46)		p-value
	n or Median	(%) or IQR	n or Median	(%) or IQR	
Male sex	19	(79%)	30	(65%)	0.23
Age at follow-up	8.46	(8.08 – 9.14)	9.24	(8.50 – 9.75)	0.02
Infant birthweight (Kg)	3.38	(3.01 – 3.69)	3.54	(3.36 - 3.87)	0.30
Gestational age	39.1	(38.2 – 40.5)	40.1	(39.6 – 41)	0.09
Maternal age	32	(29.3 – 34.8)	32	(30 – 34.3)	0.88
Inhaled steroids (child use)	0	(0%)	5	(11%)	0.09

This table presents the basic clinical characteristics of the children whose samples from the 7-10 year assessment were analysed. The age at follow up is significantly different between groups; ANCOVA was used to adjust for differences in age at time of sampling. Other important potential confounders such as steroid use were not found to differ between groups.

Proteomics signature of Autism in cord blood

We profiled 680 proteins in cord blood for differential expression between 22 cases and 44 controls in the BASELINE cohort. After filtration, 558 proteins were present in >70% of samples and were taken forward for differential expression analysis. We identified 41 proteins as significantly differentially expressed (prior to FDR correction) between Autism cases and controls ($p < 0.05$) (see Figure 22, Figure 23, Figure 24, Figure 25). No compounds retained significance following correction for false discovery at 5%. The full list of differentially expressed proteins, along with p-values and fold changes are listed in supplementary information (Supplementary

Table 1). The 41 significantly altered proteins were uploaded into the STRING database to identify the pathways and processes altered in Autism. The top networks implicated mapped to keratin signalling (n = 5), cell adhesion molecules (n = 8), metabolism, cell signalling (n = 19), complement (n = 2), Heme degradation (n = 2) and hemoglobin/oxygen transport (n = 2), with some overlap between pathways Figure 26.

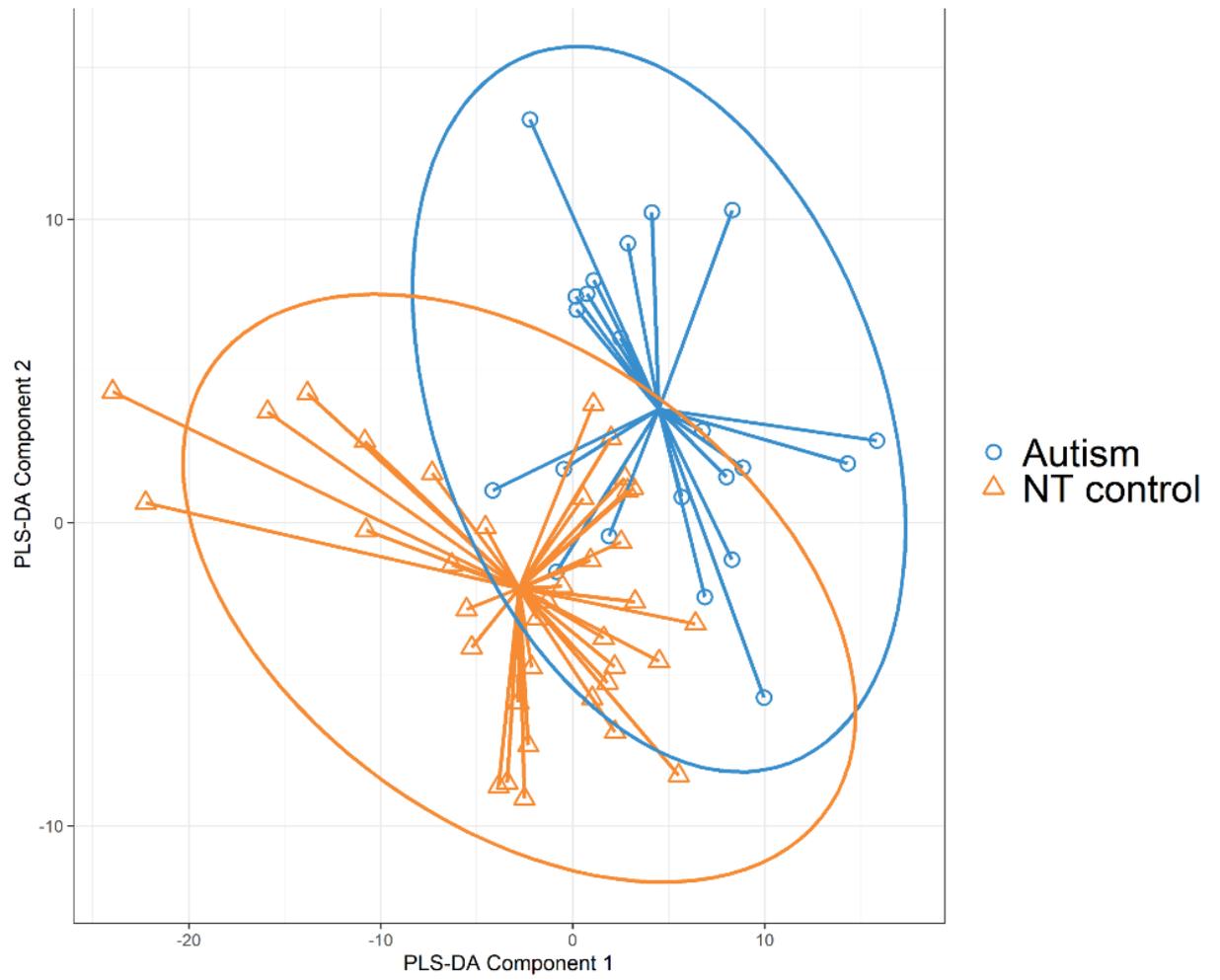


Figure 22: PLS-DA (Partial least squares-discriminant analysis)

Cord Blood analysis. PLS-DA plot of samples with clustering ellipses. We observed no clear separation between groups.



Figure 23: Autism vs control and changes in protein expression:

Y-axis is \log_{10} p-values and X-axis is \log_2 FC. Blue dots above the horizontal midline are significantly altered. Compounds to the left on the graph are reduced in the autism cohort, and those on the right are increased. Volcano plot of all proteins identified and measured in proteomics analysis of cord blood plasma samples. We profiled 680 proteins for differential expression, 41/680 were identified as significant at a $p < 0.05$. No compounds retained significance after applying a 5% FDR

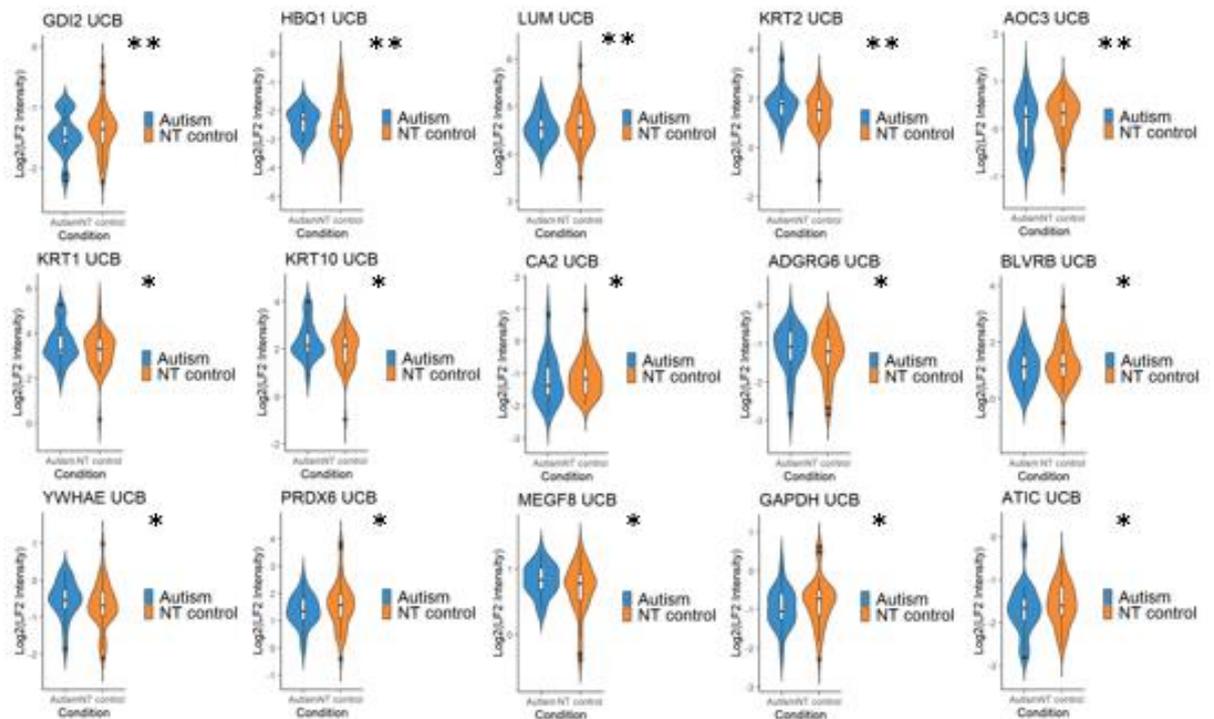


Figure 24: Violin plots of altered protein expression in autism cohort versus controls.

**indicated p value < 0.01 , and * indicates p -value < 0.05 . These plots are the top 15/41 proteins identified as significant prior to FDR correction

ROC Curve: Logistic Regression - Baseline Proteomics All Significant

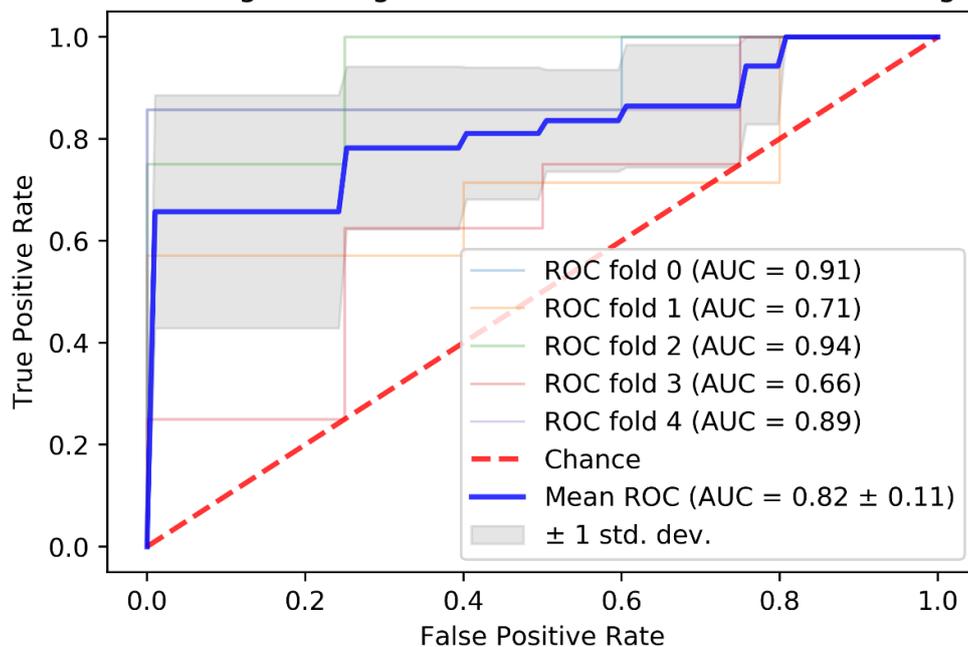


Figure 25: Proteomic prediction model of ASD.

Receiver Operator Characteristic (ROC) curve showing predictive performance of the random forest model for the prediction of autism using significantly altered proteins (n=41). Mean AUROC of 0.82 indicates strong discriminatory potential for the identification of autism from proteomic analysis of cord blood.

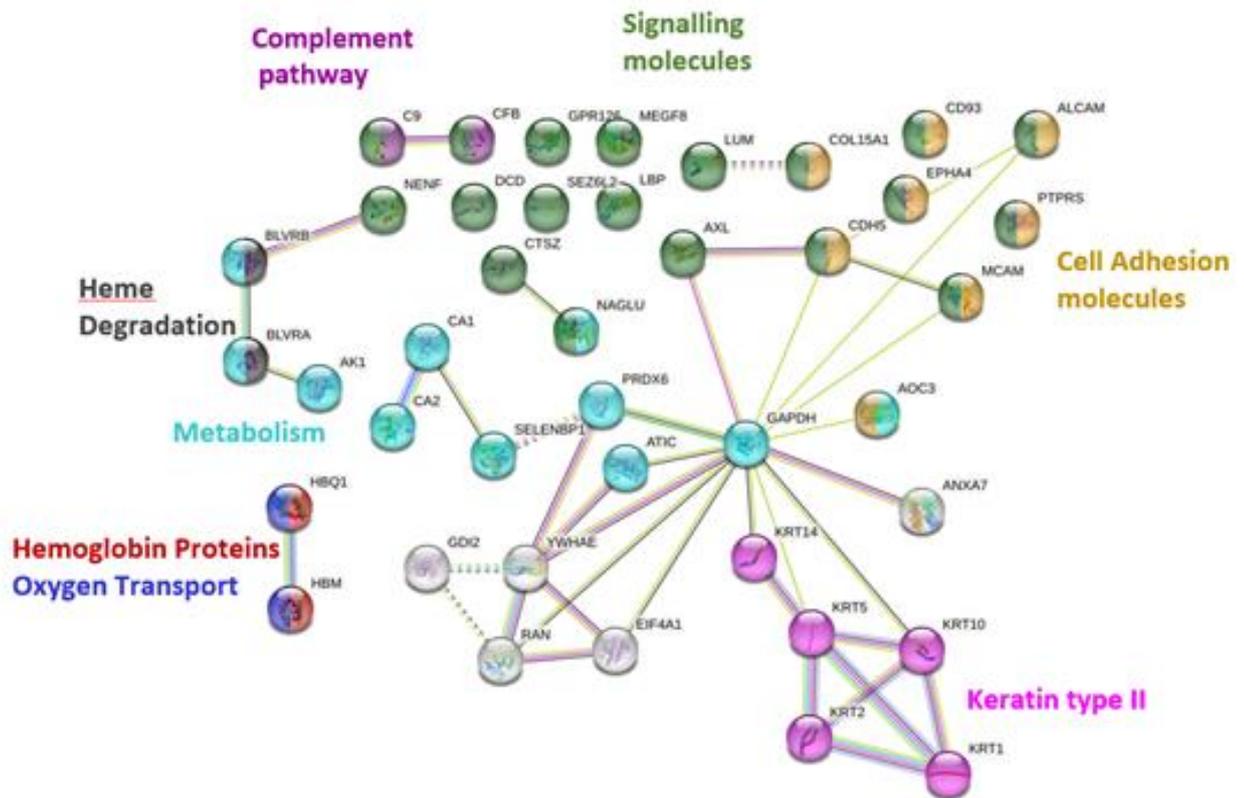


Figure 26: STRING analysis of the 41 differentially expressed proteins in cord blood in Autism.

String pathway analysis showing protein-protein interactions, and the molecular and biological processes implicated.

Proteomic signature of Autism in serum from children

We profiled 508 proteins in serum from 24 children with Autism and 48 controls for differential expression changes between diagnostic groups. Of the 508 proteins profiled, 404 proteins were present in >70% of samples, and we identified 11 proteins as significantly differentially expressed in children with Autism in comparison to controls (P value <0.05; Supplementary Figure 2). Furthermore, we observed altered expression of GAPDH, SELENBP1 and BLVRB proteins in serum in children with Autism in comparison to controls. Findings were in keeping with proteins differentially

expressed in cord blood, although opposite effects in terms of the direction of change was observed between cases and controls. The full list of differentially expressed proteins are listed in supplementary information (Supplementary Table 2).

Metabolomics signature of Autism in cord blood

Following post-filtering for coefficient of variation and missing values, discovery MS analysis successfully profiled 1782 metabolites for differential expression. PCA and PLS-DA analyses were completed to assess for outliers in samples as well as to determine if autism cases and controls displayed clear separation. A PLS-DA sample plot displayed partial overlap between cases and controls with no clear separation being displayed Figure 27. We identified 32 metabolites were identified as significantly altered between 24 children with Autism and 48 controls (P value < 0.05; Figure 28 and Figure 29). No compounds retained significance following correction for false discovery at 5% (Supplementary Table 3). Of the 32 significant compounds, seven (7) are classified as steroid and steroid derivatives (Supplementary Table 3).

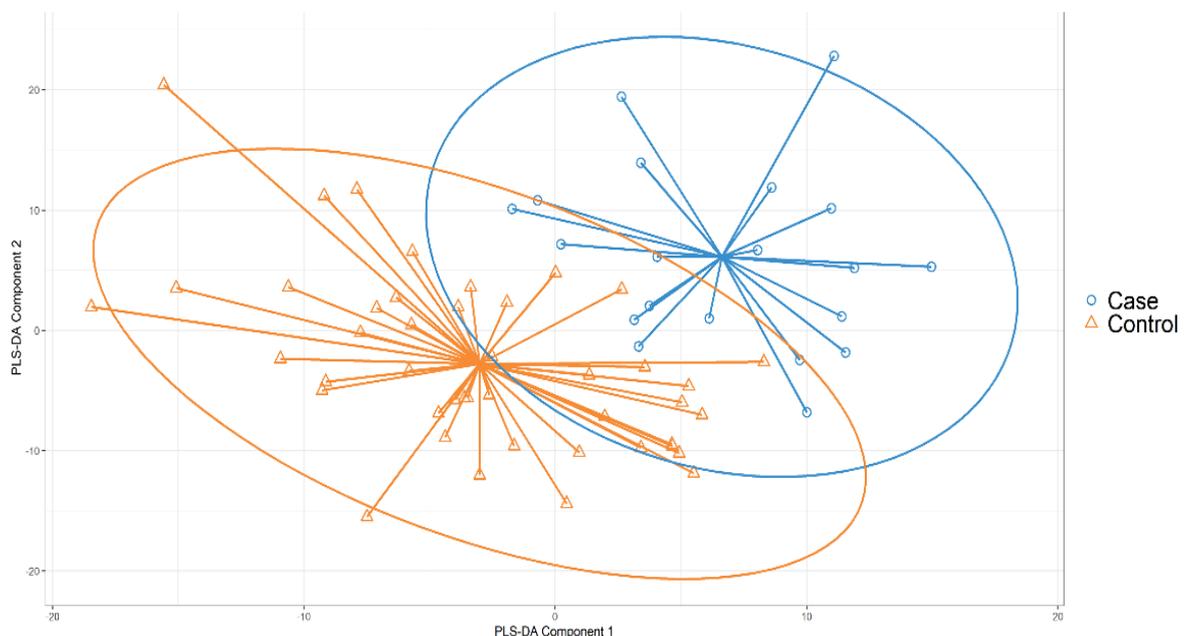


Figure 27: Results from discovery metabolomics analysis.

PLS-DA plot of samples with clustering ellipses. We observed no clear separation between groups.

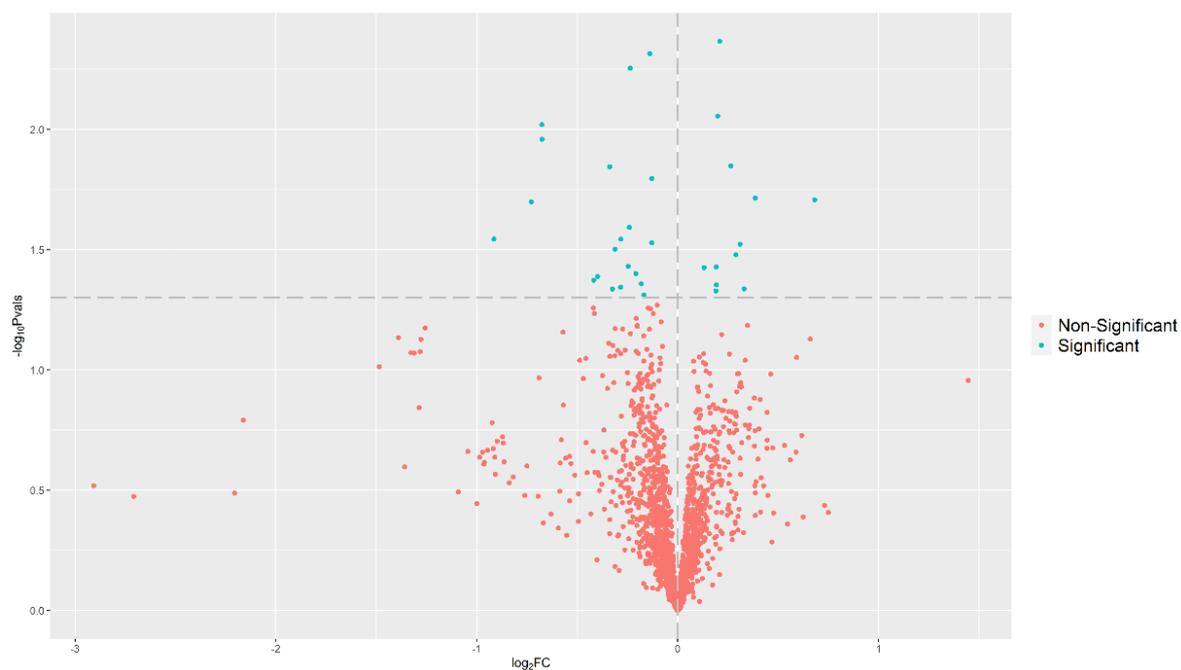


Figure 28: Volcano plot of all compounds measured in discovery metabolomics analysis.

Discovery metabolomics on the available cord blood samples resulted in the profiling of 2540/2612 distinct metabolites after data quality and filtering steps were completed. We identified 42 of 2540 quantified metabolites as significantly altered at $p < 0.05$ following a student's t -test. After the application of the Benjamini-Hochberg procedure no compounds were found to be significantly altered ($\text{FDRate} > 0.05$).

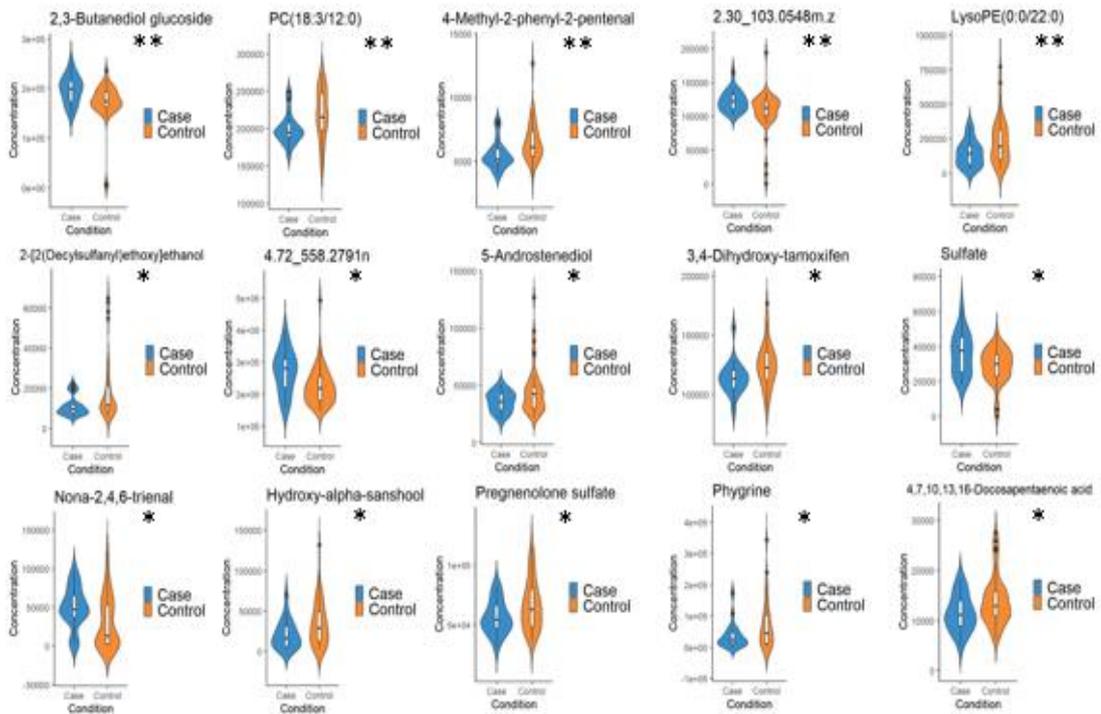


Figure 29: Violin plots of the top 15/32 significant compounds identified prior to FDR correction.

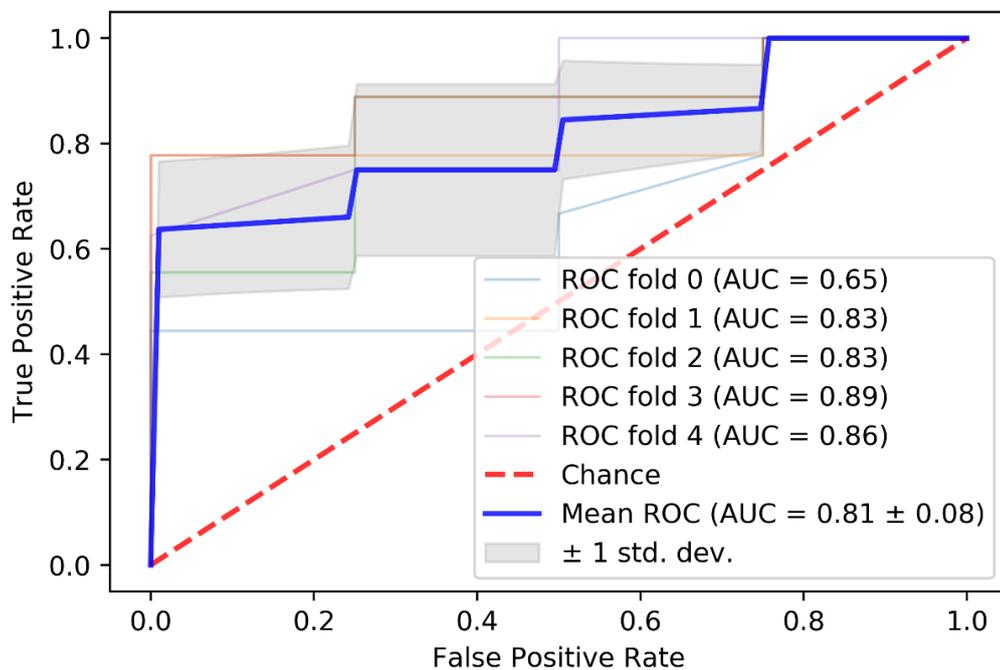


Figure 30: Receiver Operator Characteristic (ROC) curve

Showing predictive performance of the random forest model for the prediction of autism using significantly altered discovery metabolites ($n=42$)

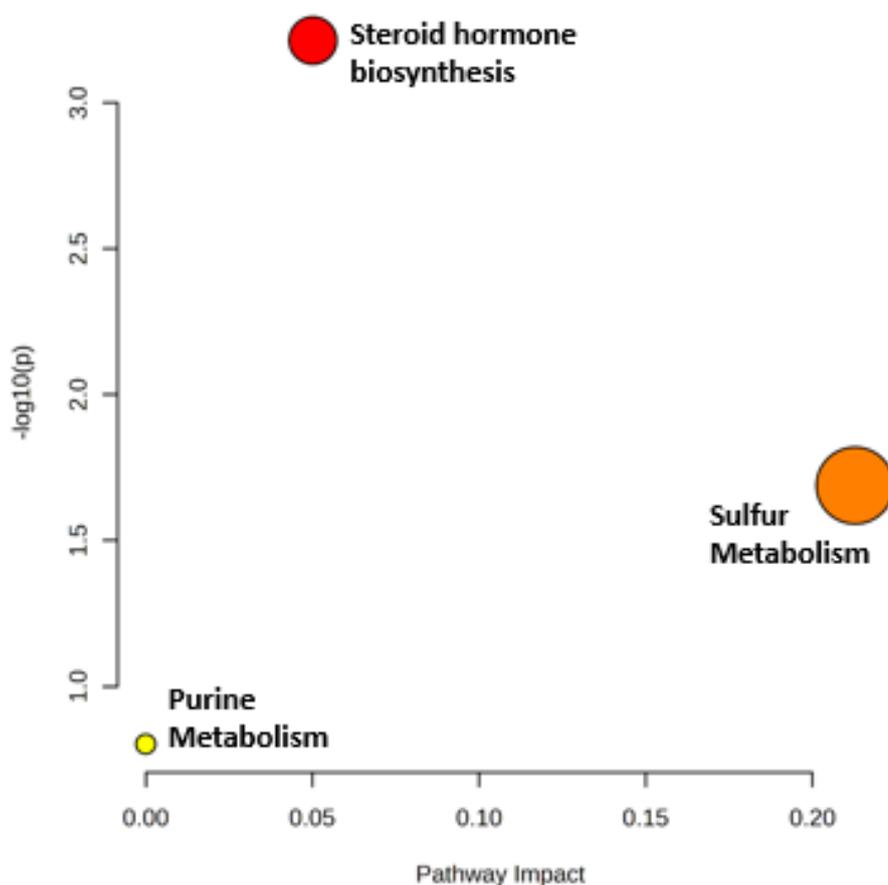


Figure 31: Pathway analysis plot

Displaying pathways identified from the significant metabolites for which a putative ID was obtained.

Cord blood and childhood steroid measurements in Autism

Based on our findings of altered steroids and steroid derivatives, targeted Multiple Reaction Monitoring (MRM) acquisition was employed for quantitation of key metabolites that map to the steroid pathway (Figure 32). Thirteen (13) steroid metabolites, which mapped to the steroid synthesis pathway, were simultaneously quantified in cord blood and child serum samples using MRM mass spectrometry. Ratios of measured steroid metabolites were also assessed for differential expression. Androstenedione and the ratio of androstenedione to dehydroepiandrosterone sulfate (DHEAS) were significantly decreased in cord blood in Autism cases in comparison to controls ($P=0.034$ and $P=0.008$, respectively), see Figure 33 and Supplementary Table 4). All other steroid metabolites and ratios

assessed, including testosterone displayed no significance between diagnostic groups.

To test for gender effects, post-hoc analysis was conducted in male subjects only (n = 18 Cases, n = 36 Controls) and female subjects (n = 4 Cases, n = 8 Controls), significance of androstenedione was lost in both groups (P>0.1) although we acknowledge these comparisons were underpowered. Spearman's correlation coefficient identified strong correlations between androstenedione and corticosterone in the Autism cases that were not observed in neurotypical controls (Figure 34). Furthermore, we observed a positive correlation between androstenedione and cortisol while a negative correlation was present in controls.

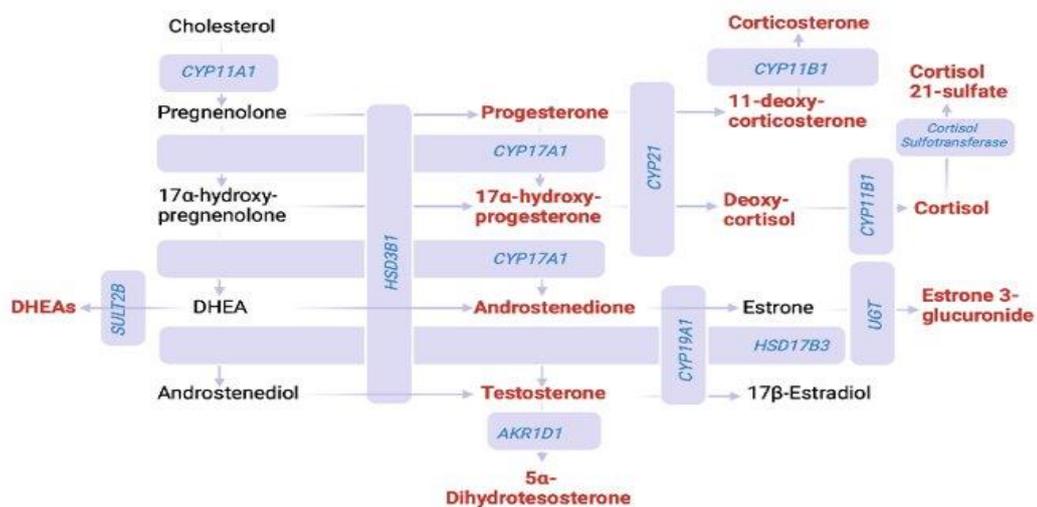


Figure 32: Steroid biosynthesis pathway:

Graphical display of the steroid synthesis metabolic pathway. Metabolites targeted in our MRM analysis are highlighted in red.

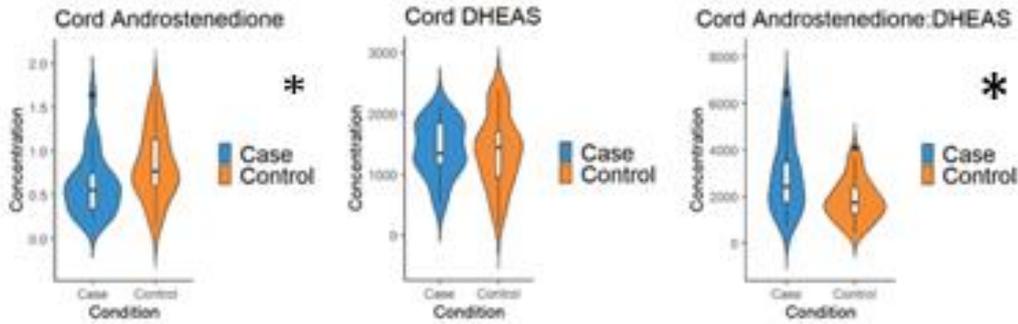


Figure 33: Cord blood steroid metabolites altered in the autism cohort.

Violin plots displaying the expression of steroid metabolites (androstenedione and DHEAS) in the cord blood samples. * = $p < 0.05$.

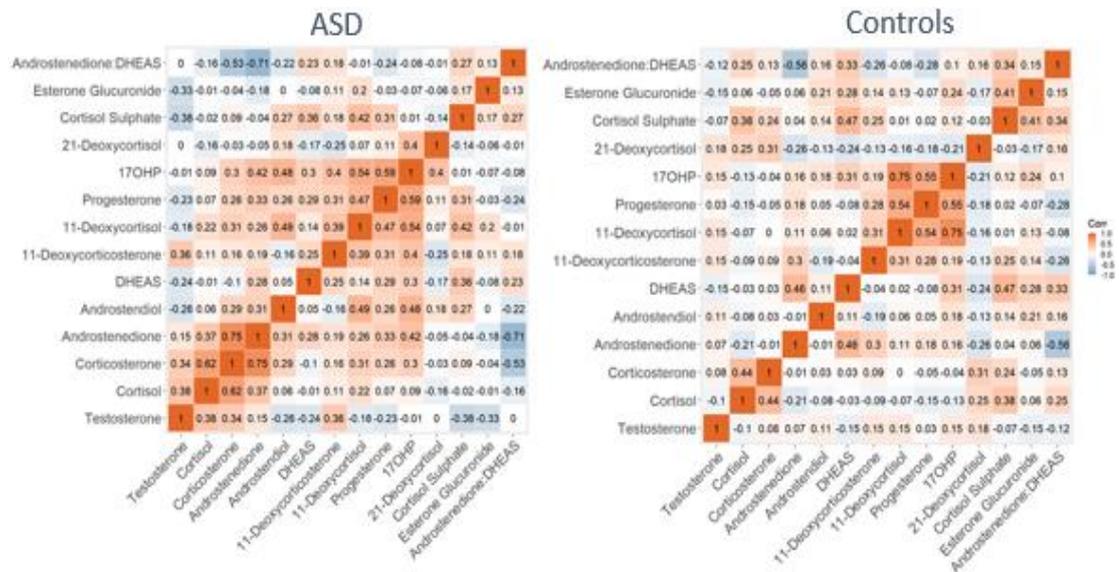


Figure 34: Heat map

Displaying pairwise Spearman's correlation coefficient of all measured metabolites in cord blood of ASD cases (left) and neurotypical controls (right).

Of the 13 steroid metabolites targeted in the serum samples from children in the PiRAMid cohort, just six steroids (cortisol, 11-deoxycortisol, corticosterone, estrone glucuronide, DHEAS, androstenedione, testosterone) were above the limit of quantitation and were tested for differential expression. No significant differences were observed between Autism cases ($n = 24$) and neurotypical controls ($n = 42$). Analysis of metabolite ratios did not identify significant differences between cases and controls (Supplementary Table 4). To test if childhood steroid levels were

confounded by age at which samples were taken (7-10 years old), ANCOVA was undertaken and again, no significant differences in steroid levels were observed between cases and controls (Supplementary Table 3).

Proteomic and metabolomics prediction models of Autism

Cord blood proteomics

Using the full set of measured proteins (n=558) an area under the receiver operator characteristic (AUROC) curve of 0.59 and 0.52 was observed in the logistic regression and random forest models respectively. Good predictive performance was observed when modelling with the significantly altered proteins (n=41). This resulted in an AUROC curve of 0.82 and 0.75 in the logistic regression and random forest models respectively (Figure 25). Results of feature ranking and all ML model testing for the cord blood predictions (proteomic) can be found in Supplementary Figure 3.

Child serum proteomics

ML using the full set of proteins quantified in serum samples (n=404) resulted in an AUROC of 0.57 and 0.40 in the random forest and logistic regression models, respectively. ML was also applied to assess the performance of the proteins identified as significantly altered in the Autism cases and these models achieved improved predictive performance with an AUROC of 0.78 and 0.74 in the random forest and logistic regression models, respectively. For full ML ranking and modelling results see Supplementary Figure 4.

Cord blood metabolomics

ML using the full set of metabolites quantified in cord blood (n = 1782) resulted in an AUROC of 0.38 and 0.36 in the random forest and logistic regression models, respectively (Supplementary figure 5). Predictive performance was improved when modelling with significantly altered metabolites achieving an AUROC of 0.77 and 0.81 in the logistic regression and random forest models, respectively (Figure 30 and supplementary figure 5).

Cord blood steroid measurements

Targeted MRM analysis of the steroid synthesis pathway quantified 13 steroid metabolites in cord blood, which were used to develop predictive models (Figure 33 and Figure 34). Machine learning modelling resulted in an AUROC of 0.66 (+0.07)

and 0.65 (+/-0.08) in the logistic regression and random forest models respectively (Supplementary Figure 5). The significantly altered features were also the top random forest features with androstenedione:DHEAS ratio and androstenedione ranking as the 1st and 2nd most predictive features (Supplementary Figure 5B and D).

DISCUSSION

Our study provides evidence for altered protein and metabolite molecular profiles in cord blood, which precede a diagnosis of Autism. The identification of a cord blood signature associated with Autism risk is consistent with recent findings in a Norwegian Autism Birth Cohort, which reported a pronounced cytokine signature in cord blood plasma, and maternal mid-gestational plasma, from children later diagnosed with Autism (473). While there is considerable evidence for maternal immune activation in Autism, our findings build on this by further implicating proteomic and metabolomic systemic molecular processes such as altered sulfur and steroid metabolism in the pathophysiology of Autism. In addition, this is first study to test directly the prediction that in utero cord blood biomarkers of Autism can persist into childhood. In a diagnostic follow-up of children between the ages of 7 and 10 years, we confirmed altered expression of GAPDH, SELENBP1 and BLVRB in serum in children with Autism in comparison to controls. Although our study size was modest, our results point to a persistent peripheral molecular signature in Autism, with perturbations in protein expression being more pronounced at parturition in comparison to childhood. Machine learning algorithms were developed using both proteomic and metabolomic signatures in cord blood to predict Autism outcome, with metabolomic features demonstrating greater predictive value (AUROC 0.86), than proteomic features (AUROC 0.82). Our findings have broad relevance to child-onset neurodevelopmental disorders where early intervention is of critical importance for improving clinical outcomes. We also lack early markers of vulnerability (pre-symptom development), our findings could promote earlier interventions should evidence based therapies targeting very young children emerge. Facilitating increased surveillance within the first years of life.

Discovery proteomic profiling identified 41 proteins as significantly differently expressed in cord blood from children with Autism in comparison to NT controls Figure 22. Of interest, SELENBP1 a transporter of selenium was decreased in Autism profiles at birth. SELENBP1 has been previously linked with schizophrenia and autism

(474). It is a key protein in sulfur metabolism and a crucial enzyme responsible for biosynthesis of hydrogen sulfide (475). Impaired sulfate metabolism is an oft-replicated biochemical endophenotype associated with Autism, where it has been widely documented that children with Autism have a reduced ability to metabolise sulfate (110, 476, 477, 478). SELENBP1 has also been implicated as a biomarker of neurological impairment (479) and is associated with major psychiatric disorders (480, 481).

In addition, we observed reduced levels of two haemoglobin degradation proteins BLVRB and BLVRA, and two haemoglobin/oxygen transport proteins HBQ1 and HBM in Autism cord blood plasma. These findings indicate an underlying metabolic disturbance contributing to Iron deficiency anaemia, which has been consistently implicated in Autism (155, 482).

Further, we identified five proteins which map to Keratin signalling, where keratin has been shown to be vital for an effective and robust materno-fetal interface including extraembryonic tissue development (483).

Furthermore, levels of GAPDH were decreased in cord blood in our Autism cohort. GAPDH, a moonlighting protein, is ubiquitous throughout human body tissues, including the fetal brain. GAPDH has established roles in glycolysis, metabolic function, modulation of the cytoskeleton, and acts as a molecular switch under oxidative stress (484, 485, 486, 487). Recently, using immunoglobulins harvested from mothers of children with Autism, GAPDH protein was identified as a novel autoantibody target in archived fetal brain samples (488). Autoantibodies to GAPDH have previously been linked to other psychopathologies including depression and schizophrenia (488, 489).

Finally, we found eight Cell Adhesion Molecules or CAMs (Lumican, AXL, COL15A1, ALCAM, MCAM, CDH5, CD93, PTPRS, EPHA4), to be differentially expressed in cord blood. Genetic studies have reported the involvement of CAMs in Autism, which have an integral role in cell-cell and cell-extracellular matrix interactions, neuronal development, axonal guidance and synaptic formation and function (485).

Proteomic analysis of serum samples from the follow up PiRAMiD cohort observed altered expression of GAPDH, SELENBP1 and BLVRB proteins in serum from children with Autism versus controls. These findings illustrate the persistence of the previously identified in utero protein signatures into pre-pubertal childhood. Interestingly, while these proteins were elevated in serum from the Autism cohort in late childhood, their expression was decreased in cord blood at parturition. We speculate these persistent and coordinated systemic fluctuations in GAPDH, SELENBP1 and BLVRB proteins point to key molecular pathways implicated in Autism aetiology, which manifest (in-utero) within the perinatal environment and likely oscillate throughout childhood. It is also noteworthy that the proteomic signature was less pronounced in childhood serum samples in comparison to cord blood samples, with respect to the number of proteins differentially expressed and effect sizes between Autism cases and controls. These results suggest cord blood is a valuable bio-fluid for biomarker discovery, as it captures a snapshot of perinatal molecular mechanisms implicated in Autism, and constructs the overlap between genes and environment.

Discovery metabolomics profiling identified 32 metabolites as significantly differentially expressed in cord blood from children with Autism in comparison to controls (Figure 3A-E). Although no features passed adjustment for multiple testing, 7 metabolites were classified as steroid and steroid derivatives (5-Androstenediol, 3,4-Dihydroxy-tamoxifen, Pregnenolone sulfate, 19-Hydroxydeoxycorticosterone, Tetrahydrodeoxycorticosterone, 19Hydroxyandrost-4-ene-3,17-dione, and 6beta-Hydroxytestosterone), all of which were reduced in Autistic cord blood samples versus controls. There is substantial evidence for prenatal steroid dysregulation in the aetiology of autism (490, 491), and numerous targeted steroid investigations have reported elevated androstenedione and testosterone levels in amniotic fluid (11-17 weeks gestation) where children developed autism (492, 493, 494, 495). There is some evidence to suggest that elevated exposure to testosterone in fetal development leads to a specific phenotype with altered vocabulary, eye gaze, restriction of interests and increased attention to detail (493). Unlike amniotic fluid which captures this perinatal testosterone surge between 11 and 17 weeks gestation,

cord blood samples represent prenatal exposure at the time of parturition (491). Previous studies exploring/measuring testosterone levels in cord blood directly did not demonstrate an association with Autism outcome or symptomology (496, 497, 498). Our measurements in cord blood concur with these observations. While testosterone was not specifically altered, we confirmed reduced androstenedione in cord blood in Autism cases in comparison to controls. Androstenedione is an endogenous androgen steroid hormone that is a precursor to testosterone, as well other androgens and estrogens (Figure 32)(499). Broadly, our discovery results indicate depleted androstenedione and steroid-derivatives (conjugated steroids) in Autistic children, suggesting altered steroid hormone biosynthesis at parturition. This supports previous findings for an association between prenatal androgen dysregulation and Autism outcome. Although previous investigations report elevated serum androstenedione levels in pre-pubertal children (500), as well as adults with Autism (501), and teenagers with ADHD (502), we did not find altered androstenedione levels in Autistic children between 7 and 10 years in this study.

Collectively, proteomics and metabolomics profiling converged on altered systemic metabolism, including sulfur metabolism and glycolysis. We observed reduced SELENBP1, a key protein involved in sulfur metabolism, which coincided with increased sulfate levels in cord blood from autistic children. Maternal circulating sulfate levels have been reported to increase two-fold during the third trimester of pregnancy, illustrating the importance of sulfate in fetal development (477). Sulfate has many roles in the body, and there is robust evidence to suggest steroid sulfation and desulfation plays a crucial role in controlling steroid action (503). In this study, we confirmed a reduction in androstenedione and observed a general depletion of steroid derivatives in cord blood in Autism cases versus controls, which we speculate may be associated with our sulfur metabolism findings. In further support of converging molecular networks, steroid biosynthesis and the immune response are intrinsically linked during pregnancy (491). Equally the complement system is one the most important defences mechanisms of the innate immune system. We identified two complement proteins C9 and CFB as differentially expressed in Autistic cord blood samples, and although the detection/quantification of cytokines is below the

limit of detection using mass spectrometry methods employed here, it is highly likely that perturbations in cytokine levels are present in the background proteome of cord blood and autism outcome (473). Taken together, our results provide concurrent evidence for the role of steroid biosynthesis, sulfur metabolism, and complement related immune responses in Autism. Alterations of which are likely to converge, creating a sub-optimal prenatal environment and perturb fetal neurodevelopment.

Study strengths and limitations

Strengths of this study extend to the sourcing of cases and controls from a clinically based study of 2,137 mother-infant dyads with continuous diagnostic follow-up from pregnancy to childhood (7 – 10 years). This longitudinal follow up allowed analysis of cord blood plasma from parturition, and follow up serum samples collected in childhood (age 7 to 10 years). Longitudinal proteomic and metabolomics profiling in this manner is novel and may allow identification of mechanistic or at least persistent dysregulation of proteins and metabolites during childhood in autism. Protocols for blood collection, processing, and biobanking were all quality controlled by the accredited INFANT biobank. The biobank adheres to the International Society for Biological and Environmental Repositories (ISBER) best practices, the Molecular Medicine Ireland Guidelines for Standardised Biobanking, and OECD Guidelines for Human Biobanks and Genetic Research Databases. As expected, we identified a significant male predominance in our Autism cohorts. Male predominance in Autism is a well-recognised phenomenon as it is three to four times more likely to arise in males (59). The diagnostic groups were matched in a 2:1 ratio (controls: cases) on age and birthdate for analysis of the cord blood samples, but due to the Covid-19 pandemic recruitment of controls was incomplete and it was not possible to maintain stringent matching. This may be a potential confounder in our follow up omics analysis. Our study utilised inter-omics profiling and state of the art mass spectrometry approach's including data independent acquisition (DIA) coupled with Fragpipe search engine for DIA-Neural Network (NN) analysis. This is the current gold standard in mass spectrometry. While discovery metabolomics profiling has the benefit of allowing the evaluation of many unique compounds, but a major drawback is the high dimensional data generated, and subsequent challenges of experimentally confirming the identification of measured compounds. Although putative

identifications were obtainable, the confidence in these identifications varies. Therefore, targeted validation was undertaken using MRM assays of the steroid metabolites, for which mass spectrometry standards were available. We were cautious in our statistical approaches, our cord blood samples were matched as closely as possible between cases and controls, we adjusted for multiple comparisons, and report both p-value and FDR adjusted p-values in the proteomic and metabolomics results tables. This study is limited by relatively small sample sizes for the development of a prediction model, and due to male predominance in Autism, female participation was too small to compare gender specific effects. We employed two different ML algorithms (random forest and logistic regression) to assess convergences across prediction models, and applied 5-fold cross validation to minimize the risk of overfitting. Access to longitudinal biological samples for molecular profiling in clinical disease is incredibly rare, and this highlights the importance of large cohort studies with biobanking of valuable samples in a longitudinal manner. This study successfully demonstrates molecular changes in SELENBP1, GAPDH, and BLVRB in cord blood from infants with Autism at parturition, and in serum from children with Autism between the ages of 7 and 10 years, and future research groups taking a similar approach may prove to be a fruitful endeavor.

CONCLUSION

Our study extends the findings of research in Autism and supports the idea that the early prenatal and feto-maternal environment plays a role in the development of Autism. We uncovered concurrent evidence for dysregulation of proteins and metabolites in cord blood, which map to sulfur metabolism, glycolysis, complement signalling, and steroid:androstenedione networks. We hypothesise these processes play a role in the prenatal neurobiological components/pathways implicated in Autism aetiology.

Using machine-learning approaches, we developed diagnostic signatures with encouraging performance in test sets to distinguish Autism cases from controls, which suggest both proteomic and metabolomic measures may help predict Autism outcomes in larger cohort studies. Future work should elaborate on the mechanistic underpinnings of how SELENBP1, GAPDH, and BLVRB drive changes in the developing

brain, and determine if these systemic measures can serve as longitudinal biomarkers of Autism severity. While longitudinal age-related deficiencies in androstenedione measurements were not observed in this cohort, this research provided fundamental evidence that cord blood should be considered a valuable bio-fluid. Cord blood has the potential to identify disease specific biomarkers for prognosis, which may also serve as systemic therapeutic targets for treating symptoms of Autism. Collectively, our data suggests cord blood molecular signatures of Autism are evident a birth. In combination with clinical data and cytokine profiling, these measures may be useful in precision risk screening of infants most likely to develop Autism, or further facilitate surveillance of children at risk in order to direct early therapeutic intervention in all cases.

DATA AVAILABILITY STATEMENT

Data are not publicly available because they contain sensitive patient information. Requests for access can be made directly to INFANT centre at infant@ucc.ie.

AUTHOR CONTRIBUTIONS

K.D and D.R. undertook the discovery metabolomics and targeted MRM steroids analysis. A.N. performed HPLC immuno-depletions, proteomic sample preparation and some bioinformatics analysis, along with C.S. who undertook all proteomic LC-MS/MS. A.N. also co-wrote the manuscript and prepared figures. D.O.B. undertook statistical analysis of the metabolomics and proteomics datasets, performed the ML analysis, and prepared associated figures. M.C. undertook clinical follow-up of each child in the PiRAMiD cohort, confirming their Autism diagnosis, performing developmental assessments, collecting and processing maternal and child blood samples from the late childhood visit, and contributed to preparation and writing of the manuscript, prepared tables and aided interpretation of results. A.K., L.G., B.H.B., T.B.H., and C.M. were involved in study design, interpretation of results, and student supervision. D.M. is the PI of Baseline and PiRAMiD and was involved in the ethical approval, data management, study design, interpretation of results, and student supervision. J.E conceived the study, was involved in study design, data managed and analysis, interpretation of results, student supervision, and co-wrote the manuscript. All authors critically reviewed the manuscript and approved the final version for submission.

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COMPETING INTERESTS

The authors report no financial interests or potential conflicts of interest.

Chapter 6

Title: Characterising the temporal evolution of emotional and behavioural problems in autistic children during early childhood

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Characterising the temporal evolution of emotional and behavioural problems in autistic children during early childhood.

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Abstract:

Children with autistic spectrum disorder (ASD) are at increased risk of developing emotional and behavioural problems beyond the core features that define the disorder. Co-morbid emotional and behavioural problems occur, and include symptoms of aggression, depression, anxiety, and social withdrawal. These maladaptive behaviours are less studied than the core ASD features, yet they are important modifiers of mental health for both child and family. High levels of EBPs can compound the core autism symptoms and even limit a child's ability to engage in therapy.

We identified a nested sub-cohort within a large mother-child birth cohort based on a confirmed multi-disciplinary diagnosis of autism before the age 10 years. We matched this group with neurotypical controls from the same cohort. Participants completed psychometric testing with the Child Behavioral Checklist (CBCL) at 2 and 5 years, and the Kaufman Brief Intelligence Test (KBIT-2) and Social Communication Questionnaire (SCQ) in late childhood.

From a birth cohort of 2137 children, thirty-four autistic children and fifty-one controls were studied. We found that the development of emotional and behavioural problems in autistic children differed significantly from the neurotypical group at 2 years (mean total problem

score was 35.3 vs 23.7 $p = 0.01$), and deviated further by the age of five (mean total problem score 59.9 vs 24.6 $p = <0.01$).

Our results show that EBPs start early and are pervasive in the paediatric autistic population. They are a reminder of the need for a continued and sustained focus on interventions aimed at improving not only autism core features, but also EBPs. Our findings support the need for targeted consideration of the emotional and behavioural health of autistic children starting in early childhood, and continuing right through the range of their development.

Keywords:

ASD · Autism · Emotional problems · Behavioural problems · Early intervention · Developmental disorders

Introduction:

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by social communication and interaction deficits and restricted, repetitive patterns of interests and behaviour that are evident in early childhood. The prevalence of ASD has been progressively increasing for decades (50, 51, 52), and is now ranging from 0.39% to 2.6% in different jurisdictions (17, 55, 56, 57).

Healthcare costs associated with ASD represent an onerous burden. A 2014 estimate of annual costs of routine ASD care and intervention in the UK and USA put the figure at £3.1 billion per annum and \$61 billion per annum respectively (450). The development of methods for the early identification of ASD are paramount to improving outcomes, and to reducing associated healthcare expenditure. Earlier identification allows earlier intervention, and consequent improvements in communication, cognitive and behavioural outcomes in autistic children (504, 505). Studies examining outcomes between infant groups displaying early signs of ASD found that targeted early intervention significantly reduced the odds of a persistent ASD diagnosis at 3 years (10). Yet in spite of such findings, autism services continue to be under-resourced (212) and diagnoses are chronically delayed (448, 449).

Children and adolescents with Autism Spectrum Disorders (ASD) frequently suffer from emotional and behavioural problems (EBP) (226). More specifically, it is reported that children with ASD have an increased prevalence of both internalizing/emotional problems (social difficulties, anxiety, depression, and withdrawal symptoms); and externalising/behavioural problems (attention problems, hyperactivity, and conduct disorders) (226, 227). Using the Child Behavior Checklist (CBCL) others report that scores on the Social, Thought, and Attention problems scales were more than two standard deviations higher in a cohort of children with autism compared to a neuro-typical sample (228). A CBCL ASD profile, consisting of high scores on the Withdrawn, Social problems and Thought problems scales has been previously described (229, 230). Children with ASD often exhibit maladaptive behaviours, defined as co-occurring, internalising and externalising behavioural problems that negatively affect everyday activities (231). These behaviours are sometimes more distressing to caregivers than the core autistic symptoms themselves (232). EBPs can restrict remedial interventions, and negatively affect long-term outcomes of ASD affected children.

While other authors have highlighted the occurrence of EBPs in various ASD groups and at various time points, consensus on how and when EBP present and evolve over time is lacking

(2, 3). Our aim is to illustrate the temporal evolution of EBPs in our ASD population using serial CBCL scoring, highlighting their development in early childhood and how these maladaptive problems evolve over the important early childhood window. The identification of EBPs may be possible in children as young as 2 years and may help to identify those with features of ASD or specific EBP syndromes, allowing more effective and timely intervention.

Methods:

Participants:

Children were recruited from the Cork Birth Cohort Study, BASELINE (Babies after SCOPE: Evaluating the Longitudinal Impact on Neurological and Nutritional Endpoints) (www.baselinestudy.net) (458). We assessed early environmental exposures and early developmental outcome as previously outlined (458). Clinical assessments of the children were undertaken at 2, 6, 12, 24 months and 5 years of age. The research team performed specific developmental assessments at 24 months and 5 years of age, and the parents completed a Child Behaviour Checklist (CBCL) questionnaire at both time-points. At 5 years, the children also completed a cognitive assessment, the KBIT-2, administered by a trained research nurse.

The clinical research fellow contacted the children with a confirmed or suspected ASD diagnosis at 5 years and invited them to attend a further developmental assessment in late childhood (children aged between 8 and 11 years at the time of this follow up assessment). Next, we matched each confirmed case based on the child's sex, birth gestation, birth weight and maternal BMI at 15 weeks' gestation, with neurotypical controls recruited from the same birth cohort. We matched two controls per each case at the onset of the study, however safety restrictions and social distancing protocols encountered during the Covid-19 pandemic reduced our ability to assess controls. At completion of recruitment, each case matched with at least one control, and 17 were matched with two. Following matching, controls also attended a late childhood developmental assessment.

Clinical Diagnosis:

The majority of children received their ASD diagnosis through the Health Service Executive (HSE) Early Intervention Team (EIT). The standard tests utilised in this setting are the Autism Diagnostic Observation Schedule, Second Edition (ADOS-2), and parent report via, either the Diagnostic Interview for Social & Communication Disorders (DISCO) or Autism Diagnostic Interview-Revised (ADI-R) questionnaires. A small number of children received their initial diagnosis through private multidisciplinary teams (MDTs) using the same assessment tools. All of these children later received a confirmatory diagnosis with the EIT service.

Measure of behaviour, maladaptive and emotional problems (24 month and 5-year visits):

We measured CBCL scores at the 24 month and 5 year assessments. The CBCL (1.5 – 5 years version) questionnaire (506) provides scores for three summary scales (i.e., Internalizing, Externalizing and Total Problems). It also provides scores for five DSM IV-Oriented scales (i.e., Affective Problems, Anxiety Problems, Pervasive Developmental Problems, Attention Deficit/Hyperactive Problems and Oppositional Defiant Problems), and seven syndrome scales (i.e., Emotionally Reactive, Anxious/Depressed, Somatic Complaints, Withdrawn, Sleep Problems, Attention Problems, and Aggressive Behaviour) (see Figure 35).

Children with scores in the prescribed clinical and borderline ranges (whereby any score that falls below the 93rd percentile is considered normal, a score between the 93–97th percentile is borderline clinical, and any score above the 97th percentile is in the clinical range) are at higher risk for developing or experiencing emotional and behavioural problems (EBP) / maladaptive problems. Higher scores indicate greater emotional and behaviour problems. The yield describes a child's behaviour and emotional state over the prior two-month period.

Emotionally Reactive (ER), Anxious/Depression (AD), Somatic Complaints (SC), and Withdrawn (WD) scales combine to yield the Internalising Problems (IP) score. The Attention Problems and Aggressive Behaviour scales combine to yield the Externalising Problems (EP) composite score (506, 507, 508). The sleep problem scale does not fit within either the internalising or externalising categories. In this study, one of the primary caregivers (typically the child's mother) completed the 100-item questionnaire for each participant.

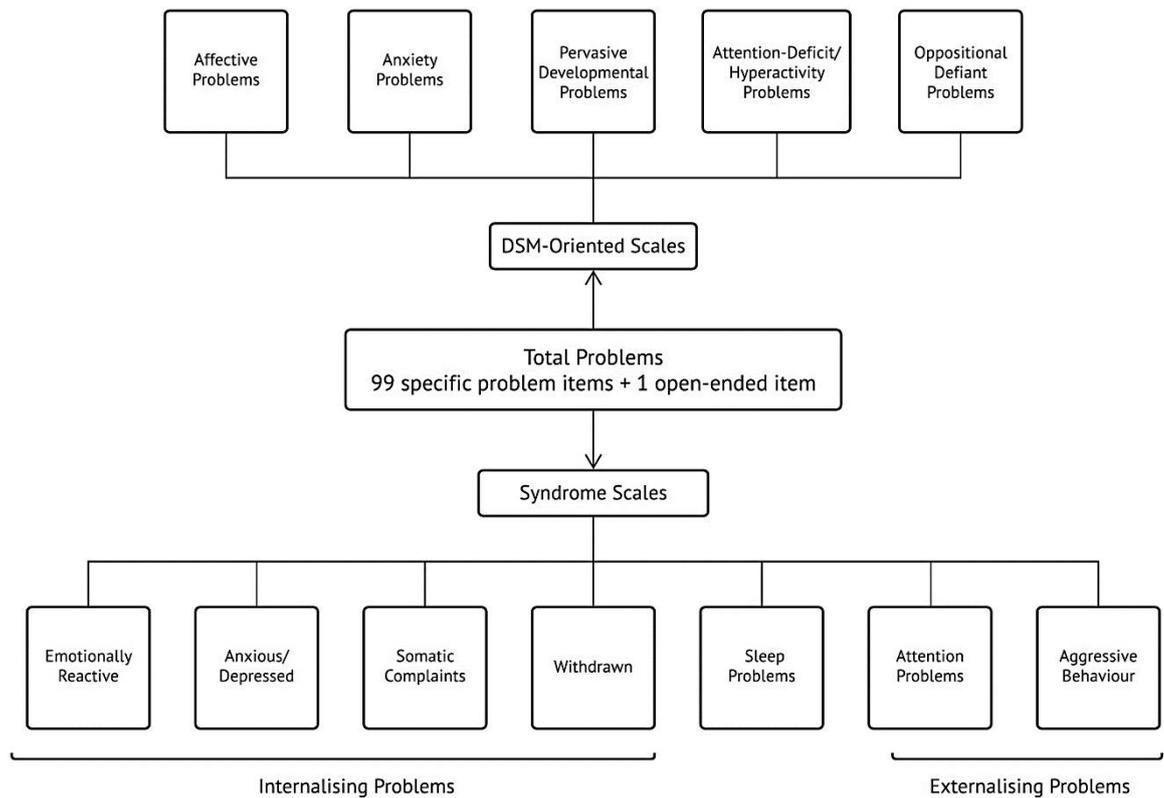


Figure 35: Layout of the subscales scored in the CBCL (1.5 - 5 years).

Syndrome scales combine to form the internalising and externalising problems scores.

Measure of ASD symptomology (Late childhood visit):

Active ASD symptomology was assessed using the Social Communication Questionnaire (SCQ) as part of the later childhood visit (509). Previously known as the Autism Screening Questionnaire, the SCQ is a brief instrument that helps to evaluate the communication skills and social functioning of an individual who may have an autism spectrum disorder (510). Aside from its use as a screener to determine if an individual requires referral for formal ASD evaluation, we can use the SCQ to compare ASD symptoms and severity across groups. The questionnaire has two forms; Current and Lifetime forms; and three subscales, communication difficulties, restrictive and repetitive behaviours, and social reciprocity. We utilised the Lifetime Form, which examines the child's entire developmental history. The SCQ consists of 40 items with "yes" or "no" answers. The first item is untallied and clarifies if the participant is verbal or non-verbal. Total scores range from 0 – 39 in verbal participants (and 0 – 33 in non-verbal participants). Higher scores represent greater social-communication impairment. The quoted cut-off score for ASD risk is 15 or greater. In younger age groups, various lower cut-offs have been proposed (511). Our focus was on children aged between approximately 8 - 11 years, and we used a cut-off of ≥ 15 as clinically significant. We

administered the SCQ to all participants, to ensure the absence of undetected clinical ASD symptomology in our control group.

Measure of cognitive ability (5-year and late childhood visits):

The Kaufman Brief Intelligence Test, Second Edition (KBIT – 2) (512) provides a brief, individualised format for the measurement of verbal and nonverbal intelligence in children and adults from the ages of four to 90 years. The test consists of three distinct subtests, two of which are Verbal subtests, the other, Nonverbal. As a brief intelligence test, it takes approximately 30 minutes to administer. The KBIT – 2 assesses verbal and nonverbal intelligence independently, providing a Verbal scaled score, a Nonverbal scaled score, and a Composite IQ score. Standard scores have a mean of 100, standard deviation of 15. The basal standard score for the each subset is 40. Standard scores can be categorised as lower extreme (≤ 69), below average (70-84), average (85-115), above average (116-130), or upper extreme (≥ 131) based on the standard score ranges. The KBIT – 2 manual allows for administration to individuals with autism, as well as those with poor verbal skills or intellectual disability. However, there are no normative data for these populations provided.

Clinical visit in later childhood (8 – 11 years):

Appointments were organised via email and telephone communication with the parents of participants. A formal ASD diagnosis was confirmed prior to attendance, and in the case of controls, the absence of ASD was confirmed. Each participant completed a follow up cognitive assessment (KBIT-2), a study-specific demographic and general medical questionnaire, a measure of ASD symptomology (Social Communication Questionnaire (SCQ)), and anthropometrics (height, weight and BMI). We discussed the appointment in detail with parents prior to attendance. For some children, we used “social stories” to help prepare them for the appointment (513). We emailed and posted each personalised story to the participants in advance. The story contained images of the venue, the clinical research fellow and the equipment that would be required. We outlined the sequence of the appointment in clear language, and the children were encouraged to express concerns or worries, which we addressed remotely over the phone and in person on the day.

Statistical Analysis:

We compared ASD cases with neuro-typical controls. Where required, data were normalised using Log transformation prior to analysis and we performed statistical analysis using IBM SPSS Statistics 26 (SPSS Statistics, Chicago, IL). Continuous or quantitative data were analysed using independent t-tests or Mann-Whitney U-tests depending on the normality of the data.

Categorical data were analysed using the Chi Square test (χ^2). We measured the relationships between individual assessments using Spearman correlations. We used mixed model analysis to examine the changes in CBCL scores over time between groups. Group (Cases, Controls), Time (24 months, 5 years) and the interaction (Group*Time) were fixed effects in the model and each subject was a random effect. If the interaction Group*Time was statistically significant indicating that changes over time differed between the ASD group and the Control group, only then pairwise comparisons were performed (see sleep and oppositional defiant scales). The pairwise comparisons were between groups at each time-point and between time-points for each group. Statistical significance (2-tailed) was <0.05.

Ethical Approval:

Ethical approval for both the Cork BASELINE Birth Cohort Study (ECM3 (x) 05/04/19) and this study (ECM 3 (k) 03/12/19) were provided locally by the Cork Research Ethics Committee (CREC). We obtained written informed consent from the mothers of each participant recruited. All children received age-specific study information and completed signed informed assent forms where their cognitive ability allowed. In children with ID, their mother acted in surrogate to complete consent.

Results:

Study population:

We outline the details of participant recruitment in Figure 36. The SCOPE Ireland pregnancy cohort (www.scopestudy.net) formed the basis of initial recruitment of infants to BASELINE [n = 1537] and an additional 600 infants were recruited after delivery providing a total sample of 2137 children who were followed from birth. At the 5-year appointment, 22 children already had a formally ASD diagnosis. A further 26 children either, scored in the “at risk/borderline range” for Autism Spectrum Disorder/Pervasive Development Disorder on the CBCL, parents expressed specific concerns regarding the possibility of ASD, or the child was reported to have an ASD associated developmental delay (most commonly speech delay). The clinical research fellow, between May 2019 and March 2020, contacted all 22 children who had a confirmed ASD diagnosis at the 5-year assessment, confirming their ASD diagnosis. The fellow also verified if an interval diagnosis of ASD had been made in the 26 additional children identified as being at risk of ASD at 5-year follow up. Of those, 15 children had received an interval ASD diagnosis, meaning the ASD cohort totalled 37 children. Ultimately, of the children with a confirmed ASD diagnosis (n = 37), 34 (92%) were able to participate. Two families could not attend due to childcare or work difficulties, while we excluded one child due to an underlying genetic syndrome. We drew healthy, neurotypical controls from the same BASELINE cohort and matched controls with cases for (i) Infant sex, (ii) Gestational age, (iii) Birthweight and (iv) Maternal BMI at 15-week visit.

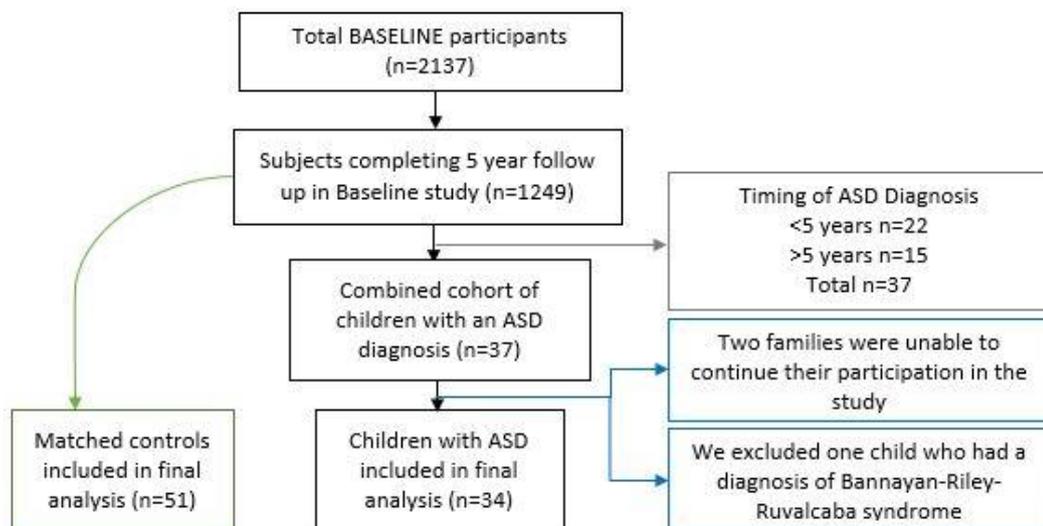


Figure 36: Recruitment flow chart.

We recruited all participants (cases and controls) from the Baseline cohort

Participant characteristics:

In Table 15, we display the clinical characteristics of the study participants. Twenty-six (76%) of those with ASD were of male sex, while 35 (69%) of controls were. Neither birthweight nor gestational age differed between groups. Maternal age did not differ between groups.

Table 15: Demographic characteristics of the whole cohort

Variable	Case (n=34)		Control (n=51)		p-value
	M	SD	M	SD	
Birthweight (grams)	3372	501	3556	518	0.11
Gestational age (weeks)	39.4	1.45	39.9	1.47	0.18
Maternal age at delivery (years)	31.32	5.33	31.8	4.29	0.65
	<i>n</i>	%	<i>n</i>	%	
18 – 28 years	9	(26.5)	7	(13.7)	0.34
29 – 39 years	24	(70.6)	42	(82.4)	
>40 years	1	(2.9)	2	(3.9)	
Male sex	26	(76)	35	(69)	0.43
Maternal BMI at 15 weeks*	11	(40)	19	(43)	0.84
Birth Season					0.23
Spring (Mar – May)	7	(20.6)	18	(35.3)	
Summer (June – Aug)	6	(17.6)	10	(19.6)	
Autumn (Sept – Nov)	14	(41.2)	11	(21.6)	
Winter (Dec – Feb)	7	(20.6)	12	(23.5)	

We calculated all p-values using the Pearson Chi square χ^2 for categorical data, and independent samples t-test for matched cases and controls. There are no significant differences demonstrated between the groups in any of the matching variables. We present the data as mean and standard deviation (SD) with continuous variables or number (n) and percentage (%) with categorical ones. *Maternal BMI was not available in all participants. 27 of 34 cases (and 44 of 51 controls) had maternal BMI documented. In those cases without maternal BMI data, we matched based on only the three child-specific variables of sex, gestational age, and birthweight.

Temporal evolution of Emotional and Behavioural Problems:

In Table 16, we outline the mean CBCL scores for cases and controls at each time point of CBCL assessment (24 months and 5 years), and compare the means using mixed model analysis. In total, there are eight participants (<10% of all participants) absent from the 24 months analysis (1 case and 7 controls), and there are four (<5% of all participants) absent from the 5 year data (all controls). As the numbers absent are relatively small (<10% of the total), it is unlikely any significant bias was introduced. All participants completed at least one of the CBCL assessments, and all subjects included in this analysis remain enrolled in the study.

CBCL Summed Scores:

When we examined the summed scores, we found that in the Total Problem Score, at 24-months, the ASD group scored significantly higher than controls (35.3 vs 23.7 $p = 0.01$), and at 5-years, the ASD group again scored significantly higher than controls (59.9 vs 24.6 $p < 0.01$). We found that in the Total Internalising scores, at 24-months, the ASD group scored similarly to controls (8.2 vs 5.6 $p = 0.11$), but at 5-years, the ASD group scored significantly higher than controls (19.7 vs 7.4 $p < 0.01$). We found that in the Total Externalising scores, at 24-months, the ASD group scored significantly higher than controls (13.5 vs 9.5 $p = 0.03$), and at 5-years, the ASD group again scored significantly higher than controls (19.4 vs 8.1 $p = < 0.01$), see Table 16.

Individual CBCL Syndrome Scales:

Significant differences between cases and controls were evident in three scales at both time points, emotionally reactive, withdrawn and attention problems. Significant differences between the groups were seen in three more scales at the 5-year time point only: Anxious/Depressed, Somatic Complaints and Aggression. As with the summed scores, when scores differed between groups, the autism group scores were higher. There was no significant difference between groups in the Sleep problems scale therefore these scores were not included in pairwise analysis (See Table 16).

DSM IV Oriented scales:

Scoring differed between groups, in four out of five of the DSM IV scales. In the autism group, scores significantly increased with age (indicating worse symptomology), see Table 16 and Table 17. These scales were Affective, Anxiety, Pervasive Developmental Disorder and Attention Deficit Hyperactivity Disorder. Oppositional Defiant Disorder scale showed no statistical difference between groups and did not progress to pairwise analysis.

Table 16: Mixed Model Analysis and pairwise comparison of CBCL scores per cases versus controls at two time points (24 months and 5 years)

Subscales (range)	Fixed Effect	CBCL 24 months			CBCL 5-years		
		Case (n=33)	Control (n=44)		Case (n=34)	Control (n=47)	
Syndrome scale scores	Group * time	Mean (CI95)	Mean (CI95)	p	Mean (CI95)	Mean (CI95)	p
Emotional Reactivity (0 – 18)	<0.01	2.7 (2 – 3.5)	1.5 (0.9 – 2.2)	0.02	6.7 (5.5 – 8)	2.3 (1.2 – 3.3)	<0.01
Anxious/ Depressed (0 – 16)	<0.01	1.6 (0.8 – 2.3)	1.5 (0.8 – 2.1)	0.85	4.1 (3.1 – 4.9)	2.2 (1.4 – 3)	<0.01
Somatic Complaints (0 – 22)	<0.01	2 (1.2 – 2.7)	1.7 (1.1 – 2.3)	0.51	3.6 (2.9 – 4.4)	1.4 (0.8 – 2.1)	<0.01
Withdrawn (0 – 16)	<0.01	1.9 (1.2 – 2.4)	0.9 (0.4 – 1.4)	0.02	5.2 (4.5 - 6)	1.4 (0.8 – 2.1)	<0.01
Sleep Probs (0 – 14)	0.08	2.7 (1.9 – 3.6)	2.5 (1.7 – 3.2)		3.7 (2.7 - 4.8)	2.3 (1.4 – 3)	
Attention probs (0 – 10)	<0.01	3.2 (2.5 – 3.9)	1.9 (1.2 – 2.5)	0.01	5.2 (4.4 – 5.9)	1.7 (1 – 2.3)	<0.01
Aggressive (0 – 38)	<0.01	10.3 (8.1 – 12.6)	7.6 (5.6 – 9.5)	0.07	14.2 (11.8 – 16.5)	6.4 (4.4 – 8.4)	<0.01

CBCL Summed scores							
Total Internal	<0.01	8.2	5.6	0.11	19.7	7.4	<0.01
(0 – 72)		(5.8 – 10.6)	(3.6 – 7.7)		(16.7 – 22.6)	(4.9 – 9.8)	
Total External	<0.01	13.5	9.5	0.03	19.4	8.1	<0.01
(0 – 48)		(10.7 – 16.3)	(7 – 11.9)		(16.4 – 22.2)	(5.6 – 10.5)	
Total Probs	<0.01	35.3	23.7	0.01	59.9	24.6	<0.01
(0 – 200)		(28.5 – 42)	(17.8 – 29.5)		(52 – 67.8)	(17.9 – 31.3)	
DSM IV subscales							
Affective	<0.01	2.4	1.5	0.12	4.6	1.7	<0.01
(0 – 20)		(1.6 – 3.1)	(0.8 – 2.2)		(3.7 – 5.6)	(0.9 – 2.5)	
Anxiety	<0.01	2.4	2.1	0.64	5.8	2.9	<0.01
(0 – 20)		(1.5 – 3.2)	(1.3 – 2.9)		(4.6 – 7)	(1.9 – 3.9)	
PDD	<0.01	4.5	1.9	<0.01	8.6	2.5	<0.01
(0 – 24)		(3.5 – 5.6)	(1 – 2.8)		(7.4 – 9.8)	(1.5 – 3.5)	
ADHD	<0.01	5.5	3.9	0.03	6.6	2.9	<0.01
(0 – 12)		(4.4 – 6.5)	(3 – 4.8)		(5.5 – 7.6)	(2 – 3.8)	
Opp. Def.	0.18	3.9	2.6		4.8	2.6	
(0 – 12)		(3.1 – 4.7)	(1.9 – 3.3)		(3.8 – 5.8)	(1.8 – 3.4)	

All data are presented as mean scores with 95% confidence interval following in parentheses (CI95). We calculated p values using mixed model analysis (see also

Table 17). Those variables, which did not reach significance for the Fixed effects interaction, group*time did not proceed to additional pairwise analysis (Sleep and Oppositional Defiant). We consider p-values as significant <0.05.

In Table 17, we demonstrate the relationship between mean CBCL scores within each group and how they change over time. In the ASD group, across all domains except sleep problems, oppositional defiant, and ADHD subscales, there is a statistically significant change over time. All changes in the ASD group indicate a worsening of symptoms between 24-month and 5-

year appointments. In the control group, only two subscales demonstrate significant change from 24 months to 5 years, the Anxious/Depressed and ADHD subscales. Anxious/Depressed showed a worsening of symptoms, while the ADHD subscale showed improvement.

At 5 years, the gap between cases and controls widened with differences between groups reaching significance across a greater spread of the CBCL subscales. The ASD group scored significantly higher than the control group across all domains. This reflects a general deterioration in EBP scores in the ASD group over early childhood (24 months – 5 years). The pairwise comparison mirrors this within the ASD group over time, which demonstrates statistically significant change between the 24-month and 5 year time points. The controls however, show no such deterioration, and show a significant improvement in the anxious/depressed, total internalising and ADD subscales (see Table 17).

Table 17: Mixed models analysis of CBCL scores within each group over time i.e. how the mean scores within each group changed over time (at 2 and 5 years of age).

Subscale (Range)	Fixed Effects	Pairwise comparison of the change in score per group between two time points, 24 months and 5-years			
Syndrome scores	Group*time	Mean ΔT Cases	p-values	Mean ΔT Controls	p-values
Emotionally Reactive (0 – 18)	<0.01	+4 (3 – 5)	<0.01	+0.7 (-0.2 – 1.6)	0.11
Anxious/ Depressed (0 – 16)	<0.01	+2.5 (1.6 – 3.3)	<0.01	+0.7 (-0.5 – 1.5)	0.02
Somatic Complaints (0 – 22)	<0.01	+1.7 (0.8 – 2.5)	<0.01	-0.2 (-1 – 0.5)	0.47
Withdrawn (0 – 16)	<0.01	+3.4 (2.7 – 4.1)	<0.01	+0.5 (-0.1 – 1.1)	0.11
Sleep Problems (0 – 14)	0.08	+1 (0 - 2)		-0.2 (-1 – 0.7)	
Attention Problems (0 – 10)	<0.01	+2 (1.2 – 2.8)	<0.01	-0.2 (-0.9 – 0.5)	0.53
Aggressive	<0.01	+3.8	<0.01	-1.2	0.33

(0 – 38)		(1.2 – 6.5)		(-3.5 – 1.2)	
CBCL Summed scores					
Total Internal	<0.01	+11.5	<0.01	+1.7	0.13
(0 – 72)		(8.9 - 14)		(-0.5 – 4)	
Total External	<0.01	+5.8	<0.01	-1.4	0.33
(0 – 48)		(2.6 - 9)		(-4.1 – 1.4)	
Total Problems	<0.01	+24.6	<0.01	+0.9	0.78
(0 – 200)		(17.1 – 32.1)		(-5.6 – 7.5)	
DSM IV subscales					
Affective	<0.01	+2.3	<0.01	+0.2	0.63
(0 – 20)		(1.2 – 3.3)		(-0.7 – 1.1)	
Anxiety	<0.01	+3.4	<0.01	+0.8	0.06
(0 – 20)		(2.5 – 4.3)		(0 – 1.6)	
PDD	<0.01	+4	<0.01	+0.6	0.21
(0 – 24)		(2.9 - 5)		(-0.4 – 1.5)	
ADHD	<0.01	+1.1	0.06	-1	<0.05
(0 – 12)		(0 – 2.2)		(-2 – 0)	
Opp. Def.	0.18	+0.9		0	
(0 – 12)		(-0.1 – 1.9)		(-0.9 – 0.9)	

All data are presented as p-values calculated using mixed model analysis. We have examined the CBCL scores using Pairwise comparison when the group*time interaction was statistically significant. This indicates mean scoring differences between the case and control groups at each time-point (Mean difference at 24 months and 5 years), and the mean scoring change over the time-period from 24 months – 5 years within each group (Mean difference in case/control scores over time). p-values are significant at <0.05. Certain subscales (Anxious/Depressed, Somatic Complaints, Sleep problems, Total Internalising problems, Affect and Anxious) were log transformed prior to analysis in order to ensure data was parametric. ΔT (delta time) is the change in score over time. (+) scores mean that the absolute score increased (worsening symptoms), and (-) meaning that the score decreased (improved).

We graphically display changes in CBCL scores over time in Figure 37 and Figure 38, demonstrating a greater burden of EBP comorbidities affecting the ASD group versus controls, particularly at the 5 years assessment. In Figure 37, for the total internalising scale

at 24 months, three (9%) individuals from the autism group and two (5%) from the controls are in the clinical range (one (3%) and four (10%) in the borderline range respectively). At 5 years, this number increase to 17 (50%) individuals from the autism group and decreases to only two (4%) from the controls (six (18%) and eight (17%) were borderline respectively).

For the total externalising scale at 24 months, two (6%) individuals from the autism group and two (5%) from the controls are in the clinical range (three (9%) and three (7%) in the borderline range respectively). At 5 years, this number increase to nine (26%) individuals from the autism group and decreases to only one (2%) from the controls (five (15%) and three (6%) were borderline respectively).

For the total problem scale at 24 months, three (9%) individuals from the autism group and two (5%) from the controls are in the clinical range (two (6%) and three (7%) in the borderline range respectively). At 5 years, this number increase to 18 (53%) individuals from the autism group and decreases to only two (4%) from the controls (three (9%) and five (11%) were borderline respectively).

In Figure 38, the line graphs demonstrate obviously higher median scores in the ASD group at both 2 and 5 years, and stable scoring among controls at both time-points relative to cases.

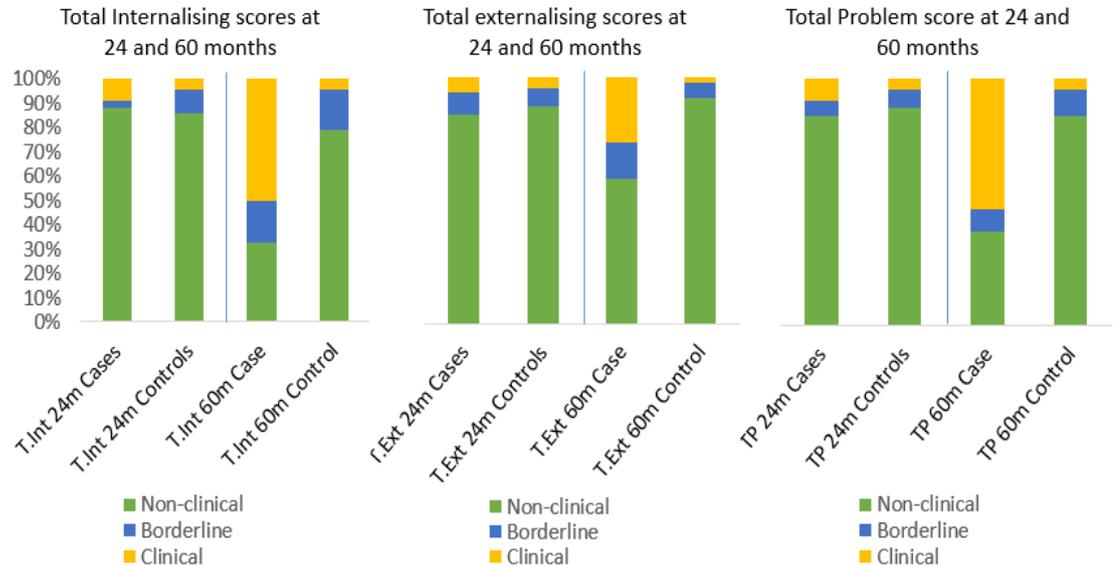


Figure 37: Scoring cut-offs in the “total” or summed subscales.

On the left, total internalising (T.Int) score, in the middle, (T.Ext) total externalising score and to the right, (TP) total problem score. These bar charts demonstrate the proportion of each group in the non-clinical, borderline or clinical ranges (as defined by the CBCL literature (see Methods)) at 24-month and 5-year assessment.

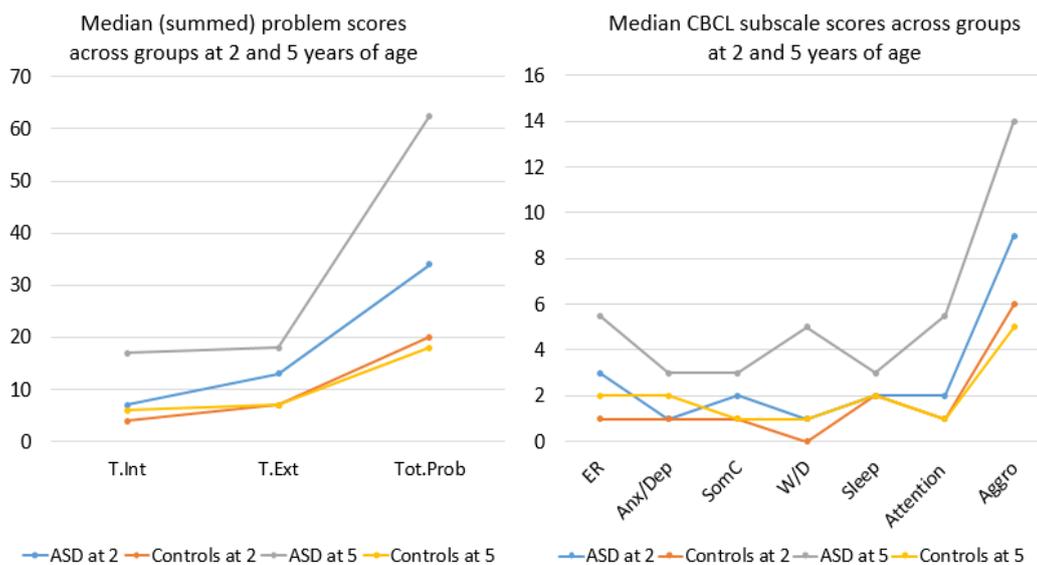


Figure 38: Trend graph depicting the median scores of participants at 24 month and 5-year assessments. Left, the “summed” total scores, and Right, the individual subscale scores.

Cognitive ability and educational needs at later childhood follow up (8 - 11 years):

Regarding the ASD group alone, the mean age at diagnosis was 4.97 years (SD 1.97) and ranged from 2.3 to 10.2 years. Children with ASD reported receiving their initial diagnosis via specialist HSE services in 23 cases (67%), a private individual practitioner in 7 cases (21%) and through a private MDT in 4 cases (12%).

In Table 18, we present ASD specific data and contrast some of the group’s psychometric characteristics arising from the late childhood visit. Child’s age at the late childhood visit differed significantly between groups, the mean age was 8.88 (SD 0.97) years in the ASD group versus 9.26 years (SD 0.88 years) in the control group ($p = 0.03$). The number of mothers who identified as homemakers or carers differed significantly between groups, with 18 (53%) of the ASD group mothers identified as carers or homemakers whilst only six (12%) of the control group mothers self-identified as such ($p = 0.01$).

Table 18: Psychometric and developmental characteristics collected at late childhood visit

Variable	Case (n = 34)		Control (n = 51)		p
	M/n	SD/ (%)	M/n	SD/ (%)	
Age at late childhood visit (years)	8.88	0.97	9.26	0.88	0.03
At least one sibling with ASD					0.32
Male Sibling	5	(15)	3	(6)	
Female Sibling	3	(9)	3	(6)	
Mothers educated to degree or higher	23	(68)	35	(69)	0.62
Current maternal working status					<0.01
Full time/Part time	15	(44)	43	(84)	
Homemaker/Carer	18	(53)	6	(12)	
Sickness beneficiary	1	(3)	2	(4)	
KBIT-2 Scoring[#]	Median	IQR	Median	IQR	p
Verbal IQ	107	97 – 122	119	113 – 125	<0.01
Nonverbal IQ	107	89 – 118	115	109 – 122	0.11
Composite IQ	111	90 - 124	115	108 - 124	<0.01
Absolute SCQ scores	18.5	14 – 24	3	1 – 5	<0.01
SCQ scores (cut-offs)	n	%	n	%	
ASD risk score ≥15	23	68	0	0	<0.01
ASD risk score ≥13	29	85	0	0	<0.01
Schooling					<0.01
Mainstream	6	18	50	98	
Mainstream with support	14	41	1	2	

ASD unit	8	23	0	0	
Special Education/Home tuition	6	18	0	0	
Developmental Co-morbidities					
Total (child may have ≥ 1 co-morbidity)	18	53	5	10	<0.01
ADHD	7	21	0	0	<0.01
Anxiety	0	0	1	2	0.41
Learning Disability (LD)	6	18	0	0	0.01
SPD	7	21	1	2	<0.01

Data are represented as numbers (n), means (M) or medians with percentages (%), standard deviations (SD) or interquartile ranges (IQR). We calculated p-values using Chi Square (χ^2) for categorical data and either t-tests or the Mann-Whitney U-test for continuous data, depending on the normality of the data in question. p – values are significant <0.05. #Three participants from the case group (9%) were unable to attend for KBIT-2 cognitive assessment. All controls participated.

All controls attend mainstream school, while twenty (59%) of the autism group attend mainstream school, with more than two thirds of these children requiring added educational supports. Of the remaining ASD group children, eight (23%) attend a dedicated ASD unit, five children (15%) attend a Special Educational school, and the remaining child is home schooled. Six children (18%) report a learning disability (LD) (all in the ASD group $p=0.01$), five of whom attend special education school, and one attends an ASD unit. Five of the six children who reported LD completed the KBIT-2 assessment. Composite IQ scores in those with LD varied; the two children with reported mild LD scored 90 and 77 (in the average and below average range respectively). Three children with moderate LD who completed the KBIT-2 assessment, scored in the lower extreme range with composite IQ scores of 64, 51 and 46.

Generally, both case and control groups scored within the average or above average ranges in the KBIT-2 assessment. There was no difference between groups in the nonverbal domain, but scores did deviate significantly in the verbal and composite IQ domains $p < 0.001$. If we exclude children with reported LD however, and only examine the cases without reported LD, then only the verbal domain remains significantly different ($p = <0.01$), with composite and non-verbal IQ scores not demonstrating statistical significance between groups (see

Table 18). Children with ASD have a significantly higher burden of developmental comorbidities when compared to their neuro-typical peers. Reports of ADHD, learning disability, and sensory processing disorder were all significantly greater in the autism group (see Table 18).

Measure of ASD symptomology:

Median SCQ scores were significantly different between cases and controls. In the ASD group median SCQ scores were 18.5 (IQR 14 - 24) and control group median SCQ scores were 3.0 (IQR 1 – 5) p-value <0.01. None of the control group fell within the SCQ at risk range (Table 18). EBPs at 5 years correlated significantly, strongly and positively with SCQ symptomatology, across both total SCQ scores and each individual SCQ subdomain (see Table 19).

Table 19: Correlation of ASD symptomatology (at 8 – 11 years) versus emotional and behavioural problems at 5 years.

CBCL summed scales	Total SCQ	Restricted behaviours	Communication difficulties	Social reciprocity
Total Problems	0.663*	0.648*	0.663*	0.557*
Total internalising	0.606*	0.641*	0.587*	0.480*
Total externalising	0.595*	0.546*	0.639*	0.533*

We calculated correlation co-efficient using the spearman (ρ) test. *correlation is significant at the <0.01 level.

As four controls did not complete the 5-year CBCL, the number of participants was 34 cases and 47 controls. All participants completed the SCQ.

Discussion:

Key Findings

We have shown that children affected by ASD experience increasing difficulties with EBPs in the early years of life. From 24 months, the EBP profile of ASD affected children deviated significantly from that of neurotypical 24 month olds. At 5 years, children with ASD experienced a worsening of reported EBP symptoms, with more than half, scoring in the clinical range for Total Problem and Internalising Problem scores, with another 25% in the clinical range for Externalising Problems. During the same period, neurotypical peers displayed a general stabilisation of, or improvement in, their EBP scores.

Subscales that did not differ significantly between groups at the 24-month time point (anxious/depressed, somatic complaints, aggression, total internalising problems, affective problems and anxiety problems) differed significantly at 5 years, suggesting a deterioration in the EBP symptoms experience by the ASD group versus their peers. Overall, the data demonstrates a greater burden of EBP comorbidities affecting the ASD group versus controls beginning in early childhood. This greater EBP burden affecting the ASD group further deteriorates by the age of 5 years.

Previous studies have demonstrated variable levels of EBPs in ASD affected children (2, 3, 514). While these studies often involved large ASD cohorts, the age ranges of the participants were often broad (2, 3). We contend that little is known about EBPs in early pre-schoolers, before ASD diagnosis, and no other study has mapped the course of these behavioural issues from such a young age, while EBPs occurring in later childhood and adolescence are much better characterised (507, 515). Overall, the literature on EBPs in children with ASD is inconclusive and limited. Our findings indicate that age will significantly affect the rates of reported EBPs and that EBPs begin to develop in early childhood, worsening into the early school-going years. It is not sufficient to coalesce large groups of ASD affected children, rather assessments should occur serially and at different age points, and should be monitored pre and post intervention.

Family and Social Elements

We recognise that EBPs worsen stress and Quality of Life scores in families of children with ASD (515, 516). Pervasive and severe social, emotional and behavioural deficits in children with ASD are also important modifiers of outcome. EBPs present further difficulties for caregivers, including elevated stress, mental and physical health problems, financial strain,

higher rates of divorce and lower overall family well-being (8, 9). More than half of parents describe as “very stressful” the time from first medical contact right up until completion of the ASD diagnostic process. A process, which can take more than 5 years. Reported parental stress is the single most important determinant of parental satisfaction with the ASD diagnostic process (448). Counterintuitively, stress is often worse if the child has a milder phenotype (likely due to significant uncertainty around the diagnosis). The negative parental and family effects of EBPs can mutually and adversely affect the child and may stymie the positive effects of behavioural interventions or family centred therapies (8, 448).

We get a clearer indication of the socioeconomic effect of ASD through our questionnaire responses. Strikingly, more than half of mothers to ASD affected children, report either homemaker or carer roles as their current job situation. Only 12% of NT mothers reported similarly. Despite equal educational attainment, mothers in the control group are far more likely to self-report as being in part-time or full-time employment. This suggests that there may be long-lasting familial and financial ramifications for these families, with mothers in particular likely to suffer a loss in productivity and often becoming a permanent loss to the workforce.

Raising a child with ASD can be an all-encompassing experience for families. Negative feelings towards children with ASD are common in parents. Negativity directed towards the child reduces the child's social capacity and functioning (517, 518). Negative emotions can worsen symptoms of ASD and lead to comorbid emotional and behavioural problems (EBP) (519, 520). A chronic lack of institutional support for families and children affected by ASD is both a reason for, and potentiator of this negative spiral. Unfortunately, the assessment of outcomes for ASD interventions is usually centred exclusively on the child. Parent and family dynamics that may influence both the immediate and long-term effects of therapy are overlooked (8).

Early parent acceptance of ASD diagnosis and engagement with interventions has been shown to have significant protective collateral effects on adjusting to the diagnosis. Early diagnosis and intervention before EBPs become prominent can improve parental wellbeing and so too their ability to provide care for their child. There is evidence that once established, EBPs persist and become recalcitrant to treatment (521).

[Neurodiversity: A confident and new terminology](#)

Over the past decade, there has been a cultural shift in the way adults and young people view their own ASD. There is more emphasis put on their point of difference or diversity,

rather than them having a deficit or a disorder (522). They have taken ownership of the descriptor “Autistic” and cast Autism in a positive light. This mind-set and the peer support structures that have built up around it can form a crucial psychological support system for young people with ADS and their families. It provides a sense of their own uniqueness but also of belonging to a wider, vibrant neurodiversity community. Parents of younger autistic children can find a voice through these organisations and are strong advocates for positive change in terms of services and accommodations for autistic people. This positive self-image can even be seen in newer linguistic descriptions of autism, the Irish, *Uathachas* (meaning singular, unique) and the Maori, *Takiwātanga* (meaning in their own time and space) putting a more positive slant of the original Greek-derived term, *Autism* (meaning self-ism).

At the later childhood follow up, none of the ASD group reported symptoms of or a diagnosis of anxiety and only one child in the control group did. These health questionnaire responses however, are at odds with the 5-year CBCL scores. According to the CBCL (at 5 years), three ASD participants scored in the borderline category and five in the clinical range for the Anxious/Depressed subscale. Only one child from the control group scored in the borderline range (the child with parent reported anxiety at follow up). This may suggest a tendency to underreport psychopathology and other developmental comorbidities in the setting of children with ASD. The time interval between CBCL scoring and appointment follow-up is lengthy (up to 6 years in some cases). The disparity between the CBCL and questionnaire responses may be due to an amelioration of anxiety symptoms in the period between 5 years of age and the 8 – 11 years age group. This is consistent with findings by other authors in older school age children (523). Alternatively, while we recognise anxiety as a common comorbidity in ASD, perhaps it is not identified readily outside the clinical sphere (524). Caregivers may tend to attribute behaviours or emotional problems to underlying ASD core symptoms rather than to a distinct anxiety problem.

While other authors have found that ASD symptomology does not correlate with EBPs or maladaptive behaviours (2, 3), we found that our measure of ASD symptomology (SCQ) correlated positively and strongly with the CBCL summed scores. Both total problem (TP) and total internalising scores correlated strongly and positively, and total externalising scores correlated moderately and positively. This may suggest that higher levels of ASD symptomology predispose to a higher likelihood of EBPs. The converse may also be true; EBPs that develop at a young age and worsen over time may impede therapy and contribute to worse functional outcomes.

The SCQ proved extremely effective at ensuring our control group was free of clinically significant ASD symptomology, and as a measure of ASD symptomology, the SCQ has proven to be a sensitive tool in our cohort. Using both the ≥ 15 and ≥ 13 cut-offs, we found that none of the controls scored in the clinical range. In this age group (8 – 11 years), it is an extremely sensitive measure of ASD symptomology. When using the SCQ as directed (with a cut-off of 15), and analysing the raw SCQ scores with non-parametric Receiver Operating Characteristic (ROC) curve analysis, we calculated an extremely high Area under curve (AUC) of 0.98, 95% Confidence Interval (0.96 – 1.0), $p = <0.001$. If we further examine the ROC curve, we find that even with the cut-off set at 12, the sensitivity marginally improves to 85.3% while maintaining a specificity of 100%. This suggests that SCQ screening in this age group may be improved by considering a lower cut-off (i.e. 12 or 13), than that suggested by the original authors. Some authors have recommended use of cut-off scores as low as 11 in the setting of young children aged under 4 years (511, 525). Our findings suggest that perhaps the same provisions may help SCQ performance in this older school-going age bracket.

Our findings add to the existing ASD literature by exploring the importance of assessing EBP in conjunction with ASD symptoms in ASD pre-schoolers. We present a well-characterised patient cohort with confirmed formal ASD diagnoses and follow up recorded at three distinct time-points (24 months, 5 years, and later childhood (8 – 11 years)). Participants were assessed using validated and robust measures of ASD symptomology, cognition and behaviour. These measures, at different time-points, allow us to examine the temporal evolution of EBPs in our small, but well-characterised cohort.

Limitations

Our small numbers under power the study for detailed examination of societal or family factors, which may affect EBPs scores in children. It is difficult to infer whether early detection of EBPs and dedicated interventions may have ameliorated outcomes in our cohort. Child centred approaches neglect the impact of ASD on families, and metrics of outcome and intervention should include elements to address not only the child, but also the family unit and prevailing environments.

It is worth noting that the mean age of autism diagnosis was almost 5 years, by which time over 50% of the ASD group had clinically significant EBP symptoms. As with early intervention improving clinical outcomes in ASD, early intervention has been found to improve the emotional and behavioural outcomes of autistic children. Future studies should focus on serial assessment at well-defined time-points and using larger cohort sizes. More reliable

data pertaining to onset, and evolution of EBPs will allow children with ASD to receive not only specific individualised ASD interventions, but also, where appropriate, behavioural and emotional therapy.

Conclusion:

We have shown that emotional and behavioural disorders are extremely common in ASD cohorts, and that they deteriorate with age. They are important modifiers of outcomes in children with ASD. EBPs co-develop with ASD symptoms in early childhood and can go under-reported by caregivers. Early co-assessment of and intervention in EBPs in autistic children is vital to ensure improved outcomes for children with ASD and their families.

Chapter 7

Discussion

Discussion

Overview of presented work and aims

Despite the recent progress in basic research, and the huge strides taken in the study of genetics in ASD, we still define ASD based solely on the clinical observation of certain recognised behaviours. The lack of early and objective diagnostic measures is one of the greatest challenges facing autism intervention today. Recent research has focussed on the identification of specific biological abnormalities in autism, which might aid early diagnosis and inform treatment. Objective and measurable biomarkers can offer the means to identify and quantify biological abnormalities, not only for screening and diagnosis, but also for surveillance and measurement of treatment response. Antenatal biomarkers to stratify autism risk during pregnancy may inform selection of at risk candidates for early intervention (the period when it is most efficacious), and biomarkers to predict treatment response may, for those already diagnosed, optimise access to therapy and improve symptoms and functional outcomes. While myriad biological, pathological, and psychological anomalies have been associated with ASD, none has yet translated to a generalizable metric or marker that may assist in diagnosis or treatment. With this in mind, the primary focus of this thesis was to use a multimodal approach to identify abnormalities of cytokine, protein, and metabolite expression, which may help to better understand the pathological processes underlying ASD and offer a validated biological marker with utility in ASD screening, diagnosis and treatment.

Given that the PiRAMiD project combined a laboratory and a clinical approach, it was crucial to direct a secondary focus on important clinical facets of autism that are remediable with current therapies, and improvement in which may ameliorate quality of life in affected children and their families. Emotional and behavioural problems are prevalent among autistic individuals and can negatively affect family life, and even a patient's ability to

participate in therapy. Questions remain regarding the age at which EBPs first emerge, and how they can magnify the core symptoms of ASD. Interventions for EBPs can be effective, yet the administration of and access to targeted intervention in many health jurisdictions remains inconsistent. The secondary aim of this thesis was to understand when EBPs first arise in children, how they can modify ASD outcomes, and the role of early screening in minimising their deleterious effects. To achieve this, I have outlined my specific aims and objectives below.

Aims:

The general aim of this thesis was,

1. To investigate, in a well-characterised cohort of autistic children, the potential contributory role of maternal immune activation and early protein or metabolic dysregulation in ASD aetiology, with the aim of identifying a potential biomarkers which may aid in the early identification of, and timely management of, the disorder.

A secondary aim of this work was,

2. To characterise the clinical features of this ASD cohort with a focus on the temporal development of emotional and behavioural comorbidities and the timing of their onset in this group.

Objectives:

I achieved these aims via the following objectives:

1. Examination of mid-gestation cytokine profiles in mothers of children with an ASD diagnosis at two mid-gestation time points (15 and 20 weeks) across two sites as part of a large multi-centre pregnancy study with the aim of identifying a potential gestational ASD biomarker.

2. Validation of candidate maternal cytokines (including an ultrasensitive assay of interleukin 17) at a single specific mid-gestational time-point (20-weeks' gestation) in a carefully characterised expanded birth cohort.
3. Discovery analysis of proteomic and metabolomic signatures in cord blood plasma and late childhood serum samples from the ASD cohort versus matched controls
4. Characterise clinically the development of emotional and behavioural problems (EBPs) in our ASD cohort with an emphasis on the evolution of EBPs over time and the timing of onset.
5. Development a maternal and child genetic biobank/repository of bio-samples taken from mother-child dyads enrolled in the study. Children included with ASD had either an early (<5 years old) or late (>5 years old) ASD diagnosis and samples were stored for interval RNA, genetic and HLA-G analysis.

In the discussion section that follows, I will summarise the findings from the PiRAMiD project, indicate how the expressed aims and objectives were achieved, and discuss the implications of my findings for future work in the field of autism biomarker research.

Summary of main findings:

The initial aim of this thesis was to investigate the role of maternal immune activation in ASD aetiology through quantification of inflammatory, protein and metabolomic molecules. Identification of potential biomarkers through these methods may allow earlier detection and intervention in autism. With regard to this aim, we found altered expression of specific cytokines (interleukins 17, and 4) at 20 weeks' gestation in archived serum from mothers of autistic children (Chapter 3 and 4). In our initial experiment we examined a combined autism cohort with cases from Ireland, and New Zealand, all with an early autism diagnosis (<5 years). To determine whether there was a difference in inflammatory markers between groups at either 15 or 20 weeks gestation, we performed electrochemiluminescence assays.

Of the initial multiplex panel of eight cytokines and chemokines (IFN- γ , IL-16, Eotaxin, MCP-1, IL-1 β , IL-8, IL-6 and IL-17A), one cytokine was significantly altered. We found that IL-17A levels were significantly reduced in the autism group versus controls at 20 weeks gestation (following adjustment for sex, mode of delivery and maternal folate intake at 15 weeks). This finding identifies IL-17A as a potential cytokine biomarker of autism risk measurable at mid-gestation. This novel finding adds to the growing evidence that in-utero exposure to maternal immune activation and resultant cytokine dysfunction is associated with an increased risk of ASD in offspring. Very few other human studies have examined cytokine levels in mid-gestation, and the only other group to examine IL-17 specifically, found it to be elevated in their autism group (34). Elevated levels of IL-17A have been reported in the blood of autistic individuals, and these have correlated positively with severity of ASD behavioural symptoms (27, 271). While there is further evidence from animal models that IL-17, and the Th17 cells that produce it, may play a key role in MIA mediated autism risk (24, 142).

Following on from the initial experiment, we wanted to replicate our findings. To that end, we further reviewed the literature to update our list of candidate cytokines. Adhering to the most current evidence at the time, we focused our experiment on quantification of eight specific cytokines, IL-17A, GM-CSF, IFN- γ , IL-1 β , IL-4, IL-6, IL-8 and TNF α . We replaced Eotaxin, IL-16 and MCP-1 with GM-CSF, TNF α , and IL-4 (119, 120). In this experiment, in an effort to validate our previous findings, we included a separate single-analyte ultrasensitive assay, specifically for IL-17A. We also quantified IL-17A as part of the regular multiplex assay along with the other analytes. We re-examined the Irish cohort, and expanded the numbers with children who were diagnosed later (>5 years), these children were captured during recruitment for the PiRAMiD study. We failed to reproduce the IL-17 data in either the multiplex or the ultrasensitive assays. We did however demonstrate altered expression of IL-4 in mid-gestational serum between the autistic and control groups at 20 weeks' gestation (Chapter 4). That we found alterations in IL-4 is intriguing. Only a small number of human-

based studies have examined mid-gestational serum of mothers to autistic children. IL-4 is the only cytokine to demonstrate altered expression across all of these studies (33, 34, 181). Surprisingly, while previous authors found elevated levels of IL-4 in autism groups versus controls, we found the opposite. IL-4 is a pleiotropic and generally anti-inflammatory cytokine; we find it at the feto-maternal interface throughout pregnancy (444). In normal pregnancy, levels of IL-4 persist and increase as normal pregnancy progresses (445). Previously, low circulating IL-4 levels during pregnancy are linked with negative obstetric and neonatal outcomes (296, 446, 447). Given the typically anti-inflammatory effects of IL-4, it is reasonable to assume, that depleted levels of IL-4 may lead to a more pro-inflammatory fetal environment with the potential to affect negatively, both fetal and maternal health.

Moving away from the specific characterisation of cytokine profiles, In Chapter 5, we examined comprehensively the proteome and metabolome of our cohort. When we studied cord blood plasma comparing cases and controls, we found profiles of altered protein and metabolite expression, which precede the autism diagnosis. Discovery proteomic analysis identified 41 proteins that were significantly altered between autism and neurotypical groups in cord blood, while discovery in the metabolomic analysis yielded 32 metabolites that were significantly altered between groups. Using machine-learning techniques, these profiles were found to predict autism in offspring with excellent test quality (AUROC) scores of 0.82 and 0.86, in the proteomic and metabolomic analyses respectively. In identifying, a cord blood signature associated with autism risk; our findings are consistent with those of the recent Norwegian Autism Birth Cohort study, who report a pronounced cytokine signature in cord blood plasma, and maternal mid-gestational plasma, from children who would go on to develop autism (473).

Proteomic analysis of child serum from the follow up PiRAMiD cohort observed altered expression of GAPDH, SELENBP1 and BLVRB proteins in the autism group versus controls.

These proteins were also altered in the cord blood analysis. These findings illustrate the persistence of our previously identified birth protein signature into pre-pubertal childhood. Interestingly, while these proteins were elevated in serum from the Autism cohort in late childhood, their expression was decreased in cord blood at parturition. GAPDH is pleiotropic protein that is ubiquitous throughout human body tissues, including the fetal brain. It has established roles in glycolysis, metabolic function, modulation of the cytoskeleton, and it acts as a molecular switch under oxidative stress (484, 485, 487). Researchers recently identified GAPDH as a novel autoantibody target in archived fetal brain samples; while previously, autoantibodies to GAPDH have been linked to other psychopathologies including depression and schizophrenia (488, 489). SELENBP1, a transporter of selenium, has been linked with schizophrenia and autism. It is a key protein in sulphur metabolism and a crucial enzyme responsible for biosynthesis of hydrogen sulphide (475). Impaired sulphate metabolism is a replicated biochemical endo-phenotype associated with Autism, and is widely documented in autistic children (476, 477, 478).

We speculate that these persistent and coordinated systemic fluctuations in GAPDH and SELENBP1 may point to key molecular pathways implicated in Autism aetiology, which manifest within the prenatal environment and likely oscillate throughout childhood. We believe that this work is the first to test directly the prediction that birth/in utero markers (cord blood signatures) of autism can persist into childhood. Collectively, these findings suggests cord blood molecular signatures of autism are evident from birth, and in combination with clinical data and cytokine profiling, these processes may be useful in precision screening of “at risk” infants, and in streaming them to targeted early interventions.

Future work might aim to elucidate the direct mechanistic underpinnings of how SELENBP1 and GAPDH drive changes in the developing brain, and determine if these systemic measures

can serve as longitudinal biomarkers of Autism severity or risk. Additionally, this research provided fundamental evidence that we should consider cord blood to be a valuable bio-fluid in autism risk studies. Cord blood has the potential to identify disease specific prognostic biomarkers, which may also serve as systemic therapeutic targets for treating and targeting aspects of Autism symptomology.

Finally, Chapter 6 focuses on the secondary of our initial aims. Through the clinical characterisation of our autism cohort, we addressed how elements of their diagnosis affects theirs, and their family's lives. We showed that autistic children experience increasing difficulties with emotional and behavioural problems (EBP) in the early years of life. From 24 months, the EBP profile of autistic children deviated significantly from that of neurotypical peers. At 5 years, children with ASD experienced a worsening of reported EBP symptoms. During the same period, neurotypical matches displayed a general stabilisation of, or improvement in, their EBP scores. Subscales that did not differ significantly between groups at the 24-month time point (anxious/depressed, somatic complaints, aggression, total internalising problems, affective problems and anxiety problems) differed significantly at 5 years, suggesting a deterioration in the EBP symptoms experience by the ASD group versus their peers. Overall, the data demonstrates a greater burden of EBP comorbidities affecting the ASD group versus controls, with an onset in early childhood, and well established before starting school. While EBPs arise of themselves as co-morbid problems in autistic children, they are also modified by important environmental factors such as parental stress, negative feelings towards the child and parental failure to accept the autism diagnosis (517, 519). These factors are often compounded by delays in the initial diagnosis, parental dissatisfaction with the diagnostic process and poor access to therapy or services once diagnosed (448). Amelioration of these factors is possible, improvements in diagnostic wait times and engagement with therapy after diagnosis are basic aspirations, while earlier screening for EBPs would allow earlier, and parent-led interventions.

Other authors have demonstrated variable levels of EBPs in autistic children, and findings overall are inconsistent (2, 3, 514). Larger studies, in order to recruit large numbers, often involve participants from a broad range of ages, meaning that conclusions are generalised and imprecise (2, 3). Even in larger cohort, information regarding EBPs in the very young is lacking. While larger cohort numbers are desirable, it is not sufficient to coalesce large groups of autistic children. In future, assessments of EBPs should occur serially and at different age points; this would allow longitudinal analysis and stratification of cohorts depending on age. Ideally, health departments should incorporate EBP screening into early stage assessments of ASD, given they are so frequently co-morbid. Coupled with improved and equitable access to diagnostics and therapies, this would see holistic improvements in ASD and EBP symptomology, family wellbeing, and mental health.

Limitations and Strengths:

Limitations of this thesis

We recruited both the autism and control samples from the same cohort of children enrolled in the BAELINE birth cohort study. The autism group consisted of 22 children with a formal ASD diagnosis at 5 year follow up, and a further 15 children who were formally diagnosed during the period between the 5-year and later childhood follow up appointments. They were originally identified as “at risk” as they had either scored in the “at risk/borderline range” for the Pervasive Development Disorder subscale on the CBCL, or their parents had expressed specific concerns regarding the possibility of ASD, or the child was reported to have an ASD associated developmental delay (most commonly speech delay). A small number of later diagnosed children did not reach the “at risk” criteria at 5 years, but still received a formal ASD diagnosis in the community. These became apparent during selection and recruitment of matched controls, four children in the original control group had received an interim autism diagnosis. They had not met the “at risk” criteria at the 5-year appointment and so escaped capture. These participants were re-categorised as cases, and matched with

new controls. It is likely then that other cases in the total sample of more than 2000 children also escaped detection. Overall, the prevalence of autism in our cohort was approximately 1.7%; this is consistent with recent reports from other groups, and suggests that recruitment to the autism group was near total. In order to remediate this problem, one could contact and question all original participants regarding any interval diagnoses. This was not possible given the time constraints of the thesis.

One of the primary limitations, highlighted throughout this thesis is the small sample size. We expect this limitation, to a degree, in longitudinal autism studies with serial bio-sample analysis, due to the relative scarcity of such cohorts. In our cohort, analysis of IL-4 levels in the groups yielded results on only 16 individuals (6 cases and 10 controls). This was due to an initial small case cohort as well as methodological issues such as attrition of viable samples, which arose due to a combination of the low absolute concentrations of IL-4 in the samples, and concentrations at or below the sensitivity (Lower Limit of Detection LLOD) of the MSD multiplex format. One should interpret these results with caution, as it is difficult to make meaningful inferences from results in samples this small. Larger scale group analyses are warranted.

In devising the overarching PiRAMiD plan for recruitment and follow up, one oversight was our failure to perform a further CBCL at the age 8 – 11 years appointment. This would have allowed further characterisation of EBPs in the cohort as well as helping to advance our understanding of the temporal evolution of such comorbidities.

Perhaps the most important limitation encountered in this thesis is a methodological one. Unfortunately, many of the bio-samples used in these studies fall well outside the ideal sample age for accurate cytokines quantification and analysis (185). To my mind, this is the single most important limitation confronting studies of this nature. The shelf life of archived samples is finite, and even samples handled under the strictest protocols and stored in long-

term ultra-low temperature storage (-80°C) suffer from significant cytokine and chemokine degradation over time (185, 440). The effect of long-term storage seems somewhat less pronounced in metabolomic (526, 527) and proteomic studies (528) but studies still demonstrate significant degradation over longer timeframes. Use of tissues or bio-fluids as early as possible following sampling is always preferable. Retrospective sample analysis, such as in Chapters 3, 4, and 5, would present an excellent opportunity to study cytokine, proteomic and metabolomic aberrations in ASD, if samples were used in a timely fashion. This will remain difficult to achieve as long as ASD services continue to be under-resourced (212) and diagnoses chronically delayed (449). Under current conditions, our experience of retrospective analysis of archival samples suggests that this style of study design is not well suited to addressing this question. Even large-scale population based studies would suffer from the same issues of sample fidelity over longer periods.

Remediation of limitations

Future study designs should be prospective, and concentrate on early ASD case identification or screening. Early identification should be paramount, the diagnostic stability of ASD is reliably fixed from as early as 14 months old (208) so screening and identification within the first 2 years of life is possible. If possible, cytokines should be analysed contemporaneously to limit degradation which will occur within 3 years even in -80°C freezer conditions (185). Basic handling of samples and initial processing requires optimisation to ensure the risk of sample degradation is minimised:

- (i) Store samples at ultra-low temperatures (-80°C),
- (ii) Initial processing should be rapid (<1 hour from venepuncture to freezer storage),
- (iii) Freeze-thaws cycles should be minimised, and

- (iv) Samples should be aliquoted to reduce exposure to freeze-thaw cycles, and facilitate more rapid freezing.

With robust methods of early screening in place, early confirmatory diagnosis within the first 2 years, and analysis of gestational samples within 3 years, it should be feasible to increase the yield and validity of such studies, and greatly reduce cytokine, metabolome and proteomic degradation through prolonged storage. This approach would best suit the study of children presenting with the earliest signs of ASD, or in targeted high-risk groups, for example, in ASD affected siblings.

Finally, given that we had a small sample size to begin with, sampling of blood samples from as many participants as possible was critical to improving the validity of our results. In Figure 39, we can see the successful sampling rates for participants at the late childhood visit. We used these child samples in the metabolomic and proteomic analysis. Unfortunately, in spite of my best efforts, only 73% of the autistic children who attended allowed venepuncture. The success rate was better in the control group (91%). In an effort to improve venepuncture rates, I introduced individualised social stories to prepare children from the autistic group before they came to the clinics. These social stories were prepared in accordance with the 10:2 criteria developed by Carol Gray et al (513), however it is unclear if this intervention had any impact as it was used for a handful of especially nervous children. Other authors have reported venepuncture compliance rates >90% in autistic children with extensive cognitive behavioural therapy based, parent-administered, preparation material (including social stories) before a clinical visit and venepuncture (529). Future studies would benefit from such a tailored, prophylactic, preparatory approach to venepuncture and other painful procedures.

Autistic children	Maternal	Controls
<ul style="list-style-type: none">• 26 Males• 8 Females• 25/34 (73.5% success) gave blood	<ul style="list-style-type: none">• 89/90 gave blood (99% success rate)	<ul style="list-style-type: none">• 37 Males• 19 Females• 51/56 (91.1% success) gave blood

Figure 39: Blood sampling in our cohort.

Strengths of this thesis

Summarily, I hope that the strengths of the work in this thesis outweigh the limitations. To help ensure the success of the project, I have endeavoured to combined robust clinical assessment tools with validated and innovative laboratory and analytical techniques. This, all in an attempt to help decipher the underlying machinations of autism and to potentially uncover novel insights into the pathophysiology of ASD.

Perhaps the greatest strength of this work is the extensive clinical characterisation of the mother and child cohorts. Participants have been followed from the prenatal period with serial, longitudinal, blood sampling in the mid-gestational (maternal), natal (umbilical cord blood), later childhood (8-11 years) periods. Coupled with these valuable biochemical samples, the SCOPE study documented the health characteristics of each mother during pregnancy in fine detail, as well as critical information related to parturition and neonatal outcomes. The children, through the BASELINE study, had comprehensive clinical and psychometric assessments at 2 year, 5 years, before further clinical and psychometric assessments in later childhood as part of the PiRAMiD study. The clinical research fellow, Dr Michael Carter, conducted the PiRAMiD follow up appointments. During the course of the visit, the child had a number of general and specific assessments:

- (i) anthropometric measurements (height, weight, BMI and centiles)
- (ii) completion of a general health and demographic questionnaire which included information regarding current maternal, paternal, and child health, use of medications and further details concerning their ASD diagnosis and relevant family and educational history
- (iii) a general paediatric physical examination to ensure that there was no significant current infections
- (iv) completion of the ASD screening tool, the social communication questionnaire (SCQ)
- (v) completion of the Kaufman Brief Intelligence Test, second edition (KBIT-2)

(vi) and finally venepuncture to include serum/plasma, EDTA and RNA blood sampling from the child-mother dyad

This detailed characterisation is very useful as it reduces the risk of introducing potential confounders to the data. During the cytokine analysis for examples, we were able to ensure none of the participants were actively unwell or carrying an infection while the blood samples were taken, while we could also document any recent anti-inflammatory or steroid use, which may suppress the cytokine milieu artificially. Using the PiRAMiD data, and the previous data from SCOPE and BASELINE we were able to develop a longitudinal picture of each participant, both in terms of their blood biochemistry and their phenotype and symptomology. This allowed us to examine the temporal evolution of emotional and behavioural comorbidities as well as examine the proteome and metabolome in a longitudinal fashion.

Another strength was adherence to strict Standard Operating Procedures for handling, processing and storage of all biological samples. Cord blood plasma from parturition and follow up serum samples collected in childhood (age 7 to 10 years) were used for the longitudinal proteomic and metabolomics profiling. The samples were collected, processed, and bio-banked according to the quality controlled INFANT biobank protocols. The ISO accredited biobank adheres to the International Society for Biological and Environmental Repositories (ISBER) best practices, and the Molecular Medicine Ireland Guidelines for Standardised Biobanking and OECD Guidelines for Human Biobanks and Genetic Research Databases. This ensures that, as much as possible, the fidelity of samples is maintained and the risk of sample or tissue degradation is minimised

Finally, in adopting a blended multi-omics approach, we were able to offer a comprehensive, serial snapshot of important biological processes at different time-points in the child's life. Firstly, using innovative processes such as Electro-chemo-luminescence (Figure 40), we were

able to examine the mid-gestation (20 weeks) cytokine milieu in our cohort of mothers to autistic children and find altered expression of interleukin-17, a result that we were able to validate in a separate cohort of autism children from New Zealand. In an expanded local cohort, including children with a later ASD diagnosis (>5 years), when we examined mid-gestation (20-weeks) cytokine levels, we found altered expression of interleukin-4 versus controls. We could not replicate the original finding of altered interleukin-17, even when using an ultrasensitive assay, this may have reflected differences between the original cohort which all had an early diagnosis (<5 years), and the expanded cohort which included those with a later diagnosis.

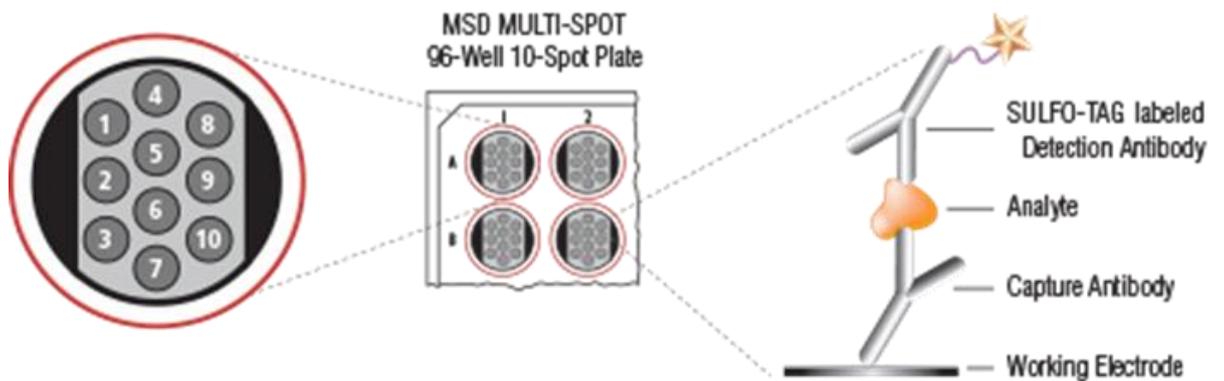


Figure 40: Electro-chemo-luminescence:

Biological samples are added to the 96 well plate. Electrochemiluminescent labels generate light when stimulated by electricity in the correct chemical environment. This reaction is incorporated into immunoassays and provides a light signal that is used to measure target proteins and other molecules. Samples are analysed using a specific MSD mesoscale instrument. Each well can measure up to 10 individual cytokines (multiplex). Ultrasensitive assays (S-plex) are also available and significantly improve detection of low concentration analytes, with sensitivity in the femtogram/ml range.

Secondly, our Omics study utilised inter-omics profiling and state of the art mass spectrometry and machine-learning approaches including data independent acquisition (DIA) coupled with Frag-pipe search engine utility for DIA-Neural Network (NN) analysis. This is the current gold standard in mass spectrometry for proteomics. Targeted validation of the metabolomic data was undertaken using MRM (Multiple reaction monitoring). These MRM

assays allowed highly selective and sensitive monitoring and quantification of steroid pathway biomolecules, with mass spectrometry standards available. The pioneering use of multiple Omics platforms and machine learning allowed us to be the first group to identify persistent altered expression of specific molecules which were present in umbilical cord blood, and which persisted in to later childhood. This is a novel finding.

Conclusions and Future Directions:

Summary

In summary, we have found that IL-17A levels were significantly reduced in our autism cohort versus controls at 20 weeks gestation. This result is novel in that only one other study has identified altered IL-17A in mid-gestational serum. While they found levels of IL-17A to be increased, we found reduced levels in maternal serum. That we drew our cohort from two geographically distinct locations (Ireland and New Zealand) adds to the validity of our findings. Our results identify IL-17A as a potential cytokine biomarker of autism risk measurable at mid-gestation and adds to the growing evidence that in-utero exposure to maternal immune activation is associated with an increased risk of ASD in offspring.

We also demonstrated altered expression of IL-4 in mid-gestational serum between the autistic and control groups at 20 weeks' gestation. Again, only a small number of human-based studies have examined mid-gestational serum of mothers to autistic children, yet all have identified altered expression of IL-4. While previous authors found elevated levels of IL-4 in their autism cohort, again, we found the opposite. IL-4 is critical for normal homeostasis in pregnancy, and its expression increases as natural, healthy pregnancy progresses. Deviation from this norm, gives added credence to our findings. It is conceivable that low levels of circulating IL-4 lead to a pre-inflammatory fetal environment with the potential for negative neonatal outcomes. These findings further add to the evidence in the growing body of mid-gestation cytokine literature.

In our inventive use of multiple -Omics platforms and machine learning technology we are the first group to identify persistent altered expression of specific molecules present in umbilical cord blood, and which persisted in to later childhood in autistic children. This is truly a novel finding, and adds to the rapidly expanding literature utilising multiomics research in search of potential autism biomarkers. This line of inquiry may prove particularly

useful in the identification of underlying mechanistic pathways, such as those pathways, we identified involving sulfur and steroid metabolism, and glycolysis. Multiomics research may have applications, for not only screening and early diagnosis but also therapeutics.

Finally, there is conflicting evidence as to the age at which autistic children first develop emotional and behavioural problems. We have shown that many autistic children develop (EBP) within the first two years of life, and that these problems worsen throughout early childhood. EBPs become apparent years before the typical age of autism diagnosis (5 years), such that, by the age of five many autistic children experience a significant worsening of their EBP symptoms. EBPs correlate moderately with ASD symptomology, which suggests that they may contribute to core ASD symptoms. This may occur through any number of ways, including increased anxiety, social withdrawal, poor engagement with therapy, and low self-esteem. In finding that EBPs develop at such an early stage, we have further evidenced the crucial importance of early screening and intervention. These problems can be mitigated with therapy, but they must be identified first. Early intervention is only possible if children are diagnosed in a timely fashion rather than waiting until their fifth or sixth year before receiving an autism diagnosis, by which point many will have established co-morbid EBPs. It is incumbent upon national health systems to develop means of early screening and therapy in at risk children thereby allowing mitigation against the development of refractory mental and emotional health problems, which may be preventable.

[Improving Future Research](#)

In order to do better in the future, researchers investigating blood-based biomarkers of autism should examine large and diverse pregnancy and birth cohorts. Not only that, but samples should be examined in a timely fashion. While proteins and metabolic molecules have demonstrated more durability, cytokine and chemokines have proven to be particularly vulnerable to degradation over time in spite of best practice processing and storage.

Cytokine analysis thus, should occur as soon as possible following initial sampling, to ensure the fidelity of results.

Clinical cohorts of autistic children, when examining co-morbidities and EBPs should stratify their cohorts according to age, and include children aged under 2 years in their analysis. Symptoms of ASD and EBPs can be identified in the very young, and tools exist for the examination of these children. Efforts to identify EBPs at a younger age may help identify children with actual autism sooner, and allow early intervention targeting EBPs, ASD or both, when it is most effective.

Advocacy and Service Development

Previously, we indicated how ASD diagnosis is chronically delayed and access to services is variable and often poor. Those children with the greatest clinical need often arise in social circumstances, which further disadvantage them socially and financially. Universal screening for ASD as part of early years monitoring is feasible, with enormous potential to identify autistic children in their early years. Early co-screening for EBPs is also plausible, and instruments exist which can screen for both ASD and EBPs in the same assessment (CBCL). Healthcare providers and public health nurses would require very little additional training to tally and score these assessments, meaning that screening could be incorporated into the existing early years developmental follow up programme.

Any screening programme is only as effective as its follow on therapeutic service, and critical to any early screening programme will be clinical engagement after diagnosis. Interaction with ASD services cannot begin and end with the diagnosis. The current reality for many children in Ireland is that they engage with services only to receive a diagnosis, and then are discharged without significant medical, psychological or occupational therapy oversight. Yet this regrettable situation is remediable. Family-based, parent-led and psychology delivered therapies are effective in treating ASD and EBPs, and help foster a more holistic approach to

therapy. Stakeholders in Ireland should focus first on the development of core therapeutic infrastructure, and then on elaboration of universal ASD and concurrent EBP screening as part of routine early childhood follow up. As public awareness and the prevalence of ASD continue to trend upwards, and with ongoing population growth, the need for such basic services will only become more pressured. Practicable solutions exist, and it is only a matter of instituting them.

Bibliography:

1. Lord C, Brugha TS, Charman T, Cusack J, Dumas G, Frazier T, et al. Autism spectrum disorder. *Nature reviews Disease primers*. 2020;6(1):5.
2. Guerrera S, Menghini D, Napoli E, Di Vara S, Valeri G, Vicari S. Assessment of Psychopathological Comorbidities in Children and Adolescents With Autism Spectrum Disorder Using the Child Behavior Checklist. *Frontiers in psychiatry*. 2019;10:535.
3. Hartley SL, Sikora DM, McCoy R. Prevalence and risk factors of maladaptive behaviour in young children with Autistic Disorder. *Journal of intellectual disability research : JIDR*. 2008;52(10):819-29.
4. Sikora DM, Vora P, Coury DL, Rosenberg D. Attention-deficit/hyperactivity disorder symptoms, adaptive functioning, and quality of life in children with autism spectrum disorder. *Pediatrics*. 2012;130 Suppl 2:S91-7.
5. American Psychiatric Association. *DSM-V*. 5th ed. Washington DC:2013.
6. Birtwell KB. Social, Cognitive, and Behavioral Development of Children and Adolescents With Autism Spectrum Disorder. In: McDougle C, editor. *Autism Spectrum Disorder*. Section 1, Chapter 2. Feb 2016 ed. UK: Oxford Press; 2016.
7. Magiati I, Ong C, Lim XY, Tan JW, Ong AY, Patricia F, et al. Anxiety symptoms in young people with autism spectrum disorder attending special schools: Associations with gender, adaptive functioning and autism symptomatology. *Autism : the international journal of research and practice*. 2016;20(3):306-20.
8. Karst JS, Van Hecke AV. Parent and Family Impact of Autism Spectrum Disorders: A Review and Proposed Model for Intervention Evaluation. *Clinical Child and Family Psychology Review*. 2012;15(3):247-77.
9. Kerns CM, Newschaffer CJ, Berkowitz S, Lee BK. Brief Report: Examining the Association of Autism and Adverse Childhood Experiences in the National Survey of Children's Health: The Important Role of Income and Co-occurring Mental Health Conditions. *Journal of Autism and Developmental Disorders*. 2017;47(7):2275-81.
10. Whitehouse AJO, Varcin KJ, Pillar S, Billingham W, Alvares GA, Barbaro J, et al. Effect of Preemptive Intervention on Developmental Outcomes Among Infants Showing Early Signs of Autism: A Randomized Clinical Trial of Outcomes to Diagnosis. *JAMA Pediatrics*. 2021;175(11):e213298-e.
11. Gaugler T, Klei L, Sanders SJ, Bodea CA, Goldberg AP, Lee AB, et al. Most genetic risk for autism resides with common variation. *Nature Genetics*. 2014;46(8):881-5.
12. Vorstman JAS, Parr JR, Moreno-De-Luca D, Anney RJL, Nurnberger JI, Jr., Hallmayer JF. Autism genetics: opportunities and challenges for clinical translation. *Nature reviews Genetics*. 2017;18(6):362-76.
13. de la Torre-Ubieta L, Won H, Stein JL, Geschwind DH. Advancing the understanding of autism disease mechanisms through genetics. *Nature medicine*. 2016;22(4):345-61.
14. Fernandes IR, Cruz ACP, Ferrasa A, Phan D, Herai RH, Muotri AR. Genetic variations on SETD5 underlying autistic conditions. *Developmental neurobiology*. 2018;78(5):500-18.
15. Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, et al. Genetic Heritability and Shared Environmental Factors Among Twin Pairs With Autism. *Archives of General Psychiatry*. 2011;68(11):1095-102.
16. Ronald A, Happé F, Bolton P, Butcher LM, Price TS, Wheelwright S, et al. Genetic Heterogeneity Between the Three Components of the Autism Spectrum: A Twin Study. *Journal of the American Academy of Child & Adolescent Psychiatry*. 2006;45(6):691-9.

17. Kim YS, Leventhal BL, Koh YJ, Fombonne E, Laska E, Lim EC, et al. Prevalence of autism spectrum disorders in a total population sample. *The American journal of psychiatry*. 2011;168(9):904-12.
18. Christensen DL, Baio J, Van Naarden Braun K, Bilder D, Charles J, Constantino JN, et al. Prevalence and Characteristics of Autism Spectrum Disorder Among Children Aged 8 Years--Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2012. *Morbidity and mortality weekly report Surveillance summaries (Washington, DC : 2002)*. 2016;65(3):1-23.
19. F NG, Gallagher L, Lopez LM. Autism spectrum disorder genomics: The progress and potential of genomic technologies. *Genomics*. 2020;112(6):5136-42.
20. Banerjee S, Bhat M, Riordan M. Genetic aspects of autism spectrum disorders: insights from animal models. *Frontiers in Cellular Neuroscience*. 2014;8(58).
21. Janecka M, Mill J, Basson MA, Goriely A, Spiers H, Reichenberg A, et al. Advanced paternal age effects in neurodevelopmental disorders-review of potential underlying mechanisms. *Translational psychiatry*. 2017;7(1):e1019.
22. Sandin S, Lichtenstein P, Kuja-Halkola R, Larsson H, Hultman CM, Reichenberg A. The Familial Risk of Autism. *JAMA*. 2014;311(17):1770-7.
23. Sandin S, Lichtenstein P, Kuja-Halkola R, Hultman C, Larsson H, Reichenberg A. The Heritability of Autism Spectrum Disorder. *Jama*. 2017;318(12):1182-4.
24. Choi GB, Yim YS, Wong H, Kim S, Kim H, Kim SV, et al. The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science (New York, NY)*. 2016;351(6276):933-9.
25. Estes ML, McAllister AK. Maternal immune activation: Implications for neuropsychiatric disorders. *Science (New York, NY)*. 2016;353(6301):772-7.
26. Careaga M, Murai T, Bauman MD. Maternal Immune Activation and Autism Spectrum Disorder: From Rodents to Nonhuman and Human Primates. *Biological psychiatry*. 2017;81(5):391-401.
27. Akintunde ME, Rose M, Krakowiak P, Heuer L, Ashwood P, Hansen R, et al. Increased production of IL-17 in children with autism spectrum disorders and co-morbid asthma. *Journal of neuroimmunology*. 2015;286:33-41.
28. Ashwood P, Wills S, Van de Water J. The immune response in autism: a new frontier for autism research. *Journal of leukocyte biology*. 2006;80(1):1-15.
29. Al-Ayadhi LY, Mostafa GA. Elevated serum levels of interleukin-17A in children with autism. *Journal of neuroinflammation*. 2012;9:158.
30. Bryn V, Aass HC, Skjeldal OH, Isaksen J, Saugstad OD, Ormstad H. Cytokine Profile in Autism Spectrum Disorders in Children. *Journal of molecular neuroscience : MN*. 2017;61(1):1-7.
31. Ahmad SF, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, Alsaad AMS, et al. Upregulation of interleukin (IL)-31, a cytokine producing CXCR1 peripheral immune cells, contributes to the immune abnormalities of autism spectrum disorder. *Journal of neuroimmunology*. 2020;349:577430.
32. Ahmad SF, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, Attia SM. Elevated IL-16 expression is associated with development of immune dysfunction in children with autism. *Psychopharmacology*. 2019;236(2):831-8.
33. Goines PE, Croen LA, Braunschweig D, Yoshida CK, Grether J, Hansen R, et al. Increased midgestational IFN-gamma, IL-4 and IL-5 in women bearing a child with autism: A case-control study. *Molecular autism*. 2011;2:13.
34. Jones KL, Croen LA, Yoshida CK, Heuer L, Hansen R, Zerbo O, et al. Autism with intellectual disability is associated with increased levels of maternal cytokines and chemokines during gestation. *Molecular psychiatry*. 2017;22(2):273-9.
35. Idle JR, Gonzalez FJ. *Metabolomics*. *Cell metabolism*. 2007;6(5):348-51.

36. Glinton KE, Elsea SH. Untargeted Metabolomics for Autism Spectrum Disorders: Current Status and Future Directions. *Frontiers in psychiatry*. 2019;10:647.
37. Baieli S, Pavone L, Meli C, Fiumara A, Coleman M. Autism and phenylketonuria. *J Autism Dev Disord*. 2003;33(2):201-4.
38. Diaz-Stransky A, Tierney E. Cognitive and behavioral aspects of Smith-Lemli-Opitz syndrome. *American journal of medical genetics Part C, Seminars in medical genetics*. 2012;160c(4):295-300.
39. Panner Selvam MK, Baskaran S, Agarwal A. Chapter Six - Proteomics of reproduction: Prospects and perspectives. In: Makowski GS, editor. *Advances in Clinical Chemistry*. 92: Elsevier; 2019. p. 217-43.
40. Schwarz E, Guest PC, Rahmoune H, Wang L, Levin Y, Ingudomnukul E, et al. Sex-specific serum biomarker patterns in adults with Asperger's syndrome. *Molecular psychiatry*. 2011;16(12):1213-20.
41. Corbett BA, Kantor AB, Schulman H, Walker WL, Lit L, Ashwood P, et al. A proteomic study of serum from children with autism showing differential expression of apolipoproteins and complement proteins. *Molecular psychiatry*. 2007;12(3):292-306.
42. English JA, Lopez LM, O'Gorman A, Focking M, Hryniewiecka M, Scaife C, et al. Blood-Based Protein Changes in Childhood Are Associated With Increased Risk for Later Psychotic Disorder: Evidence From a Nested Case-Control Study of the ALSPAC Longitudinal Birth Cohort. *Schizophrenia bulletin*. 2018;44(2):297-306.
43. Presumey J, Bialas AR, Carroll MC. Complement System in Neural Synapse Elimination in Development and Disease. *Advances in immunology*. 2017;135:53-79.
44. Kanner L. Autistic disturbances of affective contact. *Acta paedopsychiatrica*. 1968;35(4):100-36.
45. Bleuler E. *Dementia praecox or the group of schizophrenias*. 1950.
46. Organization WH. ICD-11 revision 2019 [Available from: <https://icd.who.int/en>].
47. Lotter V. Epidemiology of autistic conditions in young children. *Social psychiatry*. 1967;1(4):163-73.
48. Wing JK, O'Connor N, Lotter V. Autistic conditions in early childhood: a survey in middlesex. *British medical journal*. 1967;3(5562):389-92.
49. Bell CC. DSM-IV: Diagnostic and Statistical Manual of Mental Disorders. *JAMA*. 1994;272(10):828-9.
50. Baio J, Wiggins L, Christensen DL, Maenner MJ, Daniels J, Warren Z, et al. Prevalence of Autism Spectrum Disorder Among Children Aged 8 Years - Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2014. *Morbidity and mortality weekly report Surveillance summaries (Washington, DC : 2002)*. 2018;67(6):1-23.
51. Lundstrom S, Reichenberg A, Anckarsater H, Lichtenstein P, Gillberg C. Autism phenotype versus registered diagnosis in Swedish children: prevalence trends over 10 years in general population samples. *BMJ (Clinical research ed)*. 2015;350:h1961.
52. Hansen SN, Schendel DE, Parner ET. Explaining the increase in the prevalence of autism spectrum disorders: the proportion attributable to changes in reporting practices. *JAMA Pediatr*. 2015;169(1):56-62.
53. Wing L, Potter D. The epidemiology of autistic spectrum disorders: is the prevalence rising? *Mental retardation and developmental disabilities research reviews*. 2002;8(3):151-61.
54. Fombonne E. Epidemiology of pervasive developmental disorders. *Pediatric research*. 2009;65(6):591-8.
55. Kogan MD, Vladutiu CJ, Schieve LA, Ghandour RM, Blumberg SJ, Zablotsky B, et al. The Prevalence of Parent-Reported Autism Spectrum Disorder Among US Children. *Pediatrics*. 2018;142(6).

56. Boilson AM, Staines A, Ramirez A, Posada M, Sweeney MR. Operationalisation of the European Protocol for Autism Prevalence (EPAP) for Autism Spectrum Disorder Prevalence Measurement in Ireland. *J Autism Dev Disord.* 2016;46(9):3054-67.
57. Wang F, Lu L, Wang SB, Zhang L, Ng CH, Ungvari GS, et al. The prevalence of autism spectrum disorders in China: a comprehensive meta-analysis. *International journal of biological sciences.* 2018;14(7):717-25.
58. Treffert DA. Epidemiology of infantile autism. *Arch Gen Psychiatry.* 1970;22(5):431-8.
59. Maenner MJ, Shaw KA, Bakian AV, Bilder DA, Durkin MS, Esler A, et al. Prevalence and Characteristics of Autism Spectrum Disorder Among Children Aged 8 Years - Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2018. *Morbidity and mortality weekly report Surveillance summaries (Washington, DC : 2002).* 2021;70(11):1-16.
60. Coo H, Ouellette-Kuntz H, Lloyd JE, Kasmara L, Holden JJ, Lewis ME. Trends in autism prevalence: diagnostic substitution revisited. *J Autism Dev Disord.* 2008;38(6):1036-46.
61. Kerr-Gaffney J, Halls D, Harrison A, Tchanturia K. Exploring Relationships Between Autism Spectrum Disorder Symptoms and Eating Disorder Symptoms in Adults With Anorexia Nervosa: A Network Approach. *Frontiers in psychiatry.* 2020;11.
62. Liptak GS, Benzoni LB, Mruzek DW, Nolan KW, Thingvoll MA, Wade CM, et al. Disparities in diagnosis and access to health services for children with autism: data from the National Survey of Children's Health. *Journal of developmental and behavioral pediatrics : JDBP.* 2008;29(3):152-60.
63. Zeidan J, Fombonne E, Scora J, Ibrahim A, Durkin MS, Saxena S, et al. Global prevalence of autism: A systematic review update. *Autism Research.* 2022;15(5):778-90.
64. Windham GC, Anderson MC, Croen LA, Smith KS, Collins J, Grether JK. Birth prevalence of autism spectrum disorders in the San Francisco Bay area by demographic and ascertainment source characteristics. *J Autism Dev Disord.* 2011;41(10):1362-72.
65. Lord C, Elsabbagh M, Baird G, Veenstra-Vanderweele J. Autism spectrum disorder. *Lancet (London, England).* 2018;392(10146):508-20.
66. Elsabbagh M, Divan G, Koh YJ, Kim YS, Kauchali S, Marcín C, et al. Global prevalence of autism and other pervasive developmental disorders. *Autism research : official journal of the International Society for Autism Research.* 2012;5(3):160-79.
67. Wang K, Gaitsch H, Poon H, Cox NJ, Rzhetsky A. Classification of common human diseases derived from shared genetic and environmental determinants. *Nature Genetics.* 2017;49(9):1319-25.
68. Tick B, Bolton P, Happé F, Rutter M, Rijdsdijk F. Heritability of autism spectrum disorders: a meta-analysis of twin studies. *Journal of child psychology and psychiatry, and allied disciplines.* 2016;57(5):585-95.
69. Werling DM, Geschwind DH. Recurrence rates provide evidence for sex-differential, familial genetic liability for autism spectrum disorders in multiplex families and twins. *Molecular autism.* 2015;6:27.
70. Palmer N, Beam A, Agniel D, Eran A, Manrai A, Spettell C, et al. Association of Sex With Recurrence of Autism Spectrum Disorder Among Siblings. *JAMA Pediatr.* 2017;171(11):1107-12.
71. Dennison CA, Legge SE, Pardiñas AF, Walters JTR. Genome-wide association studies in schizophrenia: Recent advances, challenges and future perspective. *Schizophrenia research.* 2020;217:4-12.
72. Anttila V, Bulik-Sullivan B, Finucane HK, Walters RK, Bras J, Duncan L, et al. Analysis of shared heritability in common disorders of the brain. *Science (New York, NY).* 2018;360(6395).

73. Havdahl KA, von Tetzchner S, Huerta M, Lord C, Bishop SL. Utility of the Child Behavior Checklist as a Screener for Autism Spectrum Disorder. *Autism research : official journal of the International Society for Autism Research*. 2016;9(1):33-42.
74. Grove J, Ripke S, Als TD, Mattheisen M, Walters RK, Won H, et al. Identification of common genetic risk variants for autism spectrum disorder. *Nat Genet*. 2019;51(3):431-44.
75. Satterstrom FK, Kosmicki JA, Wang J, Breen MS, De Rubeis S, An JY, et al. Large-Scale Exome Sequencing Study Implicates Both Developmental and Functional Changes in the Neurobiology of Autism. *Cell*. 2020;180(3):568-84.e23.
76. Fu JM, Satterstrom FK, Peng M, Brand H, Collins RL, Dong S, et al. Rare coding variation provides insight into the genetic architecture and phenotypic context of autism. *Nature Genetics*. 2022;54(9):1320-31.
77. Sanders SJ, He X, Willsey AJ, Ercan-Sencicek AG, Samocha KE, Cicek AE, et al. Insights into Autism Spectrum Disorder Genomic Architecture and Biology from 71 Risk Loci. *Neuron*. 2015;87(6):1215-33.
78. Sanders SJ, Murtha MT, Gupta AR, Murdoch JD, Raubeson MJ, Willsey AJ, et al. De novo mutations revealed by whole-exome sequencing are strongly associated with autism. *Nature*. 2012;485(7397):237-41.
79. Lim ET, Raychaudhuri S, Sanders SJ, Stevens C, Sabo A, MacArthur DG, et al. Rare complete knockouts in humans: population distribution and significant role in autism spectrum disorders. *Neuron*. 2013;77(2):235-42.
80. Kumar RA, KaraMohamed S, Sudi J, Conrad DF, Brune C, Badner JA, et al. Recurrent 16p11.2 microdeletions in autism. *Human Molecular Genetics*. 2007;17(4):628-38.
81. Gaugler T, Klei L, Sanders SJ, Bodea CA, Goldberg AP, Lee AB, et al. Most genetic risk for autism resides with common variation. *Nature genetics*. 2014;46(8):881-5.
82. Iossifov I, O'Roak BJ, Sanders SJ, Ronemus M, Krumm N, Levy D, et al. The contribution of de novo coding mutations to autism spectrum disorder. *Nature*. 2014;515(7526):216-21.
83. Lombardo MV, Moon HM, Su J, Palmer TD, Courchesne E, Pramparo T. Maternal immune activation dysregulation of the fetal brain transcriptome and relevance to the pathophysiology of autism spectrum disorder. *Molecular psychiatry*. 2018;23(4):1001-13.
84. Rees E, Creeth HDJ, Hwu H-G, Chen WJ, Tsuang M, Glatt SJ, et al. Schizophrenia, autism spectrum disorders and developmental disorders share specific disruptive coding mutations. *Nature Communications*. 2021;12(1):5353.
85. Bailey A, Luthert P, Bolton P, Le Couteur A, Rutter M, Harding B. Autism and megalencephaly. *Lancet (London, England)*. 1993;341(8854):1225-6.
86. Pirozzi F, Nelson B, Mirzaa G. From microcephaly to megalencephaly: determinants of brain size. *Dialogues in clinical neuroscience*. 2018;20(4):267-82.
87. Hampson DR, Blatt GJ. Autism spectrum disorders and neuropathology of the cerebellum. *Frontiers in neuroscience*. 2015;9:420.
88. Zengeler KE, Lukens JR. Innate immunity at the crossroads of healthy brain maturation and neurodevelopmental disorders. *Nature reviews Immunology*. 2021;21(7):454-68.
89. Juric-Sekhar G, Hevner RF. Malformations of Cerebral Cortex Development: Molecules and Mechanisms. *Annual review of pathology*. 2019;14:293-318.
90. Courchesne E, Pierce K, Schumann CM, Redcay E, Buckwalter JA, Kennedy DP, et al. Mapping early brain development in autism. *Neuron*. 2007;56(2):399-413.
91. Schumann CM, Bloss CS, Barnes CC, Wideman GM, Carper RA, Akshoomoff N, et al. Longitudinal magnetic resonance imaging study of cortical development through early childhood in autism. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2010;30(12):4419-27.

92. Lukito S, Norman L, Carlisi C, Radua J, Hart H, Simonoff E, et al. Comparative meta-analyses of brain structural and functional abnormalities during cognitive control in attention-deficit/hyperactivity disorder and autism spectrum disorder. *Psychological medicine*. 2020;50(6):894-919.
93. Weissberg O, Elliott E. The Mechanisms of CHD8 in Neurodevelopment and Autism Spectrum Disorders. *Genes*. 2021;12(8).
94. Earl RK, Turner TN, Mefford HC, Hudac CM, Gerdtts J, Eichler EE, et al. Clinical phenotype of ASD-associated DYRK1A haploinsufficiency. *Molecular autism*. 2017;8:54.
95. Courchesne E, Pramparo T, Gazestani VH, Lombardo MV, Pierce K, Lewis NE. The ASD Living Biology: from cell proliferation to clinical phenotype. *Molecular psychiatry*. 2019;24(1):88-107.
96. Skefos J, Cummings C, Enzer K, Holiday J, Weed K, Levy E, et al. Regional Alterations in Purkinje Cell Density in Patients with Autism. *PLOS ONE*. 2014;9(2):e81255.
97. Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial activation and neuroinflammation in the brain of patients with autism. *Annals of neurology*. 2005;57(1):67-81.
98. Voineagu I, Wang X, Johnston P, Lowe JK, Tian Y, Horvath S, et al. Transcriptomic analysis of autistic brain reveals convergent molecular pathology. *Nature*. 2011;474(7351):380-4.
99. Cornell J, Salinas S, Huang HY, Zhou M. Microglia regulation of synaptic plasticity and learning and memory. *Neural regeneration research*. 2022;17(4):705-16.
100. Dziabis JE, Bilbo SD. Microglia and Sensitive Periods in Brain Development. *Current topics in behavioral neurosciences*. 2022;53:55-78.
101. Dougherty CC, Evans DW, Myers SM, Moore GJ, Michael AM. A Comparison of Structural Brain Imaging Findings in Autism Spectrum Disorder and Attention-Deficit Hyperactivity Disorder. *Neuropsychology Review*. 2016;26(1):25-43.
102. Blackmon K. Structural MRI biomarkers of shared pathogenesis in autism spectrum disorder and epilepsy. *Epilepsy & behavior : E&B*. 2015;47:172-82.
103. Modabbernia A, Velthorst E, Reichenberg A. Environmental risk factors for autism: an evidence-based review of systematic reviews and meta-analyses. *Molecular autism*. 2017;8:13.
104. Lyall K, Croen L, Daniels J, Fallin MD, Ladd-Acosta C, Lee BK, et al. The Changing Epidemiology of Autism Spectrum Disorders. *Annual review of public health*. 2017;38:81-102.
105. Bölte S, Girdler S, Marschik PB. The contribution of environmental exposure to the etiology of autism spectrum disorder. *Cellular and molecular life sciences : CMLS*. 2019;76(7):1275-97.
106. Hviid A, Hansen JV, Frisch M, Melbye M. Measles, Mumps, Rubella Vaccination and Autism: A Nationwide Cohort Study. *Annals of internal medicine*. 2019;170(8):513-20.
107. Li Y. Modern epigenetics methods in biological research. *Methods (San Diego, Calif)*. 2021;187:104-13.
108. Panisi C, Guerini FR, Abruzzo PM, Balzola F, Biava PM, Bolotta A, et al. Autism Spectrum Disorder from the Womb to Adulthood: Suggestions for a Paradigm Shift. *Journal of personalized medicine*. 2021;11(2).
109. Lombardi LM, Baker SA, Zoghbi HY. MECP2 disorders: from the clinic to mice and back. *The Journal of clinical investigation*. 2015;125(8):2914-23.
110. Hagerman RJ, Berry-Kravis E, Hazlett HC, Bailey DB, Jr., Moine H, Kooy RF, et al. Fragile X syndrome. *Nature reviews Disease primers*. 2017;3:17065.
111. Tremblay MW, Jiang Y-h. DNA Methylation and Susceptibility to Autism Spectrum Disorder. *Annual Review of Medicine*. 2019;70(1):151-66.

112. Han VX, Patel S, Jones HF, Dale RC. Maternal immune activation and neuroinflammation in human neurodevelopmental disorders. *Nature Reviews Neurology*. 2021;17(9):564-79.
113. Conway F, Brown AS. Maternal Immune Activation and Related Factors in the Risk of Offspring Psychiatric Disorders. *Frontiers in psychiatry*. 2019;10.
114. Jonakait GM. The effects of maternal inflammation on neuronal development: possible mechanisms. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience*. 2007;25(7):415-25.
115. Müller N, Weidinger E, Leitner B, Schwarz MJ. The role of inflammation in schizophrenia. *Frontiers in neuroscience*. 2015;9(372).
116. Kim Y-K, Jung H-G, Myint A-M, Kim H, Park S-H. Imbalance between pro-inflammatory and anti-inflammatory cytokines in bipolar disorder. *Journal of Affective Disorders*. 2007;104(1):91-5.
117. Vogelzangs N, Beekman ATF, de Jonge P, Penninx BWJH. Anxiety disorders and inflammation in a large adult cohort. *Translational psychiatry*. 2013;3(4):e249-e.
118. Davoli-Ferreira M, Thomson CA, McCoy KD. Microbiota and Microglia Interactions in ASD. *Frontiers in Immunology*. 2021;12.
119. Masi A, Glozier N, Dale R, Guastella AJ. The Immune System, Cytokines, and Biomarkers in Autism Spectrum Disorder. *Neuroscience bulletin*. 2017;33(2):194-204.
120. Masi A, Quintana DS, Glozier N, Lloyd AR, Hickie IB, Guastella AJ. Cytokine aberrations in autism spectrum disorder: a systematic review and meta-analysis. *Molecular psychiatry*. 2015;20(4):440-6.
121. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah IN, Van de Water J. Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders. *Journal of neuroimmunology*. 2011;232(1-2):196-9.
122. Fernández de Cossío L, Guzmán A, van der Veldt S, Luheshi GN. Prenatal infection leads to ASD-like behavior and altered synaptic pruning in the mouse offspring. *Brain, Behavior, and Immunity*. 2017;63:88-98.
123. Bauman MD, Iosif AM, Smith SE, Bregere C, Amaral DG, Patterson PH. Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. *Biological psychiatry*. 2014;75(4):332-41.
124. Jiang HY, Xu LL, Shao L, Xia RM, Yu ZH, Ling ZX, et al. Maternal infection during pregnancy and risk of autism spectrum disorders: A systematic review and meta-analysis. *Brain Behav Immun*. 2016;58:165-72.
125. Chess S. Follow-up report on autism in congenital rubella. *Journal of autism and childhood schizophrenia*. 1977;7(1):69-81.
126. Atladóttir HO, Thorsen P, Ostergaard L, Schendel DE, Lemcke S, Abdallah M, et al. Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. *J Autism Dev Disord*. 2010;40(12):1423-30.
127. Jash S, Sharma S. Pathogenic Infections during Pregnancy and the Consequences for Fetal Brain Development. *Pathogens*. 2022;11(2):193.
128. DeLong GR, Bean SC, Brown FR, 3rd. Acquired reversible autistic syndrome in acute encephalopathic illness in children. *Archives of neurology*. 1981;38(3):191-4.
129. Atladóttir HÓ, Henriksen TB, Schendel DE, Parner ET. Autism After Infection, Febrile Episodes, and Antibiotic Use During Pregnancy: An Exploratory Study. *Pediatrics*. 2012;130(6):e1447.
130. Zerbo O, Iosif AM, Walker C, Ozonoff S, Hansen RL, Hertz-Picciotto I. Is maternal influenza or fever during pregnancy associated with autism or developmental delays? Results from the CHARGE (CHildhood Autism Risks from Genetics and Environment) study. *J Autism Dev Disord*. 2013;43(1):25-33.

131. Han VX, Patel S, Jones HF, Nielsen TC, Mohammad SS, Hofer MJ, et al. Maternal acute and chronic inflammation in pregnancy is associated with common neurodevelopmental disorders: a systematic review. *Translational psychiatry*. 2021;11(1):71.
132. Ferreira LMR, Meissner TB, Tilburgs T, Strominger JL. HLA-G: At the Interface of Maternal–Fetal Tolerance. *Trends in Immunology*. 2017;38(4):272-86.
133. Guerini FR, Bolognesi E, Chiappedi M, Ripamonti E, Ghezzi A, Zanette M, et al. HLA-G coding region polymorphism is skewed in autistic spectrum disorders. *Brain Behav Immun*. 2018;67:308-13.
134. Buyon JP, Kim MY, Guerra MM, Laskin CA, Petri M, Lockshin MD, et al. Predictors of Pregnancy Outcomes in Patients With Lupus: A Cohort Study. *Annals of internal medicine*. 2015;163(3):153-63.
135. Léger J, Delcour C, Carel JC. Fetal and Neonatal Thyroid Dysfunction. *The Journal of clinical endocrinology and metabolism*. 2022;107(3):836-46.
136. Martin LA, Ashwood P, Braunschweig D, Cabanlit M, Van de Water J, Amaral DG. Stereotypies and hyperactivity in rhesus monkeys exposed to IgG from mothers of children with autism. *Brain Behav Immun*. 2008;22(6):806-16.
137. Bauman MD, Iosif AM, Ashwood P, Braunschweig D, Lee A, Schumann CM, et al. Maternal antibodies from mothers of children with autism alter brain growth and social behavior development in the rhesus monkey. *Translational psychiatry*. 2013;3(7):e278.
138. Gata-Garcia A, Diamond B. Maternal Antibody and ASD: Clinical Data and Animal Models. *Frontiers in Immunology*. 2019;10.
139. Jones KL, Van de Water J. Maternal autoantibody related autism: mechanisms and pathways. *Molecular psychiatry*. 2019;24(2):252-65.
140. Matelski L, Van de Water J. Risk factors in autism: Thinking outside the brain. *Journal of autoimmunity*. 2016;67:1-7.
141. Fu B, Tian Z, Wei H. TH17 cells in human recurrent pregnancy loss and pre-eclampsia. *Cellular & molecular immunology*. 2014;11(6):564-70.
142. Wong H, Hoeffler C. Maternal IL-17A in autism. *Experimental neurology*. 2018;299(Pt A):228-40.
143. Yockey LJ, Iwasaki A. Interferons and Proinflammatory Cytokines in Pregnancy and Fetal Development. *Immunity*. 2018;49(3):397-412.
144. Thaxton JE, Nevers T, Lippe EO, Blois SM, Saito S, Sharma S. NKG2D blockade inhibits poly(I:C)-triggered fetal loss in wild type but not in IL-10^{-/-} mice. *Journal of immunology (Baltimore, Md : 1950)*. 2013;190(7):3639-47.
145. Curran EA, O'Keeffe GW, Looney AM, Moloney G, Hegarty SV, Murray DM, et al. Exposure to Hypertensive Disorders of Pregnancy Increases the Risk of Autism Spectrum Disorder in Affected Offspring. *Molecular neurobiology*. 2018;55(7):5557-64.
146. Knuesel I, Chicha L, Britschgi M, Schobel SA, Bodmer M, Hellings JA, et al. Maternal immune activation and abnormal brain development across CNS disorders. *Nature reviews Neurology*. 2014;10(11):643-60.
147. Yang X, Li M, Haghiac M, Catalano PM, O'Tierney-Ginn P, Hauguel-de Mouzon S. Causal relationship between obesity-related traits and TLR4-driven responses at the maternal-fetal interface. *Diabetologia*. 2016;59(11):2459-66.
148. Boulanger-Bertolus J, Pancaro C, Mashour GA. Increasing Role of Maternal Immune Activation in Neurodevelopmental Disorders. *Frontiers in Behavioral Neuroscience*. 2018;12(230).
149. Minakova E, Warner BB. Maternal immune activation, central nervous system development and behavioral phenotypes. *Birth Defects Research*. 2018;110(20):1539-50.

150. Garay PA, Hsiao EY, Patterson PH, McAllister AK. Maternal immune activation causes age- and region-specific changes in brain cytokines in offspring throughout development. *Brain Behav Immun*. 2013;31:54-68.
151. Smith SE, Li J, Garbett K, Mirnics K, Patterson PH. Maternal immune activation alters fetal brain development through interleukin-6. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2007;27(40):10695-702.
152. Smith SE, Elliott RM, Anderson MP. Maternal immune activation increases neonatal mouse cortex thickness and cell density. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*. 2012;7(3):529-32.
153. Le Belle JE, Sperry J, Ngo A, Ghochani Y, Laks DR, López-Aranda M, et al. Maternal inflammation contributes to brain overgrowth and autism-associated behaviors through altered redox signaling in stem and progenitor cells. *Stem cell reports*. 2014;3(5):725-34.
154. Broek JA, Brombacher E, Stelzhammer V, Guest PC, Rahmoune H, Bahn S. The need for a comprehensive molecular characterization of autism spectrum disorders. *The international journal of neuropsychopharmacology*. 2014;17(4):651-73.
155. Gunes S, Ekinci O, Celik T. Iron deficiency parameters in autism spectrum disorder: clinical correlates and associated factors. *Italian journal of pediatrics*. 2017;43(1):86.
156. Suzuki K, Matsuzaki H, Iwata K, Kameno Y, Shimmura C, Kawai S, et al. Plasma cytokine profiles in subjects with high-functioning autism spectrum disorders. *PLoS One*. 2011;6(5):e20470.
157. Morato Torres CA, Wassouf Z, Zafar F, Sastre D, Outeiro TF, Schüle B. The Role of Alpha-Synuclein and Other Parkinson's Genes in Neurodevelopmental and Neurodegenerative Disorders. *International journal of molecular sciences*. 2020;21(16).
158. Zou M, Li D, Wang L, Li L, Xie S, Liu Y, et al. Identification of Amino Acid Dysregulation as a Potential Biomarker for Autism Spectrum Disorder in China. *Neurotoxicity research*. 2020.
159. Borish LC, Steinke JW. 2. Cytokines and chemokines. *Journal of Allergy and Clinical Immunology*. 2003;111(2, Supplement 2):S460-S75.
160. Estes ML, McAllister AK. IMMUNOLOGY. Maternal TH17 cells take a toll on baby's brain. *Science (New York, NY)*. 2016;351(6276):919-20.
161. Smith SEP, Li J, Garbett K, Mirnics K, Patterson PH. Maternal Immune Activation Alters Fetal Brain Development through Interleukin-6. *The Journal of Neuroscience*. 2007;27(40):10695-702.
162. Vezzani A, Viviani B. Neuromodulatory properties of inflammatory cytokines and their impact on neuronal excitability. *Neuropharmacology*. 2015;96(Pt A):70-82.
163. Goines P, Van de Water J. The immune system's role in the biology of autism. *Current opinion in neurology*. 2010;23(2):111-7.
164. Blauvelt A. Ixekizumab: a new anti-IL-17A monoclonal antibody therapy for moderate-to severe plaque psoriasis. *Expert opinion on biological therapy*. 2016;16(2):255-63.
165. Wendling D, Racadot E, Wijdenes J. Treatment of severe rheumatoid arthritis by anti-interleukin 6 monoclonal antibody. *The Journal of rheumatology*. 1993;20(2):259-62.
166. Fatemi SH, Pearce DA, Brooks AI, Sidwell RW. Prenatal viral infection in mouse causes differential expression of genes in brains of mouse progeny: a potential animal model for schizophrenia and autism. *Synapse (New York, NY)*. 2005;57(2):91-9.
167. Chua JSC, Cowley CJ, Manavis J, Rofe AM, Coyle P. Prenatal exposure to lipopolysaccharide results in neurodevelopmental damage that is ameliorated by zinc in mice. *Brain, Behavior, and Immunity*. 2012;26(2):326-36.
168. Meyer U, Feldon J. To poly(I:C) or not to poly(I:C): advancing preclinical schizophrenia research through the use of prenatal immune activation models. *Neuropharmacology*. 2012;62(3):1308-21.

169. Parker-Athill EC, Tan J. Maternal immune activation and autism spectrum disorder: interleukin-6 signaling as a key mechanistic pathway. *Neuro-Signals*. 2010;18(2):113-28.
170. Knuesel I, Chicha L, Britschgi M, Schobel SA, Bodmer M, Hellings JA, et al. Maternal immune activation and abnormal brain development across CNS disorders. *Nature Reviews Neurology*. 2014;10(11):643-60.
171. Bilbo SD, Block CL, Bolton JL, Hanamsagar R, Tran PK. Beyond infection - Maternal immune activation by environmental factors, microglial development, and relevance for autism spectrum disorders. *Experimental neurology*. 2018;299(Pt A):241-51.
172. Money KM, Barke TL, Serezani A, Gannon M, Garbett KA, Aronoff DM, et al. Gestational diabetes exacerbates maternal immune activation effects in the developing brain. *Molecular psychiatry*. 2018;23(9):1920-8.
173. Careaga M, Taylor SL, Chang C, Chiang A, Ku KM, Berman RF, et al. Variability in PolyIC induced immune response: Implications for preclinical maternal immune activation models. *Journal of neuroimmunology*. 2018;323:87-93.
174. Rahimian R, Belliveau C, Chen R, Mechawar N. Microglial Inflammatory-Metabolic Pathways and Their Potential Therapeutic Implication in Major Depressive Disorder. *Frontiers in psychiatry*. 2022;13:871997.
175. Afkham A, Eghbal-Fard S, Heydarlou H, Azizi R, Aghebati-Maleki L, Yousefi M. Toll-like receptors signaling network in pre-eclampsia: An updated review. *Journal of cellular physiology*. 2019;234(3):2229-40.
176. Farrugia M, Baron B. The Role of Toll-Like Receptors in Autoimmune Diseases through Failure of the Self-Recognition Mechanism. *International journal of inflammation*. 2017;2017:8391230.
177. Qu X, Yu X, Liu J, Wang J, Liu J. Pro-Inflammatory Cytokines are Elevated in Pregnant Women with Systemic Lupus Erythematosus in Association with the Activation of TLR4. *Clinical laboratory*. 2016;62(4):535-44.
178. Dinarello CA. Historical insights into cytokines. *European journal of immunology*. 2007;37 Suppl 1(Suppl 1):S34-45.
179. Zhang JM, An J. Cytokines, inflammation, and pain. *International anesthesiology clinics*. 2007;45(2):27-37.
180. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et biophysica acta*. 2014;1843(11):2563-82.
181. Irwin JL, Yeates AJ, Mulhern MS, McSorley EM, Strain JJ, Watson GE, et al. Maternal Gestational Immune Response and Autism Spectrum Disorder Phenotypes at 7 Years of Age in the Seychelles Child Development Study. *Molecular neurobiology*. 2019;56(7):5000-8.
182. Abdallah MW, Larsen N, Grove J, Nørgaard-Pedersen B, Thorsen P, Mortensen EL, et al. Amniotic fluid inflammatory cytokines: potential markers of immunologic dysfunction in autism spectrum disorders. *The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry*. 2013;14(7):528-38.
183. Abdallah MW, Pearce BD, Larsen N, Greaves-Lord K, Nørgaard-Pedersen B, Hougaard DM, et al. Amniotic fluid MMP-9 and neurotrophins in autism spectrum disorders: an exploratory study. *Autism research : official journal of the International Society for Autism Research*. 2012;5(6):428-33.
184. Nørgaard-Pedersen B, Hougaard DM. Storage policies and use of the Danish Newborn Screening Biobank. *Journal of Inherited Metabolic Disease*. 2007;30(4):530-6.
185. de Jager W, Bourcier K, Rijkers GT, Prakken BJ, Seyfert-Margolis V. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. *BMC Immunology*. 2009;10(1):52.
186. Weichhart T, Hengstschläger M, Linke M. Regulation of innate immune cell function by mTOR. *Nature Reviews Immunology*. 2015;15(10):599-614.

187. Varghese M, Keshav N, Jacot-Descombes S, Warda T, Wicinski B, Dickstein DL, et al. Autism spectrum disorder: neuropathology and animal models. *Acta Neuropathol.* 2017;134(4):537-66.
188. Petrasek T, Vojtechova I, Klovrc O, Tuckova K, Vejmla C, Rak J, et al. mTOR inhibitor improves autistic-like behaviors related to Tsc2 haploinsufficiency but not following developmental status epilepticus. *J Neurodev Disord.* 2021;13(1):14-.
189. Johnson T, Saatci D, Handunnetthi L. Maternal immune activation induces methylation changes in schizophrenia genes. *PLoS One.* 2022;17(11):e0278155.
190. Banik A, Kandilya D, Ramya S, Stünkel W, Chong YS, Dheen ST. Maternal Factors that Induce Epigenetic Changes Contribute to Neurological Disorders in Offspring. *Genes.* 2017;8(6).
191. Baron-Cohen S, Auyeung B, Nørgaard-Pedersen B, Hougaard DM, Abdallah MW, Melgaard L, et al. Elevated fetal steroidogenic activity in autism. *Molecular psychiatry.* 2015;20(3):369-76.
192. Baron-Cohen S. The extreme male brain theory of autism. *Trends in cognitive sciences.* 2002;6(6):248-54.
193. Auyeung B, Lombardo MV, Baron-Cohen S. Prenatal and postnatal hormone effects on the human brain and cognition. *Pflugers Archiv : European journal of physiology.* 2013;465(5):557-71.
194. Lombardo MV, Ashwin E, Auyeung B, Chakrabarti B, Lai MC, Taylor K, et al. Fetal programming effects of testosterone on the reward system and behavioral approach tendencies in humans. *Biological psychiatry.* 2012;72(10):839-47.
195. Chakrabarti B, Dudbridge F, Kent L, Wheelwright S, Hill-Cawthorne G, Allison C, et al. Genes related to sex steroids, neural growth, and social-emotional behavior are associated with autistic traits, empathy, and Asperger syndrome. *Autism research : official journal of the International Society for Autism Research.* 2009;2(3):157-77.
196. Azziz R, Carmina E, Chen Z, Dunaif A, Laven JS, Legro RS, et al. Polycystic ovary syndrome. *Nature reviews Disease primers.* 2016;2:16057.
197. Cesta CE, Månsson M, Palm C, Lichtenstein P, Iliadou AN, Landén M. Polycystic ovary syndrome and psychiatric disorders: Co-morbidity and heritability in a nationwide Swedish cohort. *Psychoneuroendocrinology.* 2016;73:196-203.
198. Lee BK, Arver S, Widman L, Gardner RM, Magnusson C, Dalman C, et al. Maternal hirsutism and autism spectrum disorders in offspring. *Autism research : official journal of the International Society for Autism Research.* 2017;10(9):1544-6.
199. Gasser BA, Buerki SF, Kurz J, Mohaupt MG. Hyperandrogenism? Increased 17, 20-Lyase Activity? A Metanalysis and Systematic Review of Altered Androgens in Boys and Girls with Autism. *International journal of molecular sciences.* 2021;22(22).
200. Ruta L, Ingudomnukul E, Taylor K, Chakrabarti B, Baron-Cohen S. Increased serum androstenedione in adults with autism spectrum conditions. *Psychoneuroendocrinology.* 2011;36(8):1154-63.
201. Broek JA, Guest PC, Rahmoune H, Bahn S. Proteomic analysis of post mortem brain tissue from autism patients: evidence for opposite changes in prefrontal cortex and cerebellum in synaptic connectivity-related proteins. *Molecular autism.* 2014;5:41.
202. Steeb H, Ramsey JM, Guest PC, Stocki P, Cooper JD, Rahmoune H, et al. Serum proteomic analysis identifies sex-specific differences in lipid metabolism and inflammation profiles in adults diagnosed with Asperger syndrome. *Molecular autism.* 2014;5(1):4.
203. Hayashi-Takagi A, Vawter MP, Iwamoto K. Peripheral biomarkers revisited: integrative profiling of peripheral samples for psychiatric research. *Biological psychiatry.* 2014;75(12):920-8.
204. Ristori MV, Mortera SL, Marzano V, Guerrera S, Vernocchi P, Ianiro G, et al. Proteomics and Metabolomics Approaches towards a Functional Insight onto AUTISM

- Spectrum Disorders: Phenotype Stratification and Biomarker Discovery. *International journal of molecular sciences*. 2020;21(17).
205. Green RM, Travers AM, Howe Y, McDougle CJ. Women and Autism Spectrum Disorder: Diagnosis and Implications for Treatment of Adolescents and Adults. *Current psychiatry reports*. 2019;21(4):22.
206. Lord C, Rutter M, DiLavore P, Risi S, Gotham K, Bishop S. Autism diagnostic observation schedule—2nd edition (ADOS-2). Los Angeles, CA: Western Psychological Corporation. 2012;284.
207. Wing L, Leekam SR, Libby SJ, Gould J, Locombe M. The Diagnostic Interview for Social and Communication Disorders: background, inter-rater reliability and clinical use. *Journal of child psychology and psychiatry, and allied disciplines*. 2002;43(3):307-25.
208. Pierce K, Gazestani VH, Bacon E, Barnes CC, Cha D, Nalabolu S, et al. Evaluation of the Diagnostic Stability of the Early Autism Spectrum Disorder Phenotype in the General Population Starting at 12 Months. *JAMA Pediatr*. 2019;173(6):578-87.
209. Georgiades S, Bishop SL, Frazier T. Editorial Perspective: Longitudinal research in autism – introducing the concept of ‘chronogeneity’. *Journal of Child Psychology and Psychiatry*. 2017;58(5):634-6.
210. Lai M-C, Lombardo MV, Baron-Cohen S. Autism. *The Lancet*. 2014;383(9920):896-910.
211. Segev A, Weisskopf MG, Levine H, Pinto O, Raz R. Incidence time trends and socioeconomic factors in the observed incidence of autism spectrum disorder in israel: A nationwide nested case–control study. *Autism Research*. 2019;12(12):1870-9.
212. Iadarola S, Hetherington S, Clinton C, Dean M, Reisinger E, Huynh L, et al. Services for children with autism spectrum disorder in three, large urban school districts: Perspectives of parents and educators. *Autism : the international journal of research and practice*. 2015;19(6):694-703.
213. Bishop-Fitzpatrick L, Kind AJH. A Scoping Review of Health Disparities in Autism Spectrum Disorder. *J Autism Dev Disord*. 2017;47(11):3380-91.
214. Paterson SJ, Wolff JJ, Elison JT, Winder-Patel B, Zwaigenbaum L, Estes A, et al. The Importance of Temperament for Understanding Early Manifestations of Autism Spectrum Disorder in High-Risk Infants. *J Autism Dev Disord*. 2019;49(7):2849-63.
215. Tanner A, Dounavi K. The Emergence of Autism Symptoms Prior to 18 Months of Age: A Systematic Literature Review. *J Autism Dev Disord*. 2021;51(3):973-93.
216. Zwaigenbaum L, Bauman ML, Fein D, Pierce K, Buie T, Davis PA, et al. Early Screening of Autism Spectrum Disorder: Recommendations for Practice and Research. *Pediatrics*. 2015;136(Supplement_1):S41-S59.
217. Eilenberg JS, Kizildag D, Blakey AO, Cardona ND, Oberoi A, Broder-Fingert S, et al. Implications of Universal Autism Screening: Perspectives From Culturally Diverse Families With False-Positive Screens. *Academic pediatrics*. 2022;22(2):279-88.
218. Carter M, Casey S, O'Keeffe GW, Gibson L, Gallagher L, Murray DM. Maternal Immune Activation and Interleukin 17A in the Pathogenesis of Autistic Spectrum Disorder and Why It Matters in the COVID-19 Era. *Frontiers in psychiatry*. 2022;13:823096.
219. Baraskewich J, von Ranson KM, McCrimmon A, McMorris CA. Feeding and eating problems in children and adolescents with autism: A scoping review. *Autism : the international journal of research and practice*. 2021;25(6):1505-19.
220. MacDonald M, Lord C, Ulrich DA. Motor skills and calibrated autism severity in young children with autism spectrum disorder. *Adapted physical activity quarterly : APAQ*. 2014;31(2):95-105.
221. Chen H, Yang T, Chen J, Chen L, Dai Y, Zhang J, et al. Sleep problems in children with autism spectrum disorder: a multicenter survey. *BMC psychiatry*. 2021;21(1):406.

222. Lukmanji S, Manji SA, Kadhim S, Sauro KM, Wirrell EC, Kwon CS, et al. The co-occurrence of epilepsy and autism: A systematic review. *Epilepsy & behavior : E&B*. 2019;98(Pt A):238-48.
223. Boltri M, Sapuppo W. Anorexia Nervosa and Autism Spectrum Disorder: A Systematic Review. *Psychiatry research*. 2021;306:114271.
224. Moore K, Bullard A, Sweetman G, Ahearn WH. Assessing and Treating Anxiety in Individuals with Autism. *Behavior modification*. 2022;46(6):1279-313.
225. Dziuk MA, Gidley Larson JC, Apostu A, Mahone EM, Denckla MB, Mostofsky SH. Dyspraxia in autism: association with motor, social, and communicative deficits. *Developmental medicine and child neurology*. 2007;49(10):734-9.
226. Hoffmann W, Weber L, König U, Becker K, Kamp-Becker I. The role of the CBCL in the assessment of autism spectrum disorders: An evaluation of symptom profiles and screening characteristics. *Research in Autism Spectrum Disorders*. 2016;27:44-53.
227. Mattila ML, Hurtig T, Haapsamo H, Jussila K, Kuusikko-Gauffin S, Kielinen M, et al. Comorbid psychiatric disorders associated with Asperger syndrome/high-functioning autism: a community- and clinic-based study. *J Autism Dev Disord*. 2010;40(9):1080-93.
228. Bölte S, Dickhut H, Poustka F. Patterns of parent-reported problems indicative in autism. *Psychopathology*. 1999;32(2):93-7.
229. Ooi YP, Rescorla L, Ang RP, Woo B, Fung DSS. Identification of Autism Spectrum Disorders Using the Child Behavior Checklist in Singapore. *Journal of Autism and Developmental Disorders*. 2011;41(9):1147-56.
230. Duarte CS, Bordin IA, de Oliveira A, Bird H. The CBCL and the identification of children with autism and related conditions in Brazil: pilot findings. *J Autism Dev Disord*. 2003;33(6):703-7.
231. Brereton AV, Tonge BJ, Einfeld SL. Psychopathology in Children and Adolescents with Autism Compared to Young People with Intellectual Disability. *Journal of Autism and Developmental Disorders*. 2006;36(7):863-70.
232. Lecavalier L, Leone S, Wiltz J. The impact of behaviour problems on caregiver stress in young people with autism spectrum disorders. *Journal of Intellectual Disability Research*. 2006;50(3):172-83.
233. Pilling S, Baron-Cohen S, Megnin-Viggars O, Lee R, Taylor C. Recognition, referral, diagnosis, and management of adults with autism: summary of NICE guidance. *BMJ (Clinical research ed)*. 2012;344.
234. Green J. Intervention during the prodromal stages of autism spectrum disorders. *Autism Spectrum Disorder in the First Years of Life: Research, Assessment and Treatment: Guilford Press; 2020. p. 247-75.*
235. Whitehouse AJO. Elizabeth Usher Memorial Lecture: Rethinking the clinical pathway for autism spectrum disorder and challenging the status quo. *International journal of speech-language pathology*. 2017;19(3):208-17.
236. Parr J. Autism. *BMJ clinical evidence*. 2010;2010.
237. Dawson G. Early intensive behavioral intervention appears beneficial for young children with autism spectrum disorders. *The Journal of pediatrics*. 2013;162(5):1080-1.
238. Nevill RE, Lecavalier L, Stratis EA. Meta-analysis of parent-mediated interventions for young children with autism spectrum disorder. *Autism : the international journal of research and practice*. 2018;22(2):84-98.
239. Green J, Pickles A, Pasco G, Bedford R, Wan MW, Elsabbagh M, et al. Randomised trial of a parent-mediated intervention for infants at high risk for autism: longitudinal outcomes to age 3 years. *Journal of child psychology and psychiatry, and allied disciplines*. 2017;58(12):1330-40.

240. Maglione MA, Gans D, Das L, Timbie J, Kasari C, Technical Expert Panel, et al. Nonmedical interventions for children with ASD: Recommended guidelines and further research needs. *Pediatrics*. 2012;130(Supplement_2):S169-S78.
241. White SW, Simmons GL, Gotham KO, Conner CM, Smith IC, Beck KB, et al. Psychosocial Treatments Targeting Anxiety and Depression in Adolescents and Adults on the Autism Spectrum: Review of the Latest Research and Recommended Future Directions. *Current psychiatry reports*. 2018;20(10):82.
242. Wood JJ, Kendall PC, Wood KS, Kerns CM, Seltzer M, Small BJ, et al. Cognitive Behavioral Treatments for Anxiety in Children With Autism Spectrum Disorder: A Randomized Clinical Trial. *JAMA psychiatry*. 2020;77(5):474-83.
243. Estes A, Munson J, Rogers SJ, Greenson J, Winter J, Dawson G. Long-Term Outcomes of Early Intervention in 6-Year-Old Children With Autism Spectrum Disorder. *Journal of the American Academy of Child and Adolescent Psychiatry*. 2015;54(7):580-7.
244. Landa RJ. Diagnosis of autism spectrum disorders in the first 3 years of life. *Nature clinical practice Neurology*. 2008;4(3):138-47.
245. Rogers SJ, Estes A, Lord C, Vismara L, Winter J, Fitzpatrick A, et al. Effects of a brief Early Start Denver model (ESDM)-based parent intervention on toddlers at risk for autism spectrum disorders: a randomized controlled trial. *Journal of the American Academy of Child and Adolescent Psychiatry*. 2012;51(10):1052-65.
246. Chess S. Autism in children with congenital rubella. *Journal of autism and childhood schizophrenia*. 1971;1(1):33-47.
247. Xuan ICY, Hampson DR. Gender-Dependent Effects of Maternal Immune Activation on the Behavior of Mouse Offspring. *PLOS ONE*. 2014;9(8):e104433.
248. Haddad FL, Patel SV, Schmid S. Maternal Immune Activation by Poly I:C as a preclinical Model for Neurodevelopmental Disorders: A focus on Autism and Schizophrenia. *Neuroscience and biobehavioral reviews*. 2020;113:546-67.
249. Casanova MF, El-Baz AS, Kamat SS, Dombroski BA, Khalifa F, Elnakib A, et al. Focal cortical dysplasias in autism spectrum disorders. *Acta Neuropathologica Communications*. 2013;1(1):67.
250. Varghese M, Keshav N, Jacot-Descombes S, Warda T, Wicinski B, Dickstein DL, et al. Autism spectrum disorder: neuropathology and animal models. *Acta neuropathologica*. 2017;134(4):537-66.
251. Kugelberg E. Neuroimmunology: IL-17A mediates a path to autism. *Nature reviews Immunology*. 2016;16(4):205.
252. Chang YC, Cole TB, Costa LG. Behavioral Phenotyping for Autism Spectrum Disorders in Mice. *Current protocols in toxicology*. 2017;72:11.22.1-11.22.1.
253. Hornig M, Bresnahan MA, Che X, Schultz AF, Ukaigwe JE, Eddy ML, et al. Prenatal fever and autism risk. *Molecular psychiatry*. 2018;23(3):759-66.
254. Mueller FS, Polesel M, Richetto J, Meyer U, Weber-Stadlbauer U. Mouse models of maternal immune activation: Mind your caging system! *Brain, Behavior, and Immunity*. 2018;73:643-60.
255. Li J, Robinson M, Malacova E, Jacoby P, Foster J, van Eekelen A. Maternal life stress events in pregnancy link to children's school achievement at age 10 years. *The Journal of pediatrics*. 2013;162(3):483-9.
256. Luan W, Hammond LA, Vuillermot S, Meyer U, Eyles DW. Maternal Vitamin D Prevents Abnormal Dopaminergic Development and Function in a Mouse Model of Prenatal Immune Activation. *Scientific Reports*. 2018;8(1):9741.
257. Rovira N, Alarcon A, Iriando M, Ibañez M, Poo P, Cusi V, et al. Impact of histological chorioamnionitis, funisitis and clinical chorioamnionitis on neurodevelopmental outcome of preterm infants. *Early human development*. 2011;87(4):253-7.

258. Lee I, Neil JJ, Huettner PC, Smyser CD, Rogers CE, Shimony JS, et al. The impact of prenatal and neonatal infection on neurodevelopmental outcomes in very preterm infants. *Journal of Perinatology*. 2014;34(10):741-7.
259. Shobokshi A, Shaarawy M. Maternal serum and amniotic fluid cytokines in patients with preterm premature rupture of membranes with and without intrauterine infection. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics*. 2002;79(3):209-15.
260. Rounioja S, Räsänen J, Glumoff V, Ojaniemi M, Mäkikallio K, Hallman M. Intra-amniotic lipopolysaccharide leads to fetal cardiac dysfunction. A mouse model for fetal inflammatory response. *Cardiovascular research*. 2003;60(1):156-64.
261. Ricci S, Businaro R, Ippoliti F, Lo Vasco VR, Massoni F, Onofri E, et al. Altered cytokine and BDNF levels in autism spectrum disorder. *Neurotoxicity research*. 2013;24(4):491-501.
262. Chez MG, Dowling T, Patel PB, Khanna P, Kominsky M. Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children. *Pediatric neurology*. 2007;36(6):361-5.
263. Eftekharian MM, Ghafouri-Fard S, Noroozi R, Omrani MD, Arsang-Jang S, Ganji M, et al. Cytokine profile in autistic patients. *Cytokine*. 2018;108:120-6.
264. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah I, Van de Water J. Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. *Brain Behav Immun*. 2011;25(1):40-5.
265. Kordulewska NK, Kostyra E, Piskorz-Ogórek K, Moszyńska M, Cieślińska A, Fiedorowicz E, et al. Serum cytokine levels in children with spectrum autism disorder: Differences in pro- and anti-inflammatory balance. *Journal of neuroimmunology*. 2019;337:577066.
266. Heuer LS, Croen LA, Jones KL, Yoshida CK, Hansen RL, Yolken R, et al. An Exploratory Examination of Neonatal Cytokines and Chemokines as Predictors of Autism Risk: The Early Markers for Autism Study. *Biological psychiatry*. 2019;86(4):255-64.
267. Kutuk MO, Tufan E, Gokcen C, Kilicaslan F, Karadag M, Mutluer T, et al. Cytokine expression profiles in Autism spectrum disorder: A multi-center study from Turkey. *Cytokine*. 2020;133:155152.
268. Nishimoto N, Kishimoto T. Interleukin 6: from bench to bedside. *Nature clinical practice Rheumatology*. 2006;2(11):619-26.
269. Wei H, Zou H, Sheikh AM, Malik M, Dobkin C, Brown WT, et al. IL-6 is increased in the cerebellum of autistic brain and alters neural cell adhesion, migration and synaptic formation. *Journal of neuroinflammation*. 2011;8:52.
270. Murphy SP, Tayade C, Ashkar AA, Hatta K, Zhang J, Croy BA. Interferon gamma in successful pregnancies. *Biology of reproduction*. 2009;80(5):848-59.
271. Moaaz M, Youssry S, Elfatry A, El Rahman MA. Th17/Treg cells imbalance and their related cytokines (IL-17, IL-10 and TGF- β) in children with autism spectrum disorder. *Journal of neuroimmunology*. 2019;337:577071.
272. Ahmad SF, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, Alasmari AF, et al. Involvement of CD45 cells in the development of autism spectrum disorder through dysregulation of granulocyte-macrophage colony-stimulating factor, key inflammatory cytokines, and transcription factors. *International immunopharmacology*. 2020;83:106466.
273. Casey S, Carter M, Looney AM, Livingstone V, Moloney G, O'Keeffe GW, et al. Maternal Mid-Gestation Cytokine Dysregulation in Mothers of Children with Autism Spectrum Disorder. *Journal of Autism and Developmental Disorders*. 2021.

274. van der Zwaag B, Franke L, Poot M, Hochstenbach R, Spierenburg HA, Vorstman JA, et al. Gene-network analysis identifies susceptibility genes related to glycobiochemistry in autism. *PLoS One*. 2009;4(5):e5324.
275. Chehimi M, Vidal H, Eljaafari A. Pathogenic Role of IL-17-Producing Immune Cells in Obesity, and Related Inflammatory Diseases. *Journal of clinical medicine*. 2017;6(7).
276. Hill AP, Zuckerman KE, Fombonne E. Obesity and Autism. *Pediatrics*. 2015;136(6):1051-61.
277. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic acids research*. 2019;47(D1):D607-D13.
278. Kimura A, Kishimoto T. IL-6: Regulator of Treg/Th17 balance. *European journal of immunology*. 2010;40(7):1830-5.
279. Deverman BE, Patterson PH. Cytokines and CNS development. *Neuron*. 2009;64(1):61-78.
280. Wu WL, Hsiao EY, Yan Z, Mazmanian SK, Patterson PH. The placental interleukin-6 signaling controls fetal brain development and behavior. *Brain Behav Immun*. 2017;62:11-23.
281. Wright JF, Bennett F, Li B, Brooks J, Luxenberg DP, Whitters MJ, et al. The Human IL-17F/IL-17A Heterodimeric Cytokine Signals through the IL-17RA/IL-17RC Receptor Complex. *The Journal of Immunology*. 2008;181(4):2799.
282. Yang XO, Chang SH, Park H, Nurieva R, Shah B, Acero L, et al. Regulation of inflammatory responses by IL-17F. *Journal of Experimental Medicine*. 2008;205(5):1063-75.
283. Nadeem A, Ahmad SF, Attia SM, Bakheet SA, Al-Harbi NO, Al-Ayadhi LY. Activation of IL-17 receptor leads to increased oxidative inflammation in peripheral monocytes of autistic children. *Brain, Behavior, and Immunity*. 2018;67:335-44.
284. Wong H, Hoeffler C. Maternal IL-17A in autism. *Experimental neurology*. 2018;299(Pt A):228-40.
285. Nadeem A, Ahmad SF, Attia SM, Al-Ayadhi LY, Bakheet SA, Al-Harbi NO. Oxidative and inflammatory mediators are upregulated in neutrophils of autistic children: Role of IL-17A receptor signaling. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. 2019;90:204-11.
286. Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *Journal of Biological Chemistry*. 2007;282(13):9358-63.
287. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. 2006;126(6):1121-33.
288. Ahmad SF, Zoheir KMA, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, et al. Dysregulation of Th1, Th2, Th17, and T regulatory cell-related transcription factor signaling in children with autism. *Molecular neurobiology*. 2017;54(6):4390-400.
289. Zhu C, Zhang A, Huang S, Ding G, Pan X, Chen R. Interleukin-13 inhibits cytokines synthesis by blocking nuclear factor- κ B and c-Jun N-terminal kinase in human mesangial cells. *Journal of biomedical research*. 2010;24(4):308-16.
290. Hall SL, Baker T, Lajoie S, Richgels PK, Yang Y, McAlees JW, et al. IL-17A enhances IL-13 activity by enhancing IL-13-induced signal transducer and activator of transcription 6 activation. *The Journal of allergy and clinical immunology*. 2017;139(2):462-71.e14.
291. Molloy CA, Morrow AL, Meinzen-Derr J, Schleifer K, Dienger K, Manning-Courtney P, et al. Elevated cytokine levels in children with autism spectrum disorder. *Journal of neuroimmunology*. 2006;172(1):198-205.

292. Akintunde ME, Rose M, Krakowiak P, Heuer L, Ashwood P, Hansen R, et al. Increased production of IL-17 in children with autism spectrum disorders and co-morbid asthma. *Journal of neuroimmunology*. 2015;286:33-41.
293. Bloodworth MH, Newcomb DC, Dulek DE, Stier MT, Cephus JY, Zhang J, et al. STAT6 Signaling Attenuates Interleukin-17-Producing $\gamma\delta$ T Cells during Acute Klebsiella pneumoniae Infection. *Infection and immunity*. 2016;84(5):1548-55.
294. Cooney LA, Towery K, Endres J, Fox DA. Sensitivity and Resistance to Regulation by IL-4 during Th17 Maturation. *The Journal of Immunology*. 2011;187(9):4440-50.
295. Ahmad SF, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, Alasmari AF, et al. Involvement of CD45 cells in the development of autism spectrum disorder through dysregulation of granulocyte-macrophage colony-stimulating factor, key inflammatory cytokines, and transcription factors. *International Immunopharmacology*. 2020;83:106466.
296. Chatterjee P, Chiasson VL, Bounds KR, Mitchell BM. Regulation of the Anti-Inflammatory Cytokines Interleukin-4 and Interleukin-10 during Pregnancy. *Front Immunol*. 2014;5:253.
297. Couper KN, Blount DG, Riley EM. IL-10: The Master Regulator of Immunity to Infection. *The Journal of Immunology*. 2008;180(9):5771.
298. Moaaz M, Youssry S, Elfatratry A, El Rahman MA. Th17/Treg cells imbalance and their related cytokines (IL-17, IL-10 and TGF- β) in children with autism spectrum disorder. *Journal of Neuroimmunology*. 2019;337:577071.
299. Abdallah MW, Larsen N, Mortensen EL, Atladóttir HÓ, Nørgaard-Pedersen B, Bonefeld-Jørgensen EC, et al. Neonatal levels of cytokines and risk of autism spectrum disorders: An exploratory register-based historic birth cohort study utilizing the Danish Newborn Screening Biobank. *Journal of neuroimmunology*. 2012;252(1):75-82.
300. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity*. 1994;1(5):405-13.
301. Ahmad SF, Nadeem A, Ansari MA, Bakheet SA, Attia SM, Zoheir KMA, et al. Imbalance between the anti- and pro-inflammatory milieu in blood leukocytes of autistic children. *Molecular Immunology*. 2017;82:57-65.
302. Sanders SK, Giblin PA, Kavathas P. Cell-cell adhesion mediated by CD8 and human histocompatibility leukocyte antigen G, a nonclassical major histocompatibility complex class 1 molecule on cytotrophoblasts. *The Journal of experimental medicine*. 1991;174(3):737-40.
303. Shin Yim Y, Park A, Berrios J, Lafourcade M, Pascual LM, Soares N, et al. Reversing behavioural abnormalities in mice exposed to maternal inflammation. *Nature*. 2017;549(7673):482-7.
304. Arenella M, Cadby G, De Witte W, Jones RM, Whitehouse AJ, Moses EK, et al. Potential role for immune-related genes in autism spectrum disorders: Evidence from genome-wide association meta-analysis of autistic traits. *Autism : the international journal of research and practice*. 2022;26(2):361-72.
305. Watchorn J, Huang DY, Joslin J, Bramham K, Hutchings SD. Critically ILL COVID-19 Patients With Acute Kidney Injury Have Reduced Renal Blood Flow and Perfusion Despite Preserved Cardiac Function; A Case-Control Study Using Contrast Enhanced Ultrasound. *Shock (Augusta, Ga)*. 2020.
306. Kumar A, Kumar P, Dungdung A, Kumar Gupta A, Anurag A, Kumar A. Pattern of liver function and clinical profile in COVID-19: A cross-sectional study of 91 patients. *Diabetes & metabolic syndrome*. 2020;14(6):1951-4.
307. Jacob F, Pather SR, Huang WK, Zhang F, Wong SZH, Zhou H, et al. Human Pluripotent Stem Cell-Derived Neural Cells and Brain Organoids Reveal SARS-CoV-2 Neurotropism Predominates in Choroid Plexus Epithelium. *Cell stem cell*. 2020.

308. Rifino N, Censori B, Agazzi E, Alimonti D, Bonito V, Camera G, et al. Neurologic manifestations in 1760 COVID-19 patients admitted to Papa Giovanni XXIII Hospital, Bergamo, Italy. *Journal of neurology*. 2020:1-8.
309. Peltzer B, Manocha KK, Ying X, Kirzner J, Ip JE, Thomas G, et al. Outcomes and Mortality Associated with Atrial Arrhythmias Among Patients Hospitalized with COVID-19. *Journal of cardiovascular electrophysiology*. 2020.
310. Nakamura Y, Shimizu M, Yamaki T, Kushimoto K, Yamashita A, Hayase K, et al. Myocardial injury in a patient with severe coronavirus disease: A case report. *Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy*. 2020.
311. Organisation WH. WHO Coronavirus disease (COVID-19) dashboard 2020 [Available from: <https://covid19.who.int/>].
312. Ye Q, Wang B, Mao J. The pathogenesis and treatment of the 'Cytokine Storm' in COVID-19. *J Infect*. 2020;80(6):607-13.
313. Chousterman BG, Swirski FK, Weber GF. Cytokine storm and sepsis disease pathogenesis. *Seminars in immunopathology*. 2017;39(5):517-28.
314. Wu Z, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. *JAMA*. 2020;323(13):1239-42.
315. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet (London, England)*. 2020;395(10223):497-506.
316. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Medicine*. 2020;46(5):846-8.
317. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med*. 2020;8(4):420-2.
318. Ghazavi A, Ganji A, Keshavarzian N, Rabiemajd S, Mosayebi G. Cytokine profile and disease severity in patients with COVID-19. *Cytokine*. 2021;137:155323.
319. Qi D, Yan X, Tang X, Peng J, Yu Q, Feng L, et al. Epidemiological and clinical features of 2019-nCoV acute respiratory disease cases in Chongqing municipality, China: a retrospective, descriptive, multiple-center study. *medRxiv*. 2020:2020.03.01.20029397.
320. Ouyang Y, Yin J, Wang W, Shi H, Shi Y, Xu B, et al. Downregulated Gene Expression Spectrum and Immune Responses Changed During the Disease Progression in Patients With COVID-19. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2020;71(16):2052-60.
321. Wang J, Jiang M, Chen X, Montaner LJ. Cytokine storm and leukocyte changes in mild versus severe SARS-CoV-2 infection: Review of 3939 COVID-19 patients in China and emerging pathogenesis and therapy concepts. *Journal of leukocyte biology*. 2020;108(1):17-41.
322. Moore JB, June CH. Cytokine release syndrome in severe COVID-19. *Science (New York, NY)*. 2020;368(6490):473-4.
323. Wu D, Yang XO. TH17 responses in cytokine storm of COVID-19: An emerging target of JAK2 inhibitor Fedratinib. *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi*. 2020;53(3):368-70.
324. Bulat V, Situm M, Azdajic MD, Likic R. Potential role of IL-17 blocking agents in the treatment of severe COVID-19? *Br J Clin Pharmacol*. 2021;87(3):1578-81.
325. Chen L, Liu HG, Liu W, Liu J, Liu K, Shang J, et al. [Analysis of clinical features of 29 patients with 2019 novel coronavirus pneumonia]. *Zhonghua Jie He He Hu Xi Za Zhi*. 2020;43(0):E005.

326. Gu J, Gong E, Zhang B, Zheng J, Gao Z, Zhong Y, et al. Multiple organ infection and the pathogenesis of SARS. *The Journal of experimental medicine*. 2005;202(3):415-24.
327. Netland J, Meyerholz DK, Moore S, Cassell M, Perlman S. Severe acute respiratory syndrome coronavirus infection causes neuronal death in the absence of encephalitis in mice transgenic for human ACE2. *Journal of virology*. 2008;82(15):7264-75.
328. Paterson RW, Brown RL, Benjamin L, Nortley R, Wiethoff S, Bharucha T, et al. The emerging spectrum of COVID-19 neurology: clinical, radiological and laboratory findings. *Brain*. 2020.
329. Finsterer J, Stollberger C. Causes of hypogeusia/hyposmia in SARS-CoV2 infected patients. *Journal of Medical Virology*. 2020;92(10):1793-4.
330. Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, et al. Neurologic Manifestations of Hospitalized Patients With Coronavirus Disease 2019 in Wuhan, China. *JAMA neurology*. 2020;77(6):1-9.
331. Li Z, Huang Z, Li X, Huang C, Shen J, Li S, et al. Bioinformatic analyses hinted at augmented T helper 17 cell differentiation and cytokine response as the central mechanism of COVID-19-associated Guillain-Barré syndrome. *Cell Prolif*. 2021;54(5):e13024-e.
332. Puelles VG, Lütgehetmann M, Lindenmeyer MT, Sperhake JP, Wong MN, Allweiss L, et al. Multiorgan and Renal Tropism of SARS-CoV-2. *New England Journal of Medicine*. 2020;383(6):590-2.
333. Schurink B, Roos E, Radonic T, Barbe E, Bouman CSC, de Boer HH, et al. Viral presence and immunopathology in patients with lethal COVID-19: a prospective autopsy cohort study. *The Lancet Microbe*. 2020.
334. Ramani A, Müller L, Ostermann PN, Gabriel E, Abida-Islam P, Müller-Schiffmann A, et al. SARS-CoV-2 targets cortical neurons of 3D human brain organoids and shows neurodegeneration-like effects. *bioRxiv*. 2020:2020.05.20.106575.
335. Chen R, Wang K, Yu J, Chen Z, Wen C, Xu Z. The spatial and cell-type distribution of SARS-CoV-2 receptor ACE2 in human and mouse brain. *bioRxiv*. 2020:2020.04.07.030650.
336. Wool GD, Miller JL. The Impact of COVID-19 Disease on Platelets and Coagulation. *Pathobiology : journal of immunopathology, molecular and cellular biology*. 2020:1-13.
337. Chen Y, Li Z, Zhang Y-Y, Zhao W-H, Yu Z-Y. Maternal health care management during the outbreak of coronavirus disease 2019. *Journal of Medical Virology*. 2020;92(7):731-9.
338. Favre G, Pomar L, Musso D, Baud D. 2019-nCoV epidemic: what about pregnancies? *The Lancet*. 2020;395(10224):e40.
339. Wastnedge EAN, Reynolds RM, Boeckel SRv, Stock SJ, Denison FC, Maybin JA, et al. Pregnancy and COVID-19. *Physiological Reviews*. 2021;101(1):303-18.
340. Alfaraj SH, Al-Tawfiq JA, Memish ZA. Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infection during pregnancy: Report of two cases & review of the literature. *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi*. 2019;52(3):501-3.
341. Siston AM, Rasmussen SA, Honein MA, Fry AM, Seib K, Callaghan WM, et al. Pandemic 2009 influenza A(H1N1) virus illness among pregnant women in the United States. *Jama*. 2010;303(15):1517-25.
342. Lampi KM, Lehtonen L, Tran PL, Suominen A, Lehti V, Banerjee PN, et al. Risk of autism spectrum disorders in low birth weight and small for gestational age infants. *The Journal of pediatrics*. 2012;161(5):830-6.
343. Fan C, Lei D, Fang C, Li C, Wang M, Liu Y, et al. Perinatal Transmission of COVID-19 Associated SARS-CoV-2: Should We Worry? *Clinical Infectious Diseases*. 2020.
344. Chen H, Guo J, Wang C, Luo F, Yu X, Zhang W, et al. Clinical characteristics and intrauterine vertical transmission potential of COVID-19 infection in nine pregnant women: a retrospective review of medical records. *The Lancet*. 2020;395(10226):809-15.

345. Salem D, Katranji F, Bakdash T. COVID-19 infection in pregnant women: Review of maternal and fetal outcomes. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics*. 2021;152(3):291-8.
346. Liu Y, Chen H, Tang K, Guo Y. Clinical manifestations and outcome of SARS-CoV-2 infection during pregnancy. *J Infect*. 2020.
347. Wang X, Zhou Z, Zhang J, Zhu F, Tang Y, Shen X. A case of 2019 Novel Coronavirus in a pregnant woman with preterm delivery. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2020.
348. Knight M, Bunch K, Vousden N, Morris E, Simpson N, Gale C, et al. Characteristics and outcomes of pregnant women admitted to hospital with confirmed SARS-CoV-2 infection in UK: national population based cohort study. *BMJ (Clinical research ed)*. 2020;369:m2107.
349. Zhang T, Sidorchuk A, Sevilla-Cermeño L, Vilaplana-Pérez A, Chang Z, Larsson H, et al. Association of Cesarean Delivery With Risk of Neurodevelopmental and Psychiatric Disorders in the Offspring: A Systematic Review and Meta-analysis. *JAMA Network Open*. 2019;2(8):e1910236-e.
350. Papapanou M, Papaioannou M, Petta A, Routsis E, Farmaki M, Vlahos N, et al. Maternal and Neonatal Characteristics and Outcomes of COVID-19 in Pregnancy: An Overview of Systematic Reviews. *International journal of environmental research and public health*. 2021;18(2).
351. Turner MJ, Reynolds CME, McMahon LE, O'Malley EG, O'Connell MP, Sheehan SR. Caesarean section rates in women in the Republic of Ireland who chose to attend their obstetrician privately: a retrospective observational study. *BMC Pregnancy and Childbirth*. 2020;20(1):548.
352. World Health O. Born too soon: the global action report on preterm birth. Geneva: World Health Organization; 2012.
353. Dhir SK, Kumar J, Meena J, Kumar P. Clinical Features and Outcome of SARS-CoV-2 Infection in Neonates: A Systematic Review. *Journal of tropical pediatrics*. 2021;67(3).
354. Allotey J, Stallings E, Bonet M, Yap M, Chatterjee S, Kew T, et al. Clinical manifestations, risk factors, and maternal and perinatal outcomes of coronavirus disease 2019 in pregnancy: living systematic review and meta-analysis. *BMJ (Clinical research ed)*. 2020;370:m3320.
355. Turan O, Hakim A, Dashraath P, Jeslyn WJL, Wright A, Abdul-Kadir R. Clinical characteristics, prognostic factors, and maternal and neonatal outcomes of SARS-CoV-2 infection among hospitalized pregnant women: A systematic review. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics*. 2020;151(1):7-16.
356. Yoon SH, Kang JM, Ahn JG. Clinical outcomes of 201 neonates born to mothers with COVID-19: a systematic review. *European review for medical and pharmacological sciences*. 2020;24(14):7804-15.
357. Vivanti AJ, Vauloup-Fellous C, Prevot S, Zupan V, Suffee C, Do Cao J, et al. Transplacental transmission of SARS-CoV-2 infection. *Nat Commun*. 2020;11(1):3572.
358. Kirtsman M, Diambomba Y, Poutanen SM, Malinowski AK, Vlachodimitropoulou E, Parks WT, et al. Probable congenital SARS-CoV-2 infection in a neonate born to a woman with active SARS-CoV-2 infection. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne*. 2020;192(24):E647-e50.
359. Egloff C, Vauloup-Fellous C, Picone O, Mandelbrot L, Roques P. Evidence and possible mechanisms of rare maternal-fetal transmission of SARS-CoV-2. *Journal of Clinical Virology*. 2020;128:104447.
360. Steinman G. COVID-19 and autism. *Medical Hypotheses*. 2020;142:109797.

361. Shuid AN, Jayusman PA, Shuid N, Ismail J, Kamal Nor N, Mohamed IN. Association between Viral Infections and Risk of Autistic Disorder: An Overview. *International journal of environmental research and public health*. 2021;18(6):2817.
362. Libertus K, Sheperd KA, Ross SW, Landa RJ. Limited fine motor and grasping skills in 6-month-old infants at high risk for autism. *Child development*. 2014;85(6):2218-31.
363. Ozonoff S, Macari S, Young GS, Goldring S, Thompson M, Rogers SJ. Atypical object exploration at 12 months of age is associated with autism in a prospective sample. *Autism : the international journal of research and practice*. 2008;12(5):457-72.
364. Oono IP, Honey EJ, McConachie H. Parent-mediated early intervention for young children with autism spectrum disorders (ASD). *The Cochrane database of systematic reviews*. 2013(4):Cd009774.
365. Althoff CE, Dammann CP, Hope SJ, Ausderau KK. Parent-Mediated Interventions for Children With Autism Spectrum Disorder: A Systematic Review. *American Journal of Occupational Therapy*. 2019;73(3):7303205010p1-p13.
366. Association AP. *Diagnostic and statistical manual of mental disorders (DSM-5®)*: American Psychiatric Pub; 2013.
367. Lyall K, Croen L, Daniels J, Fallin MD, Ladd-Acosta C, Lee BK, et al. The changing epidemiology of autism spectrum disorders. *Annual review of public health*. 2017;38:81-102.
368. Crane L, Chester JW, Goddard L, Henry LA, Hill E. Experiences of autism diagnosis: A survey of over 1000 parents in the United Kingdom. *Autism : the international journal of research and practice*. 2015;20(2):153-62.
369. Boyd BA, McDonough SG, Bodfish JW. Evidence-based behavioral interventions for repetitive behaviors in autism. *Journal of autism and developmental disorders*. 2012;42(6):1236-48.
370. Masi A, Quintana DS, Glozier N, Lloyd AR, Hickie IB, Guastella AJ. Cytokine aberrations in autism spectrum disorder: a systematic review and meta-analysis. *Molecular Psychiatry*. 2015;20(4):440-6.
371. Malkova NV, Yu CZ, Hsiao EY, Moore MJ, Patterson PH. Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. *Brain, Behavior, and Immunity*. 2012;26(4):607-16.
372. Garay PA, McAllister AK. Novel roles for immune molecules in neural development: implications for neurodevelopmental disorders. *Frontiers in synaptic neuroscience*. 2010;2:136.
373. Patten AR, Fontaine CJ, Christie BR. A comparison of the different animal models of fetal alcohol spectrum disorders and their use in studying complex behaviors. *Front Pediatr*. 2014;2:93-.
374. Boksa P. Effects of prenatal infection on brain development and behavior: a review of findings from animal models. *Brain, behavior, and immunity*. 2010;24(6):881-97.
375. Bauman MD, Iosif A-M, Smith SE, Bregere C, Amaral DG, Patterson PH. Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. *Biological psychiatry*. 2014;75(4):332-41.
376. Machado CJ, Whitaker AM, Smith SEP, Patterson PH, Bauman MD. Maternal immune activation in nonhuman primates alters social attention in juvenile offspring. *Biological psychiatry*. 2015;77(9):823-32.
377. Maher GM, O'Keeffe GW, Dalman C, Kearney PM, McCarthy FP, Kenny LC, et al. Association between preeclampsia and autism spectrum disorder: a population-based study. *Journal of Child Psychology and Psychiatry*. 2020;61(2):131-9.
378. Stiles J, Jernigan TL. *The Basics of Brain Development*. *Neuropsychology Review*. 2010;20(4):327-48.

379. Joseph R. Fetal Brain Behavior and Cognitive Development. *Developmental Review*. 2000;20(1):81-98.
380. Prayer D, Kasprian G, Krampfl E, Ulm B, Witzani L, Prayer L, et al. MRI of normal fetal brain development. *European Journal of Radiology*. 2006;57(2):199-216.
381. Huang H, Xue R, Zhang J, Ren T, Richards LJ, Yarowsky P, et al. Anatomical Characterization of Human Fetal Brain Development with Diffusion Tensor Magnetic Resonance Imaging. *The Journal of Neuroscience*. 2009;29(13):4263.
382. Buss C, Davis EP, Muftuler LT, Head K, Sandman CA. High pregnancy anxiety during mid-gestation is associated with decreased gray matter density in 6–9-year-old children. *Psychoneuroendocrinology*. 2010;35(1):141-53.
383. Haddad FL, Patel SV, Schmid S. Maternal Immune Activation by Poly I:C as a preclinical Model for Neurodevelopmental Disorders: A focus on Autism and Schizophrenia. *Neuroscience & Biobehavioral Reviews*. 2020;113:546-67.
384. Wolff AR, Bilkey DK. Immune activation during mid-gestation disrupts sensorimotor gating in rat offspring. *Behavioural Brain Research*. 2008;190(1):156-9.
385. Jones KL, Croen LA, Yoshida CK, Heuer L, Hansen R, Zerbo O, et al. Autism with intellectual disability is associated with increased levels of maternal cytokines and chemokines during gestation. *Molecular Psychiatry*. 2017;22(2):273-9.
386. Goines PE, Croen LA, Braunschweig D, Yoshida CK, Grether J, Hansen R, et al. Increased midgestational IFN- γ , IL-4 and IL-5 in women bearing a child with autism: A case-control study. *Molecular autism*. 2011;2(1):13.
387. Abdallah MW, Larsen N, Grove J, Norgaard-Pedersen B, Thorsen P, Mortensen EL, et al. Amniotic fluid chemokines and autism spectrum disorders: an exploratory study utilizing a Danish Historic Birth Cohort. *Brain Behav Immun*. 2012;26(1):170-6.
388. Brown AS, Sourander A, Hinkka-Yli-Salomäki S, McKeague IW, Sundvall J, Surcel HM. Elevated maternal C-reactive protein and autism in a national birth cohort. *Molecular psychiatry*. 2014;19(2):259-64.
389. Galbraith C JG, Davis P, Cooper P. *New Zealand Social Economic Index 1996 Users Guide*, Statistics New Zealand, Wellington, New Zealand. 1996.
390. Cohen S, Kamarck T, Mermelstein R. Perceived stress scale. *Measuring stress: A guide for health and social scientists*. 1994;10:1-2.
391. Kenny LC, Black MA, Poston L, Taylor R, Myers JE, Baker PN, et al. Early pregnancy prediction of preeclampsia in nulliparous women, combining clinical risk and biomarkers: the Screening for Pregnancy Endpoints (SCOPE) international cohort study. *Hypertension*. 2014;64(3):644-52.
392. Smith SEP, Li J, Garbett K, Mirnics K, Patterson PH. Maternal Immune Activation Alters Fetal Brain Development through Interleukin-6. *The Journal of Neuroscience*. 2007;27(40):10695.
393. Choi GB, Yim YS, Wong H, Kim S, Kim H, Kim SV, et al. The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science (New York, NY)*. 2016;351(6276):933.
394. Pratt L, Ni L, Ponzio NM, Jonakait GM. Maternal inflammation promotes fetal microglial activation and increased cholinergic expression in the fetal basal forebrain: role of interleukin-6. *Pediatric research*. 2013;74(4):393-401.
395. Pineda E, Shin D, You SJ, Auvin S, Sankar R, Mazarati A. Maternal immune activation promotes hippocampal kindling epileptogenesis in mice. *Annals of neurology*. 2013;74(1):11-9.
396. Ahmad SF, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, Attia SM. Elevated IL-16 expression is associated with development of immune dysfunction in children with autism. *Psychopharmacology*. 2019;236(2):831-8.

397. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah IN, Van de Water J. Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders. *Journal of Neuroimmunology*. 2011;232(1):196-9.
398. Patterson PH. Maternal infection and immune involvement in autism. *Trends in Molecular Medicine*. 2011;17(7):389-94.
399. Dabito D, Margolick JB, Lopez J, Bream JH. Multiplex measurement of proinflammatory cytokines in human serum: comparison of the Meso Scale Discovery electrochemiluminescence assay and the Cytometric Bead Array. *Journal of immunological methods*. 2011;372(1-2):71-7.
400. Feng C, Wang H, Lu N, Chen T, He H, Lu Y, et al. Log-transformation and its implications for data analysis. *Shanghai Arch Psychiatry*. 2014;26(2):105-9.
401. Wiens D, DeSoto MC. Is high folic acid intake a risk factor for autism?—a review. *Brain sciences*. 2017;7(11):149.
402. Raghavan R, Riley AW, Volk H, Caruso D, Hironaka L, Sices L, et al. Maternal multivitamin intake, plasma folate and vitamin B12 levels and autism spectrum disorder risk in offspring. *Paediatric and perinatal epidemiology*. 2018;32(1):100-11.
403. Gillberg C, Cederlund M, Lamberg K, Zeijlon L. Brief report: “the autism epidemic”. The registered prevalence of autism in a Swedish urban area. *Journal of autism and developmental disorders*. 2006;36(3):429.
404. Curran EA, O'Neill SM, Cryan JF, Kenny LC, Dinan TG, Khashan AS, et al. Research Review: Birth by caesarean section and development of autism spectrum disorder and attention-deficit/hyperactivity disorder: a systematic review and meta-analysis. *Journal of Child Psychology and Psychiatry*. 2015;56(5):500-8.
405. DeVilbiss EA, Gardner RM, Newschaffer CJ, Lee BK. Maternal folate status as a risk factor for autism spectrum disorders: a review of existing evidence. *British Journal of Nutrition*. 2015;114(5):663-72.
406. Werling DM, Geschwind DH. Sex differences in autism spectrum disorders. *Current opinion in neurology*. 2013;26(2):146-53.
407. Baron-Cohen S, Lombardo MV, Auyeung B, Ashwin E, Chakrabarti B, Knickmeyer R. Why Are Autism Spectrum Conditions More Prevalent in Males? *PLOS Biology*. 2011;9(6):e1001081.
408. Yip BHK, Leonard H, Stock S, Stoltenberg C, Francis RW, Gissler M, et al. Caesarean section and risk of autism across gestational age: a multi-national cohort study of 5 million births. *International Journal of Epidemiology*. 2017;46(2):429-39.
409. Polidano C, Zhu A, Bornstein JC. The relation between cesarean birth and child cognitive development. *Scientific reports*. 2017;7(1):1-10.
410. Morais LH, Golubeva AV, Moloney GM, Moya-Pérez A, Ventura-Silva AP, Arbolea S, et al. Enduring behavioral effects induced by birth by caesarean section in the mouse. *Current Biology*. 2020;30(19):3761-74. e6.
411. Kuwabara Y, Takeda S, Mizuno M, Sakamoto S. Oxytocin levels in maternal and fetal plasma, amniotic fluid, and neonatal plasma and urine. *Archives of gynecology and obstetrics*. 1987;241(1):13-23.
412. Morais LH, Golubeva AV, Casey S, Scott KA, Ramos Costa AP, Moloney GM, et al. Early-life oxytocin attenuates the social deficits induced by caesarean-section delivery in the mouse. *Neuropsychopharmacology*. 2021.
413. Parker-Athill EC, Tan J. Maternal Immune Activation and Autism Spectrum Disorder: Interleukin-6 Signaling as a Key Mechanistic Pathway. *Neurosignals*. 2010;18(2):113-28.
414. Hsiao EY, McBride SW, Chow J, Mazmanian SK, Patterson PH. Modeling an autism risk factor in mice leads to permanent immune dysregulation. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109(31):12776-81.

415. Al-Ayadhi LY, Mostafa GA. Elevated serum levels of interleukin-17A in children with autism. *Journal of Neuroinflammation*. 2012;9(1):158.
416. Yang D, Sun Y-Y, Bhaumik SK, Li Y, Baumann JM, Lin X, et al. Blocking Lymphocyte Trafficking with FTY720 Prevents Inflammation-Sensitized Hypoxic–Ischemic Brain Injury in Newborns. *The Journal of Neuroscience*. 2014;34(49):16467.
417. Reed MD, Yim YS, Wimmer RD, Kim H, Ryu C, Welch GM, et al. IL-17a promotes sociability in mouse models of neurodevelopmental disorders. *Nature*. 2020;577(7789):249-53.
418. Erbel C, Chen L, Bea F, Wangler S, Celik S, Lasitschka F, et al. Inhibition of IL-17A Attenuates Atherosclerotic Lesion Development in ApoE-Deficient Mice. *The Journal of Immunology*. 2009;183(12):8167.
419. Ye B, Tao T, Zhao A, Wen L, He X, Liu Y, et al. Blockade of IL-17A/IL-17R Pathway Protected Mice from Sepsis-Associated Encephalopathy by Inhibition of Microglia Activation. *Mediators of Inflammation*. 2019;2019:8461725.
420. Collison J. IL-17A blockade effective for AS. *Nature Reviews Rheumatology*. 2018;14(12):684-.
421. Wei H, Zou H, Sheikh AM, Malik M, Dobkin C, Brown WT, et al. IL-6 is increased in the cerebellum of autistic brain and alters neural cell adhesion, migration and synaptic formation. *Journal of Neuroinflammation*. 2011;8(1):52.
422. Li X, Chauhan A, Sheikh AM, Patil S, Chauhan V, Li X-M, et al. Elevated immune response in the brain of autistic patients. *Journal of neuroimmunology*. 2009;207(1):111-6.
423. Yang CJ, Liu CL, Sang B, Zhu XM, Du YJ. The combined role of serotonin and interleukin-6 as biomarker for autism. *Neuroscience*. 2015;284:290-6.
424. Wei H, Chadman KK, McCloskey DP, Sheikh AM, Malik M, Brown WT, et al. Brain IL-6 elevation causes neuronal circuitry imbalances and mediates autism-like behaviors. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*. 2012;1822(6):831-42.
425. Wong H, Hoeffler C. Maternal IL-17A in autism. *Experimental neurology*. 2018;299:228-40.
426. Al-Zalabani AH, Al-Jabree AH, Zeidan ZA. Is cesarean section delivery associated with autism spectrum disorder? *Neurosciences (Riyadh, Saudi Arabia)*. 2019;24(1):11-5.
427. Curran EA, Dalman C, Kearney PM, Kenny LC, Cryan JF, Dinan TG, et al. Association Between Obstetric Mode of Delivery and Autism Spectrum Disorder: A Population-Based Sibling Design Study. *JAMA psychiatry*. 2015;72(9):935-42.
428. Morais LH, Golubeva AV, Moloney GM, Moya-Pérez A, Ventura-Silva AP, Arboleya S, et al. Enduring Behavioral Effects Induced by Birth by Caesarean Section in the Mouse. *Current Biology*. 2020;30(19):3761-74.e6.
429. Josefi O, Ryan V. Non-Directive Play Therapy for Young Children with Autism: A Case Study. *Clinical Child Psychology and Psychiatry*. 2004;9(4):533-51.
430. Schaefer GB, Mendelsohn NJ, for the Professional P, Guidelines C. Clinical genetics evaluation in identifying the etiology of autism spectrum disorders: 2013 guideline revisions. *Genetics in Medicine*. 2013;15(5):399-407.
431. Ho KS, Twede H, Vanzo R, Harward E, Hensel CH, Martin MM, et al. Clinical Performance of an Ultrahigh Resolution Chromosomal Microarray Optimized for Neurodevelopmental Disorders. *BioMed research international*. 2016;2016:3284534.
432. Mouridsen SE, Rich B, Isager T, Nedergaard NJ. Autoimmune diseases in parents of children with infantile autism: a case-control study. *Developmental medicine and child neurology*. 2007;49(6):429-32.
433. Comi AM, Zimmerman AW, Frye VH, Law PA, Peeden JN. Familial clustering of autoimmune disorders and evaluation of medical risk factors in autism. *Journal of child neurology*. 1999;14(6):388-94.

434. Gardosi J, Francis A, Turner S, Williams M. Customized growth charts: rationale, validation and clinical benefits. *American journal of obstetrics and gynecology*. 2018;218(2s):S609-s18.
435. Apgar V. A Proposal for a New Method of Evaluation of the Newborn Infant. Originally published in July 1953, volume 32, pages 250-259. *Anesthesia and analgesia*. 2015;120(5):1056-9.
436. Cohen S. Perceived stress in a probability sample of the United States. *The social psychology of health. The Claremont Symposium on Applied Social Psychology*. Thousand Oaks, CA, US: Sage Publications, Inc; 1988. p. 31-67.
437. Motulsky HJ, Brown RE. Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. *BMC bioinformatics*. 2006;7:123.
438. Chan YH. *Biostatistics 104: correlational analysis*. Singapore medical journal. 2003;44(12):614-9.
439. Rea L, Ñames, Parker RA, Allen R, editors. *Designing and conducting survey research*2016.
440. Zhou X, Fragala MS, McElhaney JE, Kuchel GA. Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. *Current opinion in clinical nutrition and metabolic care*. 2010;13(5):541-7.
441. Croft M, Swain SL. Recently activated naive CD4 T cells can help resting B cells, and can produce sufficient autocrine IL-4 to drive differentiation to secretion of T helper 2-type cytokines. *Journal of immunology (Baltimore, Md : 1950)*. 1995;154(9):4269-82.
442. Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 receptor: signaling mechanisms and biologic functions. *Annual review of immunology*. 1999;17:701-38.
443. Wang W, Wang L, Zha B. The roles of STAT6 in regulating B cell fate, activation, and function. *Immunology letters*. 2021.
444. Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. *Journal of immunology (Baltimore, Md : 1950)*. 1993;151(9):4562-73.
445. Marzi M, Vigano A, Trabattoni D, Villa ML, Salvaggio A, Clerici E, et al. Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clinical and experimental immunology*. 1996;106(1):127-33.
446. Omu AE, Al-Qattan F, Diejomaoh ME, Al-Yatama M. Differential levels of T helper cytokines in preeclampsia: pregnancy, labor and puerperium. *Acta obstetrica et gynecologica Scandinavica*. 1999;78(8):675-80.
447. Peng Y, Yin S, Wang M. Significance of the ratio interferon- γ /interleukin-4 in early diagnosis and immune mechanism of unexplained recurrent spontaneous abortion. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics*. 2020.
448. Crane L, Chester JW, Goddard L, Henry LA, Hill E. Experiences of autism diagnosis: A survey of over 1000 parents in the United Kingdom. *Autism : the international journal of research and practice*. 2016;20(2):153-62.
449. North Lee ASD services C, Ireland. *Parents' Perspectives: An Evaluation of the North Lee Autism Spectrum Disorder Service*. Report. 2016.
450. Buescher AV, Cidav Z, Knapp M, Mandell DS. Costs of autism spectrum disorders in the United Kingdom and the United States. *JAMA Pediatr*. 2014;168(8):721-8.
451. Brett D, Warnell F, McConachie H, Parr JR. Factors Affecting Age at ASD Diagnosis in UK: No Evidence that Diagnosis Age has Decreased Between 2004 and 2014. *J Autism Dev Disord*. 2016;46(6):1974-84.
452. Rogers SJ, Vismara L, Wagner AL, McCormick C, Young G, Ozonoff S. Autism Treatment in the First Year of Life: A Pilot Study of Infant Start, a Parent-Implemented

- Intervention for Symptomatic Infants. *Journal of Autism and Developmental Disorders*. 2014;44(12):2981-95.
453. Zwaigenbaum L, Bauman ML, Choueiri R, Kasari C, Carter A, Granpeesheh D, et al. Early Intervention for Children With Autism Spectrum Disorder Under 3 Years of Age: Recommendations for Practice and Research. *Pediatrics*. 2015;136 Suppl 1:S60-81.
454. Malik-Soni N, Shaker A, Luck H, Mullin AE, Wiley RE, Lewis MES, et al. Tackling healthcare access barriers for individuals with autism from diagnosis to adulthood. *Pediatric research*. 2022;91(5):1028-35.
455. Bridgemohan C, Cochran DM, Howe YJ, Pawlowski K, Zimmerman AW, Anderson GM, et al. Investigating Potential Biomarkers in Autism Spectrum Disorder. *Front Integr Neurosci*. 2019;13:31.
456. Walsh P, Elsabbagh M, Bolton P, Singh I. In search of biomarkers for autism: scientific, social and ethical challenges. *Nat Rev Neurosci*. 2011;12(10):603-12.
457. Courchesne E, Carper R, Akshoomoff N. Evidence of brain overgrowth in the first year of life in autism. *Jama*. 2003;290(3):337-44.
458. O'Donovan SM, Murray DM, Hourihane JO, Kenny LC, Irvine AD, Kiely M. Cohort profile: The Cork BASELINE Birth Cohort Study: Babies after SCOPE: Evaluating the Longitudinal Impact on Neurological and Nutritional Endpoints. *Int J Epidemiol*. 2015;44(3):764-75.
459. English JA, Fan Y, Föcking M, Lopez LM, Hryniewiecka M, Wynne K, et al. Reduced protein synthesis in schizophrenia patient-derived olfactory cells. *Translational psychiatry*. 2015;5(10):e663.
460. Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N, et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat Protoc*. 2011;6(7):1060-83.
461. Van Rossum G, Drake FL. *Python reference manual: Centrum voor Wiskunde en Informatica Amsterdam*; 1995.
462. Team R. R development core team. *r: A language and environment for statistical computing*. r foundation for statistical computing, vienna, austria; 2014. Google Scholar. 2018.
463. McKinney W, editor *Data structures for statistical computing in python*. Proceedings of the 9th Python in Science Conference; 2010: Austin, TX.
464. Harris CR, Millman KJ, Van Der Walt SJ, Gommers R, Virtanen P, Cournapeau D, et al. Array programming with NumPy. *Nature*. 2020;585(7825):357-62.
465. Hunter JD. Matplotlib: A 2D graphics environment. *Computing in science & engineering*. 2007;9(03):90-5.
466. Virtanen P, Gommers R, Oliphant TE, Haberland M, Reddy T, Cournapeau D, et al. SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nature methods*. 2020;17(3):261-72.
467. Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, et al. Scikit-learn: Machine learning in Python. *the Journal of machine Learning research*. 2011;12:2825-30.
468. Wickham H, Chang W, Wickham MH. Package 'ggplot2'. Create elegant data visualisations using the grammar of graphics Version. 2016;2(1):1-189.
469. Kassambara A, Kassambara MA. Package 'ggcorrplot'. R package version 01. 2019;3(3).
470. Wickham H, Averick M, Bryan J, Chang W, McGowan LDA, François R, et al. Welcome to the Tidyverse. *Journal of open source software*. 2019;4(43):1686.

471. Rohart F, Gautier B, Singh A, Lê Cao K-A. mixOmics: An R package for 'omics feature selection and multiple data integration. *PLoS computational biology*. 2017;13(11):e1005752.
472. O'Boyle DS, Dunn WB, O'Neill D, Kirwan JA, Broadhurst DI, Hallberg B, et al. Improvement in the prediction of neonatal hypoxic-ischemic encephalopathy with the integration of umbilical cord metabolites and current clinical makers. *The Journal of pediatrics*. 2021;229:175-81. e1.
473. Che X, Hornig M, Bresnahan M, Stoltenberg C, Magnus P, Surén P, et al. Maternal mid-gestational and child cord blood immune signatures are strongly associated with offspring risk of ASD. *Molecular psychiatry*. 2022;27(3):1527-41.
474. Li H, Xu Y, Li W, Zhang L, Zhang X, Li B, et al. Novel insights into the immune cell landscape and gene signatures in autism spectrum disorder by bioinformatics and clinical analysis. *Frontiers in Immunology*. 2023;13.
475. Randi EB, Casili G, Jacquemai S, Szabo C. Selenium-Binding Protein 1 (SELENBP1) Supports Hydrogen Sulfide Biosynthesis and Adipogenesis. *Antioxidants (Basel)*. 2021;10(3).
476. Pagan C, Benabou M, Leblond C, Cliquet F, Mathieu A, Lemièrre N, et al. Decreased phenol sulfotransferase activities associated with hyperserotonemia in autism spectrum disorders. *Translational psychiatry*. 2021;11(1):23.
477. Williams RJ. Sulfate Deficiency as a Risk Factor for Autism. *J Autism Dev Disord*. 2020;50(1):153-61.
478. O'Reilly B, Waring R. Enzyme and sulphur oxidation deficiencies in autistic children with known food/chemical intolerances. *Journal of Orthomolecular Medicine*. 1993;8:198-.
479. Seelig J, Heller RA, Haubruck P, Sun Q, Klingenberg GJ, Hackler J, et al. Selenium-binding protein 1 (SELENBP1) as biomarker for adverse clinical outcome after traumatic spinal cord injury. *Frontiers in neuroscience*. 2021;15:680240.
480. Kanazawa T, Chana G, Glatt SJ, Mizuno H, Masliah E, Yoneda H, et al. The utility of SELENBP1 gene expression as a biomarker for major psychotic disorders: replication in schizophrenia and extension to bipolar disorder with psychosis. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*. 2008;147(6):686-9.
481. Mohammadi A, Rashidi E, Amooeian VG. Brain, blood, cerebrospinal fluid, and serum biomarkers in schizophrenia. *Psychiatry research*. 2018;265:25-38.
482. Rossignol DA, Bradstreet JJ, Van Dyke K, Schneider C, Freedman SH, O'Hara N, et al. Hyperbaric oxygen treatment in autism spectrum disorders. *Medical Gas Research*. 2012;2(1):1-13.
483. Nahaboo W, Eski SE, Vermeersch M, Saykali B, Monteyne D, Magin TM, et al. Keratin dynamics govern the establishment of the maternal-fetal interface. *BioRxiv*. 2021:2021.04. 07.438772.
484. Mazzola JL, Sirover MA. Alteration of intracellular structure and function of glyceraldehyde-3-phosphate dehydrogenase: a common phenotype of neurodegenerative disorders? *Neurotoxicology*. 2002;23(4-5):603-9.
485. Ye H, Liu J, Wu JY. Cell adhesion molecules and their involvement in autism spectrum disorder. *Neuro-Signals*. 2010;18(2):62-71.
486. Rossignol E. Genetics and function of neocortical GABAergic interneurons in neurodevelopmental disorders. *Neural plasticity*. 2011;2011.
487. Lee FH, Su P, Xie YF, Wang KE, Wan Q, Liu F. Disrupting GluA2-GAPDH Interaction Affects Axon and Dendrite Development. *Sci Rep*. 2016;6:30458.
488. Mazón-Cabrera R, Liesenborgs J, Brône B, Vandormael P, Somers V. Novel maternal autoantibodies in autism spectrum disorder: Implications for screening and diagnosis. *Frontiers in neuroscience*. 2023;17:1067833.

489. Delunardo F, Soldati D, Bellisario V, Berry A, Camerini S, Crescenzi M, et al. Anti-GAPDH Autoantibodies as a Pathogenic Determinant and Potential Biomarker of Neuropsychiatric Diseases. *Arthritis Rheumatol*. 2016;68(11):2708-16.
490. Zheng H-F, Wang W-Q, Li X-M, Rauw G, Jean-Michel Le MellÃÃ, Baker GB. Neuroactive steroids and related steroids in autism spectrum disorders. *Neuropsychiatry*. 2018;8(2):468-76.
491. Worsham W, Dalton S, Bilder DA. The prenatal hormone milieu in autism spectrum disorder. *Frontiers in psychiatry*. 2021:917.
492. Lutchmaya S, Baron-Cohen S, Raggatt P. Foetal testosterone and eye contact in 12-month-old human infants. *Infant Behavior and Development*. 2002;25(3):327-35.
493. Auyeung B, Ahluwalia J, Thomson L, Taylor K, Hackett G, O'donnell KJ, et al. Prenatal versus postnatal sex steroid hormone effects on autistic traits in children at 18 to 24 months of age. *Molecular autism*. 2012;3:1-5.
494. Chapman E, Baron-Cohen S, Auyeung B, Knickmeyer R, Taylor K, Hackett G. Fetal testosterone and empathy: evidence from the empathy quotient (EQ) and the "reading the mind in the eyes" test. *Social Neuroscience*. 2006;1(2):135-48.
495. Knickmeyer R, Baron-Cohen S, Raggatt P, Taylor K. Foetal testosterone, social relationships, and restricted interests in children. *Journal of child psychology and psychiatry*. 2005;46(2):198-210.
496. Park BY, Lee BK, Burstyn I, Tabb LP, Keelan JA, Whitehouse AJ, et al. Umbilical cord blood androgen levels and ASD-related phenotypes at 12 and 36 months in an enriched risk cohort study. *Molecular autism*. 2017;8(1):1-12.
497. Jamnadass ES, Keelan JA, Hollier LP, Hickey M, Maybery MT, Whitehouse AJ. The perinatal androgen to estrogen ratio and autistic-like traits in the general population: a longitudinal pregnancy cohort study. *J Neurodev Disord*. 2015;7(1):1-12.
498. Whitehouse AJ, Mattes E, Maybery MT, Dissanayake C, Sawyer M, Jones RM, et al. Perinatal testosterone exposure and autistic-like traits in the general population: a longitudinal pregnancy-cohort study. *J Neurodev Disord*. 2012;4(1):1-12.
499. Mahesh VB, Greenblatt RB. The in vivo conversion of dehydroepiandrosterone and androstenedione to testosterone in the human. *European Journal of Endocrinology*. 1962;41(3):400-6.
500. Majewska MD, Hill M, Urbanowicz E, Rok-Bujko P, BieÅkowski P, NamysÅwska I, et al. Marked elevation of adrenal steroids, especially androgens, in saliva of prepubertal autistic children. *European child & adolescent psychiatry*. 2014;23:485-98.
501. Geier DA, Geier MR. A prospective assessment of androgen levels in patients with autistic spectrum disorders: biochemical underpinnings and suggested therapies. *Neuroendocrinology letters*. 2007;28(5):565-74.
502. Bala KA, DoÅan M, Kaba S, Mutluer T, Aslan O, DoÅan SZ. Hormone disorder and vitamin deficiency in attention deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASDs). *Journal of Pediatric Endocrinology and Metabolism*. 2016;29(9):1077-82.
503. Foster PA, Mueller JW. Insights into steroid sulfation and desulfation pathways. *J Mol Endocrinol*. 2018;61:T271-T83.
504. Warren Z, McPheeters ML, Sathe N, Foss-Feig JH, Glasser A, Veenstra-VanderWeele J. A Systematic Review of Early Intensive Intervention for Autism Spectrum Disorders. *Pediatrics*. 2011;127(5):e1303-e11.
505. Mohammadzaheri F, Koegel LK, Rezaee M, Rafiee SM. A randomized clinical trial comparison between pivotal response treatment (PRT) and structured applied behavior analysis (ABA) intervention for children with autism. *J Autism Dev Disord*. 2014;44(11):2769-77.

506. Achenbach TM, & Rescorla, L. A. Manual for the ASEBA Preschool Forms & Profiles. Burlington, VT: University of Vermont, Research Center for Children, Youth, & Families 2000.
507. Pandolfi V, Magyar CI, Dill CA. Confirmatory factor analysis of the child behavior checklist 1.5-5 in a sample of children with autism spectrum disorders. *J Autism Dev Disord*. 2009;39(7):986-95.
508. Kariuki SM, Abubakar A, Murray E, Stein A, Newton CRJC. Evaluation of psychometric properties and factorial structure of the pre-school child behaviour checklist at the Kenyan Coast. *Child and Adolescent Psychiatry and Mental Health*. 2016;10(1):1.
509. Rutter M BA, Lord C., editor. Social Communication Questionnaire: Los Angeles, CA: Western Psychological Services; 2003.
510. Berument SK, Rutter M, Lord C, Pickles A, Bailey A. Autism screening questionnaire: Diagnostic validity. *The British Journal of Psychiatry*. 1999;175:444-51.
511. Wiggins LD, Bakeman R, Adamson LB, Robins DL. The utility of the Social Communication Questionnaire in screening for autism in children referred for early intervention. *Focus on Autism and Other Developmental Disabilities*. 2007;22(1):33-8.
512. Kaufman AS, & Kaufman, N. L. Kaufman Brief Intelligence Test, Second Edition. . Bloomington, MN: Pearson, Inc. 2004b.
513. Gray C. Social stories 101. *The Morning News*. 1998;10(1):2-6.
514. Eisenhower AS, Baker BL, Blacher J. Preschool children with intellectual disability: syndrome specificity, behaviour problems, and maternal well-being. *Journal of intellectual disability research : JIDR*. 2005;49(Pt 9):657-71.
515. Bauminger N, Solomon M, Rogers SJ. Externalizing and internalizing behaviors in ASD. *Autism research : official journal of the International Society for Autism Research*. 2010;3(3):101-12.
516. Estes A, Munson J, Dawson G, Koehler E, Zhou XH, Abbott R. Parenting stress and psychological functioning among mothers of preschool children with autism and developmental delay. *Autism : the international journal of research and practice*. 2009;13(4):375-87.
517. Tomiyama S, Kikuchi M, Yoshimura Y, Hasegawa C, Ikeda T, Saito DN, et al. Changes in maternal feelings for children with autism spectrum disorder after childbirth: The impact of knowledge about the disorder. *PLoS one*. 2018;13(8):e0201862-e.
518. EuroQol--a new facility for the measurement of health-related quality of life. *Health policy (Amsterdam, Netherlands)*. 1990;16(3):199-208.
519. Dix T. The affective organization of parenting: adaptive and maladaptive processes. *Psychological bulletin*. 1991;110(1):3-25.
520. Eisenberg N, Gershoff ET, Fabes RA, Shepard SA, Cumberland AJ, Losoya SH, et al. Mothers' emotional expressivity and children's behavior problems and social competence: mediation through children's regulation. *Developmental psychology*. 2001;37(4):475-90.
521. Anderson DK, Maye MP, Lord C. Changes in Maladaptive Behaviors From Midchildhood to Young Adulthood in Autism Spectrum Disorder. *American Journal on Intellectual and Developmental Disabilities*. 2011;116(5):381-97.
522. Singer J. *Neurodiversity : the birth of an idea*. Lexington, Kentucky: [publisher not identified] Lexington, Kentucky; 2017.
523. Shattuck PT, Seltzer MM, Greenberg JS, Orsmond GI, Bolt D, Kring S, et al. Change in autism symptoms and maladaptive behaviors in adolescents and adults with an autism spectrum disorder. *J Autism Dev Disord*. 2007;37(9):1735-47.
524. Skokauskas N, Gallagher L. Mental health aspects of autistic spectrum disorders in children. *Journal of intellectual disability research : JIDR*. 2012;56(3):248-57.
525. Oosterling IJ, Wensing M, Swinkels SH, van der Gaag RJ, Visser JC, Woudenberg T, et al. Advancing early detection of autism spectrum disorder by applying an integrated two-

stage screening approach. *Journal of child psychology and psychiatry, and allied disciplines*. 2010;51(3):250-8.

526. Smith L, Villaret-Cazadamont J, Claus SP, Canlet C, Guillou H, Cabaton NJ, et al. Important Considerations for Sample Collection in Metabolomics Studies with a Special Focus on Applications to Liver Functions. *Metabolites*. 2020;10(3).

527. Wagner-Golbs A, Neuber S, Kamlage B, Christiansen N, Bethan B, Rennefahrt U, et al. Effects of Long-Term Storage at -80 °C on the Human Plasma Metabolome. *Metabolites*. 2019;9(5).

528. Ahmad S, Sundaramoorthy E, Arora R, Sen S, Karthikeyan G, Sengupta S. Progressive degradation of serum samples limits proteomic biomarker discovery. *Analytical Biochemistry*. 2009;394(2):237-42.

529. Davit CJ, Hundley RJ, Bacic JD, Hanson EM. A pilot study to improve venipuncture compliance in children and adolescents with autism spectrum disorders. *Journal of developmental and behavioral pediatrics : JDBP*. 2011;32(7):521-5.

530. Egertson JD, MacLean B, Johnson R, Xuan Y, MacCoss MJ. Multiplexed peptide analysis using data-independent acquisition and Skyline. *Nat Protoc*. 2015;10(6):887-903.

531. Teo G, Kim S, Tsou CC, Collins B, Gingras AC, Nesvizhskii AI, et al. mapDIA: Preprocessing and statistical analysis of quantitative proteomics data from data independent acquisition mass spectrometry. *Journal of proteomics*. 2015;129:108-20.

Appendices

Appendix 1: SOPs, Consents and Assents

Purpose:

This SOP describes the procedure for blood collection for extraction of RNA.

Responsibility:

It is the responsibility of the research personnel carrying out this procedure to ensure that all steps are completed both competently and safely.

Equipment/reagent requirements:

Blood collection system

Personal protective equipment; gloves, laboratory coat, protective glasses

Blood collection tube: Tempus™ Blood RNA Tubes (Applied Biosystems), BD™ serum, BD™ EDTA blood tubes.

A polystyrene container with ice to maintain temperature at 4°C for processing and /or transport to processing laboratory, or alternatively use a water-bath (plus a thermometer) with iced water to maintain the temperature at 4°C or a pre-conditioned gel pack at 4°C

Refrigerator (2-4°C) if overnight sample storage is required

Freezer -20°C/-80°C if short-term storage is required

Vortex for sample mixing

Preparation of skin:

Local anaesthetic cream or cryogestic freeze spray is to be used for each child as per manufacturer's instructions. The cream should be applied 20 minutes prior to venepuncture, or the cryospray immediately before venepuncture.

A loose tourniquet should be applied to the selected site. The site is to be cleaned for 30 secs with 70% isopropyl alcohol wipe prior to venepuncture. Blood to be taken using vacutainer 23g butterfly needle system. After collection of blood, release tourniquet, apply clean gauze over puncture site and remove needle. Dispose of the needle in a sharps bin and soiled waste should be disposed of as per infection guidelines.

Appropriate PPE (Personal Protective Equipment) should be worn while performing venepuncture i.e. gloves.

If access is proving difficult, no more than one attempt should be made on any one child, unless there is an excellent chance of success and the child and parents are agreeable.

Collection of samples:

Blood is to be collected in Vacutainers as outlined below. Samples should be filled to the indicator mark on each bottle. The ascribed volume of blood required is noted in the table below.

Blood bottles provided should be filled in turn in the following order:

Plain Serum	EDTA Plasma	Tempus RNA
Red	Lavender	Dark blue top
6 ml	6 ml	3ml

1. 6ml of blood placed into the serum (red bottle)
2. 6 ml of blood placed into the EDTA (lavender bottle)
3. 3 ml of blood placed into the Tempus (dark blue bottle) *

* **IMPORTANT:** Immediately after the Tempus tube is filled, stabilize the blood by shaking the tube vigorously for 10 seconds to ensure that the Reagent makes uniform contact with the sample, ensuring complete blood cell lysis.

Label the vacutainers with unique PiRAMiD study number, participant's DOB and the time and date the sample was taken. Following initial processing (clot formation) serum vacutainers are to be placed in the pre-refrigerated (4°C) freezer box before transfer to CUMH lab within 1 hour. Fill freezer box with frozen ice packs before bringing to CUMH laboratory by hand.

Samples must be processed and stored within 3 hours of collection (including spin down and aliquoting for biobank freezing at -80°C)

Serum – Red top

1. BD serum collection tube must be without additives
2. Allow at least 30 - 60 mins from collection for clot formation prior to processing. Keep at room temperature for clot formation. If less than 30 minutes, there is likely to be retained cellular content in the sample. If more than 60 minutes, there is a risk of cell lysis – contaminating the sample. Ensure there is clot formation prior to centrifuging. Keep bagged in cold box on ice during this period. If haemolysis (pink to red tinge in sample) is observed, this information should be recorded.

3. Centrifuge the 6ml red capped vacutainer at 2400xg for 10 min at 4°C.
4. Keep vacutainer and second spin tube on ice until aliquots are completed. Using a sterile Pasteur pipette, pipette the serum into a sterile second spin tube labelled with patient ID and marked in red to represent serum and centrifuge at 3000xg for 10 min at 4°C.
5. Using a new sterile pipette tip, transfer 250ul aliquots of the serum into the red capped barcoded micro cryotubes. Fit red caps to micro cryotubes.
6. Scan barcodes into PiRAMiD spreadsheet and store micro cryotubes in –Wilmot 96 unit racks at -80°C in the PiRAMiD allocated freezer space.

Whole blood – EDTA Lavender Top

1. Invert the EDTA tubes 5-6 times to ensure anticoagulant mixing and absence of clots.
2. Record the time of blood draw and fasting status on the blood collection form. For clinical research studies/trials, record the relevant data and document as per the study protocol.
3. The blood should be delivered to INFANT Lab, CUMH. Keep bagged at **room temperature**.
4. For whole blood analyses, samples can be aliquoted to barcoded cryovials or stored in original 6mL EDTA tube.
5. Freeze samples within 1 hour of blood draw, in the designated PiRAMiD -80°C freezers. This is located on the 5th floor, Infant Laboratory, CUMH.
6. Record time of freezing and freezer storage location on paper and electronic log sheet

RNA - Tempus Blood Tubes

1. Draw blood directly into the evacuated Tempus Blood RNA Tube. Filling the blood collection tube to the black mark on the tube label indicates that the correct amount of blood has been drawn. Under-filling or overfilling of the tube can affect laboratory results due to the incorrect blood/additive ratio.
2. Immediately after the Tempus tube is filled, stabilise the blood by shaking the tube vigorously 10 seconds ensuring that the stabilising reagent makes uniform contact with the sample. **IMPORTANT:** Failure to mix the stabilising reagent with the blood leads to inadequate stabilisation of the gene expression profile and the formation of microclots that can potentially clog the purification filter.
3. The Tempus Blood RNA tube is appropriately labelled either with a unique study identification number and/or a bar code label generated electronically.
4. Record the time that the sample was taken in the study specific documentation.
5. Maintain the tubes at 4°C using a freezer box / polystyrene container with ice. Transport tubes to the processing laboratory as soon as is practicable (same

time as serum and EDTA) or within a maximum of 24 hours for direct storage at -80°C. Tubes should be transported at 4°C in a polystyrene container on ice.

6. Record the time of processing in the study specific documentation or data management system. Note: As a general rule samples should be processed and reach the appropriate storage conditions as soon as is practicable (This is true for all sample types).

PiRAMiD - Venepuncture SOP

Purpose:

This SOP describes the procedure for blood collection from research subjects.

Safety:

1. The research personnel will greet the research subject, identify themselves, and then indicate the blood collection procedure to the research subject.
2. The research subject will be approached in a friendly calm manner and their cooperation will be gained prior to blood collection
3. The research subject will be correctly identified prior to blood collection, by asking them to give their name and date of birth.
4. The research subject will be positioned safely and comfortably in the chair provided for venepuncture, ensuring that the protective arm is in the correct position to support the research subject in the event of fainting or any other adverse event.
5. All sample containers and equipment needed to competently and efficiently carry out the venepuncture will be assembled prior to the procedure.
6. The research personnel will wear gloves at all times during venepuncture.
7. Research personnel will use appropriate barrier precautions, such as gloves, gowns, masks, and protective eyewear to prevent exposure to skin and mucus membranes when working with known infectious research subjects.
8. The research personnel will take care to prevent needle stick injuries when using and disposing of needles.
9. Blood bottles will **not** be labelled in advance of venepuncture.
10. All blood samples will be labelled immediately after venepuncture.
11. A sharps container will be placed close to the use-area as practical.

Procedure:

1. The research subject's arm will be held extended and positioned comfortably on the arm-rest of the venepuncture chair.
2. The tourniquet will be applied 3-4 inches above the selected puncture site, applied as loosely as possible while still being effective.
3. The tourniquet will not be left in position for longer than two minutes.
4. The research subject will be asked to make a fist without pumping the hand.
5. The puncture site will be cleansed with 70% alcohol swab using a circular motion from the centre to the periphery, and be allowed to air dry prior to venepuncture.
6. The needle will then be inserted through the skin, bevel edge uppermost, into the lumen of the vein. The tourniquet will be released when the last specimen tube to be drawn is filling, or sooner if good flow is established.

7. Clean dry gauze or cotton wool will be placed on the venepuncture site and the needle will be removed.
8. The research personnel will press down on the gauze/cotton wool once the needle has been drawn out of the vein applying adequate pressure to avoid formation of a haematoma.
9. The research subjects arm will not be placed in a bent position at any time following venepuncture.
10. The research subjects arm will be inspected to ensure bleeding has stopped and a waterproof plaster strip will be applied.
11. The research personnel will ensure that the research subject has not experienced any adverse events from the venepuncture and will then assist them from the chair.
12. All contaminated materials/supplies will be disposed of in the designated containers.
13. All blood specimen tubes will be labelled immediately at the research subjects bedside with the appropriate research study labels.

PiRAMiD Study: PRedicting early onset Autism through Maternal Immune Activation and proteomic Discovery.

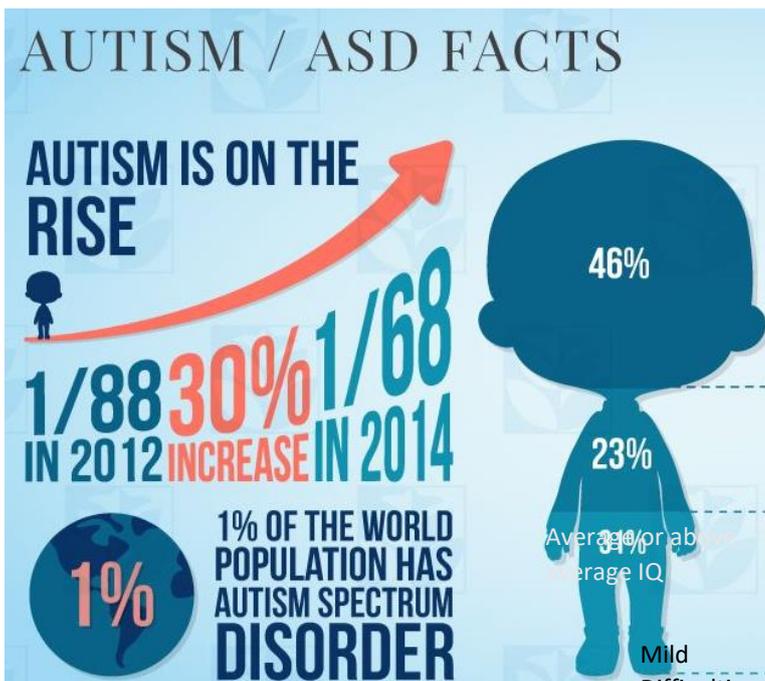
INFORMATION SHEET AND ASSENT FORM FOR CHILDREN CONTROL GROUP

(Use in conjunction with the Parent(s)/Guardian(s) ICF – Informed Consent Form)

Introduction:

You have been invited to take part in an important study that is looking for ways to help children with Autistic Spectrum Disorder (ASD). We hope to help doctors to say which children are at risk of getting ASD at as young an age as possible. ASD is a condition that can make it very hard for children to talk, and play with other children. Children with ASD can be very lonely and can find it hard to make friends. This study hopes that by doing a simple blood test, we might be able to figure out which children have ASD at a younger age and help them sooner. **While you do not have ASD, we are asking your permission to take part in this study as part of a control group (controls are healthy people who we compare with**

people who have a condition like ASD). This allows us to compare children who have (ASD) Autism Spectrum Disorder with other children that are the same age but do not have ASD.



The National Children's Research Centre has awarded us money to begin a new study and this gives us a way to try to answer some important questions about ASD. This study is called the PiRAMiD study (**PR**edicting early onset **proteomic Discovery**). CENTRE (The Irish Centre Research), University College

Autism through Maternal Immune Activation and This research is taking place in The INFANT for Fetal and Neonatal Translational Cork in Cork University Hospital.

IQ <70 Learning Difficulties

What is informed Assent?

This fact sheet will help you to understand what will happen to you during this study. When you have read it, you can decide if you would like to take part. If you do want to take part in the study, you will sign your name on the assent form at the end of this fact sheet. This is

called “informed assent”. It means that you have been told all about the study, that you understand what will happen, and that you want to take part.

We will also explain the study to your parent(s)/guardian(s) and they will decide whether they would like you to take part. They will be given a separate fact sheet called a Parent Information Leaflet and

Informed Consent Form (ICF) to sign if they are happy for you to participate. Both you and your parent(s)/guardian(s) must agree for you to take part in the study. If this information

sheet contains words or names that you do not understand, please ask your parent(s)/guardian(s) to explain them or else you can ask someone on the study team.



Do I have to take part?

It is great that you are reading this document and thinking about joining this study but we understand if you decide not to take part. It is up to you and your parent(s)/guardian(s) to decide. You can say “No” now, or at any time during the study if you change your mind for any reason. You just need to tell the people in charge of the study. This will not change the way you are treated by your doctors in the Hospital now, or in the future.

What will I have to do if I take part?

If you wish to join this study, once you have read this fact sheet you should sign the Assent Form at the end of this document. By signing this form, you allow us to look at your hospital record. You also agree to your parent(s)/guardian(s) filling out questionnaires about you, and allow us to study this information. We would also ask to take a small sample of blood from you. The blood test will be done by our experienced children’s doctor, and should only take a couple of minutes. We will study blood samples from you, your mother and your father, to try to answer our questions about what causes ASD in children. Your parent(s)/guardian(s) will be asked to complete a questionnaire about you and should take no longer than 10 minutes to do. This questionnaire asks 40 questions about your listening, talking, behaviour and emotional skills.

We would also ask you to visit the INFANT Centre at the hospital to complete another test of your growth and abilities. The test should take no longer than 1 hour in total to complete, with a break if needed.

What are the good or bad things that might happen if I take part?

The good things about taking part in this study is that it will help us to understand what causes Autism Spectrum Disorder and allow us to check your strengths and weaknesses. We hope this understanding will lead to better care for future children who have ASD.

There are no expected bad things from taking part in this study for you, but there is a blood test involved which is a little bit painful. If you are worried about anything in the study, you can ask your parent(s)/guardian(s) or you can ask the research doctor to explain things better to you.

What happens to my information collected in the study?

All the information you give us on your questionnaire will be treated in confidence (kept secret) and we will make sure not to have your name on it. If the information we get says you are at risk of harm, then we would have to act to reduce this risk. All your personal information will be kept safely at the INFANT centre and information without your name on it will be stored on the researchers' password protected computers at UCC. Once the study is finished, we will make a report that we hope will bring new information about ASD to UCC, and the world. Nobody reading the report/paper will know it is you as the report will only have the group results – these are results from everyone who took part in the study, without names, addresses or dates of birth. Your parent(s)/guardian(s) may also ask for a sum up of the results once everything is finished and it will be available on the INFANT website once ready.

What if I have any questions?

If this fact sheet contains words or information that you do not understand, please ask your parent(s)/guardian(s) to explain them or else ask the study doctor during your visit. You can keep a copy of this form and make sure that we have answered all your questions before you decide whether you want to take part in the study. Otherwise, if you are happy to take part in this research study, please fill in and sign the assent form at the end of this document.

PiRAMiD study (**PR**edicting early onset **A**utism through **M**aternal Immune **A**ctivation and proteomic **D**iscovery)

This assent form must be used in conjunction with the appropriate parent/legal guardian consent form. On its own, it does not provide informed consent for a minor to take part

Participant Name:
Participant DOB:
Study Number:

Principal Investigator: Professor Deirdre Murray Professor of Paediatrics and Child Health University College Cork

in the study.

Please circle whatever you agree with:

1. I read through the fact sheet with my parent(s)/guardian(s) and I understand the information that is given for this study.

Yes

No

2. I understand what this study is about and what I am expected to do.

Yes

No

3. I agree to information being collected about me from my parent(s)/guardian(s).

Yes

No

4. I agree to the researchers accessing my medical notes and for this to be part of this study.

Yes

No

5. I agree to the researchers taking a sample of my blood for this study

Yes

No

6. I would like to take part in this study and I understand that I don't have to if I don't want to.

Yes

No

Name of Participant (Please Print)

Signature

Date

PiRAMiD Study: PRedicting early onset Autism through Maternal Immune Activation and proteomic Discovery.

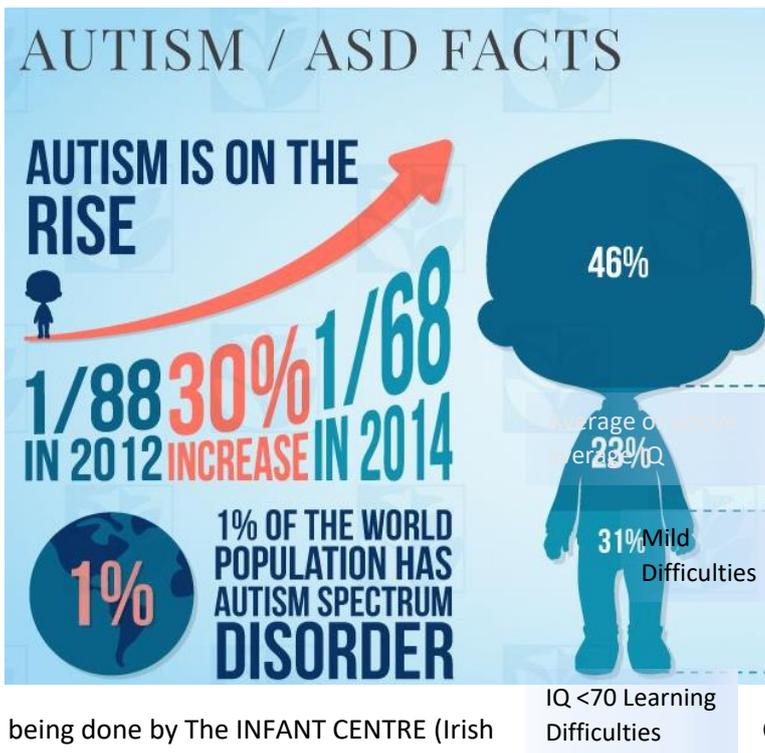
INFORMATION SHEET AND ASSENT FORM FOR CHILDREN – CASES

(Use in conjunction with the Parent(s)/Guardian(s) ICF – Informed Consent Form)

Introduction:

As a baby, you took part in a study carried out in Cork University Hospital (CUH) called the BASELINE study (**B**abies **A**fter **S**cope: **E**valuating the **L**ongitudinal **I**mpact using **N**eurological and **N**utritional **E**ndpoints). This was an important research study which is still going on, and involved you having a number of checks at different times in your childhood. You were checked at age 2 months, 6 months, 12 months, 2 years and 5 years. During these checks we were looking for information about your growth and health. The research done so far, with your help, has already led to us having a better knowledge of children's growth,

development, diet, and allergies.



We are now inviting you and your parents to take part in another study called PiRAMiD (**PR**edicting early onset **A**utism through **M**aternal **I**mmune **A**ctivation and **P**roteomic **D**iscovery). PiRAMiD is an exciting new study looking at why some of the children from BASELINE have ASD (Autism Spectrum Disorder) and others do not. This research is

being done by The INFANT CENTRE (Irish Neonatal Translational Research) in CUH (National Children's Research Centre in Dublin).

Centre for Fetal and and is paid for by the NCRC

What is Informed Assent?

This fact sheet will help you to understand what will happen to you during this study. When you have read the information, you can decide if you would like to take part. If you do want to take part in the study, you will sign your name on the Assent Form at the end of this fact sheet. This is called "informed assent". It means that you have been told all about the study, that you understand what will happen, and that you want to take part.

We will also explain the study to your parent(s)/guardian(s) and they will decide whether they would like you to take part. They will be given a separate Parent Information Leaflet



and Informed Consent Form to sign if they are happy for you to participate. Both you and your parent(s)/guardian(s) must agree that you want to take part in the study. If this fact sheet contains words or ideas that you do not understand, please ask your parent(s)/guardian(s) to explain them or else ask the study doctor.

Do I have to take part?

It is great that you are reading this document and considering taking part in this study but we understand if you decide not to take part. It is up to you and your parent(s)/guardian(s) to decide. You can say "No" now, or at any time during the study if you change your mind for any reason. You just need to tell the people in charge of the study. This will not affect the way you are treated by your doctors in the CUH now, or in the future.

What will I have to do if I take part?

If you wish to take part in this study, once you have read this information you should sign the Assent Form at the end of this document. By signing this form, you allow us to look at your hospital notes. You also agree to your parent(s)/guardian(s) filling in questionnaires about you, and allow us to study this information. Your parent(s)/guardian(s) will be asked to complete a questionnaire when you come to the INFANT centre and should take no longer than 10 minutes to do. This questionnaire has 40 questions and it tests for ASD (Autistic Spectrum Disorder). The questions are about your listening, talking, behaviour and emotional skills. During the visit we will also measure your growth and abilities, and we will take a small sample of blood from you. These checks and tests should take no longer than 1 hour in total to finish, and you can have a break if necessary (we have snacks). The blood test will be performed by our experienced children's doctor, and should only take a couple of minutes. We will study blood samples from you, your mother and your father, and try to answer our questions about what causes ASD in children.

What are the good or bad things that might happen if I take part?

The good things about taking part in this study is that it will help us to understand what causes Autism Spectrum Disorder, to try to see if there are signs of ASD in the blood, of children affected by ASD, and their parents. This may help us to predict ASD and help us to understand why it happens. This is the first step in looking for better ways to treat or prevent ASD.

There are no expected bad things from taking part in this study for you, but there is a blood test which is a little bit painful. If you are worried about anything in the study, you can ask about these with your parent(s)/guardian(s) or you can ask the research doctor.

What happens to my information collected in the study?

All the information you give us will be treated in confidence (kept secret) and we will protect your privacy (name or any personal details), we will make sure not to have your name on anything. If the information we receive suggests you are at risk of harm, then we would have to act to reduce this risk. All your personal information will be stored safely at the INFANT centre and information

without your name on it will be stored on the researchers' password protected computers at UCC. Once the study is finished, we will make a report that we hope will bring new information about ASD to UCC, and the world. Nobody reading the report/paper will know it is you as the report will only have the group results – that is results from everyone who took part in the study, without names, addresses or dates of birth. Your parent(s)/guardian(s) may also request a summary of the findings once complete and it will be available on the INFANT website once ready.

What if I have any questions?

If this fact sheet contains words or ideas that you do not understand, please ask your parent(s)/guardian(s) to explain them or else ask the study staff present during your visit. You can keep a copy of this form and make sure that we have answered all your questions before you decide whether you want to take part in the study. Otherwise, if you are happy to take part in this research study, please fill in and sign the assent form at the end of this document.

Adolescent ASSENT FORM

PiRAMiD study (**PR**edicting early onset **A**utism through **M**aternal Immune **A**ctivation and proteomic **D**iscovery)

This assent form must be used in conjunction with the appropriate parent/legal guardian consent form. On its own, it does not provide informed consent for a minor to take part

Participant Name:
Participant DOB:
Study Number:

Principal Investigator: Professor Deirdre Murray Professor of Paediatrics and Child Health University College Cork

in the study.

Please circle whatever you agree with:

7. I read through the fact sheet with my parent(s)/guardian(s) and I understand the information that is given about this study.

Yes

No

8. I understand what this study is about and what I am supposed to do.

Yes

No

9. I agree to information being collected about me from my parent(s)/guardian(s).

Yes

No

10. I agree to the researchers accessing my previous INFANT files and for my medical notes to be used in this study.

Yes

No

11. I agree to the researchers taking a sample of my blood for this study

Yes

No

12. I would like to take part in this study and I understand that I don't have to if I don't want to.

Yes

No

Name of Participant (Please Print)

Signature

Date

PiRAMiD Study: PRedicting early onset Autism through Maternal Immune Activation and proteomic Discovery.

INFORMATION SHEET AND ASSENT FORM FOR CHILDREN – CONTROL GROUP (BASELINE)

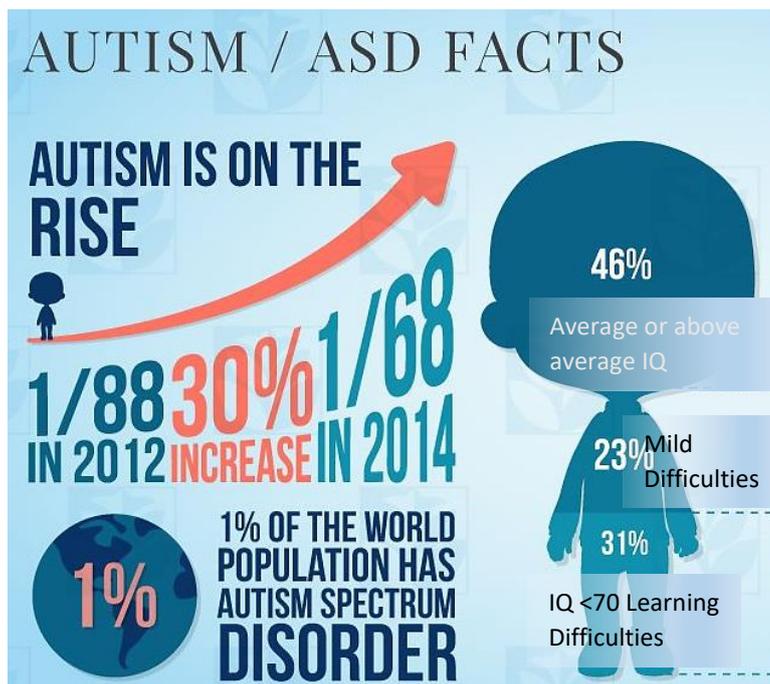
(Use in conjunction with the Parent(s)/Guardian(s) ICF – Informed Consent Form)

Introduction:

As a baby, you took part in a study in Cork University Hospital (CUH) called the BASELINE study (**Babies After Scope: Evaluating the Longitudinal Impact using Neurological and Nutritional Endpoints**) **as part of a control group** (controls are healthy people who we compare with people who have specific illnesses). During the study you had a number of tests at different times in your childhood. You were checked at age 2 months, 6 months, 12 months, 2 years and at 5 years of age. During these checks we were looking for information about your growth and health. The research we have done with you and your family's help has already helped us to understand children's growth, development, diet, and allergies

better.

Now, we are inviting you and your parents to take part in another study called PiRAMiD (**PR**edicting early onset Autism through **MA**ternal Immune Activation and **PD**roteomic Discovery). PiRAMiD is an exciting new study looking at why some of the children from BASELINE have ASD (Autism Spectrum Disorder) and others do not. This research is being done



by The INFANT CENTRE (Irish Centre for Fetal and Neonatal Translational Research) in Cork University Hospital and is paid for by the NCRC (National Children's Research Centre in Dublin).

What is informed Assent?

This fact sheet will help you understand what will happen to you during this study. When you have read the information, you can decide if you would like to take part. If you do want to take part in the study, you will sign your name on the assent form at the end of this fact

sheet. This is called “informed assent”. It means that you have been told all about the study, that you understand what will happen, and that you want to take part.

We will also explain the study to your parent(s)/guardian(s) and they will decide whether they would like you to take part. They will be given a Parent Information Leaflet and Informed Consent Form to sign if they are happy for you to participate. Both you and your parent(s)/guardian(s) must agree

that you want to take part in the study. If this fact sheet contains words or ideas that you do not understand, please ask your parent(s)/guardian(s) to explain them or else ask the study doctor.



Do I have to take part?

It is great that you are reading this document and thinking about joining this study but we understand if you decide not to take part. It is up to you and your parent(s)/guardian(s) to decide. You can say “No” now, or at any time during the study if you change your mind for any reason. You just need to tell the people in charge of the study. This will not affect the way you are treated by your doctors in the Cork University Hospital now, or in the future.

What will I have to do if I take part?

If you wish to join this study, once you have read this information you should sign the Assent Form at the end of this document. By signing this form, you allow us to look at your hospital medical notes. You also agree to your parent(s)/guardian(s) filling in

questionnaires about you, and allow us to study this information. Your parent(s)/guardian(s) will be asked to complete a questionnaire about you when you come to the INFANT centre and should take no longer than 10 minutes to do. The questions are about your listening, talking, behaviour and emotional skills. During the visit we will also measure your growth and abilities and we will take a small sample of blood from you. These checks and tests should take no longer than 1 hour in total to finish, and you can have a break if necessary (we have snacks). The blood test will be performed by our experienced children’s doctor and should only take a couple of minutes. We will study blood samples from you, your mother and your father, and try to answer our questions about what causes ASD in children.

What are the good or bad things that might happen if I take part?

The good things about taking part in this study is that it will help us to understand what causes Autism Spectrum Disorder, and allow us to check your strengths and weaknesses

against your previous appointments with us. We hope this understanding will lead to better care for future children who have ASD.

There are no expected bad things from taking part in this study for you but there is a blood test which is a little bit painful. If you are worried about anything in the study, you can ask your parent(s)/guardian(s) or you can ask the research doctor to explain things better to you.

What happens to my information collected in the study?

All the information you give us will be treated in confidence (kept secret) and we will protect your privacy (name or any personal details), we will make sure not to have your name on anything. If the

information we receive suggests you are at risk of harm, then we would have to act to reduce this risk. All your personal information will be stored safely at the INFANT centre and information without your name on it will be stored on the researchers' password protected computers at UCC. Once the study is finished, we will make a report that we hope will bring new information about ASD to UCC, and the world. Nobody reading the report/paper will know it is you as the report will only have the group results – that is results from everyone who took part in the study, without names, addresses or dates of birth. Your parent(s)/guardian(s) may also request a summary of the findings once complete and it will be available on the INFANT website once ready.

What if I have any questions?

If this fact sheet contains words or ideas that you do not understand, please ask your parent(s)/guardian(s) to explain them or else ask the study staff present during your visit. You can keep a copy of this form and make sure that we have answered all your questions before you decide whether you want to take part in the study. Otherwise, if you are happy to take part in this research study, please fill in and sign the assent form at the end of this document.

PiRAMiD study (**PR**edicting early onset **A**utism through **M**aternal Immune **A**ctivation and proteomic **D**iscovery)

This assent form must be used in conjunction with the appropriate parent/legal guardian consent form. On its own, it does not provide informed consent for a minor to take part

Participant Name:
Participant DOB:
Study Number:

Principal Investigator: Professor Deirdre Murray Professor of Paediatrics and Child Health University College Cork

in the study.

Please circle whatever you agree with:

13. I read through the fact sheet with my parent(s)/guardian(s) and I understand the information that is given about this study.

Yes

No

14. I understand what this study is about and what I am supposed to do.

Yes

No

15. I agree to information being collected about me from my parent(s)/guardian(s).

Yes

No

16. I agree to the researchers accessing my previous INFANT files and for my medical notes to be used in this study.

Yes

No

17. I agree to the researchers taking a sample of my blood for this study

Yes

No

18. I would like to take part in this study and I understand that I don't have to if I don't want to.

Yes

No

Name of Participant (Please Print)

Signature

Date:

Appendix 2: Supplementary Material from Multi-Omics study

Supplementary Figures

Figure 1: Multiomics recruitment flow chart

Flowchart: Supplementary figure 1

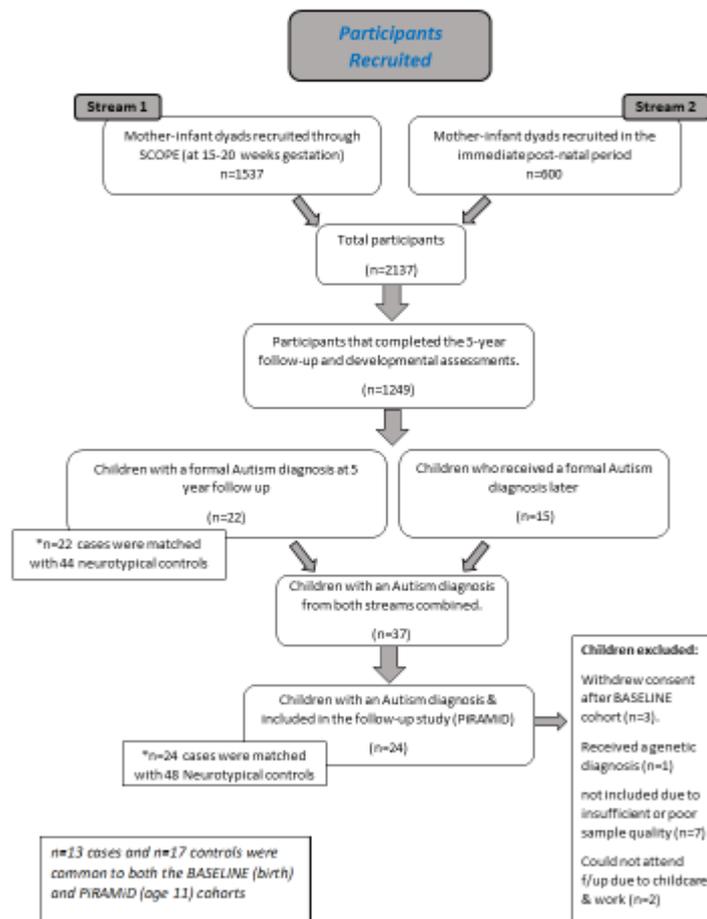


Figure 41: Recruitment flow to multi-omics study

Supplementary Figure 2: Proteomic analysis

Supplementary Figure 2: Serum Proteomics

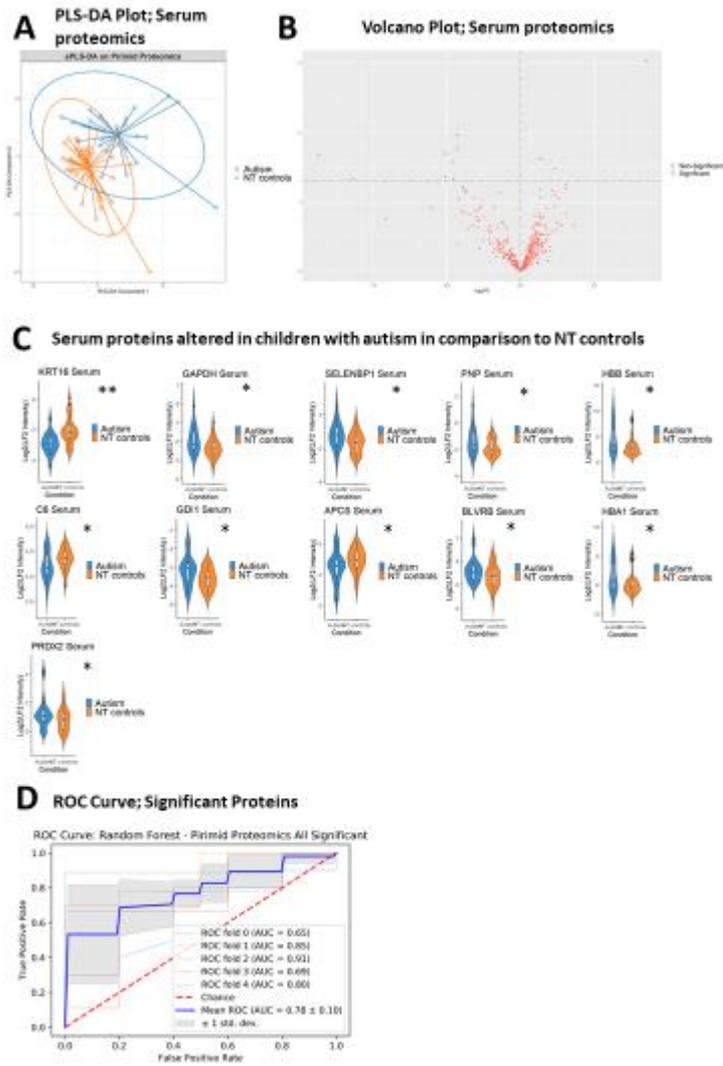


Figure 42: Serum proteomics Graphics and Outputs

Supplementary Figure 3: Cord blood Proteomics

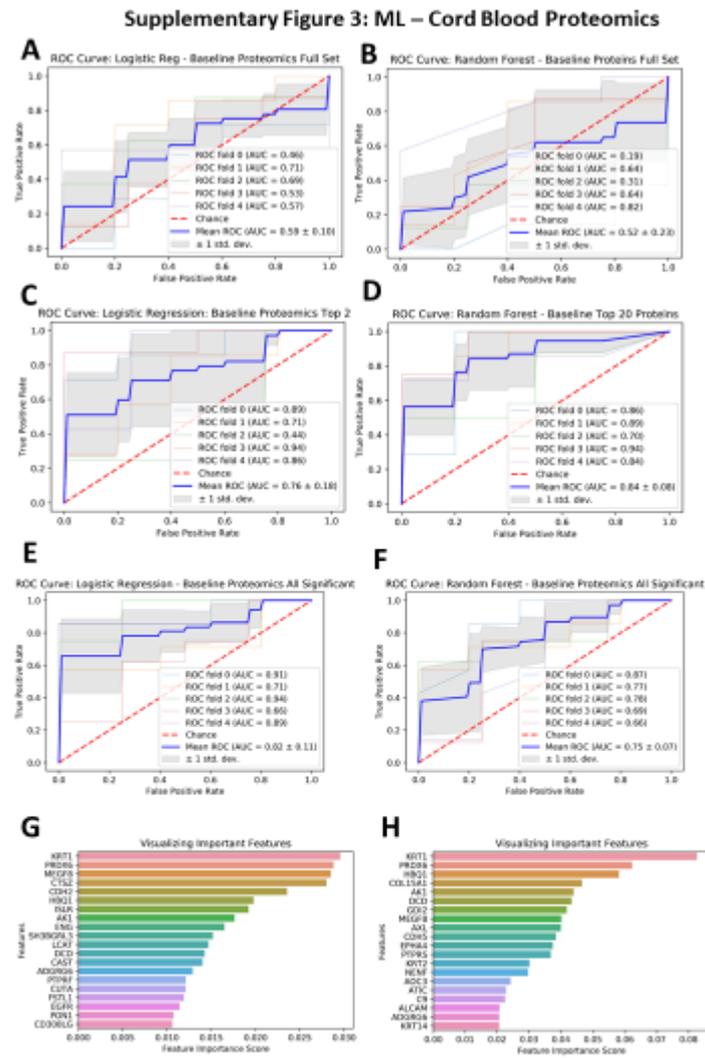


Figure 43: Cord blood Proteomics and Outputs

Supplementary Figure 4: PiRAMiD proteomics (Age 7 - 10)

Supplementary Figure 4: Pyramid Proteomics

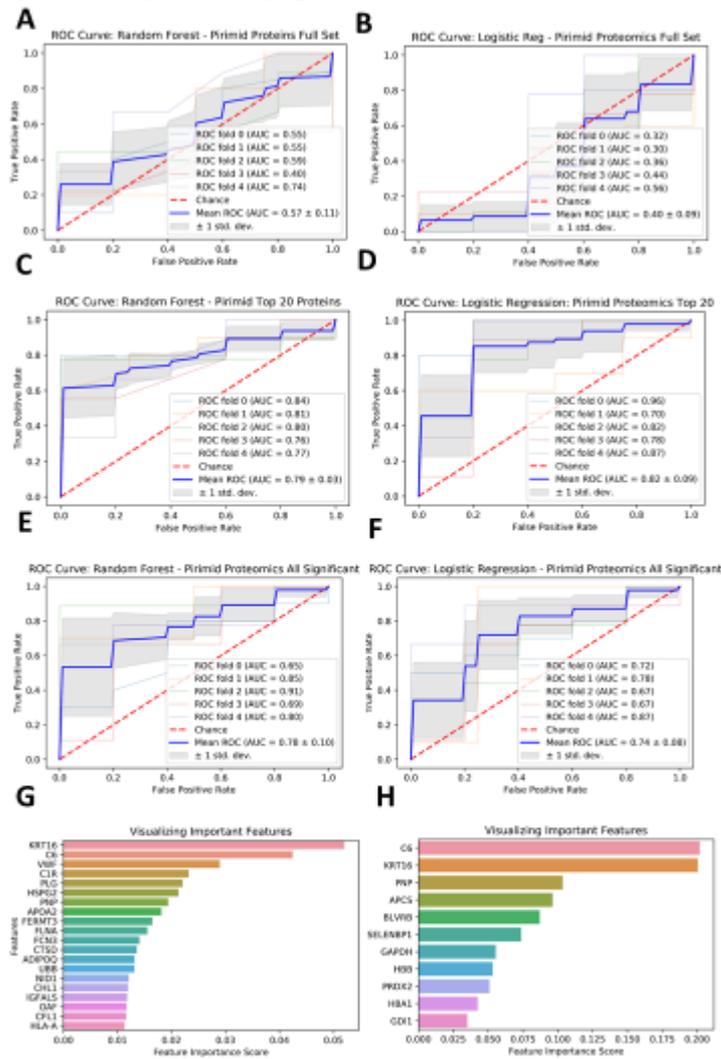


Figure 44: Late Childhood Proteomics and Outputs

Supplementary Figure 5: Cord Blood Metabolomics

Supplementary Figure 5: Cord Blood Metabolomics

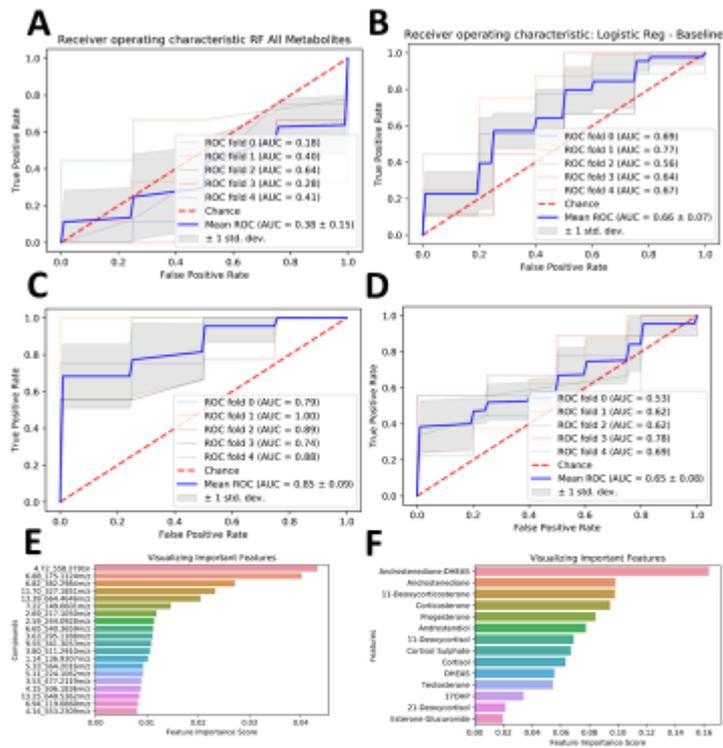


Figure 45: Cord Blood Metabolomics and Outputs

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

REAGENTS AND CHEMICALS

Proteomics

All reagents used were HPLC grade and prepared using ultrapure water with 18.2 MΩ cm resistivity (Milli-Q direct water purification system, Watford, UK). COMplete ULTRA Tablets, mini, EASYpack were purchased from Roche (Ireland). Bio-rad Bradford dye reagent was purchased from Bio-Rad (Kildare, Ireland). Sodium phosphate monobasic monohydrate, Sodium phosphate dibasic dehydrate, Acetic acid, Ammonium Bicarbonate, Bovine Serum Albumin (BSA), Tetraethylammonium bicarbonate buffer (TEAB), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), Iodoacetamide (IAA), Formic Acid (FA), and Trifluoroacetic acid (TFA) were all purchased from Sigma-Aldrich (Ireland). RapiGest SF surfactant was purchased from Waters (Wexford, Ireland). Promega Seq Grade trypsin was purchased from Promega (Madison, USA), Sodium chloride (NaCl) and Acetonitrile were purchased from Fisher Scientific (Loughborough, UK). LoBind tubes were purchased from (Merck and Sarstedt, Feltham, UK and Wexford, Ireland). 0.22µm spin filters and the Human-14 Multi Affinity Removal column were purchased from Agilent technologies (Ireland). 10kDa Molecular weight cut-off (MWCO) Amicon ultra-2 Centrifugal filter units and Zip-tip pipette tips with 0.6µl C18 resin were purchased from Merck-Millipore (Ireland). 96 well plates were purchased from Sarstedt (Wexford, Ireland).

HPLC immunodepletion: Due to the dynamic range of proteins in plasma, immunodepletions are important to remove the most highly abundant proteins from each sample. We used a Human 14 multiple affinity removal column coupled to a High performance liquid chromatography (HPLC) system, thereby targeting the top 14 most abundant proteins in plasma. The 14 proteins targeted and depleted from each sample were; Albumin, Apolipoprotein AI, Apolipoprotein AII, Complement C3, Fibrinogen, Haptoglobin, IgA, IgG, IgM, Transferrin, Transthyretin, α1-Antitrypsin, α1-Acid Glycoprotein, α2-Macroglobulin.

Firstly, each of the 66 plasma samples underwent a 1:4 sample dilution (45µl crude sample:135µl buffer A with protease inhibitor) before being transferred into a 0.22µm spin filter and spun down in the centrifuge at 16,000g and room temperature. 160µl of the filtered sample was then drawn up, injected into the HPLC system and a pre-designed method based on manufactures instructions was run.

The unbound proteins, which are allowed to 'flow through' create the LA fraction. LA fractions were used for all subsequent analytical steps outlined below. We are generally interested in the proteins which exist in lower abundances due to their potential in acting as protein signatures and potential biomarkers indicative of ASD and other diseases.

Concentration buffer exchange: LA and HA fractions are relatively dilute after HPLC immunodepletion, therefore concentration of these fractions is important for subsequent pre-processing steps. 10kDA Molecular Weight Cut-Off(MWCO) Amicon Ultra-2 centrifugal filters were used firstly, to concentrate our fractions and secondly, to perform buffer exchange steps. All steps were undertaken according to manufacturer's guidelines. Buffer exchange involved reconstituting each newly concentrated sample to its original volume using a 50mM ammonium bicarbonate solution.

A 50mM ammonium bicarbonate solution is preferable for storage of samples between steps and additionally is compatible with subsequent stages of protein digestion. Samples were stored at -20°C between immunodepletion and concentrating steps. They were then stored again at -80°C until protein quantification of all samples could take place.

Protein quantification: Protein quantification is essential to find out the total concentration of protein in each sample, when compared against a known standard. In this instance, protein quantification prior to sample digestion was determined using a Bradford assay, and Bovine Serum Albumin (BSA) was used to create standard solutions of varying concentration. A quality control (QC) was created by pooling 3ul from each sample. The QC was treated in the same way as each sample. 10ul of each reconstituted sample, QC and standard was pipetted in duplicate into a separate well of a 96 well plate. 200ul of Bradford reagent at 1:5 dilution was then added to each sample and standard. Absorbance readings were measured using a plate reader at 595nm. The standard curve was plotted using excel, and sample concentrations were determined from this graph.

Undertaking protein quantification of the QC at the same time facilitated us in determining its protein concentration, for digestion and its similarity in concentration to the other 66 plasma samples. OCs are important to condition the mass

spectrometry instrument column before and additionally at set intervals during the experiment run to monitor performance.

Protein digestion: Based on calculations from Bradford assays, the protein concentration of each sample was known. The volume of each sample corresponding to 50ug of protein was transferred into a new labelled Eppendorf for tryptic protein digestion. This is a two-day process using RapiGest, TCEP, IAA, Trypsin, FA and TFA, and is followed with desalination and sample clean-up using Zip-tips. All processes were undertaken as previously described in English et al., (2015).

LC-MS/MS analysis (discovery proteomic analysis): Following HPLC depletion, sample concentration and buffer exchange, protein quantification, and sample clean-up, all samples and internal standards were sent on dry ice to UCD for LC-MS/MS analysis.

Proteomics samples were loaded onto EvoTips and run on a timsTOF Pro mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to the EvoSep One system (EvoSep BioSystems, Odense, Denmark). The peptides were separated on a reversed-phase C₁₈ Endurance column (15cm x 150µm ID, C₁₈, 1.9 µm) using the preset 30 SPD method. Mobile phases were 0.1% (v/v) formic acid in water (phase A) and 0.1% (v/v) formic acid in acetonitrile (phase B). The peptides were separated by an increasing gradient of mobile phase B for 44 minutes using a flow rate of 0.5 uL/min. The timsTOF Pro mass spectrometer was operated in positive ion polarity with TIMS (Trapped Ion Mobility Spectrometry) and PASEF (Parallel Accumulation Serial Fragmentation) modes enabled. The accumulation and ramp times for the TIMS were both set to 100 ms., with an ion mobility (1/k0) range from 0.6 to 1.6 Vs/cm. Spectra were recorded in the mass range from 100 to 1,700 m/z. The precursor (MS) Intensity Threshold was set to 2,500 and the precursor Target Intensity set to 20,000. Each PASEF cycle consisted of one MS ramp for precursor detection followed by 10 PASEF MS/MS ramps, with a total cycle time of 1.17 s. A diaPASEF scheme consisting of 46 precursor isolation windows of 25 m/z width, covering a mass range of 300 – 1450 m/z and an ion mobility range of 0.6 to 1.6 Vs/cm, was created using the Bruker timsControl interface (2.0.53).

Bioinformatics and statistical analysis: Fragpipe (version 18.0) is a computational platform comprising a suite of tools for the analysis of proteomics mass spectrometry data, from peptide searching to validation and quantitation. Using these tools FragPipe provides multiple workflows including DIA_SpecLib_Quant which was used for the analysis of the DDA and DIA data. In this workflow MSFragger is first used to search DDA data, search results are validated by Percolator and proteins identified with ProteinProphet. A spectral library is built by EasyPQP and quantitation of DIA data is performed by the DIA-NN module. The protein groups output file (pg_matrix) contains normalised intensities and are filtered at 1% FDR. Statistical analysis was performed in Perseus (V2.0.5.0) using the pg_matrix file as input.

Proteomic analysis of serum from the PiRAMiD cohort

DIA data was processed in the open-source Skyline software tool (open-source Skyline software tool¹ (<https://skyline.gs.washington.edu>)). This tool provided the interface for visual confirmation of protein biomarkers in the samples profiled, without any file conversion. The library was constructed by searching the QC injections, which were interspersed after every ten injections throughout the run. As detailed in the online tutorials and publications by the Skyline team, the msms.txt file resulting from the MaxQuant search was used to build the library in Skyline. For our peptide targets, mass chromatograms were extracted for +2 and +3 precursor charge states and their associated fragment ions. Based on our proteomic discovery results, a skyline target list was created for SERBINBP1, GAPDH, BLVRB protein candidates according to the detailed protocol of Egertson et al (2015)(530). For our dataset, the *m/z* tolerance was <10ppm and the average retention time window was 2 minutes. All parent and fragment level data was visually confirmed across the samples, and peak editing was undertaken where necessary, using the peptide Retention Time (RT), dotproduct (idop), mass accuracy (<20ppm), and a confirmed library match to reliably identify and quantify peptides across the DIA runs. For statistical analysis, peak areas of the fragment level data was filtered from the Skyline document grid for analysis in mapDIA, an open source bioinformatics tool for pre-processing and quantitative analysis of DIA data (531). Total Ion Sum (TIS) intensity normalisation procedure was applied, followed by peptide fragment selection using 2 standard deviation threshold for outlier detection, in the independent sample setup. Differential expression analysis was based on a Bayesian latent variable model, as described (531).

Metabolomics

REAGENTS AND CHEMICALS

Metabolomics Ultrapure water with 18.2 M Ω cm resistivity (Milli-Q direct water purification system, Watford, UK) was used to prepare all mobile phase components. LC grade methanol and estrone glucuronide standard were purchased from Merck (Feltham, UK). Cortisol sulfate was purchased from Generon. LC-MS grade acetonitrile (ACN), methanol and formic acid were purchased from Fisher Scientific (Loughborough, UK). LC-MS glass vials, Ostro 96 well phospholipid and protein removal plates plus 2 mL collection plates, MassTrak Endocrine steroid calibrator set and ultra-performance liquid chromatography (UPLC) columns were purchased from Waters (Waters, Wexford, Ireland). Isotope-labelled steroid mix was purchased from Chromsystems. Nitrogen and argon gas were supplied by BOC gases (Dublin, Ireland). Metabolomics Ultrapure water with 18.2 M Ω cm resistivity (Milli-Q direct water purification system, Watford, UK) was used to prepare all mobile phase components. LC grade methanol and estrone glucuronide standard were purchased from Merck (Feltham, UK). Cortisol sulfate was purchased from Generon. LC-MS grade acetonitrile (ACN), methanol and formic acid were purchased from Fisher Scientific (Loughborough, UK). LC-MS glass vials, Ostro 96 well phospholipid and protein removal plates plus 2 mL collection plates, MassTrak Endocrine steroid calibrator set and ultra-performance liquid chromatography (UPLC) columns were purchased from Waters (Waters, Wexford, Ireland). Isotope-labelled steroid mix was purchased from Chromsystems. Nitrogen and argon gas were supplied by BOC gases (Dublin, Ireland).

Targeted Steroid analysis at birth and age 11

Chemical reagents, Standards and Columns

All reagents used in this study were of LC-MS grade unless specified otherwise. Acetonitrile (HPLC grade), methanol, ammonium acetate and formic acid were purchased from Sigma-Aldrich (Wicklow, Ireland). Certified calibrators as part of Mass Trak kit were purchased from Waters Corporation (Drinagh, Co. Wexford, Ireland), which contained the following steroids at appropriate concentrations. DHEA-S (61-29392 nmol/L), cortisol (3-1388 nmol/L), 21-deoxycortisol (0.14-141 nmol/L),

corticosterone (0.14-141 nmol/L), 11-deoxycortisol (0.15-144 nmol/L), androstenedione (0.17–169 nmol/L), 11-deoxycorticosterone (0.03-59 nmol/L), testosterone (0.05-74 nmol/L), DHEA (0.90-224 nmol/L), 17 a-hydroxyprogesterone (0.15-293 nmol/L), DHT (0.10-8.2 nmol/L) and progesterone (0.08-164 nmol/L). Additional standards not present in the MassTrak endocrine calibrator kit were purchased from Generon (Clontarf, Dublin, Ireland) and Merck, These included estrone glucuronide (Merck), cortisol sulfate (Generon), andepiandrosterone (Merck). These additional standards were made up to a stock solution of 1mg/ml and were diluted to appropriate concentration to be spiked into the calibrators. The internal standard (IS) in a methanol solution was purchased from Chromsystems to be spiked into relevant samples and standards. The contents of the IS consisted of aldosterone-d4, corticosterone-d8, cortisol-d4, cortisone-d8, 11-deoxycortisol-d5, 21-deoxycortisol-d8, androstendione-13C3, dehydroepiandrosterone-d5, 11-deoxycorticosterone-d8, DHEAS-d6, dihydrotestosterone-d3, estradiol-d5, 17-hydroxyprogesterone-13C3, progesterone-13C3 and testosterone-d3. All standards and stock solutions mentioned above were stored at -200C, excluding the MassTrak endocrine calibrator kit which was stored at 40C after reconstitution with water as per instructions.

Sample Extraction

10µl of IS solution and 50µl of the individual cord blood serum and paediatric plasma aliquots were added to individual wells within an Ostro Plate (Waters Corporation, Milford, MA, USA). After the addition of the IS, 150µl of 1% formic acid in acetonitrile solution was added to the samples and mixed via aspiration. The Ostro Plate was put on a positive pressure manifold at 60 psi for 5 minutes using compressed nitrogen gas and drawn through the sorbent contained at the base of each well to retain proteins and phospholipids and thus extracting the analytes of interest. The resulting solution collected in the collection plate was taken and dried down by placing in a MiVac QUATTRO concentrator vacuum centrifuge for 2 hours at 450C. The dried samples were then reconstituted in 150µl of equal parts of 2mM ammonium acetate in H₂O and 2mM ammonium acetate in methanol for injection into the LC-MS/MS.

Maximizing measurement accuracy and selectivity of endocrine steroids

As per the guidelines stated by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA), quality control within a bioanalytical method occurs through the spiking of a known concentration of analyte into a blank biological matrix of use in the assay (European Medicines Agency et al., 2017; Food and Drug Agency,

2018). Stripped serum spiked with the relevant steroid analytes of known concentrations were used and were obtained as part of the MassTrak endocrine steroid calibrator and quality control kit. QCs contained progesterone (0.1, 1.0, 35.0 ng/mL), testosterone (0.05, 0.5, 14.0 ng/mL), androstenedione (0.2, 2.0, 35.0 ng/mL), cortisol (10.0, 100.0, 350.0 ng/mL), corticosterone (0.2, 2.0, 35.0 ng/mL), 11-deoxycortisol (0.2, 2.0, 35.0 ng/mL), 21-deoxycortisol (0.2, 2.0, 35.0 ng/mL), 11-deoxycorticosterone (0.035, 0.35, 15.0 ng/ mL), 17-hydroxyprogesterone (0.2, 2.0, 70.047ng/mL), dihydrotestosterone (0.1, 1.0, 1.75 ng/mL), dehydroepiandrosterone Sulfate (200, 2000, 8750 ng/mL), dehydroepiandrosterone (0.8, 8.0, 35.0 ng/mL) and consisted of a low, medium and high QC concentration respectfully. The LC-MS/MS performance characteristics are outlined below. The limit of detection (LOD) values for each steroid was determined by the concentrations of the analyte in the serum matrix where the signal-to-noise (S/N) ratio was equal to 3. The lower and upper limits of quantification (LLOQ and ULOQ, respectively) are depicted by the linear ranges for each steroid analytes. The linearity of the calibration curves was determined by the r^2 value, where all curves had a value of ≥ 0.98 . The matrix effect (i.e. the endogenous constitution of the sample) the stability and reactivity of the steroid analytes were determined by taking blank individual cord blood and paediatric blood samples and spiking with the IS. The matrix factor was calculated by calculating the ratio of the peak area of the IS within the serum/plasma matrix to the peak area without the matrix present. The credibility of the samples after multiple freeze thaw events was measured by spiking blank pooled cord serum with the IS and measuring the peak area over the course of three days. The pooled cord serum sample was frozen and thawed at 40C each day over the course of three days. Precision of the assay was determined by performing an intra-assay on 5 replicates of pooled cord blood serum within the individual assay. To determine precision across multiple assays, an inter-assay was performed on 3 replicates of pooled cord blood over the course of three days and comparing the coefficient variance between the samples.

LC-MS/MS analysis

Quantitative analysis was performed on a Waters Acquity UPLC coupled with a triple quadrupole mass detector (Xevo TQ MS) using electrospray ionization in positive mode for all steroids analysed but DHEA-S, estrone glucuronide, cortisol and cortisol sulfate, which were analysed in negative mode. The column temperature was set to 500C, while the samples within the 96 well plate were kept at 80C. The mobile phase A used in the UPLC system was 2 mM ammonium acetate in water. Whilst mobile phase B consisted of 2 mM ammonium acetate in methanol. The flow rate of the

mobile phases through the column was 0.4 ml/min. The sample was injected using an autosampler in partial loop mode with needle overfill with a 50µL loop, the injection volume was 20 µL. The initial flow rate of the mobile phase consisted of 65% of mobile phase A, 35% of mobile phase B, ramping to 20:80(A:B) at 9 minutes. A one minute high organic flush 2:98 (A:B), followed by equilibration back to 65:35 (A:B) to complete the run with a total 11 minutes run time. Collision gas was provided through compressed tank of argon (BOC Gases Ireland, Dublin), while desolvation gas was created through a Peak nitrogen generator (Peak Scientific Instruments Ltd, Inchinnan, UK). The mass spectrometer was programmed to monitor the transitions involved in the MRM assay. Mass spectrometry programming was done through the use of the software MassLynx. In addition, MRM assay analysis of the analytes was performed using the software TargetLynx. Calibration curves were constructed and samples quantified using the ratio of analyte response/IS response with 1/x weighting. The internal standard used for each analyte was its corresponding isotopically labelled analogue where available. DHT-d3 was used as the IS for epiandrosterone and DHEAS-d6 was used as the IS for cortisol sulfate and estrone glucuronide.

SUPPLEMENTARY RESULTS

TABLES

Supplementary Table1: Discovery proteomic analysis in cord blood identified 41 proteins as significantly differentially expressed ($p < 0.05$) between ASD cases and neurotypical controls from the BASELINE birth cohort.

Table 20: Supplementary Table 1: Discovery proteomic analysis in cord blood

Gene Names	Gene/Protein Names	Protein IDs	P Value	FDR	Fold Change	Random Forest Ranking
GDI2	GDP Dissociation Inhibitor 2	P50395	0.00216 6586	0.5701 64158	0.9886	7
HBQ1	Hemoglobin Subunit Theta 1	P09105	0.00 567223 9	0.5701 64158	0.9897	3
LUM	Lumican	P51884	0.00710 925	0.5701 64158	1.0047	-
KRT2	Keratin, type II cytoskeletal 2 epidermal	P35908	0.00788 7589	0.5701 64158	0.9791	13
AOC3	Amine oxidase, copper containing 3	Q16853	0.00984 5084	0.5701 64158	1.0727	15

KRT1	Keratin, type II cytoskeletal 1	P04264	0.01007 0379	0.5701 64158	0.9904	1
KRT10	Keratin, type I cytoskeletal 10	P13645	0.01035 6839	0.5701 64158	0.9831	-
CA2	Carbonic Anhydrase 2	P00918	0.01042 5949	0.5701 64158	0.9877	-
ADGRG6	Adhesion G Protein-Coupled Receptor G6	Q86SQ4	0.01085 0719	0.5701 64158	1.0221	19
BLVRB	Biliverdin Reductase B	P30043	0.01141 9441	0.5701 64158	1.0061	-
YWHAE	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Epsilon. Provides instructions for making the 14-3-3 epsilon protein	P62258	0.01229 171	0.5701 64158	0.9577	-
PRDX6	Peroxiredoxin 6	P30041	0.01318 4028	0.5701 64158	0.9869	2
MEGF8	Multiple EGF Like Domains 8	Q7Z7M0	0.01390 0606	0.5701 64158	1.0338	8
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	P04406	0.01430 5194	0.5701 64158	0.9804	-
ATIC	5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase/IMP Cyclohydrolase	P31939	0.01709 3495	0.5826 85934	0.9963	16
AXL	Tyrosine-protein kinase receptor UFO	P30530	0.01728 5258	0.5826 85934	0.9971	9
SEZ6L2	Seizure Related 6 Homolog Like 2	Q6UXD5	0.01775 208	0.5826 85934	1.0752	-
ANXA7	Annexin A7	P20073	0.01945 8539	0.6023 13675	1.0051	-
DCD	Dermcidin	P81605	0.02238 8773	0.6023 13675	1.0065	6
EIF4A1	Eukaryotic initiation factor 4A-I	P60842	0.02499 4724	0.6023 13675	0.9932	-
RAN	ras-related nuclear protein	P62826	0.02556 2896	0.6023 13675	1.0026	-
AK1	Adenylate Kinase 1	P00568	0.02603 7085	0.6023 13675	0.9908	5
MCAM	Cell surface glycoprotein MUC18; Melanoma Cell Adhesion Molecule	P43121	0.02793 1856	0.6023 13675	1.3186	-
SELENBP1	Selenium Binding Protein 1	Q13228	0.02830 3213	0.6023 13675	0.9982	-

HBM	Hemoglobin Subunit Mu	Q6B0K9	0.02850 1608	0.6023 13675	1.1214	-
CD93	Complement component C1q receptor; Cluster of Differentiation 93	Q9NPY3	0.02891 0253	0.6023 13675	1.0068	-
NENF	Neudessin Neurotrophic Factor	Q9UMX5	0.03212 2857	0.6023 13675	1.0218	14
CA1	Carbonic anhydrase 1	P00915	0.03238 4499	0.6023 13675	0.9620	-
NAGLU	Alpha-N-acetylglucosaminidase	P54802	0.03427 295	0.6023 13675	1.0012	-
PTPRS	Protein Tyrosine Phosphatase Receptor Type S	Q13332	0.03437 2813	0.6023 13675	1.0032	12
EPHA4	Ephrin type-A receptor 4	P54764	0.03816 7904	0.6023 13675	1.2826	11
CFB	Complement factor B	P00751	0.03845 1334	0.6023 13675	1.0080	-
ALCAM	CD166 antigen; Activated Leukocyte Cell Adhesion Molecule	Q13740	0.03851 6272	0.6023 13675	1.0891	18
KRT14	Keratin, type I cytoskeletal 14	P02533	0.03858 7889	0.6023 13675	0.9410	20
COL15A1	Collagen Type XV Alpha 1 Chain	P39059	0.03914 6764	0.6023 13675	1.0323	4
LBP	Lipopolysaccharide-binding protein	P18428	0.04060 0093	0.6023 13675	1.0049	-
CDH5	Cadherin-5	P33151	0.04125 9138	0.6023 13675	1.0105	10
CTSZ	Cathepsin Z	Q9UBR2	0.04227 4464	0.6023 13675	0.9998	-
BLVRA	Biliverdin reductase A	P53004	0.04287 9132	0.6023 13675	1.0055	-
C9	Complement component C9	P02748	0.04317 6608	0.6023 13675	0.9997	17
KRT5	Keratin, type II cytoskeletal 5	P13647	0.04854 6057	0.6421 44965	0.9457	-

Supplementary table 2: Discovery proteomic analysis follow-up serum samples identified 11 proteins as significantly differentially expressed ($p < 0.05$) between ASD cases and neurotypical controls from the PiRAMiD cohort.

Table 21: Supplementary table 2: Discovery proteomic analysis follow-up serum samples

Gene Names	Gene/Protein Names	Protein ID's	p-value
KRT16	Keratin, type I cytoskeletal 16	P08779	0.000933134
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	P04406	0.010997854
SELENBP1	Selenium Binding Protein 1	Q13228	0.016770749
PNP	Purine nucleoside Phosphorylase	P00491	0.020355303
HBB	Hemoglobin Subunit Beta	P68871	0.021538221
C6	Complement component 6	P13671	0.0236205
GDI1	GDP Dissociation Inhibitor 2	P31150	0.026480433
APCS	Serum amyloid P component	P02743	0.033238926
BLVRB	Biliverdin Reductase B	P30043	0.04388492
HBA1	Hemoglobin Alpha 1	P69905	0.045904784
PRDX2	Peroxiredoxin 2	P32119	0.0474467

Supplementary Table 3: Discovery metabolomics analysis in ASD cord blood identified 45 features as significantly differentially expressed ($p < 0.05$) between ASD cases and controls from the BASELINE birth cohort. Where possible, putative annotations were checked and listed.

Table 22: Supplementary Table 3: Discovery metabolomics analysis in ASD cord blood

Compound	Description	Compound ID	P_Value	FDR	Fold Change	Random Forest Rank
2.69_217.1050m /z	2,3-Butanediol glucoside	HMDB00408 22	0.004301	0.990192	1.156236 91	4
13.39_664.4646 m/z	PC(18:3(6Z,9Z,12Z)/ 12:0)	LMGP01011 640	0.004845	0.990192	0.908641 331	1
6.88_175.1124m /z	4-Methyl-2-phenyl- 2-pentenal	HMDB00372 81	0.005565	0.990192	0.848721 83	2
2.30_103.0548m /z	UNKNOWN	UNKNOWN	0.008796	0.990192	1.148506 016	9
9.99_538.3869m /z	LysoPE(0:0/22:0)	HMDB00114 90	0.009543	0.990192	0.625783 637	-
7.27_263.2037m /z	2- [2(Decylsulfanyl)eth oxy]ethanol	CSID1798304 8	0.010991	0.990192	0.626183 356	13
4.72_558.2791n	UNKNOWN	UNKNOWN	0.014168	0.990192	1.200699 924	3

8.75_255.2111m /z	5-Androstenediol	HMDB00038 18	0.014295	0.990192	0.790952 303	14
13.04_445.2524 m/z	3,4-Dihydroxy- tamoxifen	HMDB00610 87	0.015983	0.990192	0.914750 772	8
4.53_627.3747m /z	UNKNOWN	UNKNOWN	0.019299	0.990192	1.306797 897	5
6.94_119.0860m /z	Nona-2,4,6-trienal	HMDB00324 41	0.019618	0.990192	1.603443 605	6
4.25_305.2216m /z	Hydroxy-alpha- sanshool	HMDB00295 67	0.019984	0.990192	0.603344 764	-
4.63_379.1958m /z	Pregnenolone sulfate	HMDB00007 74	0.025526	0.990192	0.846372 061	-
4.21_303.2067m /z	Phygrine	HMDB00397 54	0.028519	0.990192	0.530432 957	-
5.64_331.2632m /z	4,7,10,13,16- Docosapentaenoic acid	HMDB02466 21	0.02856	0.990192	0.821931 033	16
8.13_171.1170m /z	10beta-12,13- Dinor-8-oxo-6- eremophilen-11-al	HMDB00376 04	0.029591	0.990192	0.914740 326	-
1.14_136.9307m /z	Sulfate	HMDB00014 48	0.029977	0.990192	1.240168 358	7
4.84_347.2219m /z	19- Hydroxydeoxycorti costerone	HMDB00126 12	0.031493	0.990192	0.805471 817	10
6.65_540.3659m /z	O-{Hydroxy[(2R)-2- hydroxy-3- (icosyloxy)propoxy] phosphoryl}-L- serine	CSID1133767 68	0.033198	0.990192	1.222018 159	11
5.91_345.2069m /z	UNKNOWN	UNKNOWN	0.037099	0.990192	0.843159 518	-

12.70_774.5670 m/z	PS(18:0/18:0)	HMDB00123 78	0.03729	0.990192	1.142462 403	18
2.27_224.1287m /z	UNKNOWN	UNKNOWN	0.037552	0.990192	1.095251 356	-
5.43_317.2467m /z	Tetrahydrodeoxyco rticosterone	HMDB00008 79	0.039772	0.990192	0.865336 971	17
5.36_493.2726m /z	UNKNOWN	UNKNOWN	0.040949	0.990192	0.758581 583	-
4.51_303.1967m /z	19-Hydroxyandrost- 4-ene-3,17-dione	HMDB00039 55	0.042417	0.990192	0.748523 985	12
12.43_443.2386 m/z	Dimethyl 3- methoxy-4-oxo-5- (8,11,14- pentadecatrienyl)- 2-hexenedioate	HMDB00320 99	0.043819	0.990192	0.881774 145	-
4.96_283.1094m /z	5b-Cyprinol sulfat	HMDB00068 88	0.04431	0.990192	1.142574 47	-
4.69_305.2119m /z	6beta- Hydroxytestostero ne	HMDB00062 59	0.045265	0.990192	0.821863 415	-
5.89_511.3269n	UNKNOWN	UNKNOWN	0.045982	0.990192	1.258040 214	15
3.98_284.1849m /z	(3S,5R,6R,7E)-3,5,6- Trihydroxy-7- megastigmen-9-one	HMDB00387 36	0.046058	0.990192	0.798030 244	20
0.93_463.9451m /z	UNKNOWN	UNKNOWN	0.046887	0.990192	1.141164 805	19
14.53_383.3252 m/z	N-(1,3- Dihydroxyoctadec- 4-en-2-yl)acetamide	HMDB02477 81	0.048732	0.990192	0.890172 417	-

Supplementary Table 4. Summary statistics of targeted steroid analysis in cord blood and children. Mean values with Standard deviation are reported for cases and controls with P-value from Mann-Whitney U test. No compounds passed FDR correction.

Table 23: Supplementary Table 4. Summary statistics of targeted steroid analysis in cord blood and children

Metabolite	Cases	Controls	P-Value	Age 11 – Ancova P-value with age adjustment
Androstendiol	209.54 (77.65)	213.57(134.89)	0.5569	-
Androstenedione	0.62 (0.35)	0.84(0.39)	0.0193	0.936457
Androstenedione:DHEAS	2893(1566.92)	1873.02(907.69)	0.01	0.17715
Corticosterone	2.82 (3.78)	3.07(3.94)	0.5258	0.133795
Cortisol	77.28 (91.41)	79.48(97.09)	0.7699	0.061413
Cortisol Sulphate	1.14(0.62)	1.15(0.61)	0.8013	-
DHEAS	1420.50 (448.82)	1381.38(586.85)	0.6707	0.877
Esterone Glucuronide	0.04 (0.03)	0.05(0.04)	0.4033	0.74774
Progesterone	931.93(501.02)	945.11(382.59)	0.4866	-
Testosterone	0.09(0.08)	0.08 (0.07)	1	0.67396
11 Deoxycorticosterone	1.69 (1.18)	2.19(1.70)	0.0943	-
11 Deoxycortisol	2.93(1.77)	3.37(2.16)	0.3207	-

17OHP	25.33 (9.86)	29.34(16.83)	0.4951	-
21 Deoxycortisol	0.49(0.35)	0.88(1.35)	0.322	-

Supplementary Figure 1: Flow chart. 2,137 mother and baby pairs were recruited between August 2008-October 2011 for the BASELINE cohort. The preceding SCOPE study provided 1,537 mothers, while an additional 600 infants were recruited in the initial post-natal period. 1,249 participants completed all follow-up assessments at 5-years. A nested case:control study was created based on; children who had a formal ASD diagnosis at this follow-up period, and available cord blood plasma samples taken in the immediate post-natal period. This initial BASELINE study consisted of 22 children with ASD, matched with 44 neurotypical controls. Participants were followed-up to explore persistent blood-based changes in children with ASD versus neurotypical controls. All children were invited for follow-up assessments. The cohort of children with an early ASD diagnosis n=22 were combined with children who were diagnosed with ASD at a later timepoint (7-10 yrs old) n=15. These 15 children were part of the original larger study cohort, and had red flags for ASD but no formal diagnosis. Based on successful visits and bloods samples taken, a follow-up nested case:control PiRAMiD study cohort was designed including 24 cases:48 controls. There was an overlap of 13 cases and 17 controls between both BASELINE and PiRAMiD cohorts.

Supplementary Figure 2: Results from serum proteomic analysis. **A)** PLS-DA plot of samples with clustering ellipses. No clear separation is observed between groups. **B)** Volcano plot of all proteins measured. 11 of 405 quantified proteins were significantly altered at $p < 0.05$ following a student's t-test. After the application of the Benjamini-Hochberg procedure no compounds were found to be significantly altered (FDRate >0.05). **C)** Violin plots of the 11 significant proteins identified prior to FDR correction. **D)** Receiver Operator Characteristic (ROC) curve showing predictive performance of the random forest model for the prediction of autism using significantly

altered serum proteins (n=11). **E)** Pathway analysis plot displaying pathways identified from the significant proteins.

Supplementary Figure 3 ML Cord blood proteomics. A/B) ROC curves for logistic regression and random forest models trained on the full set of cord blood proteins quantified (n = 558). **C/D)** ROC curves for ML models trained using the top 20 cord blood proteins identified through random forest based feature ranking. **E/F)** ROC curves for models trained on the significantly altered cord blood proteins (n=41). **G)** Top 20 features ranked by random forest from the full set of quantified cord blood proteins for ability to predict autism. **H)** Rankings of top 20 significantly altered cord blood proteins for autism prediction as identified by random forest.

Supplementary figure 4 ML Serum Proteomics. A/B) ROC curves for ML models trained using the full set of quantified serum proteins (n= 404). **C/D)** ROC curves for ML models trained using the top 20 serum proteins for autism prediction as identified by random forest. **E/F)** ROC curves for ML models trained using the significantly altered serum proteins (n=11). **G)** Top 20 random forest feature rankings on all serum proteins quantified. **H)** Ranking of significantly altered serum proteins as identified by random forest feature ranking.

Supplementary Figure 5. ML Metabolomics: A/B) ROC curve for random forest model trained on the full set of discovery metabolites quantified. **B)** ROC curve for logistic regression model trained on the full set of targeted steroid metabolites quantified. **C)** ROC curve for random forest model trained on the top 20 features identified from the full set of discovery cord blood metabolites. **D)** ROC Curve for the random forest model trained using the full set of targeted steroid metabolites. **E)** Top 20 random forest feature rankings from the full set of discovery metabolomics. **F)** Random forest feature ranking from the full set of cord blood steroid metabolites.

Appendices 3: Questionnaires and Instruments

PIRAMiD study: **PR**edicting early onset **A**utism through
Maternal **I**mmune **A**ctivation and **P**roteomic **D**iscovery

Questionnaire

Date Completed: ___/___/___

Child ID: _____

Child DOB: ___/___/___

Child Gender: M / F

Mother ID: _____

Mother DOB: ___/___/___

Maternal Age: _____

Paternal ID: _____

Dad DOB: ___/___/___

Paternal Age: _____

Socio-demographics

Marital Status

- I. Single
- II. Married/Living as married
- III. In a relationship (not living together)
- IV. In a relationship (living together)
- V. Separated/Divorced
- VI. Widowed
- VII. In a registered same sex civil partnership

What is your highest level of education (mother)?

- I. No formal education
- II. Primary school
- III. Secondary school - Junior/Inter Certificate
- IV. Secondary school - Leaving Certificate
- V. Third level - Certificate
- VI. Third level - Diploma
- VII. Third level - Degree
- VIII. Third level - Higher/Graduate Diploma
- IX. Third level - Masters
- X. Third level – PhD
- XI. Other, please specify

Parents combined net income (after deductions of tax and PRSI)

Please tick one

- | | Per Week | Per Month | Per Year |
|-------|---------------------------|---------------------------|--------------------------|
| I. | Under €230... | Under €1,000... | Under €12,000 |
| II. | €231 to under €350... | €1,001 to under €1,500... | €12,001 to under €18,000 |
| III. | €351 to under €460... | €1,501 to under €2,000... | €18,001 to under €24,000 |
| IV. | €461 to under €575... | €2,001 to under €2,500... | €24,001 to under €30,000 |
| V. | €576 to under €800... | €2,501 to under €3,500... | €30,001 to under €42,000 |
| VI. | €801 to under €925... | €3,501 to under €4,000... | €42,001 to under €48,000 |
| VII. | €926 to under €1,150... | €4,001 to under €5,000... | €48,001 to under €60,000 |
| VIII. | €1,151 to under €1,500... | €5,001 to under €6,500... | €60,001 <€78,000 |
| IX. | €1,501 to under €1,850... | €6,501 to under €8,000... | €78,001 to under €96,000 |
| X. | €1,851 or more ... | €8,001 or more ... | €96,001 or more |
| XI. | Prefer not to say | | |
| XII. | Don't Know | | |

Current job situation

- I. Full time work
- II. Part time work
- III. Student
- IV. Homemaker
- V. Unemployed
- VI. Sickness beneficiary
- VII. Other

Maternal Occupation

(Please give specific details of your occupation; also if you have been unemployed for less than 6 months please give title of previous job)

Maternal Occupation:

Paternal Occupation

(Please give specific details of your occupation; also if you have been unemployed for less than 6 months please give title of previous job)

Paternal Occupation:

Health

Smoking

Mother

Do you currently smoke?

- I. Yes
- II. No
- III. eCigarettes

Father

Do you currently smoke?

- I. Yes
- II. No
- III. eCigarettes

If Yes, how often do you smoke?

- I. Daily/Almost Daily
- II. A few times per week
- III. Once a week
- IV. Less than once a week

Do you allow smoking in your house?

- I. No
- II. No, but do so outside
- III. Yes, occasionally
- IV. Yes, regularly
- V. Yes, e-cigarettes only

Child Health

Does your child have a developmental disorder?

- I. ASD
- II. Asperger's
- III. ADHD
- IV. Dyspraxia (Developmental co-ordination disorder)
- V. Sensory-Processing disorder
- VI. Dyslexia (specific learning disorder/difficult/disability - literacy)
- VII. Dyscalculia (specific learning disorder/difficult/disability - numeracy)
- VIII. Neuro-typical
- IX. No concerns
- X. Other

Developmental Disorder (child):

Do any of your **other** children have any of the above conditions?

Developmental Disorder (sibling): Age/ Sex/ Developmental disorder

How was your child diagnosed with this condition, and at what age?

- I. Specialist ASD services (COPE, Marian house)
- II. Private individual practitioners (psychologist/psychiatrist/SLT)
- III. Private (MDT) Multidisciplinary team
- IV. Suspected diagnosis – No formal diagnosis

Age at diagnosis:

If your child has a diagnosis of Autism/Asperger's/ASD. What tools/instruments were used to make this diagnosis?

- I. DISCO
- II. ADOS 2
- III. ADI-R
- IV. CARS
- V. Unknown
- VI. Other

Diagnostic tool:

Where does your child attend school?

- I. Mainstream
- II. Mainstream with support
- III. ASD unit
- IV. Special school
- V. Home tuition

Is your child unwell or do they have an infection currently?

Yes/No (please circle appropriate response)

If "yes" please elaborate

Current ill-health (Child):

Does your child have any underlying medical conditions?

Yes/No (please circle appropriate response)

- I. Asthma
- II. Eczema
- III. Epilepsy
- IV. Inflammatory Bowel Disease
- V. Dental caries/decay
- VI. Other (please specify)

Other conditions:

Is your child on any medications?

Medication:

Anthropometrics:

- I. HT (m)
- II. WT (Kg)
- III. BMI
- IV. BMI range

Maternal Health

Are you unwell or do you have an infection currently?

Yes/No (please circle appropriate response)

If "yes" please elaborate

Current ill-health (Maternal):

Do you have any underlying medical conditions?

- I. Inflammatory arthritis
- II. Inflammatory Bowel Disease
- III. Asthma/Eczema
- IV. Psoriasis
- V. Other

Other medical conditions:

Are you on any medications?

Medications:

Are you currently pregnant?

Yes/No (please circle as appropriate)

If yes,

1st Trimester

2nd Trimester

3rd Trimester

Do you have any other children?

No

Yes, 1

Yes, 2

Yes, 3+

Please document their names and ages

Anthropometrics:

- I. HT (m)
- II. WT (Kg)
- III. BMI
- IV. BMI range

Paternal Health

Are you unwell or do you have an infection currently?

Yes/No (please circle appropriate response)

If "yes" please elaborate

Current ill-health (Paternal):

Do you have any underlying medical conditions?

- I. Inflammatory arthritis
- II. Inflammatory Bowel Disease
- III. Asthma/Eczema
- IV. Psoriasis
- V. Other

Other medical conditions:

Are you on any medications?

Medications:

Do you have any other children?

- No
- Yes, 1
- Yes, 2
- Yes, 3+

Anthropometrics (paternal):

- I. HT (m)
- II. WT (Kg)
- III. BMI
- IV. BMI range

Psychometric Assessments (For researcher)

KBIT-2 results

Verbal

Non-verbal

Composite

SCQ results (lifetime)

Raw score

<15

>15

Venepuncture

Performed successfully?

Serum

Tempus

EDTA

**Social Communication Questionnaire (SCQ) – Lifetime
PC Answer Sheet**

Date:

Study ID:

Gender:

DOB:

Directions: Thank you for taking the time to complete this questionnaire. Please answer each question by selecting *yes* or *no*. A few questions ask about several related types of behavior; please select *yes* if *any* of these behaviors were present during the past 3 months. Although you may be uncertain about whether some behaviors were present or not, please answer *yes* or *no* to every question on the basis of what you think.

Item	Yes	No
1. Is she/he now able to talk using short phrases or sentences? If <i>no</i> , skip to question 8.	<input type="radio"/>	<input type="radio"/>
2. Can you have a to and fro “conversation” with her/him that involves taking turns or building on what you have said?	<input type="radio"/>	<input type="radio"/>
3. Has she/he ever use odd phrases or say the same thing over and over in almost exactly the same way (either phrases that she/he hears other people use or ones that she/he makes up)?	<input type="radio"/>	<input type="radio"/>
4. Has she/he ever use socially inappropriate questions or statements? For example, has she/he ever regularly ask personal questions or make personal comments at awkward times?	<input type="radio"/>	<input type="radio"/>
5. Has she/he ever gotten his/her pronouns mixed up (e.g., saying <i>you</i> or <i>she/he</i> for <i>I</i>)?	<input type="radio"/>	<input type="radio"/>
6. Has she/he ever use words that she/he seems to have invented or made up her/himself; put things in odd, indirect ways; or use metaphorical ways of saying things (e.g., saying <i>hot rain</i> for <i>steam</i>)?	<input type="radio"/>	<input type="radio"/>
7. Has she/he ever say the same thing over and over in exactly the same way or insist that you say the same thing over and over again?	<input type="radio"/>	<input type="radio"/>
8. Has she/he have things that she/he seems to do in a very particular way or order or rituals that she/he insisted that you go through?	<input type="radio"/>	<input type="radio"/>
9. Has her/his facial expressions usually seemed appropriate to the particular situation, as far as you can tell?	<input type="radio"/>	<input type="radio"/>
10. Has she/he ever used your hand like a tool or as if it were part of his/her own body (e.g., pointing with your finger or putting your hand on a doorknob to get you to open the door)?	<input type="radio"/>	<input type="radio"/>
11. Has she/he ever have any interests that preoccupy her/him and might seem odd to other people (e.g., traffic lights, drainpipes, or timetables)?	<input type="radio"/>	<input type="radio"/>
12. Has she/he ever seemed to be more interested in parts of a toy or an object (e.g., spinning the wheels of a car), rather than in using the object as it was intended?	<input type="radio"/>	<input type="radio"/>
13. Has she/he ever have any special interests that are <i>unusual</i> in their intensity but otherwise appropriate for his/her age and peer group (e.g., trains or dinosaurs)?	<input type="radio"/>	<input type="radio"/>
14. Has she/he ever seemed to be <i>unusually</i> interested in the sight, feel, sound, taste, or smell of things or people?	<input type="radio"/>	<input type="radio"/>
15. Has she/he ever have any mannerisms or off ways of moving her/his hands or fingers, such as flapping or moving her/his fingers in front of her/his eyes?	<input type="radio"/>	<input type="radio"/>
16. Has she/he ever have any complicated movements of her/his whole body, such as spinning or repeatedly bouncing up and down?	<input type="radio"/>	<input type="radio"/>
17. Has she/he ever injured her/himself deliberately, such as by biting her/his arm or banging her/his head?	<input type="radio"/>	<input type="radio"/>
18. Has she/he ever have any objects (<i>other</i> than a soft toy or comfort blanket) that she/he <i>had</i> to carry around?	<input type="radio"/>	<input type="radio"/>
19. Does she/he have any particular friends or a best friend?	<input type="radio"/>	<input type="radio"/>

For the following behaviors, please focus on the time period between the child’s fourth and fifth birthdays. You may find it easier to remember how things were at that time by focusing on key events, such as starting school, moving house, Christmastime, or other specific events that are particularly memorable for you as a family. If your child is not yet 4 years old, please consider her or his behavior in the past 12 months.

Item	Yes	No
20. When she/he was 4 to 5, did she/he ever talk with you just to be friendly (rather than to get something)?	<input type="radio"/>	<input type="radio"/>
21. When she/he was 4 to 5, did she/he ever <i>spontaneously</i> copy you (or other people) or what you are doing (such as vacuuming, gardening, or mending things)?	<input type="radio"/>	<input type="radio"/>
22. When she/he was 4 to 5, did she/he ever spontaneously point at things around her/him just to show you things (not because she/he wants them)?	<input type="radio"/>	<input type="radio"/>
23. When she/he was 4 to 5, did she/he ever use gestures, other than pointing or pulling your hand, to let you know what she/he wants?	<input type="radio"/>	<input type="radio"/>
24. When she/he was 4 to 5, did she/he nod her/his head to mean <i>yes</i> ?	<input type="radio"/>	<input type="radio"/>
25. When she/he was 4 to 5, did she/he shake her/his head to mean <i>no</i> ?	<input type="radio"/>	<input type="radio"/>
26. When she/he was 4 to 5, did she/he usually look at you directly in the face when doing things with you or talking with you?	<input type="radio"/>	<input type="radio"/>
27. When she/he was 4 to 5, did she/he smile back if someone smiles at her/him?	<input type="radio"/>	<input type="radio"/>
28. When she/he was 4 to 5, did she/he ever show you things that interest her/him to engage your attention?	<input type="radio"/>	<input type="radio"/>
29. When she/he was 4 to 5, did she/he ever offer to share things other than food with you?	<input type="radio"/>	<input type="radio"/>
30. When she/he was 4 to 5, did she/he ever seem to want you to join in her/his enjoyment of something?	<input type="radio"/>	<input type="radio"/>
31. When she/he was 4 to 5, did she/he ever try to comfort you if you are sad or hurt?	<input type="radio"/>	<input type="radio"/>
32. When she/he was 4 to 5, when she/he wants something or wants help, did she/he look at you and use gestures with sounds or words to get your attention?	<input type="radio"/>	<input type="radio"/>
33. When she/he was 4 to 5, did she/he show a normal range of facial expressions?	<input type="radio"/>	<input type="radio"/>
34. When she/he was 4 to 5, did she/he ever spontaneously join in and try to copy the actions in social games, such as <i>The Mulberry Bush</i> or <i>London Bridges Is Falling Down</i> ?	<input type="radio"/>	<input type="radio"/>
35. When she/he was 4 to 5, did she/he play any pretend or make-believe games?	<input type="radio"/>	<input type="radio"/>
36. When she/he was 4 to 5, did she/he seem interested in other children of approximately the same age whom she/he did not know?	<input type="radio"/>	<input type="radio"/>
37. When she/he was 4 to 5, did she/he respond positively when another child approached her/him?	<input type="radio"/>	<input type="radio"/>
38. When she/he was 4 to 5, if you come into a room and start talking to her/him without calling her/his name, did she/he usually look up and pay attention to you?	<input type="radio"/>	<input type="radio"/>
39. When she/he was 4 to 5, did she/he ever play imaginative games with another child in such a way that you can tell that each child understands what the other is pretending?	<input type="radio"/>	<input type="radio"/>
40. When she/he was 4 to 5, did she/he play cooperatively in games that need some form of joining in with a group of other children, such as hide-and-seek or ball games?	<input type="radio"/>	<input type="radio"/>

**Sample Social Story with a hypothetical patient
named Robert**

Robert is going to see Dr. Mick Carter today.





Robert and his Mum (Olga) will visit Dr. Mick today.

First, Robert will look at pictures and puzzles and answer some questions.

Then Robert will have some blood taken (if he wants to)

Having blood taken means that Dr. Mick will take a little bit of blood from Mum's, and Robert's arms.

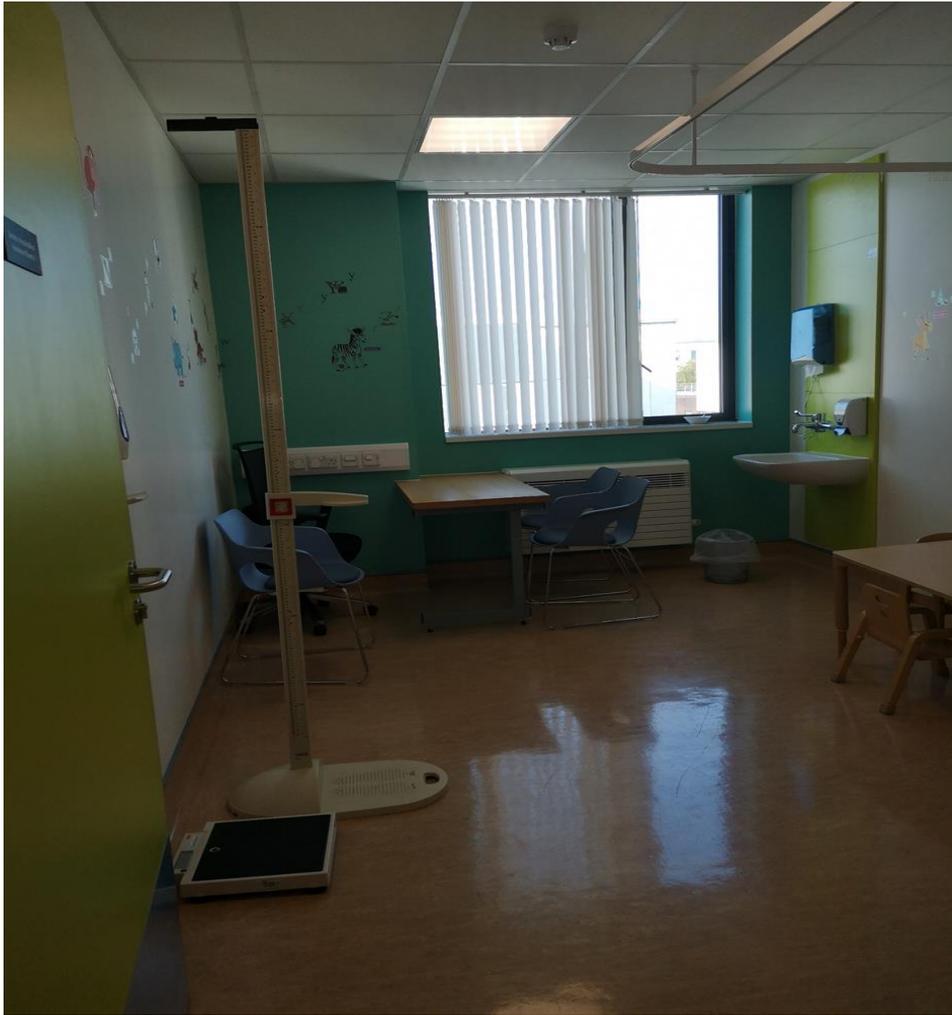
Whenever blood is taken, there are things that Dr. Mick will do.

This story will show you all the steps of having blood taken.

TIME TO GO

Robert and his parents will go to Dr. Mick.

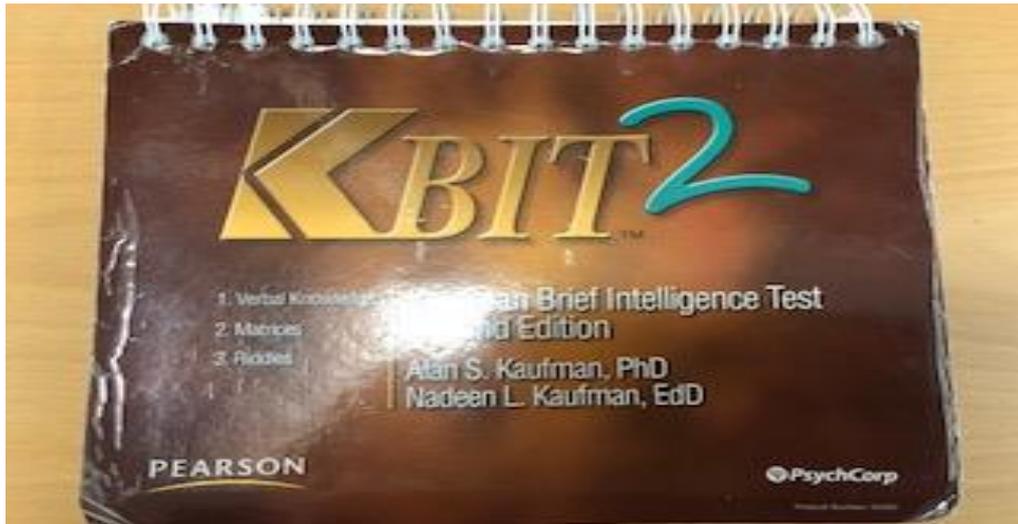




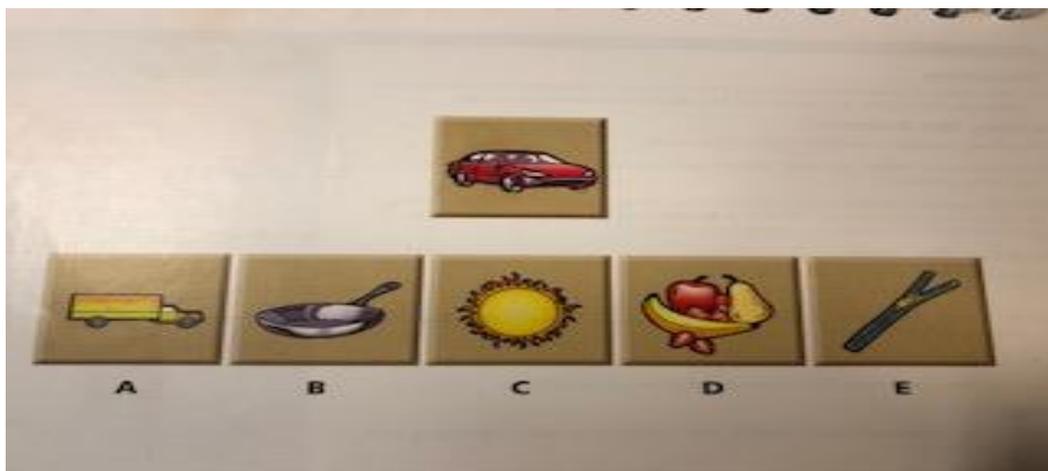
You will all go into a room like this one.



Robert will listen and look at some pictures with Dr. Mick.

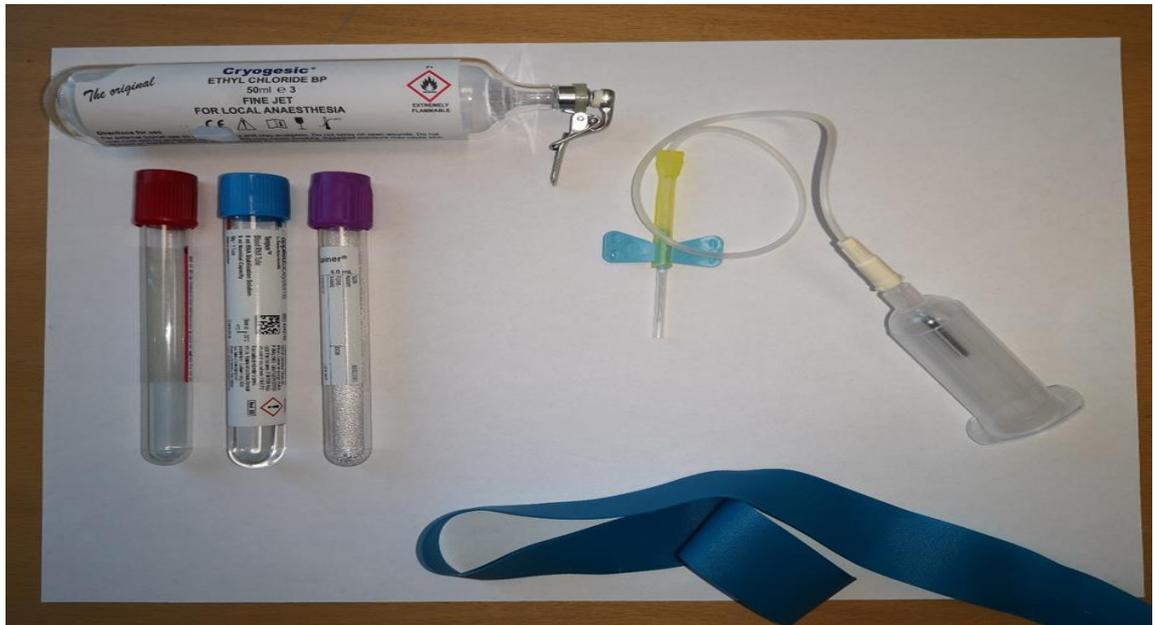


Robert will do some puzzles, and answer some questions.



Then Dr. Mick will take everyone's blood.

Dr. Mick will bring out gloves, some tubes that collect blood and the needle that helps the blood go into the tubes.



These are the things Dr. Mick will use.



Dr. Mick will put on some plastic gloves to help keep everyone safe.



Dr. Mick will wrap a band around your arm. It won't hurt.



**Dr. Mick will put a spray on your arm.
The spray may smell funny and cold on Kacey's arm.
This might feel different but it does not hurt.**

Dr. Mick will take the needle and put it into Robert's arm. It is important that Robert

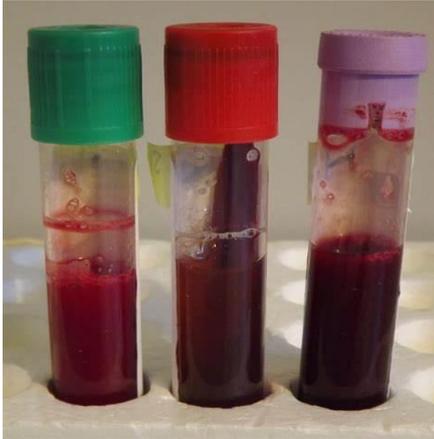
Dr. Mick will use the needle to help get Robert's blood out.

**does not move his arm.
You will need to keep
your arm still so Dr. Mick
can put the needle in
without it hurting too
much. Mum and Dad will
be there for hugs.**



**Blood goes from Robert's arm
into the needle.
Then the blood goes from the
needle and down the tube.**

Dr. Mick will fill the tubes.



Then he will put a cotton ball over the needle in Robert's arm.

Dr. Mick will take out the needle and he will gently hold the cotton ball on your arm.



**I am all done.
Well done Robert. Everyone
is so proud of you.**



Appendix 4: Published articles

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Maternal Immune Activation and Interleukin 17A in the Pathogenesis of Autistic Spectrum Disorder and Why It Matters in the COVID-19 Era

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Autism spectrum disorder (ASD) is the commonest neurodevelopmental disability. It is a highly complex disorder with an increasing prevalence and an unclear etiology. Consensus indicates that ASD arises as a genetically modulated, and environmentally influenced condition. Although pathogenic rare genetic variants are detected in around 20% of cases of ASD, no single factor is responsible for the vast majority of ASD cases or that explains their characteristic clinical heterogeneity. However, a growing body of evidence suggests that ASD

susceptibility involves an interplay between genetic factors and environmental exposures. One such environmental exposure which has received significant attention in this regard is



maternal immune activation (MIA) resulting from bacterial or viral infection during pregnancy. Reproducible rodent models of ASD are well-established whereby induction of MIA in pregnant dams, leads to offspring displaying neuroanatomical, functional, and behavioral changes analogous to those seen in ASD. Blockade of specific inflammatory cytokines such as interleukin-17A during gestation remediates many of these observed behavioral effects, suggesting a causative or contributory role. Here, we review the growing body of animal and human-based evidence indicating that interleukin-17A may mediate the observed effects of MIA on neurodevelopmental outcomes in the offspring. This is particularly important given the current corona virus disease-2019 (COVID-19) pandemic as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection during pregnancy is a potent stimulator of the maternal immune response, however the long-term effects of maternal SARS-CoV-2 infection on neurodevelopmental outcomes is unclear. This underscores the importance of monitoring neurodevelopmental outcomes in children exposed to SARS-CoV-2-induced MIA during gestation.

Keywords: ASD, autism, cytokine, maternal immune activation, MIA, interleukin-17A (IL-17A), COVID-19

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by a spectrum of deficits in social interactions and communication combined with stereotypical and repetitive behaviors. Up to 50% of those affected can have intellectual disability (ID) and limited verbal communication (1–3). In recent decades, the prevalence of ASD has consistently increased from approximately 1 in 1,000 in the 1960s (4), to 1 in 44 today in the United States (5). Increasing prevalence may in part, be explained by changes in reporting practices, increased recognition of ASD symptoms, broadening of the ASD diagnosis (1), and improved accessibility to services (6, 7). A significant ratio of 4:1 from male to female still exists with markedly differing prevalence rates between the sexes, 1/38 in males and 1/151 among females (8). Although genetic susceptibilities are recognized, the mechanism of disease development is unknown and does not follow a clear pattern of inheritance (9, 10). This suggests possible mediation by additional unknown biological or environmental factors (11). Both common and rare genetic risk factors have been identified with more than 400 diverse genes now linked to ASD. Singly, these genetic factors each convey only a modest increase in ASD risk (~1%), however collectively they can contribute to a far greater risk (12, 13). Up to 20% of individuals with ASD may possess copy number variants (CNVs) and *de novo* loss of function single nucleotide variants (SNVs) that are individually rare but in combination, increase an individual's ASD risk (12). While newer methods of genetic analysis (such as whole genome sequencing) are uncovering new candidate genes with regularity (14), the heterogeneity of the clinical and phenotypic groups within ASD strongly suggest that in those with a genetic predisposition, environmental factors may act in concert to bring about a multisystem dysfunction leading to ASD. A well-characterized environmental factor known to impact early fetal brain development and increase ASD risk is maternal inflammation during pregnancy, which is commonly called maternal immune activation (MIA). Numerous epidemiological studies have linked gestational infections with elevated risk of ASD in offspring (15–17), and animal models of MIA have simulated gestational infection resulting in MIA-induced neural and behavioral abnormalities analogous to those seen in ASD (18–20).

Focused early intervention in young children with ASD has been shown to result in normalized patterns of brain

Abbreviations: ACE-2, angiotensin-converting enzyme-2; ADHD, Attention Deficit Hyperactivity Disorder; ARDS, acute respiratory distress syndrome; ASD, autism spectrum disorder; CS, cesarean section; CD8 cell, cluster of differentiation 8, cytotoxic T-lymphocytes; CHD8, chromodomain helicase DNA binding protein 8 gene; CNV, copy number variant; COVID-19, corona virus disease-2019; FMR1, fragile X mental retardation 1 gene; GWAS, genome-wide association study; HLAG gene, human leukocyte antigen G coding gene; ID, intellectual disability; IL, interleukin; IL17A gene, interleukin 17A gene; LPS, lipopolysaccharide; MERS, Middle Eastern Respiratory Syndrome; MIA, maternal immune activation; mTor, mammalian target of rapamycin; Poly (I:C), polyinosinic:polycytidylic acid; PNS, peripheral nervous system; ROR γ t, retinoid-related orphan receptor gamma t; SARS-CoV-2, severe acute respiratory syndrome-coronavirus 2; SNV, single nucleotide variant; Th17, T helper 17 cell; TSC1/TSC 2, Tuberous sclerosis complex 2.

activity, and is associated with improved functional outcomes and reduced morbidity (21, 22). Most children affected by ASD can have a reliable and stable ASD diagnosis from as early as 14 months of age (23), yet in

spite of this, the average age of ASD diagnosis is closer to 5 years (24, 25). Numerous studies sought to identify blood-based biomarkers of ASD in affected adolescents and adults (26, 27) and have reported

alterations of molecules involved in iron transport (28), inflammation (29, 30), brain development (31), and metabolism (32). None to date has identified and validated reliable mechanistic biomarkers with the ability to improve ASD detection in the crucial early developmental period. Multiple descriptive ASD biomarkers such as characteristic MRI brain findings, abnormalities of gaze preference on eye tracking or characteristic EEG findings in infants with ASD; show promise in terms of aiding earlier ASD detection. However, none is directly involved in the pathogenesis of ASD and arises of the condition rather than contributes to it. The infant brain doubles in volume over the first year coinciding with maximal neuroplasticity and synaptogenesis. Recognition of an early mechanistic biomarker gives us the best chance of implementing strategies during this critical early childhood window allowing ASD diagnosis and intervention at the earliest possible stage.

Here, we highlight recent research in this area, both from preclinical animal studies and epidemiological human studies, along with a proposed mechanistic pathway, that we can encourage other research groups with access to suitable maternal-child cohorts to examine this question. We encourage researchers to look at the prospective study of children born during the corona virus disease-2019 (COVID-19) era, when their gestations may have been complicated by mild or even asymptomatic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Otherwise, the long-term effect, if any, of COVID-19 on the fetal brain could remain unknown for years to come.

INFLAMMATION, VIRAL INFECTION, AND ASD: WHAT ARE THE IMPLICATIONS OF THE COVID-19 PANDEMIC?

There is growing scientific evidence that aberrant immune activation occurs in ASD (27, 33) based on studies of autistic children and young adults (34, 35). As early as 1971, Stella Chess reported ASD cases associated with the 1964 Rubella outbreak in the United States (36), and in a 1977 follow up study, Chess et al. quoted ASD prevalence rates of 8–13% in children of mothers who were infected during that outbreak (16). Large epidemiological studies indicate that conditions such as maternal autoimmune disorders and mid-trimester viral infections that trigger gestational pro-inflammatory states (i.e., MIA), are linked with elevated ASD, schizophrenia, and bipolar disorder risk in offspring (16, 17, 37, 38). More recently, a range of conditions associated with proinflammatory states in pregnancy such as obesity, psychosocial stress, and pre-eclampsia were associated with increased ASD risk in children (39, 40). Thus, gestational MIA appears to play a role in the pathogenesis of the ASD phenotype in exposed offspring.

MATERNAL IMMUNE ACTIVATION AND NEURODEVELOPMENTAL OUTCOMES

We define MIA as a triggering of the maternal immune system by infectious or infectious-like stimuli resulting in an increase in measurable inflammatory markers during pregnancy (41, 42). Maternal immune activation has been most commonly simulated in preclinical rodent, murine and non-human primate (rhesus macaque) animal models by Poly (I:C) (polyinosinicpolycytidylic acid) or LPS (lipopolysaccharide) injection which, respectively, model viral and bacterial infection (18, 43, 44). Poly (I:C) is a synthetic analog of double stranded RNA, mimics the effects of viral infection (45). The triggered immune response results in offspring with behavioral, immunological, and neurological abnormalities that approximate to autistic symptoms observed in humans, notably, impaired sociability and repetitive behaviors (18, 46, 47). Offspring born to poly (I:C) treated dams have consistently, across all exposure categories [administration of varying doses of poly (I:C) and at varying gestations], shown impairment of social interaction, this is manifest as reduced communication in ultrasonic vocalizations (USV) which are usually triggered by separation from the dam in the first two postnatal weeks. Marble burying, a well-recognized behavioral paradigm to measure repetitive behaviors in rodents, again is consistently increased in murine offspring following poly (I:C) treatment (48). These offspring have proven useful in pre-clinical etiological studies as well as identification of therapeutic targets.

Cytokine dysregulation may play a causative role in observed neuronal dysfunction in pre-clinical models of MIA (20, 46, 49). In a recent study, Choi et al. convincingly demonstrated that simulated MIA in murine models leads to elevation in maternal IL-6, which in turn activates maternal Th17 cells. These maternal Th17 cells produce IL-17, which is thought to cross the placenta triggering increased expression of IL-17AR in the fetal brain and leading to cortical malformations and behavioral abnormalities (18, 50). These malformations parallel abnormalities found in brain development in children, adolescents and adults with ASD (51, 52). Poly (I:C) treatment also leads to raised IL-17A mRNA levels in placental tissue of these mice (18). Through inhibition of IL-6 and IL-17A signaling with antibody blockade of the IL-17A cytokine, Choi et

al also determined that a sustained increase in IL-17A expression seemed to be pathogenic in ASD, as IL-17A blockade prevented the development of ASD-like phenotypes (18). Specific behaviors in mice which model

core diagnostic features of ASD (including repetitive burying and increased neonatal USV) were normalized in the previously MIA-exposed offspring (53, 54).

Improved fetal resilience is associated with lower intensity of MIA. Autism spectrum disorder risk after prenatal exposure to maternal fever has been found to increase in a dose dependent manner (55, 56) and similar effects were identified in animal models of MIA (57). A balanced maternal diet seems to contribute to improved fetal resilience also (58–60). Exposure to relatively higher grades of immune activation *via* high intensity MIA (40), intrapartum infection (61, 62) and genetic risk factors lead to reduced fetal resilience, and increased likelihood of unfavorable developmental outcomes.

ALTERATIONS IN CYTOKINE EXPRESSION IN HUMAN STUDIES

While many studies have examined the cytokine profiles of individuals with ASD, only a very limited number of studies to date have examined mid-gestation cytokine levels in mothers of children who subsequently develop ASD. Three studies retrospectively analyzed maternal blood sampled during pregnancy. A 2017 study by Jones et al., reported elevated midgestation cytokines and chemokines in mothers of children with ASD associated with ID, and particularly early onset ASD (as defined by the authors as early or sustained delays in language or social skills, and excluding those showing clear skill regression) (63). Dysregulation was noted in a number of cytokines including interleukins IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, and IL-17A between 15 and 19 weeks' gestation. An earlier study noted elevations in mid-gestation serum IL-4, IL-5, and IFN-gamma levels in mothers of ASD affected children (15). While, more recently, Irwin et al. demonstrated alterations in IL-4, MCP-1, and IL-10 levels in 28-week gestation serum of mothers who birthed ASD affected children (64). Other authors have examined amniotic fluid at mid-gestation and found elevated levels of IL4, IL-10, TNF- α , and TNF- β in ASD patients vs. controls (65). Yet, amniotic fluid cytokine concentrations are more indicative of the fetal immune state rather than the maternal state (66, 67). In **Table 1**, we outline a number of the cytokines most frequently found to be dysregulated in the serum or cerebrospinal fluid (CSF) of ASD affected individuals, and gestational serum and amniotic fluid samples from mothers of ASD affected children.

A growing body of evidence supports a role in ASD pathogenesis for Th17 cells and their product cytokine, IL-17A (**Figure 1**) (79, 82). The IL17A gene itself has been identified by a small genome-wide CNV study to have amplified CNVs in ASD affected cohorts (83). Elevated levels of IL-17A have been reported in the blood of ASD affected individuals, and these correlate positively with severity of ASD behavioral symptoms (35, 63, 79). Yet, others have found high concentrations of IL-17A in individuals affected by obesity or high BMI (84), both of which are more likely in ASD groups (85). This is a potential confounder for any retrospective cohort based study designs.

STRING analysis (**Figure 2**) (86) indicates that IL-17A has proven or predicted interactions with IL-2, IL-6, IL-10, IL13, IL-17F, IL-17RA, IL-17RC, CTLA4, STAT3, and STAT6. Each of these proteins have been previously reported to have altered expression in children with ASD, as outlined below. Of these, the most persistently described, and hence, potential key player is IL-17A, along with its receptor IL17RA and receptor complex, IL17RC.

Network nodes represent proteins—each node represents all the proteins produced by a single, protein-coding gene locus. Edges (lines) represent protein-protein associations that are specific and meaningful, i.e., proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding each other. Blue connecting lines indicate that protein interaction information was derived from curated databases, pink indicates the interaction was experimentally determined, yellow indicates

TABLE 1 | Cytokine dysregulation in ASD affected individuals and in gestational serum and amniotic fluid samples of mothers with ASD affected offspring.

Cytokine	Category	Altered in blood/CSF of ASD individual	Altered in gestational blood	Altered in amniotic fluid	Cytokine characteristics relevance to ASD
TNF α	Pro-inflammatory	(29, 68–70)	(63)	(65)	Apoptosis of infected cells. Elevated in the CSF and blood of ASD affected individuals (29, 68).
IL-1 β	Pro-inflammatory	(29, 68, 71, 72)	(63)		A potent pro-inflammatory cytokine involved in acute and chronic inflammation. Correlated with ASD symptom severity (34).
IL-6	Pro-inflammatory	(29, 68, 70–74)	(63)		Induces production of acute phase proteins and stimulates B-cell antibody production (75). Pleiotropic (affects hematologic, hepatic, endocrine, and metabolic function). Thought to impact synapse formation and neuronal migration (76). Potentially mediates IL-17 linked ASD risk during pregnancy (18, 46).
IFN γ	Pro-inflammatory	(27, 29, 73)	(15, 63)		Interfaces between innate and adaptive immune response. Secreted by NK cells, and promotes cell killing. Activates macrophages, which produce IL-12 and -23, stimulating Th1 and Th17 cells, respectively. Inhibits Th2 cells. Versatile, with a role in defense against intracellular pathogens, tumor surveillance, autoimmunity, allergy, and protection of the amniotic space during pregnancy (77).
IL-17	Pro-inflammatory, Chemotactic	(29, 35, 70, 74, 78, 79)	(63)		Derived from Th17 cells, a subset of CD4 cells. Potentiates the innate PMN response through inflammation. Postulated to trigger alterations in blood brain barrier and lead to cortical dysplasia (46).
IL-4	Pro-/Anti-inflammatory, Allergy	(72)	(15, 63, 64)	(65)	A Th2 derived cytokine, often linked with asthma and allergic type inflammation (33). Dual role in pro/anti-inflammatory properties. Crucially important in mitigating inflammation during pregnancy (primarily through suppression of Th1 cells and associated cytokines (IL-2 and IFN γ)).
GM-CSF	Growth factor	(80)	(63)		A colony-stimulating factor. Produced by stromal cells, it targets bone marrow, and precursor cells mediating hematopoiesis.
IL-8	Chemotactic	(71, 73, 81)	(63)		Produced by fibroblasts, neutrophils, and macrophages. Chemo-attractant for phagocytes to site of inflammation.

The numbers in parentheses indicate the relevant references.

the interaction was determined *via* text mining, black indicates protein co-expression, and lilac indicates protein homology. Analysis was performed on 28 July 2021 *via* the string-db.org domain.

IL-17A ASSOCIATED PRO-INFLAMMATORY MEDIATORS IN ASD

Upregulation of pro-inflammatory pathways has been persistently associated with ASD. IL-6 is a versatile cytokine, with multiple functions throughout the body. It plays roles in immunity, inflammation, hematopoiesis, and oncogenesis. IL-6 works to promote pro-inflammatory Th17 cells (IL17 producers) and to downregulate anti-inflammatory Treg cells (regulatory T-Helper cells) (87, 88). Th17 cells produce cytokines that cross the placental barrier (20). This transplacental effect has been well-characterized with IL-6, which was shown to alter offspring behavior and brain development (20, 89).

Like IL-17A, IL-17F is also produced by Th17 cells (90). IL-17F is reported to be involved in the regulation of proinflammatory gene expression and responses (91). IL-17RA and IL-17RC are both members of the IL-17 receptor family. In order for IL-17A (or indeed IL-17F) to have biological effects on tissues, IL-17RA must be present (90). IL-17RA is expressed in immune cells, and some children affected by ASD appear to possess higher levels of this receptor compared to neuro-typical controls (92). IL-17RA blockade may reduce monocyte associated oxidative stress which may improve neuro-inflammation associated with ASD (92). IL-17RC is also essential for the formation of the IL-17 receptor complex (46). IL-17RC levels in neutrophils are

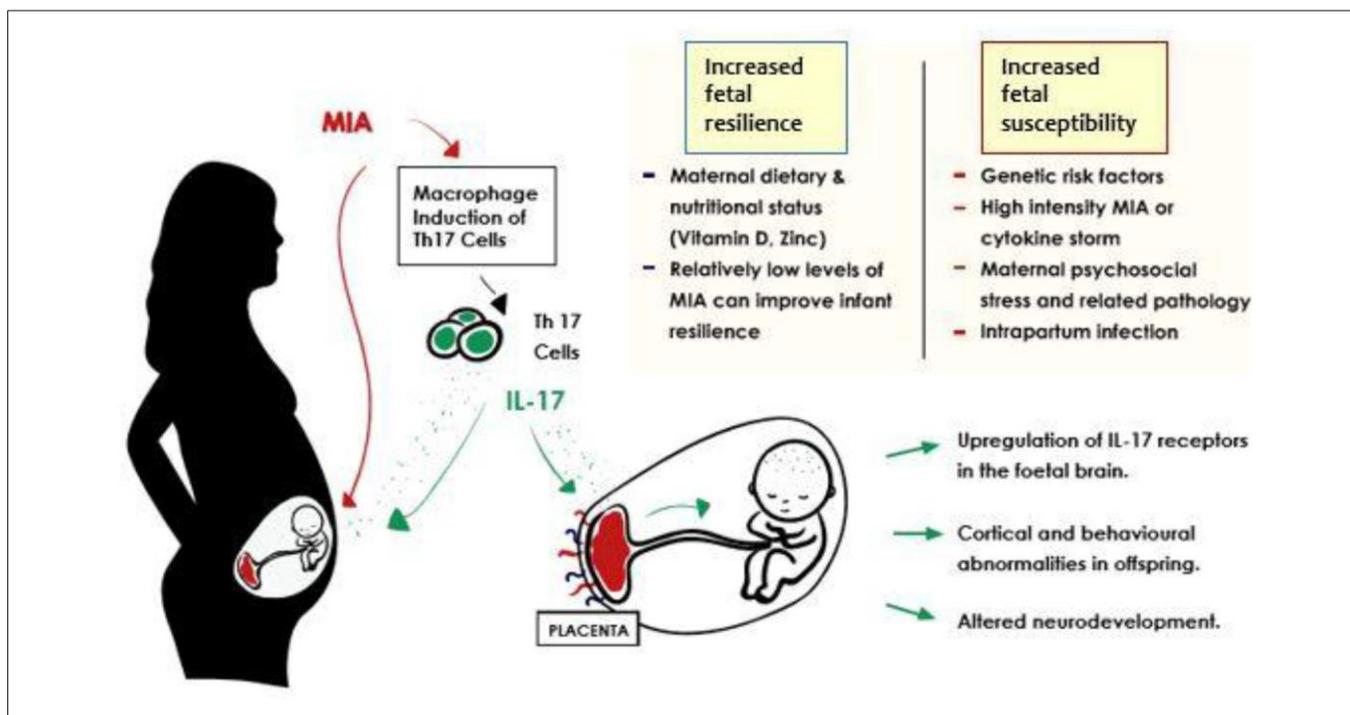


FIGURE 1 | Potential outcomes in the inflammation-exposed fetus in the context of MIA related IL-17 induction. Improved fetal resilience is associated with lower intensity of maternal immune activation. Autism spectrum disorder risk after prenatal exposure to maternal fever has been found to increase in a dose dependent manner (55, 56) and similar effects were identified in animal models of MIA (57). A balanced maternal diet seems to contribute to improved fetal resilience (58–60). Exposure to relatively higher grades of immune activation via high intensity MIA (40), intrapartum infection (61, 62), and genetic risk factors lead to reduced fetal resilience, and increased likelihood of unfavorable developmental outcomes.

raised in children with ASD compared to neuro-typical controls. In fact, expression of this receptor (mRNA and protein) was completely absent in a cohort of neuro-typical children. The presence of both IL-17A receptor subunits in ASD patients may magnify the effects of IL-17A resulting in an autistic phenotype (93).

The transcription factor STAT3 (signal transducer and activator of transcription 3) is a key player in the development of T helper cells and regulates the expression of the T helper cell specific transcriptional regulator—retinoic acid receptor related orphan receptor γ -t (ROR γ t) via IL-6 (94, 95). IL6 is a potent driver of ROR γ t activity. ROR γ t is exclusively found in lymphoid cells such as Th17 cells (CD 4 helper cells), and is required for differentiation of Tregs to Th17 cells (95). STAT3 proteins occur at elevated levels in the peripheral blood mononuclear cells (PBMCs) of children affected by ASD (96). Inhibition of STAT3 mitigates MIA associated behavioral and immunological abnormalities seen in animal models (49), while ROR γ t KO models reverse outcomes in MIA exposed mouse pups (18).

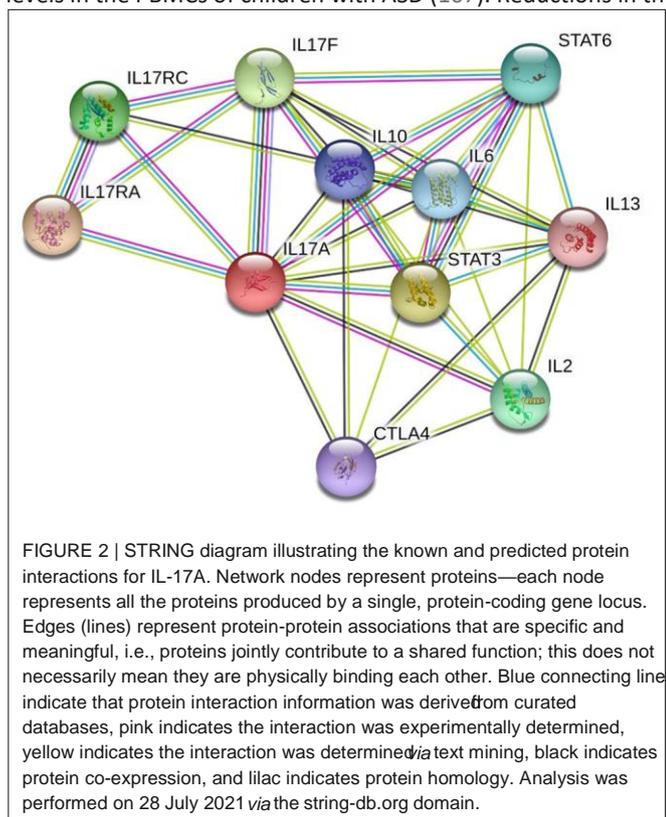
Lastly, IL-13 is a cytokine derived from T cells, which has both inflammatory and anti-inflammatory properties. IL-13 inhibits the production of other inflammatory cytokines (IL-1 α , IL-1 β , IL-6) through its effects on inflammatory macrophages (97). IL13 is recognized as a key driver in allergic and inflammatory airway disease, where its effects are potentiated by IL-17 (98). Raised IL-13 has been noted in the plasma and PBMCs of children affected by ASD (29, 99), particularly those with comorbid asthma (although IL-13 is known to be skewed in those with co-morbid atopic conditions) (35).

IL-17A ASSOCIATED ANTI-INFLAMMATORY MEDIATORS IN ASD

Another member of the STAT family, STAT6, suppresses the IL-17A inflammatory response. In certain conditions, STAT6 signaling attenuates IL-17A producing T-cells, reducing their production of IL-17A (100). IL-4 mediated inhibition of Th17 cells and IL-17A production is STAT6 dependent (101). In human studies, children with ASD reportedly have reduced levels of STAT6-expressing CD45 cells (CD45⁺STAT6⁺) in their PBMC profile compared to neuro-typical controls (80). STAT6, as part of the IL-4 signaling cascade can enhance the expression of anti-inflammatory mediators. This pathway is critical for acceptance of the fetal graft, through reduction of Th17 cells and increase of both IL-4 and Tregs in the fetal environment (102, 103).

In addition to downregulation of the STAT6 mediated pathways, downregulation of other anti-inflammatory cytokines is also reported in autism. Anti-inflammatory cytokine IL-10 acts as a “master” immuno-regulator (104) and IL-10 concentrations are significantly lower in ASD children compared with neurotypical controls (79, 105). Cytotoxic T-lymphocyte antigen 4 (CTLA4) is a glycoprotein located on T

cells (106) and is induced following T cell activation. This anti-inflammatory molecule is expressed at lower levels in the PBMCs of children with ASD (107). Reductions in the levels of these anti-inflammatory and



regulatory proteins may lead those with ASD to acquire a more pro-inflammatory state.

LINKING IMMUNITY AND GENETICS IN ASD

Bioinformatics analysis of large CNV studies suggest strongly that innate immune processes are implicated in ASD risk (108), this may indicate that immune dysfunction in ASD may be genetically driven or influenced. Maternal immune activation downregulates expression of susceptibility genes known to be highly penetrant in ASD and heavily involved in neurogenesis, cell signaling, synaptogenesis, and axonal guidance in the early stages of fetal development (108, 109). When compared with curated ASD associated gene sets [e.g., via the SFARI Gene database (<http://gene.sfari.org/>)], MIA downregulated genes were substantially enriched. The strongest enrichment of MIA downregulated genes was observed in the ASD gene categories with the highest likelihood of a link to ASD i.e., SFARI “High Confidence” or “Syndromic” ASD gene sets. This suggests that MIA may bestow increased ASD risk through downregulating the expression of the same genes that are highly penetrant in ASD during the early stages of fetal development.

Loss of function mutations in TSC1 and TSC2 genes are linked to syndromic ASD, and these genes are critical upstream regulators of the mammalian target of rapamycin (mTor) pathway. mTor has important functions in innate immunity and metabolism in particular (52, 110, 111).

Maternal immune activation also has downstream effects, in some cases influencing the transcriptome rather than the genes themselves. Fragile X mental retardation 1 gene (FMR1) and CHD8 are both highly penetrant genes for ASD, yet MIA does not seem to influence expression of these genes directly. Rather, it wields an influence on downstream gene targets such as FMRP (fragile X syndrome protein complex). This raises the possibility that MIA may act as an environmental factor disrupting crucial early developmental genomic pathways through influence on downstream gene targets (108). This might suggest that MIA could act both in a direct (genetic) and indirect fashion (epigenetic/regulatory) with the end effects converging on similar pathways.

As previously mentioned, normal pregnancy is associated with suppression of immunity, allowing the

fetus to develop inside the mother’s innate immune system. Human leukocyte antigen G coding gene antigen recognition controls the placental immune response and allows acceptance of the fetal graft.

Human leukocyte antigen G coding gene interacts with the CD8 cell surface antigen found on most cytotoxic T-lymphocytes that mediate efficient cell–cell interactions within the immune system (112). Higher rates of HLA-G mutations have been found in mothers of children with ASD (113). The Th17 pathway in particular has been identified as a likely effector of inflammatory changes on the developing fetal brain, with downstream effects on behavior and cognitive development (46, 114). We hypothesize that the physiological changes in maternal immunity during pregnancy are dysregulated in some mothers of children with ASD.

In summary, many of the inflammatory proteins reported to have altered expression in ASD are linked to pro-inflammatory Th-17 cells, their product IL-17A, and the IL-17 receptors and receptor complexes. It appears that IL-6 activation (regulated by STAT3 and STAT6 *via* ROR γ t activity) of IL-17 expression, and subsequent upregulation of IL-17 receptors and receptor complexes may have a key role in the pathogenesis of ASD. The majority of linked molecules identified above are proinflammatory and found in higher quantities in those with ASD, with a corresponding downregulation of anti-inflammatory proteins. Whether this dysregulation of IL-17 is an inherent or acquired state is unclear.

Circulating T cell and IL-17A levels are altered in a subset of children with ASD. Maternal immune activation (including IL-17A) seems to play a role in altering important developmental pathways through direct interaction with ASD susceptibility genes, and indirectly, through interaction with their gene products. Circulating levels of IL-17A are dysregulated during pregnancy in mothers of children who develop ASD and ID (63, 79, 83). Murine models support a causative role for IL-17A in the pathogenesis of ASD. We conclude from the existing evidence that IL-17A dysregulation in the mother or developing infant could play a causal role in the development of at least some subsets of ASD and may be the link between environmental exposure and genetic susceptibility. Understanding the role of IL-17A and its associated targets on neurodevelopmental outcomes is now becoming increasingly important.

WHAT IS THE RELEVANCE OF THE ONGOING COVID-19 PANDEMIC TO MIA-INDUCED ASD RISK?

Coronavirus disease 2019 (COVID-19), a disease caused by the novel coronavirus, SARS-CoV-2, has become a pandemic, affecting every corner of the globe. Although, the disease (COVID-19) affects primarily the respiratory systems of those affected, it has been found to affect and damage other organs, including the kidneys (115), liver (116), brain (117, 118), and heart (119, 120). Worldwide reported cases and COVID-19 related mortality are most likely an underestimate due to variability of public health capacities between countries, but as of August 2021, there have been almost 200 million confirmed cases of COVID-19, and over 4.2 million deaths reported to the WHO (121).

Our current knowledge of COVID-19 is based only on our limited experience with SARS-CoV-2 since December 2019 and analogously, through our experience of other coronaviruses (SARS CoV and MERS, Middle East Respiratory Syndrome). The long-term consequences of *in-utero* SARS-CoV-2 exposure and/or congenital infection are almost entirely unknown. There is clear evidence that prenatal exposure to viral infections increases the risk of adverse developmental, neurological, and psychiatric outcomes in later childhood and adult life (38, 44, 122). In this next section, we discuss the implications of the COVID-19 pandemic in the context of MIA-induced alterations in neurodevelopmental outcomes.

COVID-19 AND CYTOKINE STORM

Preclinical work shows that MIA, which stimulates interleukin17A release from Th17 cells, can establish sustained fetalplacental inflammatory responses. This inflammatory milieu can persist into childhood and affect the development of the young “primed” brain. Remarkably, in murine models, social difficulties in MIA-exposed offspring are remediable through a variety of mechanisms including IL-17 blockade (18, 46). Cytokine storm is a general term applied to maladaptive cytokine release in responses to infection and other stimuli (123). In the context of sepsis, cytokine storm is considered one of the major causes of acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS), and multi-organ failure (124, 125). In COVID-19, cytokine storm seems to play a role in disease aggravation and correlates positively with severity of disease (126). IL-17A target IL-6 and C-reactive protein (CRP) specifically, have been shown to correlate positively with increased mortality (127). Elevated numbers of Th17 cells have been isolated in the blood of individuals with fatal COVID-19 infection (128), while many authors have demonstrated significantly elevated levels of IL-17A in those with both mild and severe COVID-19 (129–131). Coronavirus infection results in macrophage, and dendritic cell activation and IL-6 release (132). This instigates an amplification cascade (JAK–STAT1/3 pathway) that results in cis signaling (binding of cell membrane bound IL-6 receptors) in lymphocytes with downregulation of Tregs and increased

differentiation of TH17 cells; as well as transsignaling (binding of soluble IL-6 receptor) effects on many other cell types (endothelial cells). This widespread immune activation and cytokine production contributes to the pathophysiology of severe COVID-19 (133). Indeed, some authors have specifically suggested therapies intended to target both Th17 cells and IL17A in COVID-19 disease (134, 135). We have already outlined how Th17 specific (T-helper 17 cell) pathways are initiated *via* activated macrophages that produce IL-6 and IL-1 β . As outlined, IL-6 in particular, is a potent potentiator and trigger for IL-17A release (123, 134, 136). IL-17A therefore, may be a key player in the COVID-19 cytokine storm.

CORONAVIRUS (SARS-CoV-2) NEUROTROPISM AND NEUROLOGICAL EFFECTS

Coronaviruses have a demonstrated specific neuro-tropism that allows them access to, and to proliferate in, the host's CNS (137, 138). Cell entry seems to occur through the angiotensin-converting enzyme-2 (ACE-2) and transmembrane protease serine 2 (TMP S2) receptors, both of which are widely expressed in the placenta and at the fetomaternal interface. While transplacental infection of the fetus is, yet to be proven conclusively, vertical transmission is certainly plausible and may lead directly to inflammatory processes in the fetal brain, in addition to indirect effects *via* the host/maternal immune response. The neurological sequelae of COVID-19 are wide-ranging and relatively common. The majority of neurological presentations so far have fallen into five categories, (i) Encephalopathy (including delirium and impaired consciousness), (ii) Inflammatory CNS disorders [including encephalitis and Acute Disseminating Encephalomyelitis (ADEM)], (iii) Cerebrovascular accident (CVA)/stroke, (iv) PNS disorders [including Guillain-Barré Syndrome (GBS) and cranial nerve palsies], (v) "Miscellaneous" central neurological disorders (such as raised intracranial pressure, seizures, and myelitis) (139). Hyposmia/Anosmia and hypogeusia (140) are recognized as two important hallmarks of acute SARS-CoV-2 infection, while more severe neurological complications have included CVAs, encephalitis, encephalopathy, and neuropsychiatric disorders (118, 141). Protein-protein network analysis for GBS and COVID-19 revealed that the combined gene set showed an increased connectivity as compared to COVID-19 or GBS alone, this was particularly true of genes related to Th17 cell differentiation. Transcriptome analysis of PBMC from patients with COVID-19 and GBS demonstrated the activation of interleukin-17 signaling in both conditions (142). Viral RNA has been isolated in clinical CSF samples in those with COVID-19 and neurological symptoms (143), and post-mortem examination of brain tissue has identified both viral RNA and neutrophilic infiltrates suggestive of aberrant immune response (144).

Recent pluripotent stem cell derived organoid models have been used to model SARS-CoV-2 infection in a wide range of tissues including gut, lung, liver, kidney, and brain (117, 145). These models demonstrate the virus' ability to infiltrate and proliferate in a variety of different cell/tissue types. Within the brain, the areas with the highest avidity for SARS-CoV2 are the choroid plexus and the hippocampus (117). This is an interesting finding, as the choroid plexuses themselves represent the interface between CSF and blood compartments (in a similar fashion to the blood-brain barrier). They are located in each of the four ventricles, and are intimately related with immediately adjacent CSF, capillary blood supply, and neural tissue. Angiotensin-converting enzyme-2 receptors also appear to be highly expressed in the choroid plexus (146). In this sense, they provide a comprehensive roadmap upon which SARS-CoV2 can potentially travel. The neurological features on COVID-19 infection are diverse and wide-ranging. Most studies to date have focused on symptomology in adult patients, but novel models of SARS-CoV-2 infection in a variety of human and animal tissues is casting new light on the mechanisms underlying COVID's infectivity and its ill-effects. There appears to be a variety of mechanisms underlying COVID's pathogenicity, not limited to direct viral effects on tissue, but also collateral effects *via* immune and thrombotic processes (147). Although there is little research on the effects of COVID on fetuses in early pregnancy, the same processes of direct viral effects and secondary immune and inflammatory effects are likely to be at play.

MATERNAL COVID-19 INFECTION AND PERINATAL EXPOSURE

Pregnant women are not thought to be more susceptible to contracting coronavirus than the general population (148), but given alterations in the pregnant immune state (103), they may be more susceptible to more severe infection (149, 150). Studies from previous pandemics, H1N1 influenza (2009), SARS (2003), and MERS (2012), suggest the possibility of significant maternal and neonatal morbidity and mortality (151, 152). Indeed, both MERS and SARS resulted in maternal death in a significant number of cases, but the

specific risk factors for a fatal outcome during pregnancy are not clear. Our experience with these previous coronaviruses indicates higher risk of adverse outcomes for the fetus and infant including fetal growth

restriction (FGR), and preterm delivery, both of which have previously been linked to increased ASD incidence (153) as well as NICU admission, spontaneous abortion, and perinatal death. As with other Coronaviruses, maternal SARS-CoV-2 infection has been associated with negative perinatal outcomes. Preterm delivery, fetal distress, stillbirth, and perinatal death have been widely reported (150, 154–156). Figures from China show that while up to 3% of pregnant women infected with COVID-19 required admission to intensive care (157, 158), a UK study showed 1% of pregnant women admitted with SARS-CoV-2 required ECMO (Extra-corporeal membrane oxygenation) and 10% Intensive Care Unit (ICU) management (159).

Cesarean section (CS) has been implicated as a risk factor for the development of ASD in offspring. The mechanisms underlying this are unclear, yet the risk of ASD is increased by approximately 33% in both elective and emergency CS procedures (160). In a systematic review of perinatal and maternal outcomes during the pandemic, CS rates were reported at extremely high levels, up to 90% in some centers (range from approximately 50–90%) (161). For comparison in work published in 2020, Turner et al. noted an all-cause national CS rate in Ireland of approximately 26% (162). These higher rates were observed in most centers in spite of recommendations from the Royal College of Obstetrics and Gynecology (RCOG) and the International Federation of Gynecology and Obstetrics (IFGO) against decisions for CS being influenced by maternal SARS-CoV-2 status.

More specifically to neonatal outcomes, the WHO quotes worldwide preterm delivery rates of approximately 10% (163). Two large review studies reported preterm delivery rates of 20–25% in SARS-CoV-2 affected pregnancies (164, 165). Women with SARS-CoV-2 seemed to be more likely to endure a preterm delivery (165). The majority of these deliveries were iatrogenic, but in some reviews, up to half were attributable to either fetal or maternal compromise (166).

Maternal and neonatal ICU admission rates were also higher in the SARS-CoV-2 affected cohorts. Maternal ICU admission and mechanical ventilation rates were high vs. age matched nonpregnant women (165). While rates of stillbirth and neonatal death appear similar to uninfected fetuses, NICU admission rates were notably higher in COVID affected pregnancies (159), commonly as a precautionary step in the care of the neonate. Neonatal morbidity was higher in the SARS-CoV-2 affected groups and was associated with preterm delivery in mothers with more severe COVID-19 primary infection. Hypoxemia and respiratory difficulties in mothers had knock on effects of reduced placenta perfusion, pre-placental hypoxemia, fetal distress, and preterm delivery (167).

Given our knowledge of the potential developmental effects of Th17 activation in pregnancy, children *in-utero* during this pandemic may have significant inflammatory exposures if maternal infection occurs. There remain unanswered questions about the impact that asymptomatic and mild maternal infection has on the fetal brain in early pregnancy. Prospective follow up studies will need to follow infants whose mothers were infected as well as health unaffected controls. There is enormous potential to leverage archived serological samples from pregnancy and neonatal cohorts to study the relationships (or associations) between markers of maternal inflammation and later neurodevelopmental outcomes in offspring born during the pandemic. While in general, the likelihood of intrauterine maternal-fetal transmission of coronaviruses is low—there have been no documented cases of vertical transmission occurring with either SARS or MERS. There are current reports of possible vertical transmission of SARS-CoV-2 in several cases of third trimester maternal infection (168–170). Little to no information exists about children exposed in the first and second trimesters yet. While generally placental seeding does not seem common, some cases have reported strong evidence of placental infection with the demonstration of high viral load and immuno-histological evidence of SARS-CoV-2 in placental tissue (168). Currently, we can only surmise what the true effect (if any) of gestational COVID-19 on the incidence of ASD will be, but already some have concerns that the incidence may increase (171, 172). No studies have yet been reported on neurodevelopmental outcomes, as the oldest offspring are still in early childhood. Still, the evidence we have outlined within this review from MIA studies examining IL-17A and its pathway members provides a strong basis to build upon our current hypothesis and ask the question; could COVID-19 induced MIA act *via* IL-17A signaling to increase the risk of ASD-like phenotypes in vulnerable offspring?

DISCUSSION: IMPROVING OUTCOMES FOR ASD AFFECTED INDIVIDUALS AND FAMILIES

We believe that in spite of the tragedy of the COVID-19 emergency, we are presented with a serendipitous opportunity to progress scientific knowledge regarding prenatal exposures and ASD risk. During the COVID-19 pandemic, we have witnessed a novel infection, affect an immunologically naïve population within an extremely well-defined period of exposure. COVID-19 is now a notifiable illness, and has been characterized and monitored more than any illness in history. Many countries have developed stringent mandatory testing protocols, and track and trace programmes. Within all this, exists an opportunity to study the longitudinal

effects of this infection on offspring of those affected by gestational COVID. Further investigation of mid-gestational cytokine profiles (IL-17A in particular) and their potential for genetic interplay could be a crucial cog in the development of actionable and cost-effective improvements in the current models of ASD care. Identification of pathways of immune dysregulation during pregnancy could lead to the identification of a risk marker of ASD that could be characterized in broader ASD cohorts. This would facilitate the identification of a predictive marker of ASD allowing earlier dedicated ASD screening in at risk children. Coupled with these potential biochemical markers, known early clinical signs of ASD exist. Crystallization of the ASD diagnosis can be as early as 14 months old according to some authors, and there are clinically detectable signs of ASD from a younger age still (23, 173, 174). The first children born of this pandemic are now reaching their toddler years, and they may represent a group with increased risk of ASD or other developmental conditions. Taken together, a postulated early biochemical marker and established early clinical markers could allow targeted early ASD screening, which would lead to earlier intervention, and improved outcomes. Therapies instituted in this age group have the potential to significantly improve clinical outcomes in ASD affected children. The timing of therapy is important with the most dramatic symptomatic and developmental improvements in those detected at an earlier age of diagnosis (175, 176).

We believe that it is the obligation of the scientific community to glean what benefit we can from this pandemic. In spite of social distancing measures, systematic national “lockdowns,” and working from home, there has been unprecedented scientific collaboration to try to counter the scourge of COVID. This has led to some outstanding success, not least in the development of two highly effective mRNA vaccines. In order to facilitate international research, the development of an international gestational COVID-19 consortium and registry would be an important step in coordinating research activities and aims. Isolation of relevant clinical bio-samples and prospective identification of patients will have already begun in some centers, and should be facilitated by the public health infrastructures that have been built up around the pandemic. Multidisciplinary collaborative follow up programmes should be established to identify, assess, and treat children with potential negative postCOVID outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

MC wrote the manuscript, reviewed the literature, and synthesized the hypothesis. SC, LGi, and LGa commented on the manuscript at all stages. GO’K commented on the manuscript and aided with literature review. DM commented on the manuscript, helped to synthesize the hypothesis, review the literature, and was the key supervisor. All authors have read and approved the final manuscript.

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REFERENCES

1. American Psychiatric Association. *DSM-V. 5th ed.* Washington, DC: American Psychiatric Association (2013).
2. Birtwell KB. Social, cognitive, and behavioral development of children and adolescents with autism spectrum disorder. In: McDougle C, editor. *Autism Spectrum Disorder. Section 1, Chapter 2.* Oxford: UK: Oxford Press (2016). p. 19–30. doi: 10.1093/med/9780199349722.003.0002
3. Magiati I, Ong C, Lim XY, Tan JW, Ong AY, Patricia F, et al. Anxiety symptoms in young people with autism spectrum disorder attending special schools: associations with gender, adaptive functioning and autism symptomatology. *Autism.* (2016) 20:306–20. doi: 10.1177/1362361315577519
4. Treffert DA. Epidemiology of infantile autism. *Arch Gen Psychiatry.* (1970) 22:431–8. doi: 10.1001/archpsyc.1970.01740290047006
5. Maenner MJ, Shaw KA, Bakian AV, Bilder DA, Durkin MS, Esler A, et al. Prevalence and characteristics of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, United States, 2018. *MWR Surveill Summ.* (2021) 70:1–16. doi: 10.15585/mmwr.ss7011a1
6. Lundstrom S, Reichenberg A, Anckarsater H, Lichtenstein P, Gillberg C. Autism phenotype versus registered diagnosis in Swedish children: prevalence trends over 10 years in general population samples. *BMJ.* (2015) 350:h1961. doi: 10.1136/bmj.h1961
7. Hansen SN, Schendel DE, Parner ET. Explaining the increase in the prevalence of autism spectrum disorders: the proportion attributable to changes in reporting practices. *JAMA Pediatr.* (2015) 169:56–62. doi: 10.1001/jamapediatrics.2014.1893
8. Baio J, Wiggins L, Christensen DL, Maenner MJ, Daniels J, Warren Z, et al. Prevalence of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, United States, 2014. *MWR Surveill Summ.* (2018) 67:1–23. doi: 10.15585/mmwr.mm6745a7
9. de la Torre-Ubieta L, Won H, Stein JL, Geschwind DH. Advancing the understanding of autism disease mechanisms through genetics. *Nat Med.* (2016). 22:345–61. doi: 10.1038/nm.4071
10. Fernandes IR, Cruz ACP, Ferrasa A, Phan D, Herai RH, Muotri AR. Genetic variations on SETD5 underlying autistic conditions. *Dev Neurobiol.* (2018) 78:500–18. doi: 10.1002/dneu.22584
11. Palmer N, Beam A, Agniel D, Eran A, Manrai A, Spettell C, et al. Association of sex with recurrence of autism spectrum disorder among siblings. *JAMA Pediatr.* (2017) 171:1107–12. doi: 10.1001/jamapediatrics.2017.2832
12. Gaugler T, Klei L, Sanders SJ, Bodea CA, Goldberg AP, Lee AB, et al. Most genetic risk for autism resides with common variation. *Nat Genet.* (2014) 46:881–5. doi: 10.1038/ng.3039
13. Vorstman JAS, Parr JR, Moreno-De-Luca D, Anney RJL, Nurnberger J Jr, Hallmayer JF. Autism genetics: opportunities and challenges for clinical translation. *Nature reviews. Genetics.* (2017) 18:362–76. doi: 10.1038/nrg.2017.4
14. F NG, Gallagher L, Lopez LM. Autism spectrum disorder genomics: the progress and potential of genomic technologies. *Genomics.* (2020). 112:5136–42. doi: 10.1016/j.ygeno.2020.09.022
15. Goines PE, Croen LA, Braunschweig D, Yoshida CK, Grether J, Hansen R, et al. Increased midgestational IFN-gamma, IL-4 and IL-5 in women bearing a child with autism: a case-control study. *Mol Autism.* (2011) 2:13. doi: 10.1186/2040-2392-2-13
16. Chess S. Follow-up report on autism in congenital rubella. *J Autism Child Schizophr.* (1977) 7:69–81. doi: 10.1007/BF01531116
17. Atladottir HO, Thorsen P, Ostergaard L, Schendel DE, Lemcke S, Abdallah M, et al. Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. *J Autism Dev Disord.* (2010) 40:1423–30. doi: 10.1007/s10803-010-1006-y
18. Choi GB, Yim YS, Wong H, Kim S, Kim H, Kim SV, et al. The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science.* (2016) 351:933–9. doi: 10.1126/science.aad0314
19. Garay PA, Hsiao EY, Patterson PH, McAllister AK. Maternal immune activation causes age- and region-specific changes in brain cytokines in offspring throughout development. *Brain Behav Immun.* (2013) 31:54–68. doi: 10.1016/j.bbi.2012.07.008
20. Smith SE, Li J, Garbett K, Mirnic K, Patterson PH. Maternal immune activation alters fetal brain development through interleukin-6. *J Neurosci.* (2007) 27:10695–702. doi: 10.1523/JNEUROSCI.2178-07.2007
21. Dawson G. Early intensive behavioral intervention appears beneficial for young children with autism spectrum disorders. *J Pediatr.* (2013) 162:1080–1. doi: 10.1016/j.jpeds.2013.02.049
22. Estes A, Munson J, Rogers SJ, Greenon J, Winter J, Dawson G. Long-term outcomes of early intervention in 6-year-old children with autism spectrum disorder. *J Am Acad Child Adolesc Psychiatry.* (2015) 54:580–7. doi: 10.1016/j.jaac.2015.04.005
23. Pierce K, Gazestani VH, Bacon E, Barnes CC, Cha D, Nalabolu S, et al. Evaluation of the diagnostic stability of the early autism spectrum disorder phenotype in the general population starting at 12 months. *JAMA Pediatr.* (2019) 173:578–87. doi: 10.1001/jamapediatrics.2019.0624
24. Landa RJ. Diagnosis of autism spectrum disorders in the first 3 years of life. *Nat Clin Pract Neurol.* (2008) 4:138–47. doi: 10.1038/ncpneu0731
25. Rogers SJ, Estes A, Lord C, Vismara L, Winter J, Fitzpatrick A, et al. Effects of a brief Early Start Denver model (ESDM)-based parent intervention on toddlers at risk for autism spectrum disorders: a randomized controlled trial. *J Am Acad Child Adolesc Psychiatry.* (2012) 51:1052–65. doi: 10.1016/j.jaac.2012.08.003
26. Broek JA, Brouwer E, Stelzhammer V, Guest PC, Rahmoune H, Bahn S. The need for a comprehensive molecular characterization of autism spectrum disorders. *Int J Neuropsychopharmacol.* (2014) 17:651–73. doi: 10.1017/S146114571300117X
27. Masi A, Quintana DS, Glozier N, Lloyd AR, Hickie IB, Guastella AJ. Cytokine aberrations in autism spectrum disorder: a systematic review and meta-analysis. *Mol Psychiatry.* (2015) 20:440–6. doi: 10.1038/mp.2014.59
28. Gunes S, Ekinci O, Celik T. Iron deficiency parameters in autism spectrum disorder: clinical correlates and associated factors. *Ital J Pediatr.* (2017) 43:86. doi: 10.1186/s13052-017-0407-3
29. Suzuki K, Matsuzaki H, Iwata K, Kameno Y, Shimmura C, Kawai S, et al. Plasma cytokine profiles in subjects with high-functioning autism spectrum disorders. *PLoS ONE.* (2011) 6:e20470. doi: 10.1371/journal.pone.0020470
30. Ashwood P, Wills S, Van de Water J. The immune response in autism: a new frontier for autism research. *J Leukoc Biol.* (2006) 80:1–15. doi: 10.1189/jlb.1205707
31. Morato Torres CA, Wassouf Z, Zafar F, Sastre D, Outeiro TF, Schüle B. The role of alpha-synuclein and other Parkinson's genes in neurodevelopmental and neurodegenerative disorders. *Int J Mol Sci.* (2020). 21:5724. doi: 10.3390/ijms21165724

32. Zou M, Li D, Wang L, Li X, Xie S, Liu Y, et al. Identification of amino acid dysregulation as a potential biomarker for autism spectrum disorder in China. *Neurotox Res.* (2020) 38:992–1000. doi: 10.1007/s12640-020-00242-9
33. Masi A, Glozier N, Dale R, Guastella AJ. The immune system, cytokines, and biomarkers in autism spectrum disorder. *Neurosci Bull.* (2017) 33:194–204. doi: 10.1007/s12264-017-0103-8
34. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah IN, Van de Water J. Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders. *J Neuroimmunol.* (2011) 232:196–9. doi: 10.1016/j.jneuroim.2010.10.025
35. Akintunde ME, Rose M, Krakowiak P, Heuer L, Ashwood P, Hansen R, et al. Increased production of IL-17 in children with autism spectrum disorders and co-morbid asthma. *J Neuroimmunol.* (2015) 286:33–41. doi: 10.1016/j.jneuroim.2015.07.003
36. Chess S. Autism in children with congenital rubella. *J Autism Child Schizophr.* (1971) 1:33–47. doi: 10.1007/BF01537741
37. Jiang HY, Xu LL, Shao L, Xia RM, Yu ZH, Ling ZX, et al. Maternal infection during pregnancy and risk of autism spectrum disorders: a systematic review and meta-analysis. *Brain Behav Immun.* (2016) 58:165–72. doi: 10.1016/j.bbi.2016.06.005
38. Conway F, Brown AS. Maternal immune activation and related factors in the risk of offspring psychiatric disorders. *Front Psychiatry.* (2019) 10:430. doi: 10.3389/fpsy.2019.00430
39. Curran EA, O’Keeffe GW, Looney AM, Moloney G, Hegarty SV, Murray DM, et al. Exposure to hypertensive disorders of pregnancy increases the risk of autism spectrum disorder in affected offspring. *Mol Neurobiol.* (2018) 55:5557–64. doi: 10.1007/s12035-017-0794-x
40. Knuesel I, Chicha L, Britschgi M, Schobel SA, Bodmer M, Hellings JA, et al. Maternal immune activation and abnormal brain development across CNS disorders. *Nat Rev Neurol.* (2014) 10:643–60. doi: 10.1038/nrneurol.2014.187
41. Boulanger-Bertolus J, Pancaro C, Mashour GA. Increasing role of maternal immune activation in neurodevelopmental disorders. *Front Behav Neurosci.* (2018). 12:230. doi: 10.3389/fnbeh.2018.00230
42. Minakova E, Warner BB. Maternal immune activation, central nervous system development and behavioral phenotypes. *Birth Defec Res.* (2018) 110:1539–50. doi: 10.1002/bdr2.1416
43. Bauman MD, Iosif AM, Smith SE, Bregere C, Amaral DG, Patterson PH. Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. *Biol Psychiatry.* (2014) 75:332–41. doi: 10.1016/j.biopsych.2013.06.025
44. Careaga M, Murai T, Bauman MD. Maternal immune activation and autism spectrum disorder: from rodents to nonhuman and human primates. *Biol Psychiatry.* (2017) 81:391–401. doi: 10.1016/j.biopsych.2016.10.020
45. Meyer U, Feldon J. To poly(I:C) or not to poly(I:C): advancing preclinical schizophrenia research through the use of prenatal immune activation models. *Neuropharmacology.* (2012) 62:1308–21. doi: 10.1016/j.neuropharm.2011.01.009
46. Wong H, Hoeffler C. Maternal IL-17A in autism. *Exp Neurol.* (2018). 299(Pt A):228–40. doi: 10.1016/j.expneurol.2017.04.010
47. Xuan ICY, Hampson DR. Gender-dependent effects of maternal immune activation on the behavior of mouse offspring. *PLoS ONE.* (2014) 9:e104433. doi: 10.1371/journal.pone.0104433
48. Haddad FL, Patel SV, Schmid S. Maternal immune activation by poly(I:C) as a preclinical model for neurodevelopmental disorders: a focus on autism and schizophrenia. *Neurosci Biobehav Rev.* (2020) 113:546–67. doi: 10.1016/j.neubiorev.2020.04.012
49. Parker-Athill EC, Tan J. Maternal immune activation and autism spectrum disorder: interleukin-6 signaling as a key mechanistic pathway. *Neurosignals.* (2010) 18:113–28. doi: 10.1159/000319828
50. Estes ML, McAllister AK. IMMUNOLOGY. Maternal TH17 cells take a toll on baby’s brain. *Science.* (2016) 351:919–20. doi: 10.1126/science.aaf2850
51. Casanova MF, El-Baz AS, Kamat SS, Dombroski BA, Khalifa F, Elnakib A, et al. Focal cortical dysplasias in autism spectrum disorders. *Acta Neuropathol Commun.* (2013) 1:67. doi: 10.1186/2051-5960-1-67
52. Varghese M, Keshav N, Jacot-Descombes S, Warda T, Wicinski B, Dickstein DL, et al. Autism spectrum disorder: neuropathology and animal models. *Acta Neuropathol.* (2017) 134:537–66. doi: 10.1007/s00401-017-1736-4
53. Kugelberg E. Neuroimmunology: IL-17A mediates a path to autism. *Nat Rev Immunol.* (2016) 16:205. doi: 10.1038/nri.2016.35
54. Chang YC, Cole TB, Costa LG. Behavioral phenotyping for autism spectrum disorders in mice. *Curr Protoc Toxicol.* (2017). 72:11.22.1–21. doi: 10.1002/cptx.19
55. Hornig M, Bresnahan MA, Che X, Schultz AF, Ukaigwe JE, Eddy ML, et al. Prenatal fever and autism risk. *Mol Psychiatry.* (2018) 23:759–66. doi: 10.1038/mp.2017.119
56. Atladóttir HÓ, Henriksen TB, Schendel DE, Parner ET. Autism after infection, febrile episodes, and antibiotic use during pregnancy: an exploratory study. *Pediatrics.* (2012) 130:e1447. doi: 10.1542/peds.2012-1107
57. Mueller FS, Polesel M, Richetto J, Meyer U, Weber-Stadlbauer U. Mouse models of maternal immune activation: Mind your caging system! *Brain Behav Immun.* (2018) 73:643–60. doi: 10.1016/j.bbi.2018.07.014
58. Li J, Robinson M, Malacova E, Jacoby P, Foster J, van Eekelen A. Maternal life stress events in pregnancy link to children’s school achievement at age 10 years. *J Pediatr.* (2013) 162:483–9. doi: 10.1016/j.jpeds.2012.09.007
59. Chua JSC, Cowley CJ, Manavis J, Rofo AM, Coyle P. Prenatal exposure to lipopolysaccharide results in neurodevelopmental damage that is ameliorated by zinc in mice. *Brain Behav Immun.* (2012) 26:326–36. doi: 10.1016/j.bbi.2011.10.002
60. Luan W, Hammond LA, Vuillermot S, Meyer U, Eyles DW. Maternal vitamin D prevents abnormal dopaminergic development and function in a mouse model of prenatal immune activation. *Sci Rep.* (2018) 8:9741. doi: 10.1038/s41598-018-28090-w
61. Rovira N, Alarcon A, Iriando M, Ibañez M, Poo P, Cusi V, et al. Impact of histological chorioamnionitis, funisitis and clinical chorioamnionitis on neurodevelopmental outcome of preterm infants. *Early Hum Dev.* (2011) 87:253–7. doi: 10.1016/j.earlhumdev.2011.01.024
62. Lee I, Neil JJ, Huettner PC, Smyser CD, Rogers CE, Shimony JS, et al. The impact of prenatal and neonatal infection on neurodevelopmental outcomes in very preterm infants. *J Perinatol.* (2014) 34:741–7. doi: 10.1038/jp.2014.79
63. Jones KL, Croen LA, Yoshida CK, Heuer L, Hansen R, Zerbo O, et al. Autism with intellectual disability is associated with increased levels of maternal cytokines and chemokines during gestation. *Mol Psychiatry.* (2017) 22:273–9. doi: 10.1038/mp.2016.77
64. Irwin JL, Yeates AJ, Mulhern MS, McSorley EM, Strain JJ, Watson GE, et al. Maternal gestational immune response and autism spectrum disorder phenotypes at 7 years of age in the Seychelles Child Development Study. *Mol Neurobiol.* (2019) 56:5000–8. doi: 10.1007/s12035-018-1424-y

65. Abdallah MW, Larsen N, Grove J, Norgaard-Pedersen B, Thorsen P, Mortensen EL, et al. Amniotic fluid inflammatory cytokines: potential markers of immunologic dysfunction in autism spectrum disorders. *World J Biol Psychiatry*. (2013) 14:528–38. doi: 10.3109/15622975.2011.639803
66. Shobokshi A, Shaarawy M. Maternal serum and amniotic fluid cytokines in patients with preterm premature rupture of membranes with and without intrauterine infection. *Int J Gynaecol Obstet*. (2002) 79:209–15. doi: 10.1016/S0020-7292(02)00238-2
67. Rounioja S, Räsänen J, Glumoff V, Ojaniemi M, Mäkilallio K, Hallman M. Intra-amniotic lipopolysaccharide leads to fetal cardiac dysfunction. A mouse model for fetal inflammatory response. *Cardiovasc Res*. (2003) 60:156–64. doi: 10.1016/S0008-6363(03)00338-9
68. Ricci S, Businaro R, Ippoliti F, Lo Vasco VR, Massoni F, Onofri E, et al. Altered cytokine and BDNF levels in autism spectrum disorder. *Neurotox Res*. (2013) 24:491–501. doi: 10.1007/s12640-013-9393-4
69. Chez MG, Dowling T, Patel PB, Khanna P, Kominsky M. Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children. *Pediatr Neurol*. (2007) 36:361–5. doi: 10.1016/j.pediatrneurol.2007.01.012
70. Eftekharian MM, Ghafouri-Fard S, Noroozi R, Omrani MD, Arsang-Jang S, Ganji M, et al. Cytokine profile in autistic patients. *Cytokine*. (2018) 108:120–6. doi: 10.1016/j.cyto.2018.03.034
71. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah I, Van de Water J. Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. *Brain Behav Immun*. (2011) 25:40–5. doi: 10.1016/j.bbi.2010.08.003
72. Kordulewska NK, Kostyra E, Piskorz-Ogórek K, Moszynska M, Cieślinska A, Fiedorowicz E, et al. Serum cytokine levels in children with spectrum autism disorder: Differences in pro- and anti-inflammatory balance. *J Neuroimmunol*. (2019) 337:577066. doi: 10.1016/j.jneuroim.2019.577066
73. Heuer LS, Croen LA, Jones KL, Yoshida CK, Hansen RL, Yolken R, et al. An exploratory examination of neonatal cytokines and chemokines as predictors of autism risk: the early markers for autism study. *Biol Psychiatry*. (2019) 86:255–64. doi: 10.1016/j.biopsych.2019.04.037
74. Kutuk MO, Tufan E, Gokcen C, Kilicaslan F, Karadag M, Mutluer T, et al. Cytokine expression profiles in autism spectrum disorder: a multi-center study from Turkey. *Cytokine*. (2020) 133:155152. doi: 10.1016/j.cyto.2020.155152
75. Nishimoto N, Kishimoto T. Interleukin 6: from bench to bedside. *Nat Clin Pract Rheumatol*. (2006) 2:619–26. doi: 10.1038/ncprheum0338
76. Wei H, Zou H, Sheikh AM, Malik M, Dobkin C, Brown WT, et al. IL6 is increased in the cerebellum of autistic brain and alters neural cell adhesion, migration and synaptic formation. *J Neuroinflammation*. (2011) 8:52. doi: 10.1186/1742-2094-8-52
77. Murphy SP, Tayade C, Ashkar AA, Hatta K, Zhang J, Croy BA. Interferon gamma in successful pregnancies. *Biol Reprod*. (2009) 80:848–59. doi: 10.1095/biolreprod.108.073353
78. Al-Ayadhi LY, Mostafa GA. Elevated serum levels of interleukin-17A in children with autism. *J Neuroinflammation*. (2012) 9:158. doi: 10.1186/1742-2094-9-158
79. Moaaz M, Youssry S, Elfataty A, El Rahman MA. Th17/Treg cell imbalance and their related cytokines (IL-17, IL-10 and TGF-β) in children with autism spectrum disorder. *J Neuroimmunol*. (2019) 337:577071. doi: 10.1016/j.jneuroim.2019.577071
80. Ahmad SF, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, Alasmari AF, et al. Involvement of CD45 cells in the development of autism spectrum disorder through dysregulation of granulocyte-macrophage colony-stimulating factor, key inflammatory cytokines, and transcription factors. *Int Immunopharmacol*. (2020) 83:106466. doi: 10.1016/j.intimp.2020.106466
81. Bryn V, Aass HC, Skjeldal OH, Isaksen J, Saugstad OD, Ormstad H. Cytokine profile in autism spectrum disorders in children. *J Mol Neurosci*. (2017) 61:1–7. doi: 10.1007/s12031-016-0847-z
82. Casey S, Carter M, Looney AM, Livingstone V, Moloney G, O’Keeffe GW, et al. Maternal mid-gestation cytokine dysregulation in mothers of children with autism spectrum disorder. *J Autism Dev Disord*. (2021). doi: 10.1007/s10803-021-05271-7. [Epub ahead of print].
83. van der Zwaag B, Franke L, Poot M, Hochstenbach R, Spierenburg HA, Vorstman JA, et al. Gene-network analysis identifies susceptibility genes related to glycobiology in autism. *PLoS ONE*. (2009) 4:e5324. doi: 10.1371/journal.pone.0005324
84. Chehimi M, Vidal H, Eljaafari A. Pathogenic role of IL-17-producing immune cells in obesity, and related inflammatory diseases. *J Clin Med*. (2017). 6:68. doi: 10.3390/jcm6070068
85. Hill AP, Zuckerman KE, Fombonne E. Obesity and autism. *Pediatrics*. (2015) 136:1051–61. doi: 10.1542/peds.2015-1437
86. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res*. (2019) 47:D607–13. doi: 10.1093/nar/gky1131
87. Kimura A, Kishimoto T. IL-6: Regulator of Treg/Th17 balance. *Eur J Immunol*. (2010) 40:1830–5. doi: 10.1002/eji.201040391
88. Deverman BE, Patterson PH. Cytokines and CNS development. *Neuron*. (2009) 64:61–78. doi: 10.1016/j.neuron.2009.09.002
89. Wu WL, Hsiao EY, Yan Z, Mazmanian SK, Patterson PH. The placental interleukin-6 signaling controls fetal brain development and behavior. *Brain Behav Immun*. (2017) 62:11–23. doi: 10.1016/j.bbi.2016.11.007
90. Wright JF, Bennett F, Li B, Brooks J, Luxenberg DP, Whitters MJ, et al. The human IL-17F/IL-17A heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex. *J Immunol*. (2008) 181:2799. doi: 10.4049/jimmunol.181.4.2799
91. Yang XO, Chang SH, Park H, Nurieva R, Shah B, Acero L, et al. Regulation of inflammatory responses by IL-17F. *J Exp Med*. (2008) 205:1063–75. doi: 10.1084/jem.20071978
92. Nadeem A, Ahmad SF, Attia SM, Bakheet SA, Al-Harbi NO, Al-Ayadhi LY. Activation of IL-17 receptor leads to increased oxidative inflammation in peripheral monocytes of autistic children. *Brain Behav Immun*. (2018) 67:335–44. doi: 10.1016/j.bbi.2017.09.010
93. Nadeem A, Ahmad SF, Attia SM, Al-Ayadhi LY, Bakheet SA, Al-Harbi NO. Oxidative and inflammatory mediators are upregulated in neutrophils of autistic children: role of IL-17A receptor signaling. *Prog Neuro Psychopharmacol Biol Psychiatry*. (2019) 90:204–11. doi: 10.1016/j.pnpbp.2018.12.002
94. Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem*. (2007) 282:9358–63. doi: 10.1074/jbc.C600321200

95. Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. (2006). 126:1121–33. doi: 10.1016/j.cell.2006.07.035
96. Ahmad SF, Zoheir KMA, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, et al. Dysregulation of Th1, Th2, Th17, and T regulatory cell-related transcription factor signaling in children with autism. *Mol Neurobiol*. (2017) 54:4390–400. doi: 10.1007/s12035-016-9977-0
97. Zhu C, Zhang A, Huang S, Ding G, Pan X, Chen R. Interleukin-13 inhibits cytokines synthesis by blocking nuclear factor- κ B and c-Jun Nterminal kinase in human mesangial cells. *J Biomed Res*. (2010) 24:308–16. doi: 10.1016/S1674-8301(10)60043-7
98. Hall SL, Baker T, Lajoie S, Richgels PK, Yang Y, McAlees JW, et al. IL17A enhances IL-13 activity by enhancing IL-13-induced signal transducer and activator of transcription 6 activation. *J Allergy Clin Immunol*. (2017). 139(2):462.e14–71.e14. doi: 10.1016/j.jaci.2016.04.037
99. Molloy CA, Morrow AL, Meinzen-Derr J, Schleifer K, Dienger K, Manning-Courtney P, et al. Elevated cytokine levels in children with autism spectrum disorder. *J Neuroimmunol*. (2006) 172:198–205. doi: 10.1016/j.jneuroim.2005.11.007
100. Bloodworth MH, Newcomb DC, Dulek DE, Stier MT, Cephus JY, Zhang J, et al. STAT6 signaling attenuates interleukin-17-producing $\gamma\delta$ T cells during acute *Klebsiella pneumoniae* infection. *Infect Immun*. (2016) 84:1548–55. doi: 10.1128/IAI.00646-15
101. Cooney LA, Towery K, Endres J, Fox DA. Sensitivity and resistance coregulation by IL-4 during Th17 maturation. *J Immunol*. (2011) 187:4440–50. doi: 10.4049/jimmunol.1002860
102. Chatterjee P, Chiasson VL, Bounds KR, Mitchell BM. Regulation of the antiinflammatory cytokines interleukin-4 and interleukin-10 during pregnancy. *Front Immunol*. (2014) 5:253. doi: 10.3389/fimmu.2014.00253
103. Jonakait GM. The effects of maternal inflammation on neuronal development: possible mechanisms. *Int J Dev Neurosci*. (2007) 25:415–25. doi: 10.1016/j.ijdevneu.2007.08.017
104. Couper KN, Blount DG, Riley EM. IL-10: The master regulator of immunity to infection. *J Immunol*. (2008) 180:5771. doi: 10.4049/jimmunol.180.9.5771
105. Abdallah MW, Larsen N, Mortensen EL, Atladóttir HÓ, Nørgaard Pedersen B, Bonefeld-Jørgensen EC, et al. Neonatal levels of cytokines and risk of autism spectrum disorders: an exploratory register-based historic birth cohort study utilizing the Danish Newborn Screening Biobank. *J Neuroimmunol*. (2012) 252:75–82. doi: 10.1016/j.jneuroim.2012.07.013
106. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity*. (1994) 1:405–13. doi: 10.1016/1074-7613(94)90071-X
107. Ahmad SF, Nadeem A, Ansari MA, Bakheet SA, Attia SM, Zoheir KMA, et al. Imbalance between the anti- and pro-inflammatory milieu in blood leukocytes of autistic children. *Mol Immunol*. (2017) 82:57–65. doi: 10.1016/j.molimm.2016.12.019
108. Lombardo MV, Moon HM, Su J, Palmer TD, Courchesne E, Pramparo T. Maternal immune activation dysregulation of the fetal brain transcriptome and relevance to the pathophysiology of autism spectrum disorder. *Mol Psychiatry*. (2018) 23:1001–13. doi: 10.1038/mp.2017.15
109. Courchesne E, Pramparo T, Gazestani VH, Lombardo MV, Pierce K, Lewis NE. The ASD living biology: from cell proliferation to clinical phenotype. *Mol Psychiatry*. (2019) 24:88–107. doi: 10.1038/s41380-018-0056-y
110. Weichhart T, Hengstschläger M, Linke M. Regulation of innate immune cell function by mTOR. *Nat Rev Immunol*. (2015) 15:599–614. doi: 10.1038/nri3901
111. Petrusek T, Vojtechova I, Klovra O, Tuckova K, Vejmla C, Rak J, et al. mTOR inhibitor improves autistic-like behaviors related to Tsc2 haploinsufficiency but not following developmental status epilepticus. *J Neurodev Disord*. (2021). 13:14. doi: 10.1186/s11689-02109357-2
112. Sanders SK, Giblin PA, Kavathas P. Cell-cell adhesion mediated by CD8 and human histocompatibility leukocyte antigen G, a nonclassical major histocompatibility complex class 1 molecule on cytotrophoblasts. *J Exp Med*. (1991) 174:737–40. doi: 10.1084/jem.174.3.737
113. Guerini FR, Bolognesi E, Chiappedi M, Ripamonti E, Ghezzi A, Zanette M, et al. HLA-G coding region polymorphism is skewed in autistic spectrum disorders. *Brain Behav Immun*. (2018) 67:308–13. doi: 10.1016/j.bbi.2017.09.007
114. Shin Yim Y, Park A, Berríos J, Lafourcade M, Pascual LM, Soares N, et al. Reversing behavioural abnormalities in mice exposed to maternal inflammation. *Nature*. (2017) 549:482–7. doi: 10.1038/nature23909
115. Watchorn J, Huang DY, Joslin J, Bramham K, Hutchings SD. Critically ill COVID-19 patients with acute kidney injury have reduced renal blood flow and perfusion despite preserved cardiac function. A case control study using contrast enhanced ultrasound. *Shock*. (2020) 55:479–87. doi: 10.2139/ssrn.3627340
116. Kumar A, Kumar P, Dungdung A, Kumar Gupta A, Anurag A, Kumar A. Pattern of liver function and clinical profile in COVID-19: a cross-sectional study of 91 patients. *Diabetes Metab Syndr*. (2020) 14:1951–4. doi: 10.1016/j.dsx.2020.10.001
117. Jacob F, Pather SR, Huang WK, Zhang F, Wong SZH, Zhou H, et al. Human pluripotent stem cell-derived neural cells and brain organoids reveal SARS-CoV-2 neurotropism predominates in choroid plexus epithelium. *Cell Stem Cell*. (2020) 27:937–50.e9. doi: 10.1101/2020.07.28.225151
118. Rifino N, Corsari B, Agazzi E, Alimonti D, Bonito V, Camera G, et al. Neurologic manifestations in 1760 COVID-19 patients admitted to Papa Giovanni XXIII Hospital, Bergamo, Italy. *J Neurol*. (2020) 268:2331–8. doi: 10.1007/s00415-020-10251-5
119. Peltzer B, Manocha KK, Ying X, Kirzner J, Ip JE, Thomas G, et al. Outcomes and mortality associated with atrial arrhythmias among patients hospitalized with COVID-19. *J Cardiovasc Electrophysiol*. (2020) 31:3077–85. doi: 10.1111/jce.14770
120. Nakamura Y, Shimizu M, Yamaki T, Kushimoto K, Yamashita A, Hayase K, et al. Myocardial injury in a patient with severe coronavirus disease: a case report. *J Infect Chemother*. (2020) 27:364–8. doi: 10.1016/j.jiac.2020.09.023
121. Organisation WH. *WHO Coronavirus Disease (COVID-19) Dashboard*. (2020). Available online at: <https://covid19.who.int/> (accessed August 30, 2021).
122. Estes ML, McAllister AK. Maternal immune activation: implications for neuropsychiatric disorders. *Science*. (2016) 353:772–7. doi: 10.1126/science.aag3194
123. Ye Q, Wang B, Mao J. The pathogenesis and treatment of the ‘Cytokine Storm’ in COVID-19. *J Infect*. (2020) 80:607–13. doi: 10.1016/j.jinf.2020.03.037
124. Chousterman BG, Swirski FK, Weber GF. Cytokine storm and sepsis disease pathogenesis. *Semin Immunopathol*. (2017) 39:517–28. doi: 10.1007/s00281-017-0639-8

125. Wu Z, McGoogan JM. Characteristics of and Important Lessons From the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese center for disease control and prevention. *Jama*. (2020) 323:1239–42. doi: 10.1001/jama.2020.2648
126. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
127. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intens Care Med*. (2020) 46:846–8. doi: 10.1007/s00134-020-05991-x
128. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med*. (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
129. Ghazavi A, Ganji A, Keshavarzian N, Rabiemajid S, Mosayebi G. Cytokine profile and disease severity in patients with COVID-19. *Cytokine*. (2021) 137:155323. doi: 10.1016/j.cyto.2020.155323
130. Qi D, Yan X, Tang X, Peng J, Yu Q, Feng L, et al. Epidemiological and clinical features of 2019-nCoV acute respiratory disease cases in Chongqing municipality, China: a retrospective, descriptive, multiple-center study. *medRxiv*. (2020) 2020.03.01.20029397. doi: 10.1101/2020.03.01.20029397
131. Ouyang Y, Yin J, Wang W, Shi H, Shi Y, Xu B, et al. Downregulated gene expression spectrum and immune responses changed during the disease progression in patients with COVID-19. *Clin Infect Dis*. (2020) 71:2052–60. doi: 10.1093/cid/ciaa462
132. Wang J, Jiang M, Chen X, Montaner LJ. Cytokine storm and leukocyte changes in mild versus severe SARS-CoV-2 infection: review of 3939 COVID-19 patients in China and emerging pathogenesis and therapy concepts. *J Leukoc Biol*. (2020) 108:17–41. doi: 10.1002/JLB.3COVR0520-272R
133. Moore JB, June CH. Cytokine release syndrome in severe COVID-19. *Science*. (2020) 368:473–4. doi: 10.1126/science.abb8925
134. Wu D, Yang XO. TH17 responses in cytokine storm of COVID-19: An emerging target of JAK2 inhibitor Fedratinib. *J Microbiol Immunol Infect*. (2020) 53:368–70. doi: 10.1016/j.jmii.2020.03.005
135. Bulat V, Situm M, Azdajic MD, Lick R. Potential role of IL-17 blocking agents in the treatment of severe COVID-19? *Br J Clin Pharmacol*. (2021) 87:1578–81. doi: 10.1111/bcp.14437
136. Chen L, Liu HG, Liu W, Liu J, Liu K, Shang J, et al. [Analysis of clinical features of 29 patients with 2019 novel coronavirus pneumonia]. *Zhonghua Jie He He Hu Xi Za Zhi*. (2020) 43:E005. doi: 10.3760/cma.j.issn.1001-0939.2020.0005
137. Gu J, Gong E, Zhang B, Zheng J, Gao Z, Zhong Y, et al. Multiple organ infection and the pathogenesis of SARS. *J Exp Med*. (2005) 202:415–24. doi: 10.1084/jem.20050828
138. Netland J, Meyerholz DK, Moore S, Cassell M, Perlman S. Severe acute respiratory syndrome coronavirus infection causes neuronal death in the absence of encephalitis in mice transgenic for human ACE2. *J Virol*. (2008) 82:7264–75. doi: 10.1128/JVI.00737-08
139. Paterson RW, Brown RL, Benjamin L, Nortley R, Wiethoff S, Bharucha T, et al. The emerging spectrum of COVID-19 neurology: clinical, radiological and laboratory findings. *Brain*. (2020) 143:3104–20. doi: 10.1093/brain/awaa240
140. Finsterer J, Stollberger C. Causes of hypogeusia/hyposmia in SARS-CoV2 infected patients. *J Med Virol*. (2020) 92:1793–4. doi: 10.1002/jmv.25903
141. Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, et al. Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. *JAMA Neurol*. (2020) 77:1–9. doi: 10.1001/jamaneurol.2020.1127
142. Li Z, Huang Z, Li X, Huang C, Shen J, Li S, et al. Bioinformatic analyses hint at augmented T helper 17 cell differentiation and cytokine response as the central mechanism of COVID-19-associated Guillain-Barré syndrome. *Cell Prolif*. (2021). 54:e13024. doi: 10.1111/cpr.13024
143. Puelles VG, Lütgehetmann M, Lindenmeyer MT, Sperhake JP, Wong MN, Allweiss L, et al. Multiorgan and renal tropism of SARS-CoV-2. *N Engl J Med*. (2020) 383:590–2. doi: 10.1056/NEJMc2011400
144. Schurink B, Roos E, Radonic T, Barbe E, Bouman CSC, de Boer HH, et al. Viral presence and immunopathology in patients with lethal COVID-19: a prospective autopsy cohort study. *Lancet Microbe*. (2020) 1: E290–9. doi: 10.1016/S2666-5247(20)30144-0
145. Ramani A, Müller L, Ostermann PN, Gabriel E, Abida-Islam P, Müller-Schiffmann A, et al. SARS-CoV-2 targets cortical neurons of 3D human brain organoids and shows neurodegeneration-like effects. *BioRxiv*. (2020) 2020.05.20.106575. doi: 10.15252/embj.2020106230
146. Chen R, Wang K, Yu J, Chen Z, Wen C, Xu Z. The spatial and cell-type distribution of SARS-CoV-2 receptor ACE2 in human and mouse brain. *BioRxiv*. (2020) 2020.04.07.030650. doi: 10.1101/2020.04.07.030650
147. Wool GD, Miller JL. The impact of COVID-19 disease on platelets and coagulation. *Pathobiology*. (2020) 88:15–27. doi: 10.1159/000512007
148. Chen Y, Li Z, Zhang Y-Y, Zhao W-H, Yu Z-Y. Maternal health care management during the outbreak of coronavirus disease 2019. *J Med Virol*. (2020) 92:731–9. doi: 10.1002/jmv.25787
149. Favre G, Pomar L, Musso D, Baud D. 2019-nCoV epidemic: what about pregnancies? *Lancet*. (2020) 395:e40. doi: 10.1016/S0140-6736(20)30311-1
150. Wastnedge EAN, Reynolds RM, Boeckel SRV, Stock SJ, Denison FC, Maybin JA, et al. Pregnancy and COVID-19. *Physiol Rev*. (2021) 101:303–18. doi: 10.1152/physrev.00024.2020
151. Alfaraj SH, Al-Tawfiq JA, Memish ZA. Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infection during pregnancy: report of two cases & review of the literature. *J Microbiol Immunol Infect*. (2019) 52:501–3. doi: 10.1016/j.jmii.2018.04.005
152. Siston AM, Rasmussen SA, Honein MA, Fry AM, Seib K, Callaghan WM, et al. Pandemic 2009 influenza A(H1N1) virus illness among pregnant women in the United States. *Jama*. (2010) 303:1517–25. doi: 10.1001/jama.2010.479
153. Lampi KM, Lehtonen L, Tran PL, Suominen A, Lehti V, Banerjee PN, et al. Risk of autism spectrum disorders in low birth weight and small for gestational age infants. *J Pediatr*. (2012) 161:830–6. doi: 10.1016/j.jpeds.2012.04.058
154. Fan C, Lei D, Fang C, Li C, Wang M, Liu Y, et al. Perinatal transmission of COVID-19 associated SARS-CoV-2: should we worry? *Clin Infect Dis*. (2020) 72:862–4. doi: 10.1093/cid/ciaa226
155. Chen H, Guo J, Wang C, Luo F, Yu X, Zhang W, et al. Clinical characteristics and intrauterine vertical transmission potential of COVID-19 infection in nine pregnant women: a retrospective review of medical records. *Lancet*. (2020) 395:809–15. doi: 10.1016/S0140-6736(20)30360-3

156. Salem D, Katranji F, Bakdash T. COVID-19 infection in pregnant women: review of maternal and fetal outcomes. *Int J Gynaecol Obstet.* (2021) 152:291–8. doi: 10.1002/ijgo.13533
157. Liu Y, Chen H, Tang K, Guo Y. Clinical manifestations and outcome of SARS-CoV-2 infection during pregnancy. *J Infect.* (2020) 2020:S01634453(20)30109-2. doi: 10.1016/j.jinf.2020.02.028
158. Wang X, Zhou Z, Zhang J, Zhu F, Tang Y, Shen X, et al. A case of 2019 novel coronavirus in a pregnant woman with preterm delivery. *Clin Infect Dis.* (2020) 71:844–6. doi: 10.1093/cid/ciaa200
159. Knight M, Bunch K, Vousden N, Morris E, Simpson N, Gale C, et al. Characteristics and outcomes of pregnant women admitted to hospital with confirmed SARS-CoV-2 infection in UK: national population based cohort study. *BMJ.* (2020) 369:m2107. doi: 10.1136/bmj.m2107
160. Zhang T, Sidorchuk A, Sevilla-Cermeño L, Vilaplana-Pérez A, Chang Z, Larsson H, et al. Association of cesarean delivery with risk of neurodevelopmental and psychiatric disorders in the offspring: a systematic review and meta-analysis. *JAMA Network Open.* (2019). 2:e1910236-e. doi: 10.1001/jamanetworkopen.2019.10236
161. Papananou M, Papaioannou M, Petta A, Routsis E, Farmaki M, Vlahos N, et al. Maternal and neonatal characteristics and outcomes of COVID-19 in pregnancy: an overview of systematic reviews. *Int J Environ Res Public Health.* (2021). 18:596. doi: 10.3390/ijerph18020596
162. Turner MJ, Reynolds CME, McMahon LE, O'Malley EG, O'Connell MP, Sheehan SR. Caesarean section rates in women in the Republic of Ireland who chose to attend their obstetrician privately: a retrospective observational study. *BMC Pregnancy Childbirth.* (2020) 20:548. doi: 10.1186/s12884-020-03199-x
163. World Health O. *Born Too Soon: The Global Action Report on Preterm Birth.* Geneva: World Health Organization (2012).
164. Dhir SK, Kumar J, Meena J, Kumar P. Clinical features and outcome of SARS-CoV-2 infection in neonates: a systematic review. *J Trop Pediatr.* (2021). 67:fmaa059. doi: 10.1093/tropej/fmaa059
165. Allotey J, Stallings E, Bonet M, Yap M, Chatterjee S, Kew T, et al. Clinical manifestations, risk factors, and maternal and perinatal outcomes of coronavirus disease 2019 in pregnancy: living systematic review and meta-analysis. *BMJ.* (2020) 370:m3320. doi: 10.1136/bmj.m3320
166. Turan O, Hakim A, Dashraath P, Jeslyn WJL, Wright A, Abdul-Kadir R. Clinical characteristics, prognostic factors, and maternal and neonatal outcomes of SARS-CoV-2 infection among hospitalized pregnant women: a systematic review. *Int J Gynaecol Obstet.* (2020) 151:7–16. doi: 10.1002/ijgo.13329
167. Yoon SH, Kang JM, Ahn JG. Clinical outcomes of 201 neonates born to mothers with COVID-19: a systematic review. *Eur Rev Med Pharmacol Sci.* (2020) 24:7804–15. doi: 10.26355/eurrev_202007_22285
168. Vivanti AJ, Vauloup-Fellous C, Prevot S, Zupan V, Suffee C, Do Cao J, et al. Transplacental transmission of SARS-CoV-2 infection. *Nat Commun.* (2020) 11:3572. doi: 10.1038/s41467-020-17436-6
169. Kirtsman M, Diambomba Y, Poutanen SM, Malinowski AK, Vlachodimitropoulou E, Parks WT, et al. Probable congenital SARS-CoV-2 infection in a neonate born to a woman with active SARS-CoV-2 infection. *CMAJ.* (2020) 192:E647–50. doi: 10.1503/cmaj.200821
170. Egloff C, Vauloup-Fellous C, Picone O, Mandelbrot L, Roques P. Evidence and possible mechanisms of rare maternal-fetal transmission of SARS-CoV-2. *J Clin Virol.* (2020) 128:104447. doi: 10.1016/j.jcv.2020.104447
171. Steinman G. COVID-19 and autism. *Med Hypotheses.* (2020) 142:109797. doi: 10.1016/j.mehy.2020.109797
172. Shuid AN, Jayusman PA, Shuid N, Ismail J, Kamal Nor N, Mohamed IN. Association between viral infections and risk of autistic disorder: an overview. *Int J Environ Res Public Health.* (2021) 18:2817. doi: 10.3390/ijerph18062817
173. Libertus K, Sheperd KA, Ross SW, Landa RJ. Limited fine motor and grasping skills in 6-month-old infants at high risk for autism. *Child Dev.* (2014) 85:2218–31. doi: 10.1111/cdev.12262
174. Ozonoff S, Macari S, Young GS, Goldring S, Thompson M, Rogers SJ. Atypical object exploration at 12 months of age is associated with autism in a prospective sample. *Autism.* (2008) 12:457–72. doi: 10.1177/1362361308096402
175. Oono IP, Honey EJ, McConachie H. Parent-mediated early intervention for young children with autism spectrum disorders (ASD). *Cochrane Database Syst Rev.* (2013) 4:Cd009774. doi: 10.1002/14651858.CD009774.pub2
176. Althoff CE, Dammann CP, Hope SJ, Ausderau KK. Parent-mediated interventions for children with autism spectrum disorder: a systematic review. *Amer J Occup Ther.* (2019). 73:7303205010p1–13. doi: 10.5014/ajot.2019.030015

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ORIGINAL PAPER

Maternal Mid- Gestation Cytokine Dysregulation in Mothers of Children with Autism Spectrum Disorder

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Abstract

Autism spectrum disorder (ASD) is a developmental disorder characterised by deficits in social interactions and communication, with stereotypical and repetitive behaviours. Recent evidence suggests that maternal immune dysregulation may predispose offspring to ASD. Independent samples t-tests revealed downregulation of IL-17A concentrations in cases, when compared to controls, at both 15 weeks ($p = 0.02$), and 20 weeks ($p = 0.02$), which persisted at 20 weeks following adjustment for confounding variables. This adds to the growing body of evidence that maternal immune regulation may play a role in foetal neurodevelopment.

Keywords IL-17A · Autism spectrum disorder · Cytokine · Inflammation · Maternal immune activation

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Introduction

ASD is an intricate continuum of neurodevelopmental disorders all of which have an onset in early childhood. These disorders are characterised by impairments in social interaction and communication, and the presence of restricted, ritualistic or repetitive interests, behaviours and activities (Birtwell, 2016; Magiati et al., 2016). To meet the diagnostic criteria, symptoms must have been present during the early developmental period, and must cause significant functional impairments (social or occupational) of varying severities (American Psychiatric Association, 2013).

It reportedly affects approximately 1.5% of the population in the developed world (Lyall et al., 2017). Although deficits can be present from infancy, diagnosis is often delayed. Classic Autism is typically formally diagnosed at an average of 5.6 years (standard deviation (SD) 4.1), and Asperger's at an average of 9.9 years (standard deviation (SD) 5.3) (Crane et al., 2015). An early, accessible biomarker which could aid early detection and intervention (Boyd et al., 2012) would be a significant step forward in the care of these children.

There is growing evidence that disturbance of inflammatory and immune responses may be a significant contributing factor behind the pathophysiology of many psychiatric disorders (Kim et al., 2007; Masi et al., 2015, 2017; Müller et al., 2015). Alterations of immune cell expression have been documented repeatedly in ASD affected children and adults as well as animals with an ASD-like phenotype (Akintunde et al., 2015; Ashwood et al., 2011; Fernández de Cossío et al., 2017), and maternal viral or bacterial infections have been found to be significantly associated with ASD in offspring (Malkova et al., 2012). Maternal immune activation (MIA) is believed to disrupt the delicate processes underlying neuronal development, increasing the risk of disordered neurodevelopment (Deverman & Patterson, 2009; Garay & McAllister, 2010).

MIA may typically be modelled in animals using lipopolysaccharide (LPS), Polyinosinic:polycytidylic

acid (Poly(I:C)), or valproic acid. MIA in rodents results in a wide array of enduring ASD-like behavioural alterations in offspring.

Neurodevelopment of the rodent brain is said to be equivalent to that noted in human mid-gestational neurodevelopment between gestational days 10–20 (Patten et al., 2014). Inflammatory insults during this time have resulted in reductions in social approach and reciprocal social behaviour, increases in repetitive and stereotypical behaviours, typically measured using a marble burying test, abnormal prepulse inhibition and ultrasonic vocalisations, impaired learning and memory, measured using a variety of maze tests, and reduced novel object recognition (Boksa, 2010; Careaga et al., 2017). Few large models of MIA induced ASD exist, though non-human primate models are more common than others, and extend findings in rodent models. A mid-gestation viral challenge in the rhesus monkey may manifest as repetitive behaviours, decreased affiliative vocalisations, inappropriate social interactions with novel animals, and impaired social attention (Bauman et al., 2014; Machado et al., 2015).

Human epidemiological studies have shown that immune disorders and mid-trimester viral illnesses which lead to a pro-inflammatory state in mothers during pregnancy, are associated with increased ASD, schizophrenia and bipolar disorder risk in offspring (Atladdottir et al., 2010; Chess, 1977; Conway & Brown, 2019; Jiang et al., 2016). In 1977, Chess noted ASD rates of 8–13% in offspring of United States (US) mothers who were infected in the 1964 Rubella outbreak (Chess, 1977). More recently, Maher et al. linked preeclampsia to increased ASD risk (Maher et al., 2020).

Midgestation in particular appears to be an important neurodevelopmental period. Some of the key processes occurring during this period include the development of the hippocampus, cortical plate, the longitudinal fissure, sulci and gyri, cerebellum, superior and inferior colliculi, primary visual, motor and sensory cortices, the cerebrospinal tract, as well as spinal cord myelination, as well as neurogenesis. The brain also significantly increases in size between

gestational weeks 13 and 21 (Huang et al., 2009; Joseph, 2000; Prayer et al., 2006; Stiles & Jernigan, 2010). Insults during this time have been found to result in neurodevelopmental and psychiatric disorders in both humans and animals (Buss et al., 2010; Haddad et al., 2020; Wolff & Bilkey, 2008).

Very few clinical studies have examined the cytokine profiles of mothers who go on to have a child with ASD. A retrospective 2017 study reported elevated levels of several circulating cytokines and chemokines in mid-gestational mothers who progressed to bear a child affected by ASD. This study was able to examine children with an early diagnosis of ASD, with and without intellectual disability. These included granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-1 α , IL-6, interferon- γ (IFN- γ), IL-8 and monocyte chemoattractant protein-1 (MCP-1) (Jones et al., 2017). An earlier study performed by Goines et al. showed dysregulation in a number of serum cytokines including IFN- γ , IL-4, IL-5 and IL-10 at a single time point between 15 and 19 weeks' gestation (Goines et al., 2011). Elevated MCP-1 has also been observed in amniotic fluid samples of ASD infants (Abdallah et al., 2012). Brown et al. identified increased levels of the inflammatory marker C-reactive protein (CRP) in prospectively collected maternal serum samples during early pregnancy (Brown et al., 2014). In recent times more conditions with a pro-inflammatory milieu, such as obesity, psychosocial stress and pre-eclampsia have also been reported to increase the risk of ASD (Curran et al., 2018; Knuesel et al., 2014; Maher et al., 2020). Thus, MIA and cytokine dysregulation during pregnancy seems to play a role in the pathogenesis of the ASD phenotype.

In the present study, we wished to examine the midgestational cytokine profiles in mothers of children with a subsequent ASD diagnosis examined at two mid-gestation time points (15 and 20 weeks) across two sites of a large multi-centre pregnancy study with the aim of identifying a gestational ASD biomarker which may aid in the timely treatment and management of the disorder.

Methods

Study Population

Maternal-child dyads were recruited to the population-based SCOPE study (www.scopestudy.net). This study used a cohort from the two SCOPE centres from which paediatric follow-up was completed. These sites were Cork, Ireland (Cork ECM5 (10) 05/02/08) and Auckland, New Zealand (SCOPE-NZ) (AKX/02/00/364). In Cork, children had detailed follow-up from birth to 5 years through the Cork BASELINE Birth Cohort Study. All children who scored below the “cutoff value” in the Ages and Stages Questionnaire (suggestive of abnormal development) were referred for paediatric assessment. Those with suspected ASD at 2 or 5 years were referred to early intervention services for full ASD assessment. Further follow-up was completed after Early Intervention Services (EIS) assessment to confirm diagnosis of ASD. Diagnosis was considered to be confirmed if made by a professional (EIS or child psychiatrist). In Auckland, telephone follow-up using standardised questionnaires was carried out as part of the Children of SCOPE study at 6 years and ASD diagnosis was by parent report.

Cases from both sites were enrolled to the cohort.

Inclusion criteria for enrolment were:

- Biobanked maternal antenatal serum samples
- Developmental follow-up completed for the child at 5 or 6 years of age (site dependant)
- Cases had a diagnosis of ASD according to the local selection criteria
- Controls had no underlying medical or developmental conditions

SCOPE-IRELAND and the Cork BASELINE Birth Cohort study was carried out with local ethical approval from the Cork Research Ethics Committee (Cork ECM5 (10) 05/02/08). Full written informed consent was obtained in all cases. SCOPE-NZ and the Children of SCOPE study was carried out with ethical approval gained from local ethics committees (New Zealand Health and Disability Ethics Committees (AKX/02/00/364 and NTX/10/10/106) and all women

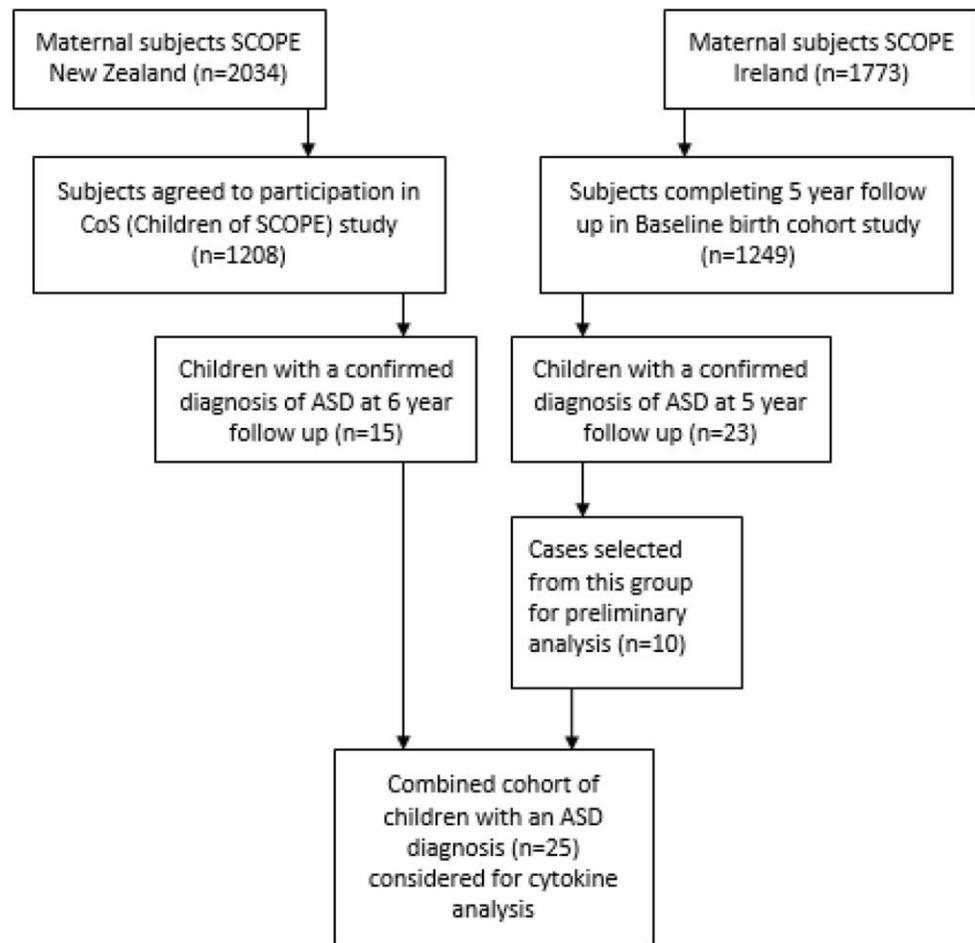
provided written informed consent. A patient recruitment flowchart is outlined in Fig. 1.

Demographic Variables

Demographic and relevant clinical data regarding the participants is presented in Table 3. ‘Age, maternal’ represents maternal age in years at the time they were approached to participate in the study whilst pregnant. ‘Birthweight, g’ is the infant’s birthweight

scores range from 27 to 40. Gestational age at delivery is presented in weeks, and APGAR (appearance, pulse, grimace, activity and respiration) scores are presented as being < 7 or ≥ 7 at both 1 and 5 min of age. Household income represents the combined household income and is quoted in New Zealand Dollars (\$) and Euros (€). Body Mass index (BMI) is categorised using the World Health Organisation (WHO) criteria and is measured in

Fig. 1 Patient recruitment flow chart outlining participant enrolment and follow up across both sites



in grams. ‘SEI’ stands for Socioeconomic Index and this variable was calculated using the New Zealand Socioeconomic Index guide. The same index was used across both locations, and Cork participants were scored based on the same criteria as their New Zealand counterparts (Galbraith et al., 1996). Perceived Stress Scores (PSS) are based on the ten question PSS questionnaire (Cohen et al., 1994). An individual’s scores on the PSS can range from 0 to 40 with higher scores indicating higher perceived stress. Low stress scores range from 0 to 13, moderate stress scores range from 14 to 26, and high stress

kilograms per metre squared. Underweight and normal BMI categories are considered together as are overweight and obese categories. Folate intake was categorised as yes or no for:

(i) any supplemental folate in the preconceptual period and (ii) at the 15 week visit.

Biofluid Collection

Serum samples were obtained from mothers recruited to the SCOPE-NZ and SCOPE-Cork studies at both 15 and 20 weeks gestation within the greater Auckland area, New Zealand and Cork University Maternity Hospital, Cork, Ireland. Biobank specimens were archived at -80°C until required. Maternal mid-pregnancy specimens from 15 and 20 weeks were retrieved from the multi-centre SCOPE study sites with ongoing paediatric follow-up. Identical protocols for collection, processing and storage of samples were followed at both sites.

Venepuncture was performed by SCOPE study specific research midwives at each of the sites in accordance with best practice guidance (SCOPE Consortium standard operating procedures (SOP)). Maternal specimens were collected in serum separator tubes (Becton–Dickinson Franklin Lakes, New Jersey) and immediately placed on ice and transported to the laboratory. Before proceeding to centrifugation, serum samples were stored at 4°C for 30 min from time of collection to allow clot formation. Presence of the clot was confirmed visually, and samples were then centrifuged at $2400\times g$ for 10 min at 4°C . Serum samples were transferred into ice cold 5 mL sterile polypropylene tubes (VWR, Radnor, Pennsylvania) via sterile Pasteur pipettes. The samples were centrifuged again at $3000\times g$ for 10 min at 4°C . Sera were then aliquoted to red capped, barcode-labelled cryovials (VWR) in volumes of 250 μL . Aliquots were logged in the SCOPE database (MedSciNet), and stored at -80°C within four hours of collection (Kenny et al., 2014). For transport of NZ serum samples to Cork, Ireland: the maternal specimens were packed on dry ice and shipped directly to the SCOPE Ireland biobank repository, where they were stored at -80°C until their use in cytokine and chemokine profiling.

Cytokine Analysis

Serologic concentrations (pg/ml) of eight cytokines, chemokines and proinflammatory proteins were investigated at 15 and 20 weeks gestation using the Mesoscale Discovery V-plex cytokine, chemokine and proinflammatory electrochemiluminescent assays (Meso Scale Diagnostics, Rockville, Maryland). Cytokines were chosen for further examination based on evidence of dysregulated expression in preclinical models (Choi et al., 2016; Pineda et al., 2013; Pratt et al., 2013; Smith et al., 2007) and autistic patients (Ahmad et al., 2019; Ashwood et al., 2011; Masi et al., 2015; Patterson, 2011).

IL-16 and IL-17A were examined using the V-plex multiplex Cytokine Panel 1 kit (KD15050D). Eotaxin and MCP-1 were examined using the V-plex multiplex Chemokine Panel 1 kit (K15047D). IFN- γ , IL-1 β , IL-6 and IL-8 were examined using the V-plex multiplex Proinflammatory Panel 1 kit (K15049D). All standards and samples were run in duplicate.

All plates were prepared according to manufacturer's instructions and analysed on the Meso QuickPlex SQ 120. Results were generated as calculated concentration means on the Mesoscale (MSD) Discovery Workbench 4.0 assay analysis software. Calibration curves used to calculate concentrations of individual cytokines are established by fitting the calibrator signals to a four-parameter logistic model with a $1/Y^2$ weighting. The MSD analysis software determines individual cytokine concentrations from electrochemiluminescent signals via backfitting to the calibration curve. Calculated concentrations are also multiplied by the dilution factor applied to the samples, which in this case, was 4. Samples were excluded if %CV was higher than 25% between duplicates as previously described (Dabir et al., 2011). Lower and upper limits of detection (LLOD and ULOD) as well as interassay coefficients of variation (CVs) for each protein are outlined in Table 1. Limits of detection represent calculated concentrations which correspond to signals 2.5 standard deviations above/below the blank (zero calibrator).

Samples were chosen due to early ASD presentation (formal diagnosis prior to 5 years) and sample

availability. Several were excluded from the individual final analyses due to either poor %CV values or concentrations reading below the LLOD for individual cytokines. Of the combined 25 cases and 38 controls, the final sample numbers for cases after all exclusions are outlined in Table 2.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 (Graphpad Software Inc., San Diego, CA) and IBM SPSS

cytokine variable under investigation. For comparisons of continuous variables between groups, independent samples t-tests were used when there were two groups and one-way ANOVAs were used when there were more than two groups. Relationships between categorical variables were investigated using the chi-squared test. Statistical significance (2-tailed) was set at $p \leq 0.05$ and all tests were two-sided.

Results

Participant Details

Of the 2034 mothers recruited to SCOPE-NZ, 1208 agreed to participate in the follow up birth cohort study, Children of SCOPE. 16 NZ children who completed developmental follow-up and had an ASD diagnosis by 6 years were selected for cytokine profiling (compared to 16 controls). While the NZ cohort was originally matched, one case was excluded from analysis due to possible chromosomal abnormality, and its corresponding control was one of only two females remaining in the cohort, so was

Table 1 Median LLOD and ULOD for each of the tested cytokines

Proteins	Median LLOD (pg/ml)	Median ULOD (pg/ml)	Interassay CV (%)
IL-17A	1.60	6560.00	8.63
IFN- γ	0.34	1400.00	10.13
Eotaxin	0.44	1820.00	12.04
MCP-1	0.13	530.00	10.43
IL-16	0.83	3400.00	6.35
IL-1 β	0.14	575.00	9.07
IL-6	0.19	765.00	8.76
IL-8	0.15	599.00	9.18

All units are pg/ml

Table 2 Final sample numbers for combined Cork and Auckland cases and controls

Proteins	Cases 15 weeks	Controls 15 weeks	Excluded Cases 15 weeks	Cases 20 weeks	Controls 20 weeks	Excluded Total weeks (below LLOD)	Total excluded (%CV > 25%)
IL-17A	20	34	9	20	31	12	10
IFN- γ	20	28	15	19	30	14	2
Eotaxin	15	23	25	18	16	29	7
MCP-1	21	32	10	19	32	12	1
IL-16	22	35	6	21	37	5	5
IL-1 β	14	19	30	14	22	27	25
IL-6	20	28	15	20	29	14	1
IL-8	22	28	13	19	29	15	0

Derived from the original 25 cases and 38 controls

Statistics 24/26 (SPSS Statistics, Chicago, IL). All cytokine variables were \log_{10} transformed prior to analysis to achieve normality (Feng et al., 2014). Independent samples t-tests were used to investigate differences between cases and controls for the cytokine variables. Multiple logistic regression models were used to assess whether cytokine concentrations can predict ASD outcome after adjusting for individual confounding variables. A confounder was defined as a variable that was associated with both case/control status and the

not excluded, resulting in 15 NZ cases total.

Of the 2183 mothers recruited to Cork’s Baseline birth cohort study, 1537 were recruited from SCOPE Ireland at the 20 weeks visit and an additional 600 children were recruited to the cohort postnatally. In total, 1249 children completed 5 year follow up assessment in the Cork BASELINE Birth Cohort Study. Of these children, 23 had a reported diagnosis of ASD, and 10 had available mid-gestation samples and were selected for cytokine profiling (compared to 22 controls). Cases selected from the Cork cohort were

contacted via telephone by the study clinical research fellow in June/ July 2019, and all cases were verbally confirmed to have ASD (diagnosed by local EIS or child psychologist). While the Cork cohort was originally matched, numerous samples were excluded, resulting in a lack of matching.

The cohort of ASD cases from NZ and Cork were combined ($n = 25$), and samples from the mothers of these children were analysed alongside those from the mothers of neurotypical controls $n = 38$.

Detailed clinical characteristics of participants and mothers from both cohorts are provided in Table 3. As previously stated, several samples from both locations were excluded from the final analysis due to either poor %CV values or concentrations reading below the LLOD for individual cytokines. This resulted in an altered male/female ratio between cases and controls and ultimately an unmatched cohort. Other significant differences between cases

and controls included mode of delivery and folate use in early pregnancy (15 weeks).

Mid-Gestational Cytokine Analysis

To determine whether there was any difference in inflammatory markers between mothers of ASD and neurotypical children at either 15 or 20 weeks gestation, electrochemiluminescent Mesoscale assays were performed.

Of the original panel of eight cytokines, one was significantly altered—IL-17A. IL-17A was significantly altered at both 15 and 20 weeks in mothers of children who went on to have a child affected by ASD, compared to controls. IL-17A concentrations were significantly different between cases (Mean (M) = -0.22 ; Standard Deviation (SD) = 0.28) and controls (M = -0.001 ; SD = 0.35) at 15 weeks ($t(52) = 2.43$; $p = 0.02$), and between cases (M = -0.26 ; SD = 0.38) and controls (M = -0.002 ; SD = 0.40) at 20 weeks ($t(49) = 2.32$;

Demographics for combined NZ and IRE cohorts ($n = 63$)

Variables	Cases ($n = 25$)	Controls ($n = 38$)	p-value
Age (maternal), years	30.4 (5.7)	30.6 (3.6)	0.9
Birthweight, g	3604.0 (666.0)	3439.0 (431.0)	0.2
Sex (infant)			0.02
Male	23 (92)	25 (66)	
Female	2 (8)	13 (34)	
Mode of delivery			0.04
Unassisted vaginal	9 (36)	16 (42)	
Assisted vaginal	4 (16)	15 (40)	
Pre-labour LSCS	1 (4)	2 (5)	
Labour LSCS	11 (44)	5 (13)	
Gestational age at delivery	39.9 (1.5)	40.0 (1.4)	0.9
1-min Apgar			0.08
< 7	2 (8)	0	
≥ 7	23 (92)	38 (100)	
5-min Apgar			*

Table 3 Demographic characteristics of participants

< 7	0	0	
≥ 7	25 (100)	38 (100)	
Ethnicity			1
Caucasian	23 (92)	35 (92)	
Non-Caucasian	2 (8)	3 (8)	
SEI (maternal)	52.6 (16.2)	49.8 (11.7)	0.4
Household income			0.4
Unknown	2 (8)	2 (5)	
< \$75 K (< €64 K)	6 (24)	11 (29)	
\$75–100 K (€64–84 K)	10 (40)	8 (21)	
> \$100 K (> €85 K)	7 (28)	17 (45)	
Smoking status in pregnancy			0.4
No, never smoked	20 (80)	24 (63)	
No, ex-smoker	4 (16)	11 (29)	
Yes, current smoker	1 (4)	3 (8)	
PSS (perceived stress score)	13.8 (7.3)	14.7 (6.7)	0.6
BMI (WHO categories)			0.2
Underweight/Normal ($\leq 25 \text{ kg/m}^2$)	14 (56)	27 (71)	
Overweight/Obese ($> 25 \text{ kg/m}^2$)	11 (44)	11 (29)	
Folate—pre-conceptual			0.6
No	9 (36)	11 (29)	
Yes	16 (64)	27 (71)	
Folate—15 week visit			0.02
No	3 (12)	15 (40)	
Yes	22 (88)	23 (61)	

Comparison is made between cases and controls across the whole cohort. p-Values are calculated using the Pearson Chi square for categorical data, and independent samples t-test where appropriate for continuous variables. Variations in local Caesarean section practices from each site likely give rise to the significant difference in Mode of Delivery rates. Eight of eleven (73%) of the ASD cases delivered by lower segment Caesarean section—“Labour LSCS” were in NZ. “Pre-labour LSCS” was excluded when identifying confounding variables due to small sample numbers ($n = 3$). There are no significant differences in birth weight, either between cases and controls, or between subjects from each site. Numbers are presented as mean (SD) or n (%)

$p = 0.02$) (Fig. 2). After adjusting for confounding by folate, IL-17A no longer showed a statistically significant association with ASD risk at 15 weeks (adjusted odds ratio [aOR] 0.17 (95% CI 0.02–1.57); $p = 0.12$). Downregulation at 20 weeks remained, as there were no changes in associations after adjustment for confounding by folate (aOR 0.14 (95% CI 0.02–0.87); $p = 0.03$).

Expression of IFN- γ , IL-16, Eotaxin, MCP-1, IL-1 β , IL-8 and IL-6 was not significantly different in mothers who went on to have a child with ASD when compared to controls at either timepoint. Therefore, levels of these cytokines were not associated with increased ASD risk.

IFN- γ was not found to be significantly different between cases ($M = 0.26$; $SD = 0.28$) and controls ($M = 0.25$; $SD = 0.31$) at 15 weeks ($t(46) = 0.19$; $p = 0.85$) or between cases ($M = 0.34$; $SD = 0.31$) and controls ($M = 0.39$; $SD = 0.41$) at 20 weeks ($t(47) = 0.51$; $p = 0.62$) (Fig. 3a). IL-16 was not significantly different between cases ($M = 2.04$; $SD = 0.16$) and controls ($M = 2.01$; $SD = 0.18$) at 15 weeks ($t(55) = 0.64$; $p = 0.52$), or between cases ($M = 2.01$; $SD = 0.19$) and controls ($M = 2.02$; $SD = 0.20$) at 20 weeks ($t(56) = 0.12$; $p = 0.92$) (Fig. 3b). Sex was found to be a confounder for IL-16 at 15 weeks, though levels remained not significantly associated with development of ASD after adjusting for confounding by sex (aOR 2.38 (95% CI 0.63–89.61); $p = 0.64$). Eotaxin was not significantly different between cases ($M = 1.50$; $SD = 0.27$) and controls ($M = 1.57$; $SD = 0.31$) at 15 weeks ($t(36) = 0.73$; $p = 0.47$), or between cases ($M = 1.61$;

$SD = 0.31$) and controls ($M = 1.62$; $SD = 0.23$) at 20 weeks ($t(32) = 0.11$; $p = 0.91$) (Fig. 3c). MCP-1 was not significantly different between cases ($M = 1.87$; $SD = 0.26$) and controls ($M = 1.87$; $SD = 0.23$) at 15 weeks ($t(51) = 0.10$; $p = 0.92$), or between cases ($M = 1.87$; $SD = 0.29$) and controls ($M = 1.90$; $SD = 0.19$) at 20 weeks ($t(49) = 0.58$; $p = 0.56$) (Fig. 3d). IL-8 was not significantly different between cases ($M = 0.56$; $SD = 0.25$) and controls ($M = 0.57$; $SD = 0.36$) at 15 weeks ($t(48) = 0.15$; $p = 0.88$), or between cases ($M = 0.54$; $SD = 0.23$) and controls ($M = 0.61$; $SD = 0.28$) at 20 weeks ($t(46) = 0.89$; $p = 0.38$) (Fig. 3e). IL-1 β was not significantly different between cases ($M = -1.39$; $SD = 0.83$) and controls ($M = -1.03$; $SD = .75$) at 15 weeks ($t(31) = 1.28$; $p = 0.21$), or between cases ($M = -1.37$; $SD = 0.89$) and controls ($M = -1.23$; $SD = 0.65$) at 20 weeks ($t(34) = 0.54$; $p = 0.59$) (Fig. 3f). Mode of delivery was found to be a confounder for IL-1 β at 15 and 20 weeks, though IL-1 β at 15 weeks (aOR 0.83 (95% CI 0.28–2.45); $p = 0.74$) and 20 weeks (aOR 0.76 (95% CI 0.26–2.23); $p = 0.61$) remained not significantly associated with development of ASD after adjusting for confounding by mode of delivery. IL-6 was not significantly different between cases ($M = -0.44$; $SD = 0.22$) and controls ($M = -0.40$; $SD = 0.24$) at 15 weeks ($t(46) = 0.54$; $p = 0.59$), or between cases ($M = -0.36$; $SD = 0.27$) and controls ($M = -0.39$; $SD = 0.19$) at 20 weeks ($t(47) = 0.50$; $p = 0.62$) (Fig. 3g). Sex was found to be a confounder for IL-6 at 15 weeks, though IL-6 at 15 weeks remained not significantly associated with development of ASD after adjusting for confounding by sex (aOR 0.30 (95% CI 0.17–5.17); $p = 0.41$).

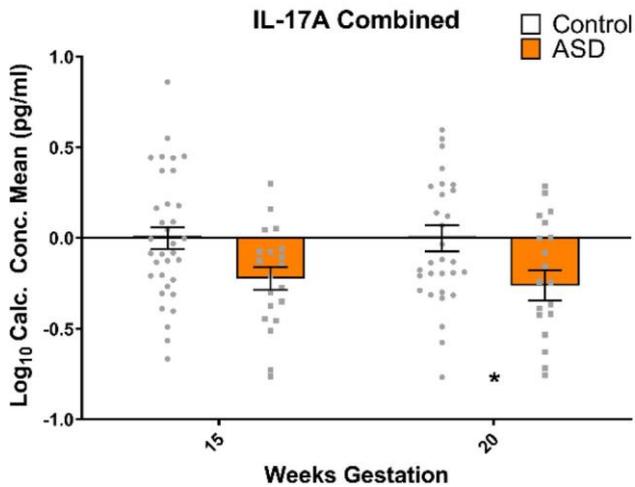


Fig. 2 IL-17A is downregulated, at 20 weeks gestation in mothers of ASD children when compared to neurotypical controls. This remains after adjusting for confounding variables—folate intake at 15 weeks. All data are mean \pm SEM; independent samples t-tests, analysed on a case vs control basis. * = $p < 0.05$. White bars represent controls, while orange bars represent cases (mothers of ASD affected offspring)

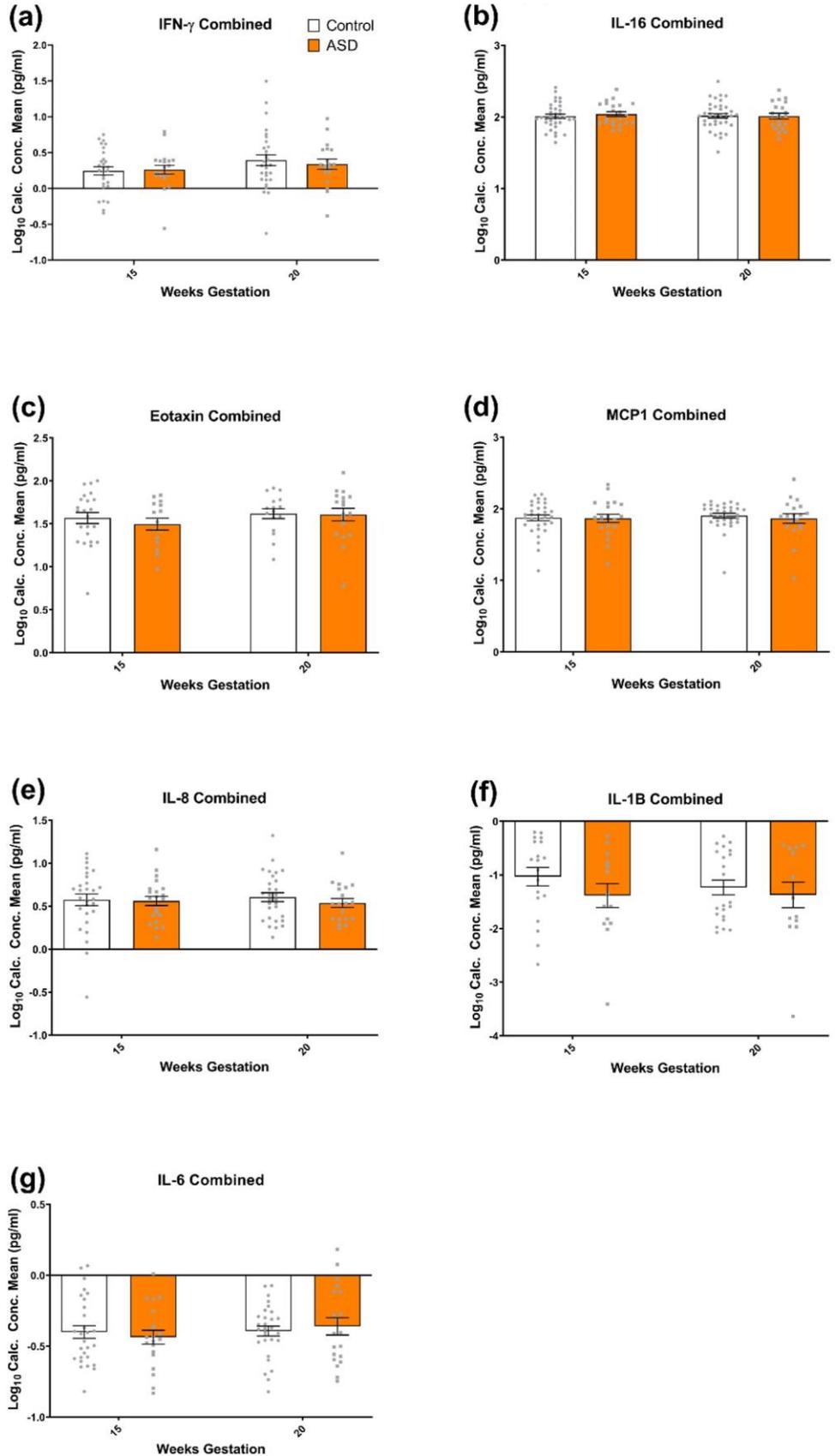
Maternal Health

To examine whether other factors might have altered maternal cytokine profiles we examined

maternal health factors and medication use during pregnancy.

None of the participants had any of the following preexisting inflammatory conditions—inflammatory bowel disease, rheumatoid or inflammatory arthritis, venous thromboembolic disease. No subjects suffered from either psoriasis or ankylosing spondylitis. The commonest reported medical condition was asthma. Several participants from each site had physician diagnosed asthma; 12 suffered from mild asthma and 3 suffered from moderate asthma. The individuals with moderately severe asthma (one case (Cork), two controls (NZ)) were being treated with regular low dose inhaled corticosteroid and long acting beta agonist or Terbutaline combination therapy. The subjects with mild asthma were 7 controls (4 Cork, 3 NZ) and 5 cases (2 Cork, 3 NZ). None of the asthmatics received oral steroid therapy at any point during pregnancy or in the preceding year. Hypothyroidism was also relatively common and occurred in three cases (2 NZ, 1 Cork) and two controls (1 NZ, 1 Cork).

Fig. 3 a IFN- γ , b IL-16, c eotaxin, d MCP1, e IL-8, f IL-1 β and g IL-6 were not significantly altered at either 15 or 20 weeks gestation in mothers of ASD children when compared to neurotypical controls. All data are mean \pm SEM; independent samples t-tests, analysed on a case vs control basis. White bars represent controls, while orange bars represent cases (mothers of ASD affected offspring)



Two of the three cases had evidence of hypothyroidism first detected during the 1st trimester and became euthyroid with treatment. Those others with a history of hypothyroidism were treated prior to pregnancy and were euthyroid throughout. Finally, a single case in Cork had Coeliac disease (on gluten free diet).

With regard to antepartum infections, between 0 and 15 weeks gestation, upper respiratory tract infections were reported in 12 subjects, 5 (4 NZ, 1 Cork) cases and 7 (5 NZ, 2 Cork) controls. Other infections were also reported in three (all NZ) cases and seven (3 NZ, 4 Cork) controls (5 gastroenteritis, 3 lower urinary tract infections (UTI), 1 case of genital herpes and another vaginal candida, treated with Clotrimazole. At 15–20 weeks no infections were reported in the NZ group, but two controls from Cork had a UTI and one case had an unspecified infection. None of the participants were taking regular anti-inflammatories and those taking paracetamol or aspirin did so only occasionally or for a specific episode. There was no significant difference between case and control groups in terms of reported paracetamol/aspirin use.

In summary, there were no significant differences in maternal health, inflammation or medication use between the two groups.

Discussion

In the present report, we have identified IL-17A as a potential cytokine biomarker whose expression is significantly reduced in mid-gestation (20 weeks) in pregnancies resulting in a child with ASD after adjusting for folate intake at 15 weeks. This novel finding adds to the growing body of evidence that in utero exposure to MIA and resultant cytokine dysfunction is associated with an increased risk of the subsequent development of ASD.

Interestingly, the potential confounders identified within this study—sex, mode of delivery and maternal folate intake—are widely discussed risk factors for the development of ASD (Curran et al., 2015a, 2015b; Gillberg et al., 2006; Raghavan et al., 2018; Wiens & DeSoto, 2017). After adjusting for maternal folate intake at midgestation, IL-17A levels at 15 weeks were no longer significantly associated with ASD development in offspring. A high number of case subjects (22) answered ‘yes’ to taking folate supplements during midgestation, while only three answered no. While no data are available on the doses of folate taken here, studies have linked both low and high dose maternal folate intake to DNA hyper/hypomethylation, gamma-aminobutyric acid (GABA), dopamine and serotonin dysfunction, and altered synaptic plasticity, neurogenesis and growth cone development. These events trigger neurodevelopmental disturbances which may lead to the development of ASD (DeVilbiss et al., 2015; Raghavan et al., 2018; Wiens & DeSoto, 2017). As previously mentioned, the current study had a larger ratio of males to females. It is widely understood that ASD is more commonly diagnosed in males. There are a number of theories on why this is the case. It appears that males may tend to externalise symptoms of the disorder, whereas females typically internalise symptoms, complicating diagnosis for females (Baron-Cohen et al., 2011; Werling & Geschwind, 2013). Mode of delivery was also identified as a confounder. Indeed, over 50% of mothers of ASD cases delivered by C-section which was initiated after the onset of labour. Emergency C-section is typically preceded by either foetal or maternal indications which may themselves be independent risk factors for ASD (Yip et al., 2017). C-section delivery has been linked to impaired cognitive and behavioural outcomes in both humans and animal models (Curran et al.,

2015a, 2015b; Morais et al., 2020; Polidano et al., 2017). Delivery by C-section has been linked to reductions in endogenous oxytocin (Kuwabara et al., 1987), and subsequent social deficits in mice. These deficits may be reversed in mice by exogenous oxytocin therapy early during the postnatal period (Morais et al., 2021).

Although this is one of the few human studies to examine maternal midgestation cytokine dysregulation linked to ASD, there is an abundance of data from animal studies on the cytokine and behavioural changes resulting from MIA. MIA has been replicated in small animal models where induction of MIA through maternal infection leads to an autistic phenotype in offspring, characterised in mice by increased self-grooming, increased marble burying behaviour (repetitive, stereotyped behaviours) and deficits in ultrasonic vocalisations (communication). These alterations maybe prevented by inhibition of specific cytokines (IL-6 and IL-17A), which suggests that the cytokines themselves may have a causative role in the resultant neuronal dysfunction (Parker-Athill & Tan, 2010; Smith et al., 2007; Wong & Hoeffler, 2018).

In the murine MIA model of ASD, Poly(I:C) treatment has been found to increase IL-17A levels in maternal blood and the postnatal brain as well as placental messenger RNA (mRNA) levels of the cytokine. To determine whether alterations in IL-17A expression are symptomatic of, or pathogenic in ASD, a recent study inhibited IL-17A signalling in Poly(I:C) treated pregnant mice and reported that ASD-like phenotypes in the offspring were prevented (Choi et al., 2016). IL-17A and IL-6 appear to work in tandem. Knockout of IL-6 in Poly(I:C) treated dams results in failure to alter IL-17A levels in offspring, which suggests IL-6 acts upstream of IL-17A (Choi et al., 2016). Poly(I:C) is a synthetic analogue of double stranded RNA which mimics the effects of viral infection when injected into test subjects (Meyer & Feldon, 2012). It is used as a model of MIA extensively in rat, mouse and non-human primate studies. Pups of MIA-exposed dams in Poly(I:C) murine models have demonstrated communication challenges, reduced social approach, increased repetitive behaviours (Choi et al., 2016) and alterations in development of the cerebral cortex and cerebellum (Garay et al., 2013; Hsiao et al., 2012).

Accumulating evidence supports a role for T-helper 17 (Th17) cluster of differentiation 4 (CD4) cells and their product cytokine IL-17A in ASD. Th17 cells have previously been implicated in the pathogenesis of a variety of autoimmune and neuroinflammatory disorders (Al-Ayadhi & Mostafa, 2012). Upstream IL-6 is also a key player in differentiation of these Th17 cells (Choi et al., 2016). Th17/IL-17 mediated immunity has been found to cause severe damage to the brain in response to inflammation-sensitised hypoxia (Yang et al., 2014). The gene for IL-17A (IL17A) has been identified in a genome-wide analysis to have enriched/overexpressed copy number variants in ASD cohorts (van der Zwaag et al., 2009). In subsets of children with ASD, IL17A has been found at elevated levels in the blood (both plasma and serum) and correlated with increased severity of behavioural symptoms (Akintunde et al., 2015; Al-Ayadhi & Mostafa, 2012). Nadeem et al. report that children affected by ASD have an increased number of IL-17A receptors in monocytes and that activation via IL-17A increases the child's oxidative inflammation. Blocking the receptor may ameliorate inflammatory effects, which suggests an interesting therapeutic option for both inflammatory and behavioural symptoms (Nadeem et al., 2018). Indeed, IL17A administration in a murine model improves sociability following MIA (Reed et al., 2020). IL-17A/IL-17A receptor blockade has also been shown to ameliorate the symptoms of other disorders such as atherosclerosis (Erbel et al., 2009), inflammation-sensitised

encephalopathy (Ye et al., 2019) and ankylosing spondylitis (Collison, 2018). IL-6 has been detected at elevated levels in cerebellar tissues of humans affected by ASD in their lifetime. Altered levels of this cytokine have been linked to dysfunctional adhesion and migration of neural cells, as well as imbalanced excitatory and inhibitory functions. This suggests that altered expression of IL-6 may contribute to the autistic phenotype and pathogenesis (Wei et al., 2011). Levels are also significantly increased in the frontal cortex and plasma of ASD patients (Li et al., 2009; Yang et al., 2015). Elevated IL-6 in the murine brain also results in an autistic behavioural phenotype, as well as abnormal dendritic morphology and distribution (Wei et al., 2012). Though we do not observe any notable alterations in IL-6 in this study, perhaps it acts at later timepoints when the nervous system is more developmentally mature.

The present study has a number of strengths which increase our confidence in the findings. It involves a multicentre, multi-national maternal cohort of over 4000 women, with very detailed maternal demography and 1st trimester health and lifestyle data at 15 weeks gestation. Of these women, 39 went on to have a child affected by ASD (~ 1% ASD rate). The rate of ASD seen in this cohort is similar to that seen across the developed world (~ 1.5%), so this study is a realistic reflection of ASD incidence. For this reason, we are confident that we have identified the majority of expected cases. Serum samples from both SCOPE study centres were collected, processed and biobanked according to identical protocols to ensure uniformity. Though it appears that our finding IL-17A downregulation goes against the previous reports regarding induced upregulation of IL-17 in animal studies (Wong & Hoeffler, 2018), one must consider that this is currently one of the only studies in humans which has examined IL-17A in midgestation, and is therefore a novel finding. There is increasing evidence that IL-17A may cross the placenta from mother to foetus (Wong & Hoeffler, 2018), which may, in theory, explain reduced levels in maternal serum and increased levels typically seen in the serum of offspring.

Though the present study has some major strengths, we must also address its limitations. One major shortcoming of the current study is its inability to replicate the findings of similar mid-gestation ASD cytokine studies (Abdallah et al., 2012; Goines et al., 2011; Jones et al., 2017). However, results are conflicting amongst the previous studies. Goines et al. reported midgestational elevation of IFN- γ in mothers of children who develop ASD, which contrasts with the current study (Goines et al., 2011). Jones et al. from the same research group detected midgestational downregulation of IL-8 and MCP-1 in mothers of children who develop ASD without intellectual disability. We did not find significant alterations in these cytokines in our cohort (Jones et al., 2017). Abdallah et al. utilised amniotic fluid to profile elevated MCP-1 in mothers of children who developed ASD. While we see very slight downregulation of MCP-1 at 20 weeks, Abdallah et al. do not specify weeks gestation at measurement in their study (Abdallah et al., 2012). The differences in findings between studies may relate to several factors: assays and measurement of cytokines (Luminex/Millipore—neither used Mesoscale assays), differences in the stage of gestation at measurement, and our small study size compared to other similar studies. The relatively small numbers of ASD cases makes it difficult to draw meaningful conclusions regarding different sub-types of ASD. A number of ASD samples were also lost due to poor quality, reflected by large but inconsistent (across multiplex plates) numbers lost due to poor %CV, further reducing our cohort size, subsequently resulting in a disproportionately large percentage of male cases compared to females. In addition to this, a large number of samples were below the LLOD for many cytokines (up to 25—Table 2), which suggests the

assay used may not have been sensitive enough. This ultimately created an unmatched cohort. The follow-up procedure was different at both sites, with a more detailed follow up at 2 and 5 years available to the Cork BASELINE study. However, the diagnosis of ASD was similar: parental report (Auckland), parental report of confirmed EIS or psychiatrist diagnosis. In Cork, children were diagnosed relatively early and so may be more on the severe end of the spectrum to that in Auckland. A large percentage of cases were delivered via Caesarean section. This may skew results as this mode of delivery has previously been associated with increased ASD incidence (Al-Zalabani et al., 2019; Curran et al., 2015a, 2015b; Morais et al., 2020). Larger, longer-term studies, which take longterm outcomes into account will be required with repeated maternal cytokine profiling to attempt to replicate and expand our findings.

Conclusion

To conclude, this study has identified dysfunctional IL-17A expression at 20 weeks gestation in mothers of ASD children. IL-17A may act as a potential early marker of maternal immune dysfunction and if validated would aid screening of high risk infants to support focused early therapeutic intervention in infancy (Josefi & Ryan, 2004). The current study provides a foundation for further investigation of IL-17A in large maternal cohorts. This multicentre study also provides novel insight into the midgestation cytokine profiles in mothers of both neurotypical and ASD offspring and is another piece in the puzzle of this elusive disorder.

Author Contributions SC wrote the manuscript, designed and performed all experimental work, and performed data analysis. MC commented on the manuscript at all stages and performed demographic analysis. AML commented on the manuscript at all stages and assisted with experimental work. VL assisted with statistical analysis. GM commented on the manuscript at all stages and provided training and technical assistance. GWOK commented on the manuscript at all stages and provided support and supervision. RST, LCK, FPMcC, LMEMcC, and JMDT commented on the manuscript at all stages, and coordinated the SCOPE study across both sites. DMM commented on the manuscript at all stages, and was involved in study design and supervision.

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Declarations

Conflict of interest None.

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References

- Abdallah, M. W., Larsen, N., Grove, J., Norgaard-Pedersen, B., Thorsen, P., Mortensen, E. L., & Hougaard, D. M. (2012). Amniotic fluid chemokines and autism spectrum disorders: An exploratory study utilizing a Danish Historic Birth Cohort. *Brain, Behavior, and Immunity*, 26(1), 170–176. <https://doi.org/10.1016/j.bbi.2011.09.003>
- Ahmad, S. F., Ansari, M. A., Nadeem, A., Bakheet, S. A., Al-Ayadhi, L. Y., & Attia, S. M. (2019). Elevated IL-16 expression is associated with development of immune dysfunction in children with autism. *Psychopharmacology*, 236(2), 831–838.
- Akintunde, M. E., Rose, M., Krakowiak, P., Heuer, L., Ashwood, P., Hansen, R., Hertz-Picciotto, I., & Van de Water, J. (2015). Increased production of IL-17 in children with autism spectrum disorders and co-morbid asthma. *Journal of Neuroimmunology*, 286, 33–41. <https://doi.org/10.1016/j.jneuroim.2015.07.003>
- Al-Ayadhi, L. Y., & Mostafa, G. A. (2012). Elevated serum levels of interleukin-17A in children with autism. *Journal of Neuroinflammation*, 9(1), 158. <https://doi.org/10.1186/1742-2094-9-158>
- Al-Zalabani, A. H., Al-Jabree, A. H., & Zeidan, Z. A. (2019). Is cesarean section delivery associated with autism spectrum disorder? *Neurosciences (Riyadh)*, 24(1), 11–15. <https://doi.org/10.17712/nsj.2019.1.20180303>
- American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders (DSM-5®)*. American Psychiatric Association.
- Ashwood, P., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Pessah, I. N., & Van de Water, J. (2011a). Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders. *Journal of Neuroimmunology*, 232(1–2), 196–199. <https://doi.org/10.1016/j.jneuroim.2010.10.025>
- Atladottir, H. O., Thorsen, P., Ostergaard, L., Schendel, D. E., Lemcke, S., Abdallah, M., & Parner, E. T. (2010). Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. *Journal of Autism and Developmental Disorders*, 40(12), 1423–1430. <https://doi.org/10.1007/s10803-010-1006-y>
- Baron-Cohen, S., Lombardo, M. V., Auyeung, B., Ashwin, E., Chakrabarti, B., & Knickmeyer, R. (2011). Why are autism spectrum conditions more prevalent in males? *PLOS Biology*, 9(6), e1001081. <https://doi.org/10.1371/journal.pbio.1001081>
- Bauman, M. D., Iosif, A.-M., Smith, S. E., Bregere, C., Amaral, D. G., & Patterson, P. H. (2014). Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. *Biological Psychiatry*, 75(4), 332–341.
- Birtwell, K. B. (2016). Social, cognitive, and behavioral development of children and adolescents with autism spectrum disorder. In C. McDougle (Ed.), *Autism spectrum disorder* (Feb 2016 ed., Vol. Section 1, Chapter 2). Oxford Press.
- Boksa, P. (2010). Effects of prenatal infection on brain development and behavior: A review of findings from animal models. *Brain, Behavior, and Immunity*, 24(6), 881–897.
- Boyd, B. A., McDonough, S. G., & Bodfish, J. W. (2012). Evidencebased behavioral interventions for repetitive behaviors in autism. *Journal of Autism and Developmental Disorders*, 42(6), 1236–1248. <https://doi.org/10.1007/s10803-011-1284-z>
- Brown, A. S., Sourander, A., Hinkka-Yli-Salomäki, S., McKeague, I. W., Sundvall, J., & Surcel, H. M. (2014). Elevated maternal C-reactive protein and autism in a national birth cohort. *Molecular Psychiatry*, 19(2), 259–264. <https://doi.org/10.1038/mp.2012.197>
- Buss, C., Davis, E. P., Muftuler, L. T., Head, K., & Sandman, C. A. (2010). High pregnancy anxiety during mid-gestation is associated with decreased gray matter density in 6–9-year-old children. *Psychoneuroendocrinology*, 35(1), 141–153. <https://doi.org/10.1016/j.psyneuen.2009.07.010>
- Careaga, M., Murai, T., & Bauman, M. D. (2017). Maternal immune activation and autism spectrum disorder: From rodents to nonhuman and human primates. *Biological Psychiatry*, 81(5), 391–401. <https://doi.org/10.1016/j.biopsych.2016.10.020>
- Chess, S. (1977). Follow-up report on autism in congenital rubella. *Journal of Autism and Childhood Schizophrenia*, 7(1), 69–81. <https://doi.org/10.1007/bf01531116>
- Choi, G. B., Yim, Y. S., Wong, H., Kim, S., Kim, H., Kim, S. V., Hoefler, C. A., Littman, D. R., & Huh, J. R. (2016). The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science*, 351(6276), 933. <https://doi.org/10.1126/science.1231144>
- Cohen, S., Kamarck, T., & Mermelstein, R. (1994). Perceived stress scale. *Measuring Stress: A Guide for Health and Social Scientists*, 10, 1–2.
- Collison, J. (2018). IL-17A blockade effective for AS. *Nature Reviews Rheumatology*, 14(12), 684–684. <https://doi.org/10.1038/s41584-018-0117-2>
- Conway, F., & Brown, A. S. (2019). Maternal immune activation and related factors in the risk of offspring psychiatric disorders. *Front Psychiatry*. <https://doi.org/10.3389/fpsy.2019.00430>
- Crane, L., Chester, J. W., Goddard, L., Henry, L. A., & Hill, E. (2015). Experiences of autism diagnosis: A survey of over 1000 parents in the United Kingdom. *Autism*, 20(2), 153–162. <https://doi.org/10.1177/1362361315573636>
- Curran, E. A., Dalman, C., Kearney, P. M., Kenny, L. C., Cryan, J. F., Dinan, T. G., & Khashan, A. S. (2015a). Association between obstetric mode of delivery and autism spectrum disorder: A population-based sibling design study. *JAMA Psychiatry*, 72(9), 935–942. <https://doi.org/10.1001/jamapsychiatry.2015.0846>

- Curran, E. A., O’Keeffe, G. W., Looney, A. M., Moloney, G., Hegarty, S. V., Murray, D. M., Khashan, A. S., & Kenny, L. C. (2018). Exposure to hypertensive disorders of pregnancy increases the risk of autism spectrum disorder in affected offspring. *Molecular Neurobiology*, *55*(7), 5557–5564. <https://doi.org/10.1007/s12035-017-0794-x>
- Curran, E. A., O’Neill, S. M., Cryan, J. F., Kenny, L. C., Dinan, T. G., Khashan, A. S., & Kearney, P. M. (2015b). Research review: Birth by caesarean section and development of autism spectrum disorder and attention-deficit/hyperactivity disorder: A systematic review and meta-analysis. *Journal of Child Psychology and Psychiatry*, *56*(5), 500–508. <https://doi.org/10.1111/jcpp.12351>
- Dabitao, D., Margolick, J. B., Lopez, J., & Bream, J. H. (2011). Multiplex measurement of proinflammatory cytokines in human serum: Comparison of the Meso Scale Discovery electrochemiluminescence assay and the Cytometric Bead Array. *Journal of Immunological Methods*, *372*(1–2), 71–77. <https://doi.org/10.1016/j.jim.2011.06.033>
- Deverman, B. E., & Patterson, P. H. (2009). Cytokines and CNS development. *Neuron*, *64*(1), 61–78.
- DeVilbiss, E. A., Gardner, R. M., Newschaffer, C. J., & Lee, B. K. (2015). Maternal folate status as a risk factor for autism spectrum disorders: A review of existing evidence. *British Journal of Nutrition*, *114*(5), 663–672. <https://doi.org/10.1017/S0007114515002470>
- Erbel, C., Chen, L., Bea, F., Wangler, S., Celik, S., Lasitschka, F., Wang, Y., Böckler, D., Katus, H. A., & Dengler, T. J. (2009). Inhibition of IL-17A attenuates atherosclerotic lesion development in ApoE-deficient mice. *The Journal of Immunology*, *183*(12), 8167. <https://doi.org/10.4049/jimmunol.0901126>
- Feng, C., Wang, H., Lu, N., Chen, T., He, H., Lu, Y., & Tu, X. M. (2014). Log-transformation and its implications for data analysis. *Shanghai Archives of Psychiatry*, *26*(2), 105–109. <https://doi.org/10.3969/j.issn.1002-0829.2014.02.009>
- Fernández de Cossío, L., Guzmán, A., van der Veldt, S., & Luheshi, G. N. (2017). Prenatal infection leads to ASD-like behavior and altered synaptic pruning in the mouse offspring. *Brain, Behavior, and Immunity*, *63*, 88–98. <https://doi.org/10.1016/j.bbi.2016.09.028>
- Galbraith, C., Jenkin, G., Davis P, & Cooper, P. (1996). *New Zealand Social Economic Index 1996 Users Guide, Statistics New Zealand, Wellington, New Zealand*. Retrieved from archive.stats.govt.nz › media › nz-socio-eco-idx-usr-guide
- Garay, P. A., Hsiao, E. Y., Patterson, P. H., & McAllister, A. K. (2013). Maternal immune activation causes age- and regionspecific changes in brain cytokines in offspring throughout development. *Brain, Behavior, and Immunity*, *31*, 54–68. <https://doi.org/10.1016/j.bbi.2012.07.008>
- Garay, P. A., & McAllister, A. K. (2010). Novel roles for immune molecules in neural development: Implications for neurodevelopmental disorders. *Frontiers in Synaptic Neuroscience*, *2*, 136.
- Gillberg, C., Cederlund, M., Lamberg, K., & Zeijlon, L. (2006). Brief report: “the autism epidemic”. The registered prevalence of autism in a Swedish urban area. *Journal of Autism and Developmental Disorders*, *36*(3), 429.
- Goines, P. E., Croen, L. A., Braunschweig, D., Yoshida, C. K., Grether, J., Hansen, R., Kharrazi, M., Ashwood, P., & Van de Water, J. (2011). Increased midgestational IFN- γ , IL-4 and IL-5 in women bearing a child with autism: A case–control study. *Molecular Autism*, *2*(1), 13. <https://doi.org/10.1186/2040-2392-2-13>
- Haddad, F. L., Patel, S. V., & Schmid, S. (2020). Maternal immune activation by Poly I:C as a preclinical model for neurodevelopmental disorders: A focus on autism and schizophrenia. *Neuroscience & Biobehavioral Reviews*, *113*, 546–567. <https://doi.org/10.1016/j.neubiorev.2020.04.012>
- Hsiao, E. Y., McBride, S. W., Chow, J., Mazmanian, S. K., & Patterson, P. H. (2012). Modeling an autism risk factor in mice leads to permanent immune dysregulation. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(31), 12776–12781. <https://doi.org/10.1073/pnas.1202556109>
- Huang, H., Xue, R., Zhang, J., Ren, T., Richards, L. J., Yarowsky, P., Miller, M. I., & Mori, S. (2009). Anatomical characterization of human fetal brain development with diffusion tensor magnetic resonance imaging. *The Journal of Neuroscience*, *29*(13), 4263. <https://doi.org/10.1523/JNEUROSCI.2769-08.2009>
- Jiang, H. Y., Xu, L. L., Shao, L., Xia, R. M., Yu, Z. H., Ling, Z. X., Yang, F., Deng, M., & Ruan, B. (2016). Maternal infection during pregnancy and risk of autism spectrum disorders: A systematic review and meta-analysis. *Brain, Behavior, and Immunity*, *58*, 165–172. <https://doi.org/10.1016/j.bbi.2016.06.005>
- Jones, K. L., Croen, L. A., Yoshida, C. K., Heuer, L., Hansen, R., Zerbo, O., DeLorenze, G. N., Kharrazi, M., Yolken, R., Ashwood, P., & Van de Water, J. (2017). Autism with intellectual disability is associated with increased levels of maternal cytokines and chemokines during gestation. *Molecular Psychiatry*, *22*(2), 273–279. <https://doi.org/10.1038/mp.2016.77>
- Josefi, O., & Ryan, V. (2004). Non-directive play therapy for young children with autism: A case study. *Clinical Child Psychology and Psychiatry*, *9*(4), 533–551. <https://doi.org/10.1177/1359104504046158>
- Joseph, R. (2000). Fetal brain behavior and cognitive development. *Developmental Review*, *20*(1), 81–98. <https://doi.org/10.1006/drev.1999.0486>
- Kenny, L. C., Black, M. A., Poston, L., Taylor, R., Myers, J. E., Baker, P. N., McCowan, L. M., Simpson, N. A., Dekker, G. A., Rodems, K., & Roberts, C. T. (2014). Early pregnancy prediction of preeclampsia in nulliparous women, combining clinical risk and biomarkers: The Screening for Pregnancy Endpoints (SCOPE) international cohort study. *Hypertension*, *64*(3), 644–652.

- Kim, Y.-K., Jung, H.-G., Myint, A.-M., Kim, H., & Park, S.-H. (2007). Imbalance between pro-inflammatory and anti-inflammatory cytokines in bipolar disorder. *Journal of Affective Disorders*, *104*(1), 91–95. <https://doi.org/10.1016/j.jad.2007.02.018>
- Knuesel, I., Chicha, L., Britschgi, M., Schobel, S. A., Bodmer, M., Hellings, J. A., Toovey, S., & Prinssen, E. P. (2014). Maternal immune activation and abnormal brain development across CNS disorders. *Nature Reviews Neurology*, *10*(11), 643–660. <https://doi.org/10.1038/nrneurol.2014.187>
- Kuwabara, Y., Takeda, S., Mizuno, M., & Sakamoto, S. (1987). Oxytocin levels in maternal and fetal plasma, amniotic fluid, and neonatal plasma and urine. *Archives of Gynecology and Obstetrics*, *241*(1), 13–23.
- Li, X., Chauhan, A., Sheikh, A. M., Patil, S., Chauhan, V., Li, X.-M., Ji, L., Brown, T., & Malik, M. (2009). Elevated immune response in the brain of autistic patients. *Journal of Neuroimmunology*, *207*(1), 111–116. <https://doi.org/10.1016/j.jneuroim.2008.12.002>
- Lyall, K., Croen, L., Daniels, J., Fallin, M. D., Ladd-Acosta, C., Lee, B. K., Park, B. Y., Snyder, N. W., Schendel, D., & Volk, H. (2017). The changing epidemiology of autism spectrum disorders. *Annual Review of Public Health*, *38*, 81–102.
- Machado, C. J., Whitaker, A. M., Smith, S. E. P., Patterson, P. H., & Bauman, M. D. (2015). Maternal immune activation in nonhuman primates alters social attention in juvenile offspring. *Biological Psychiatry*, *77*(9), 823–832. <https://doi.org/10.1016/j.biopsych.2014.07.035>
- Magiati, I., Ong, C., Lim, X. Y., Tan, J. W., Ong, A. Y., Patricia, F., Fung, D. S., Sung, M., Poon, K. K., & Howlin, P. (2016). Anxiety symptoms in young people with autism spectrum disorder attending special schools: Associations with gender, adaptive functioning and autism symptomatology. *Autism*, *20*(3), 306–320. <https://doi.org/10.1177/1362361315577519>
- Maher, G. M., O’Keeffe, G. W., Dalman, C., Kearney, P. M., McCarthy, F. P., Kenny, L. C., & Khashan, A. S. (2020). Association between preeclampsia and autism spectrum disorder: A population-based study. *Journal of Child Psychology and Psychiatry*, *61*(2), 131–139. <https://doi.org/10.1111/jcpp.13127>
- Malkova, N. V., Yu, C. Z., Hsiao, E. Y., Moore, M. J., & Patterson, P. H. (2012). Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. *Brain, Behavior, and Immunity*, *26*(4), 607–616. <https://doi.org/10.1016/j.bbi.2012.01.011>
- Masi, A., Glozier, N., Dale, R., & Guastella, A. J. (2017). The immune system, cytokines, and biomarkers in autism spectrum disorder. *Neuroscience Bulletin*, *33*(2), 194–204. <https://doi.org/10.1007/s12264-017-0103-8>
- Masi, A., Quintana, D. S., Glozier, N., Lloyd, A. R., Hickie, I. B., & Guastella, A. J. (2015). Cytokine aberrations in autism spectrum disorder: A systematic review and meta-analysis. *Molecular Psychiatry*, *20*(4), 440–446. <https://doi.org/10.1038/mp.2014.59>
- Meyer, U., & Feldon, J. (2012). To poly(I:C) or not to poly(I:C): Advancing preclinical schizophrenia research through the use of prenatal immune activation models. *Neuropharmacology*, *62*(3), 1308–1321. <https://doi.org/10.1016/j.neuropharm.2011.01.009>
- Morais, L. H., Golubeva, A. V., Casey, S., Scott, K. A., Ramos Costa, A. P., Moloney, G. M., Dinan, T. G., & Cryan, J. F. (2021). Early-life oxytocin attenuates the social deficits induced by caesarean section delivery in the mouse. *Neuropsychopharmacology*. <https://doi.org/10.1038/s41386-021-01040-3>
- Morais, L. H., Golubeva, A. V., Moloney, G. M., Moya-Pérez, A., Ventura-Silva, A. P., Arbolea, S., Bastiaanssen, T. F., O’Sullivan, O., Rea, K., Borre, Y., Scott, K. A., Patterson, E., Cherry, P., Stilling, R., Hoban, A. E., El Aidy, S., Sequeira, A. M., Beers, S., Moloney, R. D., Renes, I. B., Wang, S., Knol, J., Ross, P., O’Toole, P. W., Cotter, P. D., Stanton, C., Dinan, T. G., & Cryan, J. F. (2020). Enduring behavioral effects induced by birth by caesarean section in the mouse. *Current Biology*, *30*(19), 3761–3774 e3766. <https://doi.org/10.1016/j.cub.2020.07.044>
- Müller, N., Weidinger, E., Leitner, B., & Schwarz, M. J. (2015). The role of inflammation in schizophrenia. *Frontiers in Neuroscience*. <https://doi.org/10.3389/fnins.2015.00372>
- Nadeem, A., Ahmad, S. F., Attia, S. M., Bakheet, S. A., Al-Harbi, N. O., & Al-Ayadhi, L. Y. (2018). Activation of IL-17 receptor leads to increased oxidative inflammation in peripheral monocytes of autistic children. *Brain, Behavior, and Immunity*, *67*, 335–344. <https://doi.org/10.1016/j.bbi.2017.09.010>
- Parker-Athill, E. C., & Tan, J. (2010). Maternal immune activation and autism spectrum disorder: Interleukin-6 signaling as a key mechanistic pathway. *Neurosignals*, *18*(2), 113–128. <https://doi.org/10.1159/000319828>
- Patten, A. R., Fontaine, C. J., & Christie, B. R. (2014). A comparison of the different animal models of fetal alcohol spectrum disorders and their use in studying complex behaviors. *Frontiers in Pediatrics*, *2*, 93–93. <https://doi.org/10.3389/fped.2014.00093>
- Patterson, P. H. (2011). Maternal infection and immune involvement in autism. *Trends in Molecular Medicine*, *17*(7), 389–394. <https://doi.org/10.1016/j.molmed.2011.03.001>
- Pineda, E., Shin, D., You, S. J., Auvin, S., Sankar, R., & Mazarati, A. (2013). Maternal immune activation promotes hippocampal kindling epileptogenesis in mice. *Annals of Neurology*, *74*(1), 11–19.
- Polidano, C., Zhu, A., & Bornstein, J. C. (2017). The relation between cesarean birth and child cognitive development. *Scientific Reports*, *7*(1), 1–10.
- Pratt, L., Ni, L., Ponzio, N. M., & Jonakait, G. M. (2013). Maternal inflammation promotes fetal microglial activation and increased cholinergic expression in the fetal basal forebrain: Role of interleukin-6. *Pediatric Research*, *74*(4), 393–401.

- Prayer, D., Kasprian, G., Krampfl, E., Ulm, B., Witzani, L., Prayer, L., & Brugger, P. C. (2006). MRI of normal fetal brain development. *European Journal of Radiology*, *57*(2), 199–216. <https://doi.org/10.1016/j.ejrad.2005.11.020>
- Raghavan, R., Riley, A. W., Volk, H., Caruso, D., Hironaka, L., Sices, L., Hong, X., Wang, G., Ji, Y., Wahl, A., & Brucato, M. (2018). Maternal multivitamin intake, plasma folate and vitamin B12 levels and autism spectrum disorder risk in offspring. *Paediatric and Perinatal Epidemiology*, *32*(1), 100–111.
- Reed, M. D., Yim, Y. S., Wimmer, R. D., Kim, H., Ryu, C., Welch, G. M., Andina, M., King, H. O., Waisman, A., Halassa, M. M., Huh, J. R., & Choi, G. B. (2020). IL-17a promotes sociability in mouse models of neurodevelopmental disorders. *Nature*, *577*(7789), 249–253. <https://doi.org/10.1038/s41586-019-1843-6>
- Smith, S. E., Li, J., Garbett, K., Mirnics, K., & Patterson, P. H. (2007a). Maternal immune activation alters fetal brain development through interleukin-6. *Journal of Neuroscience*, *27*(40), 10695–10702. <https://doi.org/10.1523/jneurosci.2178-07.2007>
- Stiles, J., & Jernigan, T. L. (2010). The basics of brain development. *Neuropsychology Review*, *20*(4), 327–348. <https://doi.org/10.1007/s11065-010-9148-4>
- van der Zwaag, B., Franke, L., Poot, M., Hochstenbach, R., Spierenburg, H. A., Vorstman, J. A., van Daalen, E., de Jonge, M. V., Verbeek, N. E., Brilstra, E. H., & van't Slot, R., & Staal, W. G. (2009). Gene-network analysis identifies susceptibility genes related to glycometabolism in autism. *PLoS ONE*, *4*(5), e5324. <https://doi.org/10.1371/journal.pone.0005324>
- Wei, H., Chadman, K. K., McCloskey, D. P., Sheikh, A. M., Malik, M., Brown, W. T., & Li, X. (2012). Brain IL-6 elevation causes neuronal circuitry imbalances and mediates autism-like behaviors. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1822*(6), 831–842. <https://doi.org/10.1016/j.bbdis.2012.01.011>
- Wei, H., Zou, H., Sheikh, A. M., Malik, M., Dobkin, C., Brown, W. T., & Li, X. (2011). IL-6 is increased in the cerebellum of autistic brain and alters neural cell adhesion, migration and synaptic formation. *Journal of Neuroinflammation*, *8*(1), 52. <https://doi.org/10.1186/1742-2094-8-52>
- Werling, D. M., & Geschwind, D. H. (2013). Sex differences in autism spectrum disorders. *Current Opinion in Neurology*, *26*(2), 146–153. <https://doi.org/10.1097/WCO.0b013e32835ee548>
- Wiens, D., & DeSoto, M. C. (2017). Is high folic acid intake a risk factor for autism?—A review. *Brain Sciences*, *7*(11), 149.
- Wolff, A. R., & Bilkey, D. K. (2008). Immune activation during mid-gestation disrupts sensorimotor gating in rat offspring. *Behavioural Brain Research*, *190*(1), 156–159. <https://doi.org/10.1016/j.bbr.2008.02.021>
- Wong, H., & Hoeffler, C. (2018). Maternal IL-17A in autism. *Experimental Neurology*, *299*(Pt A), 228–240. <https://doi.org/10.1016/j.expneurol.2017.04.010>
- Yang, C. J., Liu, C. L., Sang, B., Zhu, X. M., & Du, Y. J. (2015). The combined role of serotonin and interleukin-6 as biomarker for autism. *Neuroscience*, *284*, 290–296. <https://doi.org/10.1016/j.neurosci.2014.10.011>
- Yang, D., Sun, Y.-Y., Bhaumik, S. K., Li, Y., Baumann, J. M., Lin, X., Zhang, Y., Lin, S. H., Dunn, R. S., Liu, C. Y., Shie, F. S., & Kuan, C.-Y. (2014). Blocking lymphocyte trafficking with FTY720 prevents inflammation-sensitized hypoxic–ischemic brain injury in newborns. *The Journal of Neuroscience*, *34*(49), 16467. <https://doi.org/10.1523/JNEUROSCI.2582-14.2014>
- Ye, B., Tao, T., Zhao, A., Wen, L., He, X., Liu, Y., Fu, Q., Mi, W., & Lou, J. (2019). Blockade of IL-17A/IL-17R pathway protected mice from sepsis-associated encephalopathy by inhibition of microglia activation. *Mediators of Inflammation*, *2019*, 8461725. <https://doi.org/10.1155/2019/8461725>
- Yip, B. H. K., Leonard, H., Stock, S., Stoltenberg, C., Francis, R. W., Gissler, M., Gross, R., Schendel, D., & Sandin, S. (2017). Caesarean section and risk of autism across gestational age: A multinational cohort study of 5 million births. *International Journal of Epidemiology*, *46*(2), 429–439. <https://doi.org/10.1093/ije/dyw336>

scientific reports



ADI-R	Autism diagnostic interview-revised
ADOS	Autism diagnostic observation schedule
ASD	Autism spectrum disorder
BMI	Body mass index
CBCL	Childhood behavioral checklist
CD 4 cell	Cluster of differentiation 4 cell
CREC	Cork research ethics committee
CSF	Cerebrospinal fluid
DISCO	Diagnostic interview for social and communication disorders
ECL	Electrochemiluminescence
ELISA	Enzyme-linked immunosorbent assay
GE	Gastroenteritis

GMCSF

Granulocyte–macrophage colony-stimulating factor

OPEN Mid-gestation cytokine profiles in mothers of children affected by autism spectrum disorder: a case–control study

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Autism Spectrum disorder is one of the commonest and most important neurodevelopmental conditions affecting children today. With an increasing prevalence and an unclear aetiology, it is imperative we find early markers of autism, which may facilitate early identification and intervention.

Alterations of gestational cytokine profiles have been reported in mothers of autistic children. Increasing evidence suggests that the intrauterine environment is an important determinant of autism risk. This study aims to examine the mid-gestational serum cytokine profiles of the mothers of autistic children from a well-characterised birth cohort. A nested sub-cohort within a large mother–child birth cohort were identified based on a confirmed multi-disciplinary diagnosis of autism before the age 10 years and neuro-typical matched controls in a 2:1 ratio. IFN- γ , IL-1 β , IL-4, IL-6, IL-8, IL-17A, GMCSF and TNF α were measured in archived maternal 20-week serum using MesoScale Diagnostics multiplex technology and validation of our IL-17A measurements was performed using an ultrasensitive assay. From a cohort of 2137 children, 25 had confirmed autism before 10 years and stored maternal serum from mid-gestation. We examined the sera of these 25 cases and 50 matched controls. The sex ratio was 4:1 males to females in each group, and the mean age at diagnosis was 5.09 years (SD 2.13). We found that concentrations of IL-4 were significantly altered between groups. The other analytes did not differ significantly using either multiplex or ultra-sensitive assays. In our well-characterised prospective cohort of autistic children, we confirmed mid-gestational alterations in maternal IL-4 concentrations in autism affected pregnancies versus matched

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controls. These findings add to promising evidence from animal models and retrospective screening programmes and adds to the knowledge in this field.

Abbreviations

HLA-G gene	Human leukocyte antigen G coding gene
HSE	Health service executive
ID	Intellectual disability
IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10 and IL-17A	Interleukins 1 alpha, 1 beta, 2, 4, 6, 8, 10, 17A
IL-17AR	Interleukin 17A receptor
IQR	Interquartile range
ISO	International Organization for Standardization
KBIT-2	Kaufmann brief intelligence test, second edition
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification MIA
Maternal immune activation mRNA	Messenger ribonucleic acid
MSD	MesoScale discovery
MZ	Monozygotic
Poly (I:C): Polyinosinic	Polycytidylic acid
PMN	Polymorphonuclear cells
PP	Polypropylene
PSS	Perceived stress score
ROUT	Robust regression and outlier removal
RTI	Respiratory tract infection
SCQ	Social communication questionnaire
SOP	Standard operating procedure
SRS	Social responsiveness scale
STAT6	Signal transducer and activator of transcription 6
Th1, 2 and 17	T helper 1, T helper 2, and T helper 17 cells
TNF α , TNF β	Tumour necrosis factor alpha, beta
Tregs	Regulatory T-cells

ULOQ Upper limit of quantification UTI Urinary
tract infection

Autism spectrum disorder (ASD) is an intricate continuum of neurodevelopmental disorders, all of which have an onset in early childhood and persist throughout life. These disorders are characterised by core impairments in social communication, and the presence of restricted and repetitive interests and behaviours^{1–4}. There exists within this spectrum a broad range of heterogeneity. Clinical phenotypes vary widely, aetiology remains unclear, and many different comorbidities afflict those with ASD. There is clearly a strong genetic component in many cases with heritability estimates of 50–90%^{5,6}, while the apparent male preponderance with rates exceeding that of females three to fourfold, also hints at a strong genetic foundation^{7,8}. Yet, even using newer techniques such as ASD-optimised ultrahigh resolution chromosomal microarray, we only find a single gene determinant in approximately 25% of cases^{9,10}. A recent study of monozygotic twins (MZ) (who share 100% similar copies of their genetic material) quoted ASD concurrence rates as low as 59% between MZ siblings¹¹. Some authors have found that specific HLA-DR (Human leukocyte antigen) subtypes are overexpressed in children with ASD versus controls^{12,13}. Despite these advances, we are yet to discover a single gene determinant that can account for more than a small percent of ASD cases. All this suggests that we cannot explain many cases of ASD by genetic factors alone, or at least we cannot explain them using our current understanding of ASD genetics or our current techniques of genetic analysis.

This imperfect picture of ASD genetics has led some to investigate the role of environmental exposures in the aetiology of ASD. Researchers have identified many environmental risks in ASD. Advanced parental age, foetal environmental exposures, perinatal and obstetric events, maternal medication use, smoking and alcohol use, psychosocial hardship, nutrition and toxic exposures have all been implicated as risks in the pathogenesis of ASD^{11,14}. Some authors attribute up to 17% of ASD risk to these exposures, yet the exact balance between genetic and environmental determinants and their roles in aetiology remains disputed^{11,15}. The current ASD literature suggests that mutations occurring in genes involved in synapse formation, cell adhesion molecule production (such as Cadherins), scaffolding proteins (SHANK proteins), ion channels (sodium, calcium, and potassium channels), and signaling molecules can disrupt regulatory or coding regions and affect synapse formation, plasticity and synaptic transmission¹⁶.

Cell signaling pathways such as PI3K, PTEN and mTOR interact with synapse influencing targets at multiple levels, mutations affecting these pathways lead to aberrant synaptic protein synthesis and have been shown to influence the development of monogenic (syndromic ASD) as well as non-syndromic ASD^{17,18}.

Multiple mechanisms have been proposed through which each of these exposures may exert an influence on genetic and epigenetic risk in ASD, but

there are only a handful that are likely to effect abnormal neurodevelopment. Animal models of inflammation and maternal immune activation are particularly well characterised, and have successfully modelled ASD type behaviours and social difficulties in mice, rats and non-human primates^{19–21}.

Maternal immune activation (MIA) is defined as an increase in measured levels of inflammatory markers in mothers during pregnancy, and more specifically refers to a triggering of the maternal immune system by infectious or infectious-like stimuli resulting in an increase in measurable inflammatory markers^{22,23}. Through this activation, a cytokine cascade transmits to the foetus, resulting in adverse neurodevelopmental phenotypes and even remodelling or malformations of the developing foetal brain. There have been many studies, which have profiled cytokine, chemokine, immune cell and inflammatory signatures in ASD affected individuals^{24–29}. A much smaller number of studies have characterised cytokine profiles in expectant mothers who progressed to give birth to children who develop ASD^{30,31}. The few previous studies, which have examined gestational serum, have indicated mid-gestational upregulation in specific pro-inflammatory cytokines or indeed down-regulation in anti-inflammatory cytokines. These findings arise from retrospective examination of stored serum samples from the wide 15–19 week gestational window. None of these studies confirmed a formal psychiatric or multidisciplinary team diagnosis of ASD, nor did they account for important and relevant underlying maternal inflammatory conditions such as inflammatory bowel disease³² and rheumatoid arthritis³³. Our aim in this study was to measure candidate cytokines at a single specific mid-gestational time-point (20-weeks' gestation) in a carefully characterised prospectively recruited birth cohort.

Methods

Study population. Mother and child dyads were recruited from the Cork BASELINE Birth Cohort Study (Babies after SCOPE: Evaluating the Longitudinal Impact on Neurological and Nutritional Endpoints) (www.baselinedudy.net). In total, recruitment ran for just over three years, from August 2008 to October 2011. The SCOPE Ireland pregnancy cohort (www.scopestudy.net) formed the basis of recruitment of infants to BASELINE [$n = 1537$] and an additional 600 infants were recruited after delivery providing a total sample of 2137. The research team performed assessments on day of life 2 and at 2, 6, 12, 24 and 60 months of age. Team researchers performed specific developmental assessments at 24 months (using the Ages and Stages parental questionnaire, and the Child Behaviour Checklist) and at 60 months (using the Kaufman Brief Intelligence Test, 2nd edition (KBIT-2) and the Child Behaviour Checklist). Blood and DNA samples were bio-banked at 15 and 20 weeks' gestation, at birth, and at 24 and 60 months. Children with low scores at either time-point were examined further by the study paediatrician (DM) and were referred for early intervention assessment. In this study, archived midgestational (20 weeks) serum samples were analysed. The inclusion criteria for the study were:

1. Subjects had bio-banked mid-gestational serum samples available,
2. All participants had completed 5 year follow up (ideally including developmental assessment),
3. Children who were suspected ASD cases had received a confirmed ASD diagnosis according to local practices,
4. Those participants with alternate developmental conditions (such as recognised genetic syndromes) were excluded

Clinical diagnosis. The majority of children received their ASD diagnosis through the Health Service Executive (HSE) ASD service. The standard tests utilised in this setting are the Autism Diagnostic Observation Schedule (ADOS), and parent report via either the Diagnostic Interview for Social & Communication Disorders (DISCO) or Autism Diagnostic Interview-Revised (ADI-R) questionnaires. A small number of children received their initial diagnosis through private multidisciplinary teams using the same assessment tools. All of these children later received a confirmatory diagnosis with the HSE ASD service.

Demographic variables. We have presented the demographic and relevant clinical data regarding the participants in Table 2. Male sex is indicated as a percentage in each participant group. Infant birthweight is presented in grams. Gestational age is in weeks. Customised birth centile indicates the percentile of the child's birthweight in relation to their gestational age at birth. Centiles were adjusted for mothers' height, weight at 15-week visit, ethnicity, and infant sex. The centiles were calculated using an online research calculator and were based on UK standards [https:// www. gesta tion. net/](https://www.gestation.net/)³⁴. Maternal age is presented in years and sub-categorised in to three age ranges, 18–28, 29–39, > 40 years. We present maternal BMI in kg/m² and sub-categorise according to WHO criteria, underweight BMI < 18.5, normal BMI 18.5–24.99, overweight BMI 25–29.99 and obese BMI > 30 kg/m². We present the Apgar scores³⁵ at one and five minutes as the proportion from each group with a tally less than seven. In our group there were three categories of marriage status, single, married or de facto (stable relationship akin to marriage) and finally we document smoking status in this pregnancy as (No) non-smoker, (Yes, but stopped) smoked until pregnancy was discovered, and (Still smoking) continues to smoke. The 10-question Perceived Stress Score questionnaire forms the basis for the Perceived Stress Scores (PSS). An individual's scores on the PSS can range from zero to 40 with higher scores indicating higher perceived stress. Low stress scores range from 0–13, moderate stress scores range from 14–26, and high stress scores range from 27–40³⁶. Past medical history indicates the relevant past medical history of mothers in the study, and intrapartum infections correspond to reported infections in the first 20 weeks of pregnancy.

Ethical approval and consent to participate. Ethical approval for both the SCOPE study (Cork ECM5 (10) 05/02/08) and Cork BASELINE Birth Cohort Study (ECM3 (x) 05/04/19) were provided locally by the Cork Research Ethics Committee (CREC). We obtained written informed consent from the mothers of each case and control recruited for additional enrolment in the PiRAMiD

study (Predicting early onset Autism through Maternal Immune Activation and Proteomic Discovery). Additional ethical approval for the PIRAMiD study was obtained from CREC (ECM 3 (k) 03/12/19). The Cork Research Ethic Committee (CREC) approved all research protocols in this study. Each participant gave informed consent. Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall 6 Little Hanover Street, Cork, Ireland.

Bio-fluid collection. We obtained archived serum samples of mothers recruited to the SCOPE-Cork study at 20 weeks' gestation within Cork University Maternity Hospital, Cork, Ireland. Biobank specimens were archived at -80°C in the SCOPE (Cork) ISO accredited biobank facility until required. SCOPE study specific research midwives in accordance with best practice guidance (SCOPE Consortium S.O.P.) had performed venepuncture at the 20-week visit. Maternal specimens were collected in serum separator tubes (Becton–Dickinson Franklin Lakes, New Jersey), immediately placed on ice, and transported to the laboratory. Before proceeding to centrifugation, serum samples were stored at 4°C for 30 min from time of collection to allow clot formation. Researchers confirmed the presence of the clot visually, and samples were then centrifuged at $2400\times g$ for 10 min at 4°C . Serum samples were transferred to ice cold 5 mL sterile PP (polypropylene) tubes (VWR, Radnor, Pennsylvania) via sterile Pasteur pipettes. Samples were again centrifuged at $3000\times g$ for 10 min at 4°C . Sera were then aliquoted to red capped, barcode-labelled cryovials (VWR) in volumes of 250 μl . Aliquots were logged in the SCOPE database (MedSciNet), and stored at -80°C within four hours of initial collection³⁷.

Cytokine analysis. We selected our candidate cytokines (IL-1 β , IL-4, IL-6, IL-8, IL-17A, GM-CSF, TNF α , IFN γ) based on previous literature highlighting aberrations in cytokine levels in individuals with ASD³⁸ versus healthy controls. We also reviewed the literature and focused on a number of publications which have measured mid-gestation (15–19 weeks) cytokines previously^{30,31}, and on IL-17A in particular. Much of the recent literature espouses IL-17A's potential as a key player in MIA associated neurodevelopmental outcomes^{19,39,40}. In order to quantify IL-17A more precisely, we examined IL-17A as part of a multiplex ELISA (enzyme-linked immunosorbent assay), and then individually, using a separate ultrasensitive ELISA assay (MSD S-plex).

MSD multiplex V-plex assay. We profiled the serologic concentrations (pg/ml) of eight cytokines and proinflammatory proteins at 20 weeks' gestation using the Mesoscale Discovery V-plex cytokine and proinflammatory electro-chemiluminescent (ECL) assays (Meso Scale Diagnostics, Rockville, Maryland 20850-3173, United States).

We used the V-plex multi-spot Cytokine Panel 1 (human) kit (LOT No: Z0047047) to examine IL-17A and GMCSF, and we examined IFN- γ , IL-1 β , IL-4, IL-6, IL-8 and TNF α using the V-plex multi-spot Proinflammatory Panel 1 (human) kit (LOT No: Z0047096). We ran all standards in triplicate, but we ran all participant samples in duplicate due to low sample volumes.

MSD S-plex IL-17A ultrasensitive assay. We profiled serologic concentrations (fg/ml) of IL-17A at 20 weeks' gestation using the Mesoscale Discovery S-plex (Lot No: Z00S0003) IL-17A ECL assay (Meso Scale Diagnostics, Rockville, Maryland 20850-3173, United States). We ran all standards and participant samples in triplicate (single analyte kits require a smaller volume of serum for analysis than multiplex kits).

We performed all experiments as per the manufacturer's instructions and analysed the plates on a MESO QuickPlex SQ 120 instrument. Numeric results were generated as "calculated concentration means" on the MSD Discovery Workbench 4.0 assay analysis software. Samples were excluded if the coefficient of variation (%CV) was higher than 25% between duplicates/triplicates as previously described⁴¹. We have outlined the Lower limits of detection (LLOD), lower limits of quantification (LLOQ) and the upper limits of quantification (ULOQ) as well as inter-assay CV (Coefficient of variation) for each cytokine in Table 1 for both the multiplex and ultrasensitive assays.

Statistical analysis. We compared the ASD cases (n = 25) as a whole with the neuro-typical controls (n = 50), All data were analysed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA) and IBM SPSS Statistics 26 (SPSS Statistics, Chicago, IL). ROUT analysis⁴³ was performed to remove outliers for each analyte (Q = 1%). Data arising from the cytokine analysis were analysed using Mann–Whitney U-test, as data were non-parametric. Data within the demographics table were compared using the Pearson Chi-square method for categorical data and independent t-tests or Mann–Whitney U-tests were data were parametric or non-parametric respectively. The age of the serum samples used was compared between groups using Mann–Whitney U-test. To assess for cytokine degradation over time, we correlated sample age with the concentrations of analytes using Spearman rank correlation (Rho) ρ bivariate analysis. Statistical significance (2-tailed) was set at $p < 0.05$.

Results

Of the initial 2137 recruited, 1249 completed 5-year follow up (see Fig. 1) Fig. 1: Included in the final analysis group were 75 child-mother pairs. Each mother had stored serum from 20 weeks' gestation for analysis and had no significant past medical history of inflammatory disease. The case and control split was one case to two controls (25 cases to 50 controls). We selected neuro-typical, healthy controls from the same BASELINE birth cohort, and we matched controls to cases based on:

1. Infant sex,
2. Gestational age at birth,
3. Birthweight and
4. Maternal BMI at 15-week visit.

	LLOD median pg/mL	LLOD range pg/mL	LLOQ pg/mL	ULOQ pg/mL	Inter-assay CV %
Proinflammatory panel					

IFN γ	0.37	0.21–0.62	1.76	938	8.16
IL-1 β	0.05	0.01–0.07	0.646	375	7.95
IL-4	0.02	0.01–0.03	0.218	158	6.23
IL-6	0.06	0.05–0.09	0.633	488	8.62
IL-8	0.07	0.03–0.14	0.591	375	7.8
TNF α	0.04	0.01–0.13	0.690	248	6.74
Cytokine panel					
GMCSF	0.16	0.08–0.19	0.842	750	10.78
IL-17A	0.31	0.19–0.55	3.19	3650	11.45
	fg/mL	fg/mL	fg/mL	fg/mL	%
Ultrasensitive IL-17A					
IL-17A	13	N/A	60	140,000	8.67

Table 1. Sensitivity of assays per each analyte examined. In this table LLOD, LLOQ, ULOQ for each analyte tested using the MSD proinflammatory panel 1, cytokine panel 1, and MSD S-plex Human IL-17A kits. The units of measurement used in the multiplex assays are pg/ml (10^{-12} g (picograms) per millilitre), while the units in the ultrasensitive assay are fg/ml (10^{-15} g (femtograms) per millilitre). The quantitative range of the assay lies between the LLOQ and ULOQ. Inter-assay CV is a measure of the variance between runs of sample replicates on different plates and assesses plate-to-plate consistency—inter-assay CV values < 15% were deemed acceptable⁴². All inter-assay CVs were within the permissible range, indicating a low level of plate-to-plate variability.

We identified 22 children with a confirmed diagnosis of ASD at the 5-year developmental assessments and a further 13 cases were diagnosed between 5 and 10 years. These “later” cases consisted of children who received their formal ASD diagnosis after 5 years of age. These cases were identified on review of the 5-year follow up documentation. Those with expressed parental concern about ASD, developmental assessment suggestive of ASD, or at risk ASD scoring in the Child Behavior Checklist (CBCL) were added to the cases cohort. The clinical research fellow verified these additional later ASD cases via a follow up telephone interview. Following confirmation of each ASD diagnosis, the research fellow invited each candidate and his or her parents to attend a follow on cognitive (KBIT-2) and ASD symptomology (SCQ) assessment. The cohort and their parents’ medical histories were further characterised using a study-specific health questionnaire.

In total, there were 10 case exclusions. Nine (9) cases had no stored serum from mid-gestation. We excluded these cases along with their matched controls. We excluded one further case (and matched controls) due to a genetic diagnosis of Bannayan-Riley-Ruvalcaba syndrome. We have depicted the recruitment stream in Fig. 1. The ASD prevalence of those 1249 children still enrolled at 5-years was approximately 3%, generally, in line with what others have quoted recently^{7,44}.

Cohort characteristics. In our cohort, the ratio of male to female ASD affected children was 4:1; this is consistent with most consensus of male preponderance in ASD^{8,45}. There was no difference between groups in relation to infant birthweight or gestational age at delivery. There were no significant differences between those infants with low (< 7) reported 1- and 5-min Apgar scores. The groups matched closely in terms of maternal age and maternal BMI. All mothers participating in the study were first time mothers. The groups were ethnically homogenous, with all participants bar one of Caucasian European background. The exception was a single control of Australasian descent. With regard to inflammatory conditions and potential modifiers of inflammation, no participants reported use of any anti-inflammatories or steroids during pregnancy. Each group reported approximately equal rates of smoking. Perceived stress scores (PSS) did not significantly differ between groups, though more controls reported moderate to high stress. One mother in the control group reported suffering from Psoriasis, and a mother in the cases group reported having coeliac disease. There was no significant difference between groups in the commonly reported medical conditions of anaemia (diagnosed prior to pregnancy), asthma, depression (none on active treatment) and thyroid disease. Of those with thyroid disease, one participant from each group had hyperthyroidism; the remainder were euthyroid following treatment. In the first 20 weeks of pregnancy, 44% of controls reported at least one infection (most commonly a Respiratory Tract Infection), while only 20% of cases did so, again, this was non-significant. We have detailed the participant demographics in Table 2.

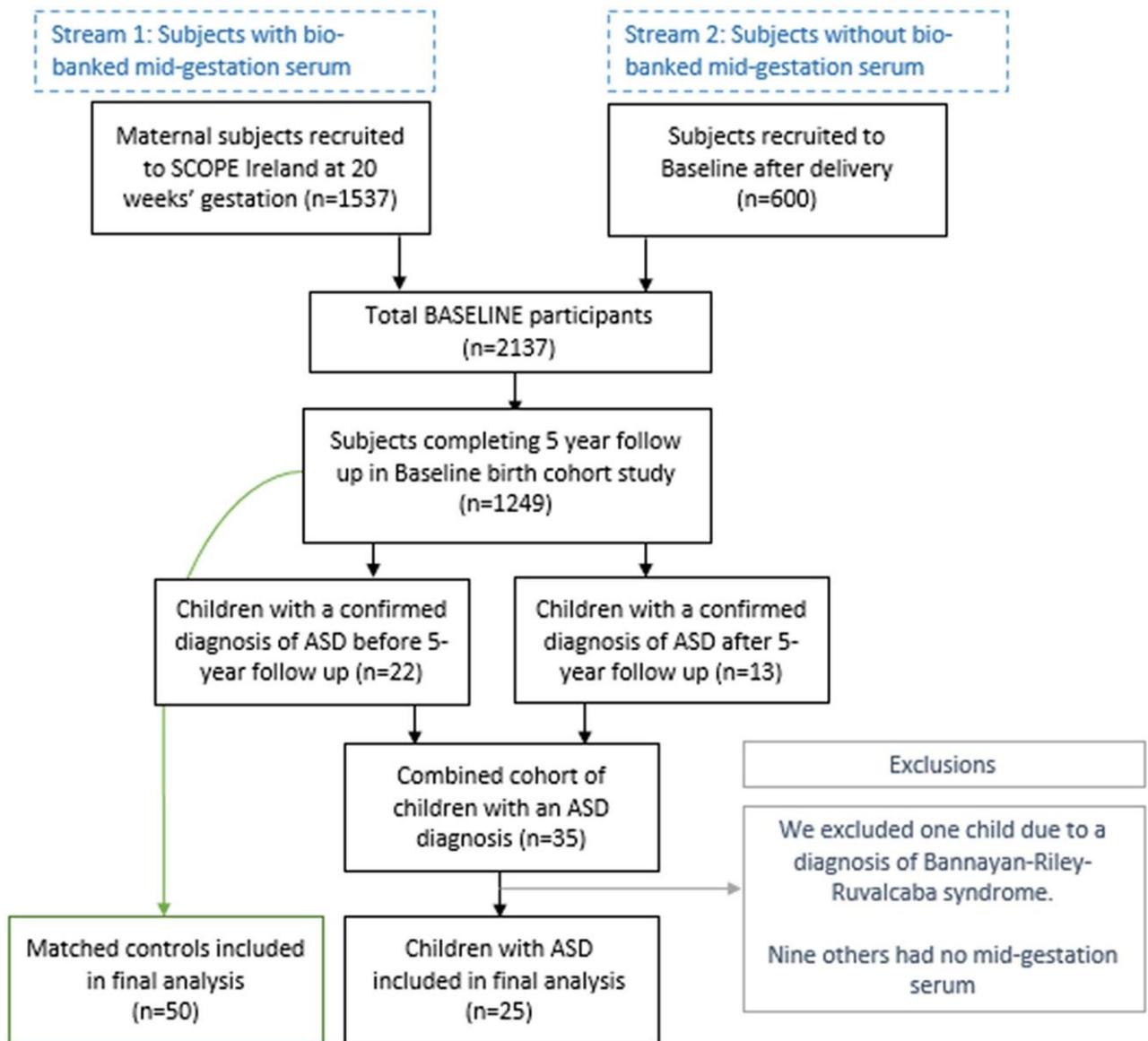


Figure 1. Recruitment numbers flow chart: Participants in Baseline were drawn from two streams, those recruited at 15-week booking appointment (n = 1537) and those recruited in the immediate post-natal period (600), totalling 2137 participants. 1249 participants completed follow up at 5 years. Of those, Twenty-two participants had a known ASD diagnosis at 5-year follow up; a further 13 were diagnosed with ASD between 5 and 10 years of age. Of the 35 participants with a diagnosis of ASD, 10 were excluded from this analysis. One child had a significant genetic diagnosis (Bannayan-Riley-Ruvalcaba syndrome); none of the other nine children had stored midgestational serum. This left 25 participants with ASD who were then matched 2:1 with healthy controls.

Cytokine analysis. To determine whether a significant difference existed between the mid-gestation (20week) inflammatory profiles of mothers of ASD and neurotypical children Meso Scale Discovery ECL assays were performed. Of the original eight cytokines examined, IL-4 was significantly

altered (p -value 0.04). Cytokine concentrations were compared using Mann–Whitney U test. Expression of the seven other cytokines did not differ significantly. In Table 3 we present the number of samples analysed and the sample attrition rates. IL-17A was examined using a multiplex system and an ultrasensitive single analyte assay, and neither indicated a significant difference between groups. A summary of the cytokine analysis results is presented in Table 4, and the individual analyte results are presented in Fig. 2 through Fig. 10.

Variable	Cases (n = 25)		Controls (n = 50)		p -value
	n or M	(%) or SD	n or M	(%) or SD	
Male sex	20	(80%)	40	(80%)	1
Infant birthweight	3488	SD 532	3496	SD 455	0.80
Gestational age	39.65	SD 1.5	39.78	SD 1.5	0.75
Customised birthweight centile	48.23	SD 26.5	51.90	SD 26.9	0.51
Maternal age	30.76	SD 5.3	31.46	SD 3.9	0.52
18–28	8	(32%)	9	(18%)	0.26
29–39	17	(68%)	39	(78%)	
> 40	0	(0%)	2	(4%)	
Maternal BMI	25.80	SD 4.9	25.23	SD 4.0	0.60
Underweight	1	(4%)	1	(2%)	0.25
Normal	13	(52%)	26	(52%)	
Overweight	7	(28%)	14	(28%)	
Obese	4	(16%)	9	(18%)	
Apgar 1 min < 7	4	(16%)	3	(6%)	0.16
Apgar 5 min < 7	1	(4%)	1	(2%)	0.61
Marital status					
Single	2	(8%)	3	(6%)	0.87
Married	20	(80%)	39	(78%)	
De facto	3	(12%)	8	(16%)	
Smoked (pregnancy)					
No	20	(80%)	37	(74%)	0.85
Yes, but stopped	2	(8%)	5	(10%)	
Still smoking	3	(12%)	8	(16%)	
PSS (moderate or high)	8	(32%)	24	(48%)	0.24
Past medical history					
Anaemia	2	(8%)	8	(16%)	0.34
Thyroid disease	4	(16%)	3	(6%)	0.26
Depression	2	(8%)	5	(10%)	0.74
Asthma	4	(16%)	5	(10%)	0.41
Intrapartum infection (< 20w)					
Respiratory tract infection (RTI)	3	(12%)	13	(26%)	0.16
Urinary tract infection (UTI)	2	(8%)	7	(14%)	0.45
Gastroenteritis (GE)	0	(0%)	2	(4%)	0.31

Table 2. Demographic characteristic of study participants. In this table, we calculated all p -values using the Pearson Chi square for categorical data, and

independent samples t-test or Mann–Whitney U-test where appropriate for continuous variables depending on the normality of the distribution. There are no significant differences demonstrated between the groups in any of the variables listed. Cases and controls are well matched with little variance between the key matching variables, infant sex, gestational age and birthweight. Data are presented as either the mean (SD) with continuous variables or n (percentage) with categorical ones.

MSD multiplex V-plex. In Fig. 2 IFN γ concentration in ASD cases versus matched controls Fig. 2, IFN γ concentration was analysed using the Mesoscale Discovery platform. Units of concentration are pictograms/millilitre (pg/ml). IFN γ was not significantly altered in mothers of ASD affected children (median 2.773) at 20 weeks' gestation compared to neuro-typical controls (median 2.763). ROUT analysis (Q = 1%) was performed to identify and exclude outliers. In total, five outliers were removed (three controls and two cases). Final analysis was performed on n = 22 cases and n = 37 controls $p = 0.99$.

Analyte	Number of samples analysed per group		Exclusions – concentration < LLOD		Exclusion – high CV	
	Cases	Control	Cases	Controls	Cases	Controls
IFN γ	24	40	0	0	1	10
IL-1 β	13	15	11	31	1	4
IL-4	7	10	15	34	3	6
IL-6	25	42	0	1	0	7
IL-8	23	41	0	0	2	9
TNF α	24	42	0	1	1	7
GMCSF	8	24	13	21	4	5
IL-17A	18	36	2	6	5	8
IL-17A ultrasensitive	25	49	0	0	0	1

Table 3. Number of samples analysed and sample losses during processing. In this table we present the number of samples analysed for each analyte. Some of the cytokines had significant samples attrition during processing. The two reasons for loss of samples from the analysis were high CV values (> 25%) and undetectable concentrations of cytokine, below the LLOD of the assay. Use of an ultrasensitive assay rectified this issue in the case of IL-17A.

	Cases		Controls		p -value < 0.05	ROUT analysis	
	Median	IQR	Median	IQR		Case	Control
IFN- γ	2.77	2.16–4.40	2.76	1.79–4.70	0.99	2	3
IL-1 β	0.03	0.01–0.05	0.07	0.02–0.11	0.09	2	0
IL-4	0.03	0.02–0.03	0.05	0.03–0.07	0.04	1	0
IL-6	0.44	0.24–0.76	0.40	0.25–0.56	0.49	0	3
IL-8	5.52	3.90–7.00	4.88	3.52–5.79	0.10	0	5
TNF α	1.13	0.85–1.69	1.11	0.92–1.46	0.69	0	2
GM-CSF	0.12	0.08–0.28	0.16	0.10–0.24	0.38	0	2

IL-17A	0.69	0.49–0.99	0.84	0.39–1.01	0.85	0	0
IL-17A (U)	3.47	3.36–3.59	3.45	3.31–3.61	0.80	0	0

Table 4. Summary table of cytokine analysis results. In this table we quote all analyte concentrations in pg/ mL except for ultrasensitive IL-17A assay (IL-17A (U)) which we quote in fg/mL. *p*-values are statistically significant at values less than 0.05. Outliers were removed using ROUT analysis on GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA). The final column “ROUT analysis” indicates the number of outliers removed from each group per analyte. We used Mann Whitney U-tests for the calculation of *p*-values as data were non-parametric.

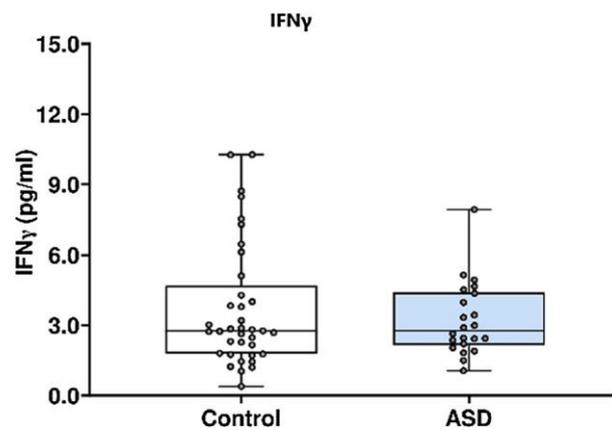


Figure 2 IFN γ concentration in ASD cases versus matched controls.

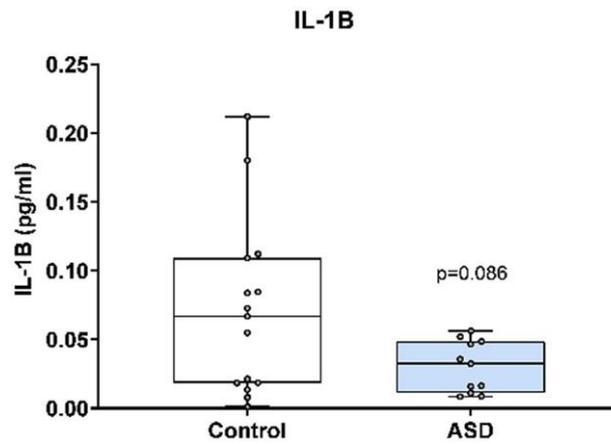


Figure 3 IL-1 β concentration in ASD cases versus matched controls.

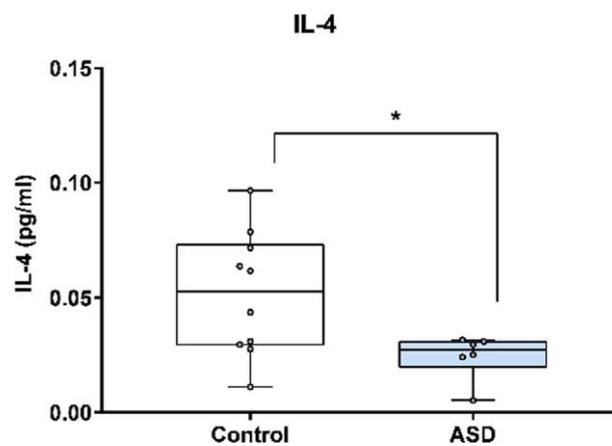


Figure 4 IL-4 concentration in ASD cases versus matched controls.

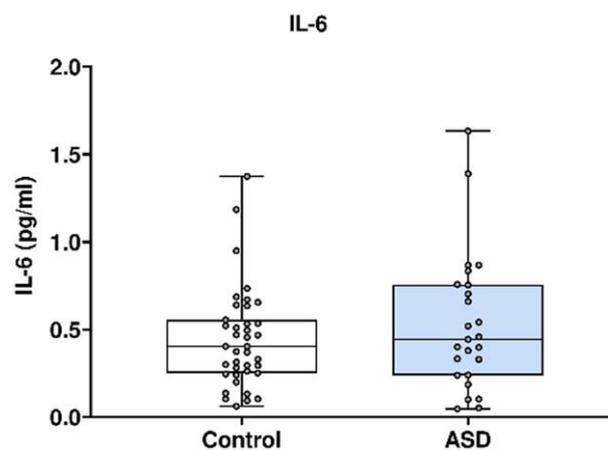


Figure 5 IL-6 concentrations in ASD cases versus matched controls.

In Fig. 3, IL-1 β concentration was analysed using the Mesoscale Discovery platform. Units of concentration are pictograms/millilitre (pg/ml). IL-1 β was not significantly altered in mothers of ASD affected children (median 0.032) at 20 weeks' gestation compared to neuro-typical controls (median 0.067).

ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. In total, two outliers were removed (two cases). Final analysis was performed on $n = 11$ cases and $n = 15$ controls $p = 0.09$.

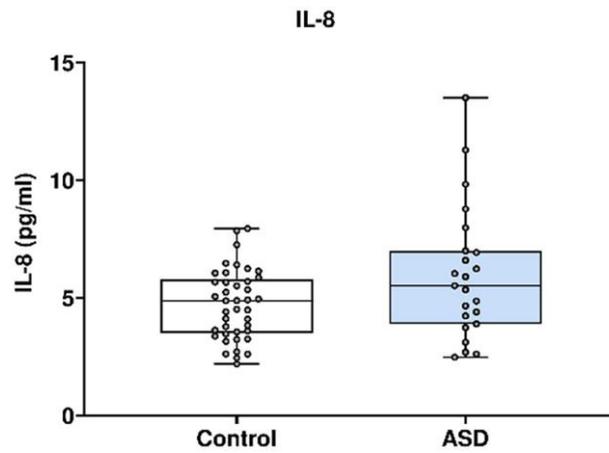


Figure 6 IL-8 concentration in ASD cases versus matched controls.

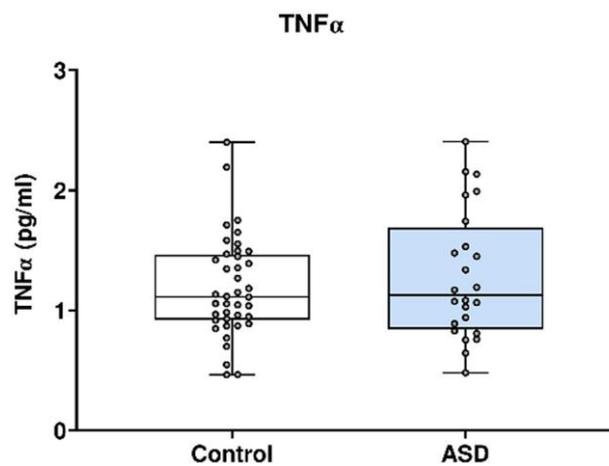


Figure 7 TNF α concentration in ASD cases versus matched controls.

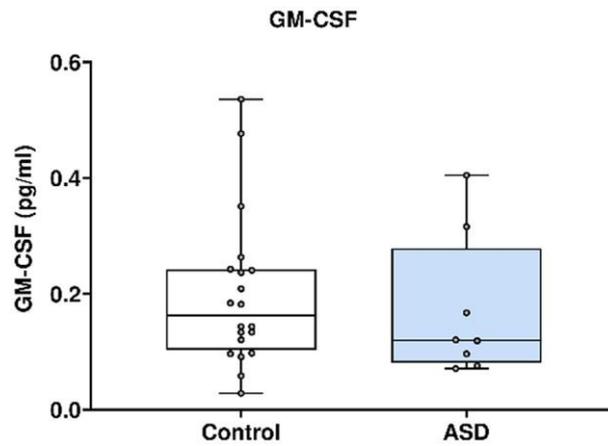


Figure 8 GM-CSF concentration in ASD cases versus matched controls.

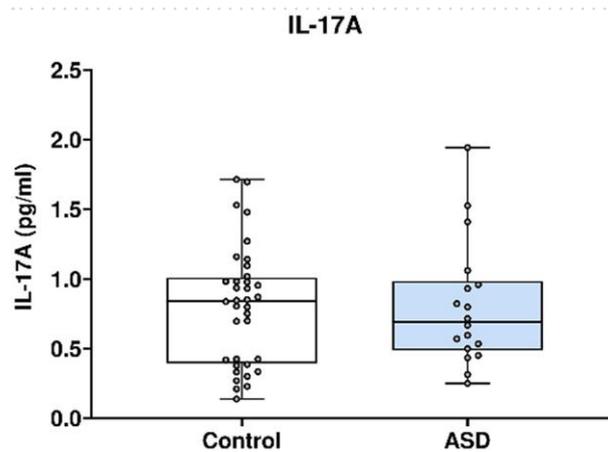


Figure 9 IL-17A concentrations from the multiplex analysis in ASD cases versus controls.

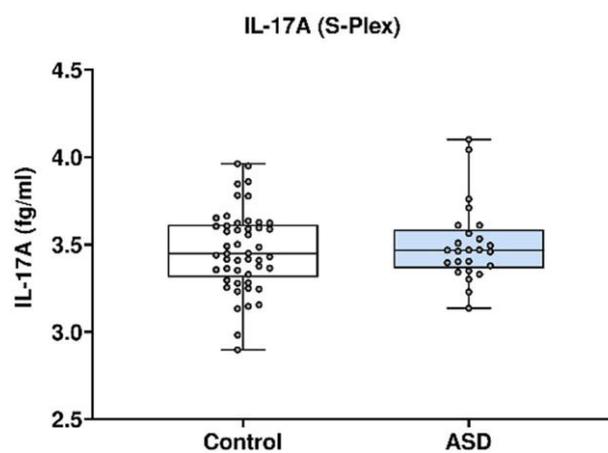


Figure 10 IL-17A (Ultrasensitive) concentrations of IL-17A in ASD cases versus controls.

In Fig. 4, IL-4 concentration was analysed using the Mesoscale Discovery platform. Units of concentration are pictograms/millilitre (pg/ml). IL-4 was significantly altered in mothers of ASD affected children (median 0.027) at 20 weeks' gestation compared to neuro-typical controls (median 0.053) $p = 0.04$. ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. one

outlier was removed (one case). Final analysis was performed on $n = 6$ cases and $n = 10$ controls. *indicates a statistically significant p value < 0.05 .

In Fig. 5, IL-6 concentration was analysed using the Mesoscale Discovery platform. Units of concentration are pictograms/millilitre (pg/ml). IL-6 was not significantly altered in mothers of ASD affected children (median 0.444) at 20 weeks' gestation compared to neuro-typical controls (median 0.404). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. three outliers were removed (three controls). Final analysis was performed on $n = 25$ cases and $n = 39$ controls. $p = 0.49$.

In Fig. 6, IL-8 concentration was analysed using the Mesoscale Discovery platform. Units of concentration are pictograms/millilitre (pg/ml). IL-8 was not significantly altered in mothers of ASD affected children (median 5.519) at 20 weeks' gestation compared to neuro-typical controls (median 4.881). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. five outliers were removed (five controls). Final analysis was performed on $n = 23$ cases and $n = 36$ controls. $p = 0.10$.

In Fig. 7, TNF α concentration was analysed using the Mesoscale Discovery platform. Units of concentration are pictograms/millilitre (pg/ml). TNF α was not significantly altered in mothers of ASD affected children (median 1.127) at 20 weeks' gestation compared to neuro-typical controls (median 1.114). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. two outliers were removed (two controls). Final analysis was performed on $n = 24$ cases and $n = 40$ controls. $p = 0.69$.

In Fig. 8, GM-CSF concentration was analysed using the Mesoscale Discovery platform. Units of concentration are pictograms/millilitre (pg/ml). GM-CSF was not significantly altered in mothers of ASD affected children (median 0.120) at 20 weeks' gestation compared to neuro-typical controls (median 0.163). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers. two outliers were removed (two controls). Final analysis was performed on $n = 8$ cases and $n = 22$ controls. $p = 0.38$.

	Total sample number	Median sample age (IQR)	p -value
Case	25	9.75 (9.48–10.71)	0.61
Control	50	10.08 (9.52–10.57)	

Table 5. Sample age (years). p -value in this table was calculated using the Mann–Whitney U test.

	IFN γ	IL-1 β	IL-4	IL-6	IL-8	TNF α	IL17A	GMCSF	*IL17A
Samples (n)	64	28	17	67	64	66	54	32	74
ρ (Rho)	–	–	–	0.104	–	–	–	0.062	– 0.194
p	0.103	0.346	0.065	0.137	0.308	0.063	0.76	0.1	0.42

Table 6. Correlation between sample age and analyte concentration. In this table, we measured correlation using Spearman's rank correlation (Rho) ρ bivariate analysis of sample age and each individual concentration of analyte per sample. Number of samples analysed (n) per analyte. Statistical

significance is considered when p value < 0.05 . *analyte measured using ultra-sensitive MSD assay.

In Fig. 9, L-17A concentration was analysed using the Mesoscale Discovery platform. Units of concentration are pictograms/millilitre (pg/ml). IL-17A was not significantly altered in mothers of ASD affected children (median 0.691) at 20 weeks' gestation compared to neuro-typical controls (median 0.842). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers, none were found. Final analysis was performed on $n = 18$ cases and $n = 36$ controls. $p = 0.85$.

MSD S-plex ultrasensitive assay. In Fig. 10, IL-17A concentration was analysed using the Mesoscale Discovery platform. Units of concentration are femtograms/millilitre (fg/ml). IL-17A was not significantly altered in mothers of ASD affected children (median 3.468) at 20 weeks' gestation compared to neuro-typical controls (median 3.449). ROUT analysis ($Q = 1\%$) was performed to identify and exclude outliers, none were identified. Final analysis was performed on $n = 25$ cases and $n = 49$ controls. $p = 0.80$.

Post hoc analysis to examine the effect of storage duration on sample quality. Our study used samples that had been stored for an extended time (ranging from 9.1 year to 11.8 years) before their analysis (Table 5: Sample age (years)). Somewhat mitigating this, the majority of samples were collected and stored for a similar duration before use. While degradation is highly likely to have occurred in each sample, we expect that, as all samples were stored under similar conditions, that the degree of cytokine degradation is comparable across all samples. To test this, we correlated sample age with the concentrations of each analyte (see Table 6). We found that seven of nine analytes correlated negatively with sample age suggesting some degradation over the period from the most recent to the earliest sampling. Two analytes, IL-6 and GM-CSF, correlated positively but the correlations were “weak” and “negligible” respectively. One analyte, TNF α demonstrated a significant “fair” negative correlation⁴⁶ with sample age ρ (Rho) $- 0.308$ ($p = 0.01$), which is also reflected by linear regression analysis ($F(1, 63) = 5.037$; $p = 0.028$. $R^2 = 0.074$ ⁴⁷). This finding confirms significant and moderate TNF α degradation in the timeframe of our sample acquisition, but no other cytokines were significantly altered in this period. While we undertook steps to reduce cytokine loss from degradation, by avoidance of freeze thaw cycles and remotely monitored ultra-low temperature storage at $- 80$ °C^{48,49}, it remains likely that sample degradation occurred irrespective of remedial action. However, both controls and ASD samples were stored for similar lengths of time.

Discussion

We have shown that the expression of IL-4 in maternal serum is altered significantly between ASD affected and matched control groups at 20 weeks'

gestation in a small, but carefully characterised cohort of mothers and children where the child has a diagnosis of ASD by age 10 years.

Previous evidence indicates that aberrations of the immune system may play a role in ASD^{31,38,50}. Some propose that alterations in cytokine expression could facilitate the classification of ASD subtypes^{24,31,51} as well as work as biomarkers of response to treatment. In the diagnosis and management of ASD, earlier is better, and identification of reliable biomarkers during pregnancy may allow for targeted behavioural interventions from early infancy. This could also aid the development of targeted pharmacological strategies which have already shown promise in animal models¹⁹, and analogues of which are currently in use in routine medicine practice^{52,53}.

Interleukin-4. In demonstrating alterations in IL-4, we have corroborated findings in the small number of existing studies that have examined mid-gestational serum of mothers to autistic children. Across all of these studies (including our own), IL-4 is the only cytokine to consistently demonstrate altered expression^{30,31,54}. Interestingly, while previous authors found levels of IL-4 to be elevated in the ASD affected group versus controls, in our study we found the opposite. Physiologically, IL-4 is a pleiotropic, generally anti-inflammatory cytokine that functions to suppress the pro-inflammatory milieu. Produced by activated T-cells, NK cells, and mast cell, IL-4 aids the conversion of naïve T helper cells into Th2 cells as well as potentiating the Th2 response^{55,56}. IL-4 also has a role in the developmental and maintenance of key regulatory T-cells (Tregs) through STAT6 signalling pathways⁵⁷. Tregs are important mediators of inflammation during pregnancy and at the foeto-maternal interface⁵⁸. We find IL-4 itself at the foeto-maternal interface throughout pregnancy⁵⁹, indeed in normal pregnancy; levels of IL-4 persist and increase as the pregnancy progresses⁶⁰. Low circulating levels of IL-4 during pregnancy have been linked with spontaneous abortions, pre-eclampsia, intra-uterine growth restriction and pre-term delivery^{61–63}. Failure of the usual pregnancy homeostasis (elevated IL-4 levels) may lead to a more pro-inflammatory pregnancy environment with subsequent effects on maternal health, obstetrics outcomes, and child health and development.

Animal-based studies. Although there are very few human studies that have examined the molecular links between MIA and ASD, many animal-based studies have addressed the question of MIA and the association of elaboration of cytokines and parallel behavioural changes in offspring. MIA has been replicated in a variety of small animal models: mouse, rat and simian phenotypes of ASD have been created through intrauterine inflammatory exposure^{64–66}. These models provide valuable insights into the effects inflammation can have on social and communicative behaviour in progeny^{64,66}. Remedial steps have been possible with improvements in and resolution of some ASD traits following blockade of specific inflammatory pathways (IL-6 and IL-17A)¹⁹. This work suggested that these two cytokines in particular are significantly involved in the neuronal dysfunction brought about through MIA^{19,65–67}. MIA-mouse models of ASD, have shown increased IL-17A levels in maternal blood, the postnatal brains of offspring⁶⁸ and in

placental mRNA levels of the IL-17A. This suggests upregulation of IL-17A activity at the feto-maternal interface. In 2016, Choi et al. demonstrated persuasively that simulated MIA in murine models leads to elevation in maternal IL-6, leading to downstream activation of maternal Th17 cells. Maternal Th17 cells produce IL-17A that is hypothesised to cross to the foetus via the placenta leading to increased expression of IL-17AR in the foetal brain, contributing to cortical malformations and behavioural abnormalities^{19,69}.

Human studies. Quite a number of human based studies have examined immune and cytokine aberrations in individuals (adults and children) affected by ASD themselves. Here, we will outline each of the analytes we have examined and discuss their overall function^{70–72} and highlight their purported role in ASD. TNF α is a proinflammatory cytokine that mediates apoptosis of virally infected cells. In previous studies, it has been demonstrated in elevated levels in the CSF and blood of ASD affected individuals^{73–76}.

IL-1 β is a potent pro-inflammatory cytokine involved in both acute and chronic inflammation. It has been positively correlated with ASD symptom severity⁵¹, as well being elevated in the serum of a number of studied ASD populations^{73,74,77,78}.

IL-6 is another pro-inflammatory cytokine with broad, pleiotropic effects throughout the body (hematologic, hepatic, endocrine and metabolic). It induces production of acute phase proteins and stimulates B-cell antibody production⁷⁹. It is thought to impact synapse formation and neuronal migration⁸⁰ as well as potentially mediating IL-17 linked ASD risk in pregnancy^{19,66}. Alterations in expression have been noted in the serum and CSF of autistic individuals^{73,74,76–78,81,82}.

IFN γ is a versatile cytokine that interfaces between innate and adaptive immune response. It is secreted by NK cells, and promotes NK killing. It activates macrophages, which in turn, produce IL-12 and -23, stimulating Th1 and Th17 cell respectively. IFN γ inhibits Th2 cells and plays a role in defence against intracellular pathogens, tumour surveillance, autoimmunity, allergy and the protection of the amniotic space during pregnancy⁸³. A number of studies have identified alteration of IFN γ in ASD groups^{38,73,81}.

IL-17 is a pro-inflammatory and chemotactic cytokine. Derived from Th17 cells, a subset of CD4 cells, IL-17 potentiates the innate polymorphonuclear cell response throughout inflammation. In ASD studies, it is postulated to trigger alterations in the blood brain barrier and lead to cortical dysplasia⁶⁶, and altered concentrations of IL-17 have been identified in the sera of ASD affected individuals^{24,26,73,76,82,84}.

GM-CSF is a colony stimulating growth factor that is produced by stromal cell. It targets bone marrow, and precursor cells, mediating haematopoiesis. In one study, altered levels have been observed in individuals with ASD⁸⁵. IL-8 is classified as a chemotactic cytokine and is produced by fibroblasts, neutrophils and macrophages. It is chemo-attractant for phagocytes at site of inflammation, and has been identified in a number of studies as altered in ASD populations versus controls^{27,77,81}. While the cytokine profiles of ASD

affected individuals have been well characterised, very few studies have investigated the relationship between mid-gestation cytokine levels and ASD risk in offspring. To our knowledge, only three human studies have examined maternal serum^{30,31,54}, and one more has examined amniotic fluid cytokine profiles in mothers of ASD affected children⁸⁶. The findings from these studies, effectively provide all of our current understanding of gestational cytokine profiles in the setting of ASD.

Previous literature on gestational samples analysis in ASD. Working from the same laboratory and using similar methods, Goines et al. (2011) and more recently, Jones et al. (2017) both demonstrated elevated mid-gestational cytokine levels between groups of ASD affected children versus controls or children without ASD. Goines et al. demonstrated elevated levels of mid-gestation (15–19 weeks' gestation) IFN γ , IL-4 and IL-5 with an associated 50% increased ASD risk. While Jones et al. showed elevated levels of mid-gestation GM-CSF, IL-6, IFN γ and IL-1 α in the ASD affected group versus children with developmental delay, but not ASD. The authors do not mention the age of the samples used in either study, but the samples used were sourced from the same birth cohort in Orange County, California between 2000 and 2003. In both studies, the samples were initially stored at room temperature and later at – 20 °C freezer conditions before long-term storage at – 80 °C. This initial handling may have contributed to some cytokine degradation. In the Goines study, ASD cases were matched with neuro-typical controls based solely on child characteristics (sex, birth month and year), something which the authors acknowledge in their limitations. Neither study had access to comprehensive maternal health information during the pregnancy (including intrapartum infections). Nor did they have a record of relevant maternal medical history, all, information important to the interpretation of their findings.

Irwin et al. (2018) demonstrated alterations in IL-4, MCP-1 and IL-10 levels in 28-week gestation serum of mothers who birthed ASD affected children⁵⁴. Specifically, IL-4 (usually anti-inflammatory or involved in allergic type inflammation⁸⁷) was increased and associated with higher ASD symptomology (as measured by the Social Communication Questionnaire (SCQ)) in offspring. Higher concentrations of IL-10 (anti-inflammatory) were associated with fewer ASD symptoms in offspring (measured by the Social Responsiveness Scale (SRS)), and finally, elevated MCP-1 was associated with fewer ASD symptoms (as measured by the SCQ). The samples used in this analysis were reported to be at least 5 years old. No controls were used in this analysis, instead a large cohort of ASD affected individuals were enrolled, and the 28-week gestation cytokine concentrations were correlated with ASD symptomology at 7 years of age. This is novel in two senses, none has previously assessed the cytokine profile in the third trimester, and none has correlated cytokine findings with severity of ASD symptomology in this way. As with previous authors, they had no access to relevant maternal pre-conceptual medical history or gestational infections data.

Finally, Abdallah et al. (2013) examined amniotic fluid samples and found elevated levels of IL-4, IL-10, TNF α , and TNF β . In a preliminary study (2012),

they also identified elevations in MMP-9 in ASD cases relative to controls⁸⁸. Advanced sample age is again an issue with the oldest samples in this analysis being 29 years old, the youngest 10 years old. The samples were stored at –20 °C according to local guidance⁸⁹. Both the storage conditions and the samples ages are likely to have contributed to significant cytokine degradation⁴⁸.

Limitations. The samples used in our study fall outside the ideal sample age for accurate analysis of cytokines⁴⁸. To our mind, this is the single most important limitation confronting studies of this nature. Unfortunately, the shelf life of archived samples is finite, and even samples in long-term ultra-low temperature storage (–80 °C) suffer from degradation of cytokines and chemokines over time^{48,49}. Retrospective sample analysis, would present an excellent opportunity to study cytokine aberrations in ASD, if the time to ASD diagnosis was shorter. One UK study found that the average delay between concerns first being noted by parents and the child receiving a diagnosis of an ASD was 4.6 years (SD 4.4 years)⁹⁰. ASD services continue to be under-resourced⁹¹ and diagnoses are chronically delayed⁹². Under current conditions, our experience of retrospective analysis of archival samples suggests that this style of study design is not well suited to addressing this question. Even largescale population based studies would suffer from the same issues of sample fidelity over longer periods.

To ensure future study designs are capable of accurate mid-gestation cytokine analysis, they should be prospective, and concentrate on early ASD case identification or screening. Early identification should be paramount, the diagnostic stability of ASD is reliably fixed from as early as 14 months old⁹³ so screening and identification within the first 2–3 years of life is possible. Cytokines should be analysed contemporaneously, acute phase reactants such as IL-1 β and IL-6 have demonstrated greater than 50% degradation within 3 years even in –80 °C freezer conditions⁴⁸. IL-4 is stable only for 3 years, while IL-17A, IFN γ , and TNF α , all suffer more than 50% degradation within 4 years at ultra-low temperature storage⁴⁸. Basic handling of samples and initial processing requires optimisation to ensure the risk of sample degradation is minimised: (i) Store samples at ultra-low temperatures, (ii) initial processing should be rapid (< 1 h from venepuncture to freezer storage) and (iii) freeze-thaws cycles should be minimised. With robust methods of early screening in place, early confirmatory diagnosis within the first 2/3 years, and analysis of gestational samples within 3 years, it should be feasible to increase the yield and validity of such studies, and greatly reduce cytokine loss through prolonged storage. While this approach would allow for study of children presenting with the earliest signs of ASD, or targeted high-risk groups (ASD affected siblings). It would likely miss those presenting later, including those who are a high-functioning phenotype or of female sex.

Finally, our small sample size is a major limitation, and results should be interpreted with caution. Analysis of IL-4 levels in the groups yielded results on only 16 individuals (6 cases and 10 controls). Attrition of the viable samples was due to a combination of the low absolute concentrations of IL-4 in the samples (likely exacerbated by advanced sample age), concentrations

at or below the sensitivity (LLOD) of the MSD multiplex format and high CV values. It is difficult to make inferences about results in samples this size, and larger scale group analysis is warranted.

Strengths. Although our study has suffered from some of the same limitations as previous studies, our study is strengthened by the quality of our cohort. Each child had a concrete specialist service ASD diagnosis, confirmed by the clinical paediatric fellow. Each child was well characterised clinically and matching was strictly observed. Matching was not only based on child characteristics (Sex, Gestational age, Birthweight), but also on an important maternal characteristic, BMI at 15 weeks' gestation. This enhanced the validity of our results. In addition to detailed child characteristics, we have also included important information regarding the past medical histories, medication or anti-inflammatory use, and pre-existing inflammatory conditions of the mothers included in the study. We present crucial information about infection rates in the first 20 weeks of pregnancy, all of which presents a major confounder to accurate analysis if this information is absent. Our methods were robust, and we identified two key issues of multiplex assay sensitivity and advanced sample age, and remedied the former through utilisation of ultrasensitive single analyte plates.

Conclusion

In conclusion, in a carefully characterised maternal-child cohort study we did not replicate the findings of similar mid-gestational studies, but did find some evidence of mid-gestational cytokine aberrations (downregulated IL-4) in the mothers of children with ASD. Reduced levels of IL-4 are linked to a pro-inflammatory state during pregnancy and negative obstetric and foetal outcomes. All studies to date have had similar and significant limitations. Future studies should focus on minimising the time between sample acquisition and analysis, use of best practice for initial sample handling, and early identification and characterisation of cases and their mothers. Future analysis should be serial and include investigation of samples taken from early in pregnancy. The first trimester, and particularly 8–12 weeks' gestation is a crucial period for organogenesis and differentiation, and analysis from this period will help complete the picture of gestational cytokine fluctuations and their effect on neurodevelopment.

Data availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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References

1. Birtwell, K. B. Social, Cognitive, and Behavioral Development of Children and Adolescents with Autism Spectrum Disorder. In *Autism Spectrum Disorder* (ed. McDougle, C.) (Oxford Press, 2016).
2. Lord, C., Elsabbagh, M., Baird, G. & Veenstra-Vanderweele, J. Autism spectrum disorder. *Lancet (London, Engl)*. **392**(10146), 508–520 (2018).

3. Magiati, I. *et al.* Anxiety symptoms in young people with autism spectrum disorder attending special schools: Associations with gender, adaptive functioning and autism symptomatology. *Autism Int. J. Res. Pract.* **20**(3), 306–320 (2016).
4. American Psychiatric Association. DSM-V. 5th ed. (Washington DC 2013).
5. Hallmayer, J. *et al.* Genetic heritability and shared environmental factors among twin pairs with autism. *Arch. Gen. Psychiatry* **68**(11), 1095–1102 (2011).
6. Ronald, A. *et al.* Genetic heterogeneity between the three components of the autism spectrum: A twin study. *J. Am. Acad. Child Adolesc. Psychiatry* **45**(6), 691–699 (2006).
7. Kim, Y. S. *et al.* Prevalence of autism spectrum disorders in a total population sample. *Am. J. Psychiatry* **168**(9), 904–912 (2011).
8. Christensen, D. L. *et al.* Prevalence and characteristics of autism spectrum disorder among children aged 8 years: Autism and developmental disabilities monitoring network, 11 sites, United States, 2012. *Morb. Mortal. Wkly. Rep. Surveill. Summ.* **65**(3), 1–23 (2016).
9. Schaefer, G. B. & Mendelsohn, N. J. Clinical genetics evaluation in identifying the etiology of autism spectrum disorders: 2013 guideline revisions. *Genet. Med.* **15**(5), 399–407 (2013).
10. Ho, K. S. *et al.* Clinical performance of an ultrahigh resolution chromosomal microarray optimized for neurodevelopmental disorders. *Biomed. Res. Int.* **2016**, 3284534 (2016).
11. Sandin, S. *et al.* The familial risk of autism. *JAMA* **311**(17), 1770–1777 (2014).
12. Ahmad, S. F. *et al.* Dysregulation of the expression of HLA-DR, costimulatory molecule, and chemokine receptors on immune cells in children with autism. *Int. Immunopharmacol.* **65**, 360–365 (2018).
13. Lee, L. C. *et al.* HLA-DR4 in families with autism. *Pediatr. Neurol.* **35**(5), 303–307 (2006).
14. Janecka, M. *et al.* Advanced paternal age effects in neurodevelopmental disorders-review of potential underlying mechanisms. *Transl. Psychiatry* **7**(1), e1019 (2017).
15. Sandin, S. *et al.* The heritability of autism spectrum disorder. *JAMA* **318**(12), 1182–1184 (2017).
16. Banerjee, S., Bhat, M. & Riordan, M. Genetic aspects of autism spectrum disorders: insights from animal models. *Front. Cell. Neurosci.* **8**, 58 (2014).
17. Hoeffer, C. A. & Klann, E. mTOR signaling: At the crossroads of plasticity, memory and disease. *Trends Neurosci.* **33**(2), 67–75 (2010).
18. Caglayan, A. O. Genetic causes of syndromic and non-syndromic autism. *Dev. Med. Child Neurol.* **52**(2), 130–138 (2010).
19. Choi, G. B. *et al.* The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science (New York, NY)*. **351**(6276), 933–939 (2016).
20. Estes, M. L. & McAllister, A. K. Maternal immune activation: Implications for neuropsychiatric disorders. *Science (New York, NY)*. **353**(6301), 772–777 (2016).
21. Careaga, M., Murai, T. & Bauman, M. D. Maternal immune activation and autism spectrum disorder: From rodents to nonhuman and human primates. *Biol. Psychiat.* **81**(5), 391–401 (2017).
22. Boulanger-Bertolus, J., Pancaro, C. & Mashour, G. A. Increasing role of maternal immune activation in neurodevelopmental disorders. *Front. Behav. Neurosci.* **12**, 230 (2018).
23. Minakova, E. & Warner, B. B. Maternal immune activation, central nervous system development and behavioral phenotypes. *Birth Def. Res.* **110**(20), 1539–1550 (2018).
24. Akintunde, M. E. *et al.* Increased production of IL-17 in children with autism spectrum disorders and co-morbid asthma. *J. Neuroimmunol.* **286**, 33–41 (2015).
25. Ashwood, P., Wills, S. & Van de Water, J. The immune response in autism: A new frontier for autism research. *J. Leukocyte Biol.* **80**(1), 1–15 (2006).
26. Al-Ayadhi, L. Y. & Mostafa, G. A. Elevated serum levels of interleukin-17A in children with autism. *J. Neuroinflamm.* **9**, 158 (2012).
27. Bryn, V. *et al.* Cytokine profile in autism spectrum disorders in children. *J. Mol. Neurosci. MN.* **61**(1), 1–7 (2017).
28. Ahmad, S. F. *et al.* Upregulation of interleukin (IL)-31, a cytokine producing CXCR1 peripheral immune cells, contributes to the immune abnormalities of autism spectrum disorder. *J. Neuroimmunol.* **349**, 577430–577431 (2020).
29. Ahmad, S. F. *et al.* Elevated IL-16 expression is associated with development of immune dysfunction in children with autism. *Psychopharmacology* **236**(2), 831–838 (2019).
30. Goines, P. E. *et al.* Increased midgestational IFN-gamma, IL-4 and IL-5 in women bearing a child with autism: A case-control study. *Mol. Autism* **2**, 13 (2011).
31. Jones, K. L. *et al.* Autism with intellectual disability is associated with increased levels of maternal cytokines and chemokines during gestation. *Mol. Psychiatry* **22**(2), 273–279 (2017).
32. Mouridsen, S. E., Rich, B., Isager, T. & Nedergaard, N. J. Autoimmune diseases in parents of children with infantile autism: A case-control study. *Dev. Med. Child Neurol.* **49**(6), 429–432 (2007).
33. Comi, A. M., Zimmerman, A. W., Frye, V. H., Law, P. A. & Peeden, J. N. Familial clustering of autoimmune disorders and evaluation of medical risk factors in autism. *J. Child Neurol.* **14**(6), 388–394 (1999).
34. Gardosi, J., Francis, A., Turner, S. & Williams, M. Customized growth charts: Rationale, validation and clinical benefits. *Am. J. Obstet. Gynecol.* **218**(2s), S609–S618 (2018).
35. Apgar, V. A proposal for a new method of evaluation of the newborn infant. *Anesthesia Analgesia* **120**(5), 1056–1059 (2015).
36. Cohen, S. *Perceived stress in a probability sample of the United States. The social psychology of health. The Claremont Symposium on Applied Social Psychology* 31–67 (Sage Publications, Inc, 1988).
37. Kenny, L. C. *et al.* Early pregnancy prediction of preeclampsia in nulliparous women, combining clinical risk and biomarkers: the Screening for Pregnancy Endpoints (SCOPE) international cohort study. *Hypertension* **64**(3), 644–652 (2014).
38. Masi, A. *et al.* Cytokine aberrations in autism spectrum disorder: A systematic review and meta-analysis. *Mol. Psychiatry* **20**(4), 440–446 (2015).

39. Conway, F. & Brown, A. S. Maternal immune activation and related factors in the risk of offspring psychiatric disorders. *Front. Psychiatry* **10**, 430 (2019).
40. Kugelberg, E. Neuroimmunology: IL-17A mediates a path to autism. *Nat. Rev. Immunol.* **16**(4), 205 (2016).
41. Dabito, D., Margolick, J. B., Lopez, J. & Bream, J. H. Multiplex measurement of proinflammatory cytokines in human serum: Comparison of the Meso Scale Discovery electrochemiluminescence assay and the Cytometric Bead Array. *J. Immunol. Methods* **372**(1–2), 71–77 (2011).
42. Günther, A., Becker, M., Göpfert, J., Joos, T. & Schneiderhan-Marra, N. Comparison of bead-based fluorescence versus planar electrochemiluminescence multiplex immunoassays for measuring cytokines in human plasma. *Front. Immunol.* **11**, 2486 (2020).
43. Motulsky, H. J. & Brown, R. E. Detecting outliers when fitting data with nonlinear regression: A new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinform.* **7**, 123 (2006).
44. Baio, J. *et al.* Prevalence of autism spectrum disorder among children aged 8 years: Autism and developmental disabilities monitoring network, 11 sites, United States, 2014. *Morb. Mortal. Wkly Rep. Surveill. Summ.* **67**(6), 1–23 (2018).
45. Palmer, N. *et al.* Association of sex with recurrence of autism spectrum disorder among siblings. *JAMA Pediatr.* **171**(11), 1107–1112 (2017).
46. Chan, Y. H. Biostatistics 104: correlational analysis. *Singapore Med. J.* **44**(12), 614–619 (2003).
47. Rea, L., Parker, R. A. & Allen, R. *Designing and Conducting Survey Research* (John Wiley and Sons, 2016).
48. de Jager, W., Bourcier, K., Rijkers, G. T., Prakken, B. J. & Seyfert-Margolis, V. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. *BMC Immunol.* **10**(1), 52 (2009).
49. Zhou, X., Fragala, M. S., McElhane, J. E. & Kuchel, G. A. Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. *Curr. Opin. Clin. Nutr. Metab. Care* **13**(5), 541–547 (2010).
50. Goines, P. & Van de Water, J. The immune system's role in the biology of autism. *Curr. Opin. Neurol.* **23**(2), 111–117 (2010).
51. Ashwood, P. *et al.* Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders. *J. Neuroimmunol.* **232**(1–2), 196–199 (2011).
52. Blauvelt, A. Ixekizumab: a new anti-IL-17A monoclonal antibody therapy for moderate-to severe plaque psoriasis. *Expert Opin. Biol. Ther.* **16**(2), 255–263 (2016).
53. Wendling, D., Racadot, E. & Wijdenes, J. Treatment of severe rheumatoid arthritis by anti-interleukin 6 monoclonal antibody. *J. Rheumatol.* **20**(2), 259–262 (1993).
54. Irwin, J. L. *et al.* Maternal gestational immune response and autism spectrum disorder phenotypes at 7 years of age in the seychelles child development study. *Mol. Neurobiol.* **56**(7), 5000–5008 (2019).
55. Croft, M. & Swain, S. L. Recently activated naive CD4 T cells can help resting B cells, and can produce sufficient autocrine IL-4 to drive differentiation to secretion of T helper 2-type cytokines. *J. Immunol.* **154**(9), 4269–4282 (1995).
56. Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J. & Paul, W. E. The IL-4 receptor: Signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* **17**, 701–738 (1999).
57. Wang, W., Wang, L. & Zha, B. The roles of STAT6 in regulating B cell fate, activation, and function. *Immunol. Lett.* **233**, 87–91 (2021).
58. Jonakait, G. M. The effects of maternal inflammation on neuronal development: Possible mechanisms. *Int. J. Dev. Neurosci. Off. J. Int. Soc. Dev. Neurosci.* **25**(7), 415–425 (2007).
59. Lin, H., Mosmann, T. R., Guilbert, L., Tuntipipat, S. & Wegmann, T. G. Synthesis of T helper 2-type cytokines at the maternalfetal interface. *J. Immunol.* **151**(9), 4562–4573 (1993).
60. Marzi, M. *et al.* Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clin. Exp. Immunol.* **106**(1), 127–133 (1996).
61. Omu, A. E., Al-Qattan, F., Diejomaoh, M. E. & Al-Yatama, M. Differential levels of T helper cytokines in preeclampsia: Pregnancy, labor and puerperium. *Acta Obstet. Gynecol. Scand.* **78**(8), 675–680 (1999).
62. Peng, Y., Yin, S. & Wang, M. Significance of the ratio interferon- γ /interleukin-4 in early diagnosis and immune mechanism of unexplained recurrent spontaneous abortion. *Int. J. Gynaecol. Obstetr. Off. Organ Int. Fed. Gynaecol. Obstetr.* **154**, 39–43 (2020).
63. Chatterjee, P., Chiasson, V. L., Bounds, K. R. & Mitchell, B. M. Regulation of the anti-inflammatory cytokines interleukin-4 and interleukin-10 during pregnancy. *Front. Immunol.* **5**, 253 (2014).
64. Bauman, M. D. *et al.* Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. *Biol. Psychiat.* **75**(4), 332–341 (2014).
65. Smith, S. E., Li, J., Garbett, K., Mirnics, K. & Patterson, P. H. Maternal immune activation alters fetal brain development through interleukin-6. *J. Neurosci. Off. J. Soc. Neurosci.* **27**(40), 10695–10702 (2007).
66. Wong, H. & Hoeffler, C. Maternal IL-17A in autism. *Exp. Neurol.* **299**(Pt A), 228–240 (2018).
67. Parker-Athill, E. C. & Tan, J. Maternal immune activation and autism spectrum disorder: Interleukin-6 signaling as a key mechanistic pathway. *Neurosignals* **18**(2), 113–128 (2010).
68. Knesel, I. *et al.* Maternal immune activation and abnormal brain development across CNS disorders. *Nat. Rev. Neurol.* **10**(11), 643–660 (2014).
69. Estes, M. L. & McAllister, A. K. Maternal TH17 cells take a toll on baby's brain. *Science (New York, NY)*. **351**(6276), 919–920 (2016).
70. Dinarello, C. A. Historical insights into cytokines. *Eur. J. Immunol.* **37**(Suppl 1), S34-45 (2007).
71. Zhang, J. M. & An, J. Cytokines, inflammation, and pain. *Int. Anesthesiol. Clin.* **45**(2), 27–37 (2007).
72. Turner, M. D., Nedjai, B., Hurst, T. & Pennington, D. J. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochem. Biophys. Acta.* **1843**(11), 2563–2582 (2014).
73. Suzuki, K. *et al.* Plasma cytokine profiles in subjects with high-functioning autism spectrum disorders. *PLoS ONE* **6**(5), e20470 (2011).
74. Ricci, S. *et al.* Altered cytokine and BDNF levels in autism spectrum disorder. *Neurotox. Res.* **24**(4), 491–501 (2013).

75. Chez, M. G., Dowling, T., Patel, P. B., Khanna, P. & Kominsky, M. Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children. *Pediatr. Neurol.* **36**(6), 361–365 (2007).
76. Eftekharian, M. M. *et al.* Cytokine profile in autistic patients. *Cytokine* **108**, 120–126 (2018).
77. Ashwood, P. *et al.* Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. *Brain Behav. Immun.* **25**(1), 40–45 (2011).
78. Kordulewska, N. K. *et al.* Serum cytokine levels in children with spectrum autism disorder: Differences in pro- and anti-inflammatory balance. *J. Neuroimmunol.* **337**, 577066–577071 (2019).
79. Nishimoto, N. & Kishimoto, T. Interleukin 6: From bench to bedside. *Nat. Clin. Pract. Rheumatol.* **2**(11), 619–626 (2006).
80. Wei, H. *et al.* IL-6 is increased in the cerebellum of autistic brain and alters neural cell adhesion, migration and synaptic formation. *J. Neuroinflamm.* **8**, 52 (2011).
81. Heuer, L. S. *et al.* An exploratory examination of neonatal cytokines and chemokines as predictors of autism risk: The early markers for autism study. *Biol. Psychiat.* **86**(4), 255–264 (2019).
82. Kutuk, M. O. *et al.* Cytokine expression profiles in Autism spectrum disorder: A multi-center study from Turkey. *Cytokine* **133**, 155152 (2020).
83. Murphy, S. P. *et al.* Interferon gamma in successful pregnancies. *Biol. Reprod.* **80**(5), 848–859 (2009).
84. Moaaz, M., Youssry, S., Elfatry, A. & El Rahman, M. A. Th17/Treg cells imbalance and their related cytokines (IL-17, IL-10 and TGF- β) in children with autism spectrum disorder. *J. Neuroimmunol.* **337**, 577071 (2019).
85. Ahmad, S. F. *et al.* Involvement of CD45 cells in the development of autism spectrum disorder through dysregulation of granulocyte-macrophage colony-stimulating factor, key inflammatory cytokines, and transcription factors. *Int. Immunopharmacol.* **83**, 106466 (2020).
86. Abdallah, M. W. *et al.* Amniotic fluid inflammatory cytokines: potential markers of immunologic dysfunction in autism spectrum disorders. *World J Biol. Psychiatry Off. J World Fed Soc Biol Psychiatry* **14**(7), 528–538 (2013).
87. Masi, A., Glozier, N., Dale, R. & Guastella, A. J. The immune system, cytokines, and biomarkers in autism spectrum disorder. *Neurosci. Bull.* **33**(2), 194–204 (2017).
88. Abdallah, M. W. *et al.* Amniotic fluid MMP-9 and neurotrophins in autism spectrum disorders: An exploratory study. *Autism Res. Off. J. Int. Soc. Autism Res.* **5**(6), 428–433 (2012).
89. Nørgaard-Pedersen, B. & Hougaard, D. M. Storage policies and use of the Danish Newborn Screening Biobank. *J. Inherit. Metab. Dis.* **30**(4), 530–536 (2007).
90. Crane, L., Chester, J. W., Goddard, L., Henry, L. A. & Hill, E. Experiences of autism diagnosis: A survey of over 1000 parents in the United Kingdom. *Autism Int. J. Res. Pract.* **20**(2), 153–162 (2016).
91. Iadarola, S. *et al.* Services for children with autism spectrum disorder in three, large urban school districts: Perspectives of parents and educators. *Autism Int. J. Res. Pract.* **19**(6), 694–703 (2015).
92. North Lee ASD services C, Ireland. Parents' Perspectives: An Evaluation of the North Lee Autism Spectrum Disorder Service. Report (2016).
93. Pierce, K. *et al.* Evaluation of the diagnostic stability of the early autism spectrum disorder phenotype in the general population starting at 12 months. *JAMA Pediatr.* **173**(6), 578–587 (2019).

Author contributions

M.C. wrote the manuscript, prepared all tables and Fig. 1. M.C. and S.C. performed all experiments jointly. G.O'K. performed statistical analysis and prepared Figs. 2, 3, 4, 5, 6, 7, 8, 9, 10. S.C., G.O'K., L.G., and D.M. reviewed and amended the document at each redraft. All authors reviewed the manuscript and authorised the final submission.

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Competing interests

The authors declare no competing interests.

Additional information

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