Understanding the role of inflammation and epigenetics in neurodevelopmental and neuropsychiatric disorders

A thesis submitted in

fulfilment of the requirements

for the degree of Doctor of Philosophy

by

Sarah Alshammery, BSc (Hons)

Children's Hospital Westmead Clinical School

Faculty of Medicine and Health

The University of Sydney

2025

Statement of originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Sarah Alshammery

November 2024

The research reported in this thesis was supported by the Research Training Program Fees Offset.

Authorship attribution statement

Sarah Alshammery was the primary author of the publication presented in Chapter 2 of this thesis. The published version of this manuscript can be found in Chapter 7.1 Appendix.

Common targetable inflammatory pathways in brain transcriptome of autism spectrum disorders and Tourette syndrome

Alshammery, S, Patel, S, Jones, H. J., Han, V. X., Gloss, B. S., Gold, W. A., Dale, R. C. (2022). *Frontiers in Neuroscience*, doi: 10.3389/fnins.2022.999346

Author contribution: I reviewed published literature, analysed the data, generated figures, drafted the manuscript and edited final manuscript for peer-review.

In addition, Sarah Alshammery is a co-author on a prepared manuscript under review, which can be found in Chapter 7.2 Appendix. This manuscript contains methods, and results from Chapter 4 of this thesis.

A/P Wendy Gold, *P* Russell Dale, and *Dr* Shrujna Patel provided considerable support in the studies included in this thesis. They had prominent roles in study conception and design, data interpretation, and critical appraisal of the content of this thesis.

As supervisors for the candidature upon which this thesis is based, we can confirm that the authorship attribution statements above are correct.

Lead supervisor:	**Co-supervisor:**	**Co-supervisor:**
A/P Wendy Gold	*P Russell Dale*	*Dr Shrujna Patel*
November 2024	November 2024	November 2024

Additional publications co-authored during candidature

Jones, H. F., Han, V. X., Patel, S., Gloss, B. S., Soler, N., Ho, A., Sharma, S., Kothur, K., Nosadini, M., Wienholt, L., Hardwick, C., Barnes, E. H., Lim, J. R., **Alshammery, S.,** Nielsen, T. C., Wong, M., Hofer, M. J., Nassar, N., Gold, W., Brilot, F., … Dale, R. C. (2021). Maternal autoimmunity and inflammation are associated with childhood tics and obsessive-compulsive disorder: Transcriptomic data show common enriched innate immune pathways. Brain, behavior, and immunity, 94, 308–317. https://doi.org/10.1016/j.bbi.2020.12.035

Han, V. X., Jones, H. F., Patel, S., Mohammad, S. S., Hofer, M. J., **Alshammery, S.,** Maple-Brown, E., Gold, W., Brilot, F., & Dale, R. C. (2022). Emerging evidence of Toll-like receptors as a putative pathway linking maternal inflammation and neurodevelopmental disorders in human offspring: A systematic review. Brain, behavior, and immunity, 99, 91–105. https://doi.org/10.1016/j.bbi.2021.09.009

Tsang, E., Han, V. X., Flutter, C., **Alshammery, S.,** Keating, B. A., Williams, T., Gloss, B. S., Graham, M. E., Aryamanesh, N., Pang, I., Wong, M., Winlaw, D., Cardamone, M., Mohammad, S., Gold, W., Patel, S., & Dale, R. C. (2024). Ketogenic diet modifies ribosomal protein dysregulation in KMT2D Kabuki syndrome. EBioMedicine, 104, 105156. Advance online publication. https://doi.org/10.1016/j.ebiom.2024.105156

Conference abstracts and presentations during candidature

2021

Presented a **talk** at the **Kids Neuroscience** group meeting titled: "Brain transcriptome of autism spectrum disorders and Tourette syndrome defines common targetable inflammatory pathways". *Sydney, September.*

2022

Presented a **poster** at the **Australasian Neuroscience Society** titled: "Targeting common inflammatory pathways in brain transcriptome of autism spectrum disorders and Tourette syndrome". *Melbourne, December.*

2023

Invited **speaker** at the **Asia-Pacific School of Neuroimmunology**. I delivered a workshop on single cell RNA sequencing analysis in collaboration with Dr Shrujna Patel and Dr Brooke Keating. In addition, I presented a **talk** titled: "Common Inflammatory pathways in brain transcriptomics". *Sydney, February.*

Presented a **poster** at the **University of Sydney Faculty of Medicine and Health HDR Conference** titled: "Toll-like receptor stimulation in Neurodevelopmental disorders" and won the Poster Award. *Sydney, July.*

Presented a **poster** at the **Westmead Research & Innovation Conference** titled: "Inflammatory pathways in brain transcriptome data of neurodevelopmental disorders". *Sydney, September.*

Presented a **joint-talk** at the **Kids Neuroscience Symposium** titled: "Proteomics in Neurodevelopmental disorders" in collaboration with Dr Jinni Yang and Erica Tsang. *Sydney, September.*

2024

Presented a ***talk*** at the **Kids Neuroscience Centre** group meeting titled: "Functional immune responses in Neurodevelopmental disorders". *Sydney, May.*

Presented a **talk** at the **University of Sydney Faculty of Medicine and Health HDR Conference** titled: "Investigating the proteome: peripheral blood mononuclear cells of children with Paediatric Acute Neuropsychiatric Syndrome". *Sydney, July.*

Acknowledgements

First and foremost, I would like to express my sincerest gratitude to my three supervisors, each of whom has played a unique and irreplaceable role in my journey. To Wendy Gold, thank you for introducing me to the world of research and for your nurturing guidance from day one. Your encouragement has been foundational, giving me the confidence and curiosity to pursue this path. I know you will remain a mentor for life. To Russell Dale, thank you for inspiring me to think boldly and to always seek more than what is readily visible. Your big ideas and vision have been a constant reminder of the limitless potential within research. To Shrujna Patel, I am profoundly thankful for your mentorship and wise counsel, which have been both grounding and transformative, shaping not only my research but my personal growth along the way. I would also like to thank Russell Dale and the Petre Foundation along with Cerebral Palsy Alliance for their generous financial support throughout this PhD project. This journey began with a deep curiosity and a commitment to research, driven by a greater aim of making a difference for children affected by neurological conditions. Their support has been invaluable in bringing this work to life.

I am also immensely thankful for my lab mates, from the two research groups I had the privilege of being a part of. Thank you for the camaraderie, collaboration, and countless shared moments that made the lab a supportive and vibrant place to work. Your insights, humour, and teamwork have been invaluable throughout this journey. Notably, thank you to Dr Velda Han for your efforts in my project, and sharing this journey alongside me. Thank you to Dr Brooke Keating, for your constant help and kind words. I would also like to acknowledge A/P Markus Hofer for his scientific input and help throughout this project. In addition, thank you to Dr Brian Gloss for walking me through the intricacies of bioinformatics since my honours.

To my family, I owe endless gratitude. To my parents—this accomplishment belongs to you as much as it does to me. Baba and Mama, thank you for your endless belief in me and for the countless sacrifices that have made this journey possible. To my siblings, thank you for your steadfast support and for always cheering me on. To my in-laws, thank you for your kindness, encouragement, and for welcoming me so warmly into your family. Finally, thank you to my friends, who have been there through every high and low. Thank you for being my source of laughter, comfort, and support.

Last but certainly not least, to my dear husband and my rock, Ammar. Who entered my life during the final stretch of this journey and yet has been my unwavering support through it all—thank you for your love, patience, and belief in me. You have been my confidant, my encourager, and my source of strength, lifting me up during late nights and early mornings, through every challenge and every success. You have filled my life with joy and perspective, offering both the quiet support I needed to focus and the encouragement to dream bigger. I am profoundly grateful, and I look forward to building every step of our journey together in the years to come.

Table of Contents

List of figures

List of tables

List of common abbreviations

ADHD	Attention deficit hyperactivity disorder
ASD	Autism spectrum disorders
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DE	Differentially expressed
DSM-V	Diagnostic and Statistical Manual of Mental Diseases V
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
GABA	Gamma-aminobutyric acid
GO	Gene Ontology
GWAS	Genome wide association studies
IF	Interferon
IL	Interleukin
LBP	LPS binding protein
LC	Liquid chromatography
LPS	Lipopolysaccharide
MIA	Maternal immune activation
MS	Mass spectrometry
NC	Neurotypical control
NDD	Neurodevelopmental disorders
PANDAS	Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections
PANS	Paediatric Autoimmune Neuropsychiatric Syndrome
PCR	Polymerase Chain Reaction
PET	Positron emission tomography
Poly I:C	Polyinosinicpolyinosinic:polycytidylic acid
RBDEP	Rubella Birth Defect Evaluation Project
SNPs	Single nucleotide polymorphisms
TLRs	Toll-like receptors

Thesis Abstract

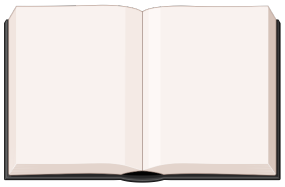
Neurodevelopmental disorders (NDD) are heterogenous neurological conditions affecting brain development between the window of early gestation and early adulthood. Neurodevelopmental disorders encompass a range of conditions including intellectual disability, Autism spectrum disorders (ASD), Attention-Deficit/Hyperactivity Disorder (ADHD), and tic disorders such as Tourette syndrome, and neuropsychiatric conditions such as Obsessive-compulsive disorder (OCD). These conditions are highly comorbid, where individuals with one NDD often suffer from one or more neurodevelopmental conditions. Paediatric Acute Neuropsychiatric Syndrome (PANS) is a neurodevelopmental disorder phenotype, dominated by OCD and is often accompanied with ASD and tic symptoms. The hallmark of PANS is an infection-provoked and abrupt, sudden onset of symptoms. It is agreed that a complex interplay of genetic, epigenetic and environmental influences contribute to the expression of neurodevelopmental disorders. A large body of evidence, including pre-clinical and clinical studies, support the involvement of maternal immune dysregulation during gestation as a risk factor for neurodevelopmental and neuropsychiatric conditions (Chapter 1), and the role of the immune system in neurodevelopmental disorders more generally.

This thesis aimed to investigate the immune and inflammatory dysregulations in individuals with NDDs. The first study utilised publicly available brain transcriptome data of individuals with ASD and Tourette syndrome (Chapter 2). Given the shared genetic heterogeneity and comorbidity of NDDs, there is an increasing need to investigate common disease pathways. As inflammation has been reported in brain transcriptomics in both ASD and Tourette syndrome, we examined for shared gene expression between the two conditions, in order to improve our understanding of the pathophysiology of NDDs and provide future potential therapeutic targets. Transcriptome data was obtained from the original authors and analysed through a pipeline investigating sample clustering, differential expression and pathway analysis. The findings from this chapter bring new evidence of shared immune signalling in ASD and Tourette syndrome brain transcriptome.

My second aim was to investigate the NDD proteome in immune cells: I performed a proteomic investigation of peripheral blood cells from two cohorts of children with PANS compared to controls. We hypothesised a dysregulated immune response within the proteome of children with PANS, using peripheral blood mononuclear cells. From this work, we highlighted dysregulated pathways involving the immune response, translation (ribosomal) and epigenetic mechanisms. This work is the first to investigate the proteome of children with PANS and sets the path for further proteomic investigations of complex NDDs.

Finally, my goal was to further investigate the immune profile of these children, and NDDs as a whole. I established a functional TLR assay to assess immune responses of peripheral blood cells from children with PANS compared to cells from neurotypical control children. Firstly, the assay was developed using control peripheral blood mononuclear cells to optimise the dosage and stimulation duration of LPS; a TLR4 bacterial agonist. Through measurement of IL-6 and TNF at the mRNA (qPCR) and protein (ELISA) level, I determined that a 500ng/ml dosage at 0, 30-minutes, 3 hours and 24 hours yielded a profile suitable for testing. Our assay highlighted a repressed immune system in children with PANS, identified by lower levels of mRNA and protein IL-6 and TNF compared to controls. This functional assay has the potential to expand and include other immune biomarkers, and explore future therapeutics, in PANS and other complex NDDs.

The work presented within this thesis overall highlights a dysregulated immune response in individuals with NDDs such as ASD, Tourette syndrome, OCD and PANS. In addition, proteomic investigations of peripheral blood cells of children with PANS has highlighted immune, translational (ribosomal) and epigenetic dysregulations. Utilising the methods and results within this thesis, with future advancements, may provide future therapeutic opportunities. Further research developing strategies presented within this body of work will streamline targeting identified dysregulated pathways in a more personalised approach to help individuals with NDDs.



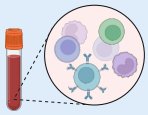
CHAPTER 1
Review of the literature

Introduction to neurodevelopmental and neuropsychiatric conditions, and the implication of the immune response, epigenetics, and multi-omic technologies.

CHAPTER 2
Original research #1 (publication)

AIM
To explore the immune and inflammatory findings in brain transcriptome of ASD and Tourette's syndrome.

OUTCOME
Confirmed previous findings of enriched immune pathways and identified a common inflammatory signal.



CHAPTER 3
Original research #2 (chapter)

AIM
To explore the proteomic signature of peripheral blood cells of children with PANS and identify dysregulated pathways.

OUTCOME
Implicated dysregulated immune and translational (ribosomal) signalling and validated in a second cohort.

CHAPTER 4
Original research #3 (chapter)

AIM
To explore the immune and inflammatory findings in peripheral blood cells of children with PANS through a functional assay.

OUTCOME
Identified a repressed immune response in cells of children with PANS following LPS stimulation at different timepoints.

CHAPTER 5
Discussion

Insights towards a deeper understanding of complex neurodevelopmental conditions: From bioinformatics to therapeutic potential.

(Figure generated using Biorender)

Chapter 1

General Introduction

1.1 Neurodevelopmental and neuropsychiatric disorders

Neurodevelopmental disorders (NDD) are heterogenous neurological conditions affecting brain development and function during the developmental window. The Diagnostic and Statistical Manual of Mental Diseases V (DSM-V) associates neurodevelopmental disorders with developmental deficits or differences within brain processes resulting in impairments of personal, social, academic, or occupational functioning.[1] Neurodevelopmental disorders encompass a range of conditions including intellectual disability, Autism spectrum disorders (ASD), Attention-Deficit/Hyperactivity Disorder (ADHD), tic disorders such as Tourette syndrome, and neuropsychiatric conditions such as obsessive-compulsive disorder (OCD).

These neurodevelopmental conditions are highly comorbid, where individuals with one NDD often suffer from one or more neurodevelopmental conditions.[2] As such, classifications of neurodevelopmental disorder symptoms and conditions is done on a continuum, where individuals may satisfy criteria for more than one diagnosis based on the symptoms exhibited. Symptoms of neurodevelopmental disorders include impairment in behaviour, emotional and social communication, memory, and learning.[1,3] The current treatment plans of these conditions can be described as 'symptom based', where symptoms are managed through pharmacological, educational, and psychotherapeutic interventions. The current limited understanding of these conditions hinders the development of targeted

management plans. Another layer of complexity is presented by the high phenotypic and aetiologic heterogeneity within individuals classified under the same disorder. Individuals diagnosed with the same NDD may exhibit a wide range of symptoms and traits, resultant from diverse genetic, epigenetic and environmental liabilities.
The cases presenting to Professor Dale's (supervisor's) clinic include children presenting with complex NDDs, where the most common comorbid diagnoses are Tourette syndrome, OCD, ASD (and ADHD). Therefore, the focus of this thesis will be on ASD, Tourette syndrome and OCD.

1.1.1 Autism spectrum disorders

ASD is an overarching term describing a neurodevelopmental disorder with heterogenous presentations, characterised by persistent deficits in cognitive symptoms, social communication, and social interaction. ASD is described with high heritability and comorbidity to other neuropsychiatric disorders.[1] Children with autism experience persistent deficits in social interaction and communication including social-emotional reciprocity, alongside repetitive patterns of behaviour. Autism spectrum disorders has a male to female ratio of at least 3:1, and onset of ASD symptoms described to present as early as 6-24 months of age, although a diagnosis can be delayed until adolescence or adulthood.[4,5] The diagnosis of ASD, based on the DSM-V criteria necessities a) persistent deficits in social communication and interaction including verbal, non-verbal and relationship communications. b) presence of repetitive patterns of behaviour, interests, and/or activities. c) presence of symptoms during development. d) impairment of symptoms on social, occupational, and other areas of function. e) impact of disorder is not better explained by intellectual disability.[1]

1.1.2 Tourette syndrome

Tourette syndrome is a neurodevelopmental disorder characterised by repetitive and rapid non-rhythmic motor movements or vocalisations that are often preceded by premonitory urges.[6] The urges are defined as feelings of energy or tension that are relieved by tic performance. Tourette syndrome is a chronic tic disorder in which tics (both motor and vocal tics) last for more than 12 months and is reported to have an onset between 4-8 years of age,[1] with a male to female ratio of 3:1.[1,7,8] The severity of disease is unpredictable, as symptoms often peak between the ages of 8-12, and gradually decrease during adulthood.[7] Symptoms can span weeks or months and vary in occurrence and intensity based on factors such as psychosocial stress. Diagnosis of Tourette syndrome is challenging as symptoms are often showcased in a familiar environment, and commonly wax and wane in severity, which not only introduces hurdles in its diagnosis, but the disorder's overall management.[9,10] The DSM-V criteria for Tourette syndrome diagnosis comprises a) the presence of motor tics and ≥ 1 vocal tics, b) persistence of tics for ≥ 1 years, c) onset before 18 years of age, d) not be attributable to physiological effects of a substance or another medical condition.[1]

1.1.3 Obsessive-compulsive disorder

OCD is a neuropsychiatric and neurodevelopmental condition governed by persistent obsessions and/or compulsions, presenting as repetitive thoughts and behaviours.[1] The main symptoms of this condition present as recurrent, intrusive thoughts, urges, or images (obsessions) which can be satisfied by performing actions to reduce the anxiety associated with the obsessions (compulsions). Typical OCD symptoms include excessive washing, repetition in checking door locks and/or electrical appliances, and thoughts of loved ones harmed. While attempts at classifying individuals with OCD based on symptoms (washing, harmful thoughts) were ineffective, an important variable for differentiation is the age-of-onset, as identified by a meta-analysis.[11] Individuals diagnosed with OCD can be stratified as

either early-onset (mean onset of 11 years) or late-onset (mean onset of 23 years). Early-onset OCD is linked with higher male rates, greater disease severity and prevalence of obsessive compulsive symptoms, and associated with poorer treatment responses. In addition, those with early-onset OCD were found to exhibit comorbid tic, other OCD, anxiety, and mood disorders. The patients seen in the Dale clinic (supervisor) typically fall within the early onset-group. The diagnosis of OCD based on the DSM-V criteria necessities (a) the presence of obsessions and/or compulsions, (b) the obsessions or compulsions consume >1 hour per day, cause distress or impairment in important areas of functioning, (c) symptoms not be attributable to effects of a substance, and (d) symptoms not be better explained by those of another mental disorder.[1]

1.1.4 Prevalence and overall burden

It is estimated that 10% of children under the age of five suffer from one or more types of neurodevelopmental disorder.[12] In 2022, ASD have been reported to affect around 290,900 Australians,[13] a number which has greatly increased in recent years possibly owing to advancements in diagnosis and awareness. A data linkage study estimated the prevalence of autism spectrum disorders in New South Wales, Australia to be 1.1% by the age of 6, and 1.3% by the age of 12 years.[14] This can be compared to a study which utilised Medicare data to estimate the autism spectrum disorder prevalence in Australian 4 year olds to be 1.1%.[15] Reported from parents and teachers, an Australian prospective cohort study identified the prevalence of autism spectrum disorders to range from 2.6%-4.4% in 12 year old children[16]. Tourette syndrome is estimated to affect 1% of all school-aged children, with a male to female ratio of 4:1.[17] The national study of mental health and wellbeing (2022) estimated that roughly 3% of individuals over 16 years old are affected by OCD in Australia.[18]

These conditions are debilitating, and often carry burdens not only on the developing child but the caregivers. Neurodevelopmental disorders cause significant impairment to daily

functioning, as well as personal, social and economic strain on families. In Australia, the diagnosis of autism spectrum disorders costs a family ~$35,000 annually.[19] The impediment of these conditions extends past the affected families, and into the healthcare where a total burden cost of $1 billion was estimated for autism spectrum disorders in Australia.[20] In addition, a study examining OCD admissions in the largest provider of children's health services in Australia estimated the admission cost of all OCD diagnoses to be over $10 billion.[21]

1.1.5 Comorbidity

Owed to the highly complex interactions of genetics, epigenetics and the environment, the comorbidity rates of neurodevelopmental disorders are high. Up to 85% of individuals with Tourette syndrome have comorbid neurodevelopmental conditions such as ADHD, OCD, and ASD.[22] Studies have shown that 22-83% of children with autism spectrum disorders satisfy requirements for an ADHD diagnosis.[23] Individuals with these conditions present with overlapping clinical symptoms, further consolidating the shared aetiologies of the conditions.

In addition, the comorbid nature of these conditions extends past NDD symptoms and comprise neuropsychiatric comorbidities such as stress, depression and anxiety. School attendance is a vital part of a developing child's life, and children exhibiting anxiety and depression symptoms were shown to miss out on more school days when compared to their peers.[24] Sleep problems are a prominent comorbidity for children with autism spectrum disorders, where up to 80% of children are affected.[25-27]

1.1.6 Aetiology and pathophysiology

While some conditions can be traced back to a single factor, most chronic health conditions are complex and can often be explained by an aggregate of influences.[28] Our genetics is intricate, where inherited genetic variations can either trigger disease pathogenesis or act as

an underlying basis for development when combined with additional factors. Instead, a synergistic or antagonistic combination of influences contribute to development of disease risk, often referred to as gene - environment interactions.

This notion is crucial for neurodevelopmental disorders where its agreed upon that a complex interplay of genetic, epigenetic and environmental influences contribute to disease development.[29-31] Although there are monogenic forms of neurodevelopmental disorders, such as Rett Syndrome, and Fragile X syndrome – these monogenic cases are often associated with significant disease risk, linked with genes highly vulnerable to disruptive variants.[32] Instead, majority of cases are now recognised to be attributed to vulnerability genes with low penetrance.[33] A large genome-wide meta-analysis identified loci with pleiotropic and antagonistic effects on multiple complex psychiatric disorders such as ASD, ADHD, Tourette syndrome, OCD, along with schizophrenia, bipolar disorder, depression and anorexia.[34] This meta-analysis shed light on the genetic influences of psychiatric conditions, mentioning genes with broad liability to the disorders by acting on early neurodevelopmental processes such as brain circuitry formation within the second trimester. That is, the same vulnerability genes can cause multiple types of neurodevelopmental or neuropsychiatric conditions. In addition, the authors hypothesised the development of these genetic risks into discrete psychiatric conditions may be linked to interactive effects of common/rare loci and environmental factors mediated by epigenetics.

Environmental perturbations to the mother during development of the foetus is a vital area of research within the field of neurodevelopmental and neuropsychiatric conditions. Inflammation during the gestational period is thought to result in increased expression of these conditions.[35] A review by our group recently covered the relevance of inflammation, including maternal infection during gestation, to development of neurodevelopmental disorders.[36] The shared aetiology between these disorders may explain

the high comorbidity rates between neurodevelopmental and neuropsychiatric conditions. A neurodevelopmental continuum has been proposed to better model the distinct disorders, where shared genetic and environmental risk often co-existing within the same individuals. This models psychiatric conditions such as schizophrenia and neurodevelopmental disorders on a continuum, taking into account shared symptoms which present during development such as cognition impairment, male predominance and developmental delays.[2,37]

1.2 The immune system in neurodevelopmental disorders

1.2.1 Introduction to neurodevelopment

Neurodevelopment is a highly orchestrated active timeline of events, beginning form the third gestational week, and extending postnatally throughout late adolescence.[38] The development of the brain is extremely organised and consists of gene expression and epigenetic processes impacted by environmental factors.[35]

The levels of connectivity and processing within the developing brain are high, and throughout development synapse levels are reduced or "pruned" to maintain a functional relationship that exists within neuronal and non-neuronal cell types. The adult human brain contains around 86 billion neurons, with an equal number of non-neuronal glial cells.[39] Neurons are the messengers of the central nervous system (CNS), forming connections and networks to process information starting at 42 days post conception (mid-first trimester). Neurons oversee the rapid proliferation and overproduction of synaptic connections within early brain development, a process known as synaptogenesis lasting past the first year of life.[40] This overproduction is followed by a process of pruning and decrease of unused and overabundant synapses, occurring at different points of life within different brain regions. Proper circuit function is reliant on a balanced and correct numbers of neurons and synapses – overproduction of cortical neurons may impact the balance of excitatory and inhibitory neurons.[41] The division of neural progenitor cells in distinct proliferative zones during this

period switch from symmetrical (two identical neural progenitor cells are produced), to asymmetrical (one neural progenitor cell and one neuron are produced). This balance is vital in neurodevelopmental disorders, with dysregulations observed in human post-mortem tissue from children with autism,[42] as well as a mouse model.[43] Concert and interconnected connections amongst cells of the CNS are made possible by productive and appropriate synapses. The neurons migrate to their final destination, the developing neocortex, while the remainder of the neural progenitor cells continue to generate vital cells of the CNS such as oligodendrocytes and astrocytes. Finally, the resident immune cells of the brain, the microglia, are derived from early erythromyeloid precursors within the yolk sac and migrate the parenchyma prior to closure of the blood brain barrier (BBB).[41,44] With the development of the CNS nearing its end, microglia adapt to resume distinct sets of physiological functions.

Coordinated teamwork from the nervous, endocrine and the immune systems are vital for proper development of the offspring.[45] The immune system is fundamental for protection from a range of insults such as infections, playing a crucial role in the development of the central nervous system by maintaining homeostasis and promoting inflammation.[46,47]

1.2.2 Overview of the immune system's involvement

Initial concepts has painted the brain as an 'immune-privileged' organ, isolated from the peripheral immune system by the BBB. However, many research efforts have proved otherwise, by finding immune cells within the CNS, and the permeability of the BBB.[48-50] While the brain reserves a certain degree of immune privilege vital for limiting damage, it is not unaffected. The immune system is able to reflect environmental alterations and prepare for changes which may occur in the future as a form of defence. The initial programming of our defence system within development influences how we respond to environmental insults during a lifetime.[51] Development *in utero* is a critical period where the gestational environment can have major consequences on the developmental outcome of the offspring.[51] A large body

of evidence, including pre-clinical and clinical studies, support the involvement of maternal immune dysregulation during gestation as a risk factor for neurodevelopmental and neuropsychiatric conditions such as ASD.[52]

The innate immune system contributes to neurodevelopmental processes such as regulation of cell proliferation, migration, and synaptogenesis through many molecules. Inflammation is a vital, protective mechanism against infection and injury, defined by increase of pro-inflammatory cytokines, recruitment and activation of cells to sites of inflammation, and local tissue damage.[51] Cytokines are signalling proteins secreted by many immune cells in response to their environment.[47] The production of cytokines marks a cascade of signalling events, and in the developing CNS are responsible for mediating neurogenesis, gliogensis and other vital processes.[41] Cytokines such as Interleukin 1 beta (IL-1β) are diversly expressed during neurodevelopment, where levels were reported to be increased during development and steady within adulthood.[53-55] The innate immune system's sensor for danger are conserved receptors within the developing brain expressed by microglia and neuron progenitor cells known as Toll-like receptors (TLRs).[41] The expression levels of TLRs and their signalling adaptors vary during different developmental timeframes. For instance, animal studies have shown that the expression of TLR4 gradually increases from the embryonic period and stabilises at high levels within the adult brain.[56]

1.2.3 Microglia and synaptic pruning

Glia are non-neuronal cells within the CNS, responsible for vital roles such as myelination, providing support to neurons, and maintaining homeostasis. Chief amongst them are the primary immune cells of the CNS, microglia. Microglia are tissue resident macrophages, fundamental players of innate immunity originating from the yolk sac.[57] Microglia is the most dominant immune cell type within the brain, comprising 80% of brain immune cells, and localising near blood vessels.[47] Increasing prominence and recognition of the importance of

microglia was made possible by recent discoveries outlining the cells' multiple critical functions. In addition to the common roles in meditating inflammation, and clearing cellular debris by process of phagocytosis, microglia are involved in complex neurodevelopmental processes such as regulating cell death, promoting synaptogenesis and functional neuronal circuits.[58] Microglia are also major surveyors of their environment within the CNS even in their resting state, where they can scan the volume of the brain over a course of few hours in adult mice.[59] In addition, microglia regulate myelination, the process of wrapping neurons with a layer of insulation to facilitate neuronal activity and communication,[40] through promoting the survival of oligodendrocyte precursor cells and their maturation into oligodendrocytes.[47]

A vital process of neurodevelopment is synaptogenesis, the formation and pruning of points of contacts between cells of the CNS known as synapses.[40] Synaptic connections are formed exuberantly by neurons during early development of the brain, many of which are pruned necessarily to achieve appropriate brain connectivity. Early studies have emphasised the importance of microglia, where functional loss alters the excitatory and inhibitory synaptic activity, along with long-term potentiation.[60] Synapse formation and maturation are influenced by microglia and their secretion of key factors such brain-derived neurotrophic factor (BDNF) and interleukin 10 (IL-10), found to be vital for the formation of inhibitory and excitatory synapses within the hippocampus of rat models.[61,62] The process of pruning synapses is dependent on microglia, the resident immune cells of the brain. Microglia have been proposed to mediate the microglia-synapse interactions within development by utilising the complement cascade.[41] Early neurons bind molecules such as complement components C1q and C3, where they're ushered into synaptic compartments to mediate synaptic pruning.[63] Subsequently, these complement proteins are phagocytosed by the resident phagocytes of the CNS, microglia. Thus, the hypothesis is that complement

proteins are able to act as a "tag" for synapse removal, to then be found by microglia expressing complement receptor 3 on the surface.[64]

Another way that microglia facilitate and maintain homeostasis is through their title of a professional phagocytes. Phagocytosis involves the recognition, engulfment and digestion of large particles including bacteria, apoptotic cells and cell debris. This processes is majorly mediated by microglia, the professional phagocytes within the CNS, however astrocytes and oligodendrocytes are also able to carry on the job.[65] Areas of the brain containing neural progenitor cells such as the sub granular zone and the subventricular zone, microglia can be found to phagocytose apoptotic cells from development throughout the lifespan of the organism.[66]

1.2.4 Maternal immune activation

Inflammation in the mother is a well-established insult during gestation with links to development of neuropsychiatric symptoms in the offspring, by affecting the vulnerable process of neurodevelopment.[35] Maternal immune activation (MIA) is a phenomena that has circulated the literature of neurodevelopmental disorders, where an active immune response during pregnancy as a result of genetic and environmental interplay in the mother, can contribute to increased risk of NDDs. This was first associated with increased rates of ASD and schizophrenia in mothers infected by the rubella virus, and further validated by additional viral outbreaks.[67]

Epidemiological data has linked various infections during pregnancy to an increased risk of neurodevelopmental disorders in the offspring. A cohort study of children in the United States named the Rubella Birth Defect Evaluation Project (RBDEP) identified an early link between development of ASD and rubella infection.[68] An increased risk of ADHD in offspring was linked to genitourinary (genital and urinary) infections during 33-36 weeks of pregnancy in a Danish cohort study.[69] To add, a case-control study identified a significant

association between ASD risk in offspring and bacterial infections during third-trimester hospital stays.[70] A meta-analysis found that maternal infections, in particular bacterial infections, during the second and third trimester in particular, was associated with ASD in offspring.[71] When compared to influenza risk, the evidence in neurodevelopmental disorders is limited. While a risk of ASD was associated to influenza infections during the second trimester in a United States cohort study, this was contingent on the mother not taking antibiotics during pregnancy.[72] Fever concomitant to prenatal infection during gestation has been proved to be associated to ASD in offspring in a meta-analysis examining the relationship of ASD diagnosis to prenatal immune activation.[73]

A chronic inflammatory state in pregnant mothers coupled with genetic and epigenetic risk factors, can result in cellular stress and further inflammation.[74] An activated immune system results in a concert of cytokine and chemokine production, which can cross the placental barrier during pregnancy and have affect on the developing foetus.[75] A United States case-control study acknowledged that increased levels of interferon-γ (IFN-γ) , IL-4 and IL5 in maternal serum during mid pregnancy were associated with increased risk of ASD in offspring.[76]

In addition to the evidence outlined above, our group has provided numerous studies focusing on maternal inflammatory states and neurodevelopmental disorder outcomes in offspring. Firstly, we reviewed the current clinical evidence associating maternal inflammatory states such as infection during pregnancy and development of childhood neurodevelopmental disorders.[36] History of chronic immune system activation during pregnancy was associated with severe social impairment in children with ASD in a cohort study by our group.[77] To add, a systematic review by our group presented association between crucial maternal risk factors such as obesity, gestational diabetes, pre-eclampsia, depression, autoimmune disease and neurodevelopmental outcomes such as ASD, ADHD

and Tourette syndrome.[78] There was a statistically significant increase in offspring NDD associated with the above maternal pro-inflammatory factors. Furthermore, previous work by the group has similarly identified that mothers of children with OCD and tics have higher rates of maternal autoimmune conditions when compared to mothers of controls.[78,79]

1.2.5 Animal evidence of maternal immune activation

A plethora of animal models have been generated to investigate maternal immune activation and have contributed vastly to what we now understand regarding NDD risk and an activated immune response during pregnancy. Injection of immune stimulants into pregnant dams during mid and late gestation produces offspring with cognitive impairments, learning defects, amongst other behaviours that mimic neurodevelopmental symptoms in humans.[80] Observation of offspring in these studies have identified febrile symptoms, along with brain inflammation, where long-lasting pro-inflammatory cytokines dysregulations such as IL-6, TNF and IL-1β were observed in the serum of mothers and offspring.[81-87] Epidemiological studies of humans modelling maternal immune activation refer to an external immune challenges during pregnancy such as infection, autoimmune conditions or psychiatric symptoms. Animal models of MIA typically utilise two main immunogenic agents to mimic an activated immune response in pregnant dams: (1) viral-like Polyinosinicpolyinosinic:polycytidylic acid (Poly I:C), and (2) bacterial-like lipopolysaccharide (LPS). These immunogens stimulate the toll like receptor pathway, and induce a well-defined response controlled by the timing and dosage. In rodents, the effect of a immunogen challenge encompasses the broad perinatal period – during gestation and ~2 weeks after birth.[88] This is due to the first few weeks of the neonatal rodent development being equivalent to the third trimester in human fetal development.[89] The timing of immune stimulation is crucial, as processes such as synaptogenesis, apoptosis, and immunogenesis occur during the third trimester.[74]

One of the most widely used immunogens to model maternal immune activation is Poly:IC – a synthetic double-stranded RNA, stimulating a viral inflammatory response through activation of toll-like receptor 3 (TLR3).[90] This agonist is recognised by TLR3, and results in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), AP-1 and IRF3. These transcription factors induce the expression of inflammatory cytokines and type 1 interferons. Within animal models utilising mice or rats, the dose of Poly:IC ranges from 250ug/kg to 20 mg/kg. The timing of injection within these animal models ranges from embryonic days 9-19, with the most reported bring E12.5 and E15.[80,91] Another common immunogen utilised to model MIA is LPS – a cell wall component of gram-negative bacteria such as Escherichia coli, stimulating an immune response through activation of toll-like receptor 4 (TLR4).[90] LPS leads to the activation of TLR4 through help of CD14 and LPS binding protein (LBP), and results in a cascade of inflammatory cytokine and interferon gene expression. Dosage of LPS within rodent and rat animal models ranges from 25ug/kg to 1mg/kg. Similar to Poly:IC, the timing of LPS administration varies from E9 – E19, with the most reported timing being E15 and E18.[80,91] Both of these immunogens have been described to be administered to animals multiple times, however, the response to the immunogens is often attenuated following a second administration, but not a third or fourth.[92] To illustrate this, Poly I:C challenge in pregnant dams mid-gestation (gestational day 9) identified methylation alterations within the WNT signalling pathway of adult offspring, a conserved pathway crucial to development of the CNS. On the other hand, methylation changes of the gamma-aminobutyric acidergic (GABAergic) in adult offspring were linked to late (gestational day 17) Poly I:C challenge of pregnant dams.[93] These findings provide evidence that the timing of the challenge may influence the type of effect in offspring.

Environmental perturbations during sensitive periods of development may alter neurodevelopmental trajectories and increase risk of NDDs. MIA is thought to act as a 'disease primer' for neuropsychiatric symptoms, increasing susceptibility to effects of other

risk factors later in life.[74] The effect of the environment must be taken into consideration when recapitulating MIA risk factors that lead to an NDD phenotype in animal models. Environmental influences such as social stress, diet, allergens and pollutants have been shown to induce an inflammatory response with ability to disrupt neural and behavioural development in NDD children.[92] A "two-hit" model exists within the MIA literature, considering the combination of environmental, genetic and epigenetic "hits" which occur during development, and increase the risk of NDD.[80,88] These models have demonstrated transgenerational effects of prenatal environmental challenges, where epigenetic alterations within the brains of second and third generation MIA offspring were linked to behavioural abnormalities such as sociability and motivation.[94,95]

It is important to mention that findings from different animal models can be multifarious, attributed due to the lack of gold standard techniques and condition that may lead to variations in offspring effects. The timing of injecting a viral or bacterial mimetic along with the route of administration can play a significant role in the outcomes of studies, particularly in abortion rates of offspring. In addition, batch differences in immunogens even from the same manufacturer can produce variabilities in outcomes, in addition to the of preparations. For example, the method of purification of LPS is important due to the down-stream effects it produces, whereby a standard preparation has the ability to co-activate TLRs 2 and 4, while ultrapure LPS is specific to TLR4 only.[87]

1.2.6 Paediatric Acute Neuropsychiatric Syndrome

Within human studies, environmental influences such as infections during the postnatal period have been linked to worsening of neuropsychiatric symptoms within children.[96] In addition, our group reviewed the current literature associating maternal inflammation and infection during pregnancy and linked these inflammatory states to increased risk of NDDs.[36] In 1998, Swedo et al., coined Paediatric Autoimmune Neuropsychiatric Disorders Associated

with Streptococcal infections (PANDAS) to describe a cohort of children presenting with acute prepubertal onset of OCD and tic disorders triggered by group A B-haemolytic streptococcal infections (GABHS).[97] These children presented with sudden-onset tic and obsessive compulsive symptoms, along with psychiatric comorbidities such as anxiety, night-time fears and cognitive deficits exacerbated by infection. The introduction of this entity sparked controversy within the scientific community due to the lack of clear described disease mechanisms and markers of diagnosis, as well as inability to distinguish with symptoms with the prototypic post-streptococcal neuropsychiatric disease, Sydenham's Chorea.[98] The authors suggested that GABHS resulted in autoimmune or immunological responses triggered due to molecular mimicry, which result in early-onset OCD or tics, however there has never been definitive proof of such an autoimmune mechanism. In addition, problems with the PANDAS hypothesis were encountered with tic disorder patients, where tics are known to wax and wane- this made it hard to discriminate patients with Tourette syndrome from patients with PANDAS who have "abrupt onset and episodic course". To add to this challenge, Group A streptococcal infections are highly prevalent in school aged children, which can result in 'false positive' associations when using streptococcal serology in the context of children with tics and OCD.

The inclusion criteria of streptococcal infections resulted in many misdiagnoses as well as missed diagnoses of PANDAS. To try to improve this diagnostic challenge, a distinct clinical subgroup that included children with abrupt onset neuropsychiatric conditions was coined. The inclusion criteria of Paediatric Acute Neuropsychiatric Syndrome (PANS) include (1) abrupt, dramatic onset of OCD or severely restricted food intake, (2) concurrent presence of additional neuropsychiatric symptoms, and (3) symptoms are not better explained by a known medical or neurologic disorder such as Sydenham's Chorea (Figure 1.1).[99] Children with PANS present with extreme compulsions such as licking shoes, barking,

motor and phonic tics such as whooping and wringing hands, behavioural aggression and episodes of extreme anxiety. While the definition of this clinical syndrome has undergone refinement, PANS is still considered contentious within the field. Early descriptions of PANS suspected the condition of autoimmune origins, however the evidence for an autoimmune diathesis is lacking. Our group's hypothesis is that PANS is part of the NDD continuum, a phenotype representing the complex interactions between gene and environment- this interaction can result in infection triggered exacerbation of neuropsychiatric symptoms. There is a lack of studies exploring the underlying disease mechanisms of PANS, combined with the lack of biomarkers at present- these factors feed into the contentious nature of PANDAS and PANS within the field.

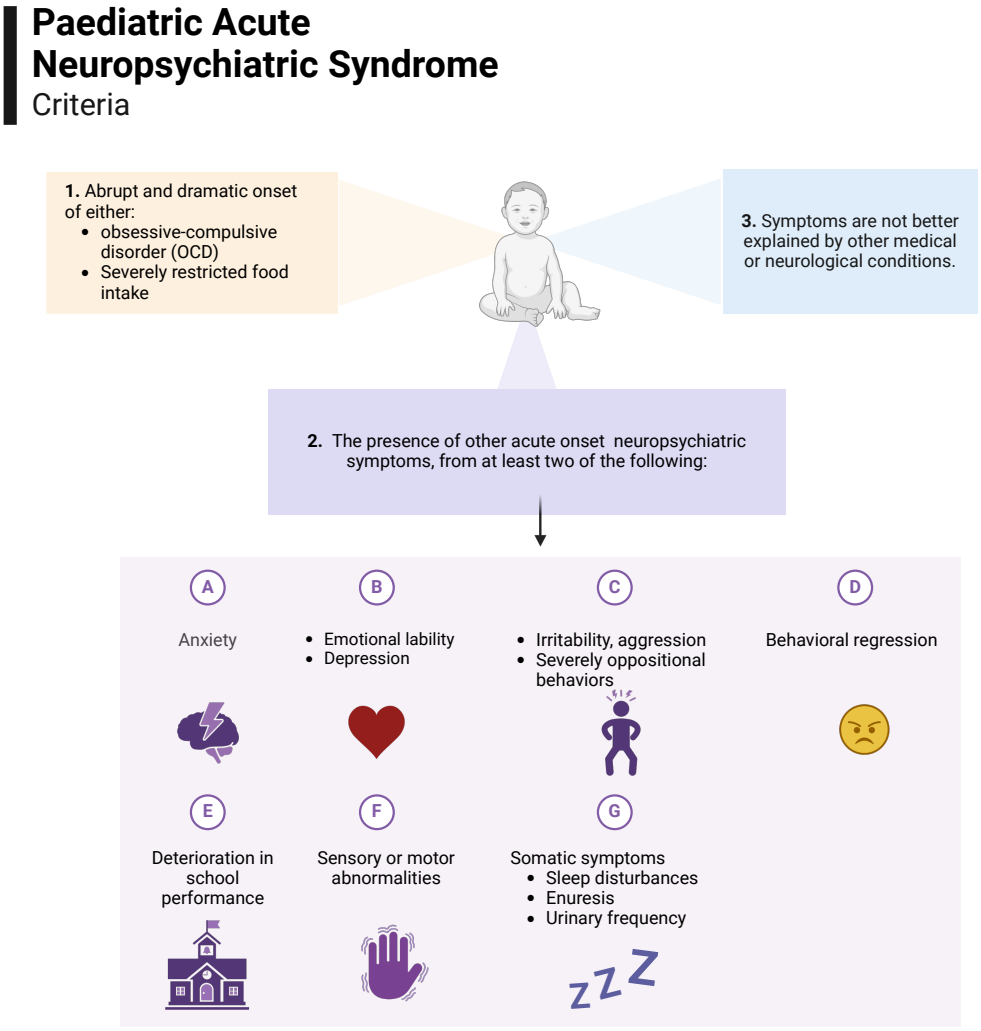


Figure 1.1 Diagnosis criteria of Paediatric Acute Neuropsychiatric Syndrome
Diagnostic criteria of Paediatric Acute Neuropsychiatric Syndrome (PANS). Criteria were utilised from Chang *et al.*,[99] Figure generated using Biorender.

Due to the limited understanding of the PANS entity, our group set out to investigate the underlying mechanism of PANS through multiple omic strategies and a functional assay. Chapter 3 of this thesis focuses on utilising mass spectrometry based proteomic analyses to investigate two cohorts of this clinical subgroup and identify dysregulated proteins and pathways. From this work, the aim is to better understanding the biology of PANS and identify abnormal networks and pathways that may lead to further insights and future investigations. In addition, Chapter 4 of this thesis utilises a cohort of children with PANS to assess the immune system in response to a bacterial challenge in a functional toll-like

receptor assay. The investigations within these two chapters aim to better our understanding of the PANS clinical entity and pave the way for further studies.

1.2.7 Clinical evidence of inflammation

Bidirectional communication of the immune system and the central nervous system are recognised as vital processes which contribute to normal brain development. As such, interactions of the two systems are implicated in the pathophysiology of neurodevelopmental disorders such as ASD, Tourette syndrome and OCD.[100] Preclinical studies have demonstrated multiple pathways are utilised for crosstalk of the immune and central nervous system.[101] Following an insult to the CNS, an immune response is initiated resulting in activation of peripheral macrophages and CNS microglia,[102,103] where signalling cascades are communicated to the BBB, leading to further changes within inflammatory, phagocytic and oxidative stress pathways.[104]

Common pro-inflammatory cytokines mentioned in this section include IL-1β, IL-6 and TNF. IL-1β is a pro-inflammatory cytokine released as a result of activated peripheral monocytes and tissue macrophages (including microglia) and promotes inflammation by indirect promotion of lymphocyte function and macrophage activation. Release of IL-1β induces the expression of mediators such as cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS). In addition, IL-1β is a potent stimulator of IL-6 production in endothelial cells. IL-6 is a pro-inflammatory cytokine produced by immune cells including B cells, T cells and macrophages (as well as endothelial and fibroblast cells) and is vital for microglia and astrocyte neuroinflammation. In addition, TNF is similarly produced by macrophages, T cells and natural killer cells, responsible for mediating the inflammatory response by recruitment of phagocytes and lymphocytes to sites of injury. These pro-inflammatory cytokines induce an acute-phase reaction whereby the body attempts to respond to local inflammation and limit damage through fever and production of protease inhibitors and corticosterone.[105]

1.2.8 Inflammation in the brain

Evidence of dysregulated cytokine expression within the CNS of individuals with NDDs supports the role of immune mediators in these complex conditions. The resident immune cells of the brain, microglia, are the primary source of cytokines and other immune molecules released within the brain.[106] One of the earliest studies to implicate the innate immune response in post-mortem brains of individuals with ASD highlighted neuroglial responses characterised by microglial and astroglia activation compared to controls. In addition, these immune activations were associated with increase of cytokines expression, particularly a global increase was identified for MCP-1.[107] Later studies investigating post-mortem brains in individuals with ASD has similarly reported microglial activation and neuroinflammation.[51,108,109] In Tourette syndrome, post-mortem analysis revealed increased microglia coupled with morphological changes consistent with activation, within the striatum of cases compared to controls.[110] Similarly, region-specific microglial activation was reported in positron emission tomography (PET) studies of individuals with ASD,[111] and similarly identified in children with ADHD and Tourette syndrome when compared to controls.[112]

Many studies implicate neuroinflammation in NDDs post-mortem tissue, where dysregulated levels of pro-inflammatory cytokines within CNS of individuals with ASD are reported.[113] Investigations utilising post-mortem brain tissue of individuals with ASD identified elevated levels of pro-inflammatory cytokines TNF, IL-6 and IFN-γ in patients compared to controls.[114-116] In addition, NF-κB was observed to be elevated in the orbitofrontal cortex of individuals with ASD compared to controls. Interestingly, increased NF-κB, a mediator of the immune response responsible for expression of inflammatory cytokines and chemokines, was observed in mature microglia and astrocytes relative to neurons.[117] An increase in proinflammatory cytokines such as IL-2, IL-12, monocyte chemoattractant protein 1 (MCP-1) and TNF have similarly been found in a post-mortem

brain analysis of Tourette syndrome patients during symptom exacerbations.[118] Another early transcriptomic investigation of Tourette syndrome post-mortem brains utilising microarrays identified an increase in the protein tyrosine phosphatase family and TNF-ligand superfamily within the putamen region.[119] Furthering the dysregulation of the neuro-immune crosstalk in NDDs, the authors validated the microarray findings through real time PCR and western blot analyses and showed 3/5 proteins had discrepancies in protein levels when compared to gene expression.[119]

1.2.9 Inflammation in the periphery

Circulating cytokines in peripheral samples such as blood, plasma or serum are a great alternative to the otherwise inaccessible brain, when investigating the underlying mechanisms of the immune response in NDDs. There have been many studies to explore the effects of the immune response by measuring circulating cytokines in peripheral samples of ASD patients. Following in the footsteps of MIA models, stimulation of peripheral blood mononuclear cells from patients with ASD stratified patients based on symptom severity through increased production of pro-inflammatory cytokines following an immune challenge.[120] Studies have also attempted to correlate the severity of ASD symptoms to levels of pro-inflammatory cytokines, where increased plasma IL-1β and IL-6 were associated with more impaired behavioural and communication symptoms.[121] A recent meta-analysis of circulating pro-inflammatory cytokines in ASD studies has confirmed the activated immune response evidence and reaffirmed higher concentrations of pro-inflammatory cytokines such as IFN- γ, IL-1β, IL-6, and TNF in patients compared to controls.[122] In addition, the authors performed meta-regression analyses in order to explore sources of heterogeneity within the studies included, and identified an interaction of age, gender and latitude. For example, differences in proportion of male patients included had a positive effect on IL-1β, but a negative effect on TNF levels.

An imbalance in pro-inflammatory cytokine levels within the periphery have been reported in individuals with OCD, with some studies describing elevated levels compared to controls and others describing decreased levels.[105,123-125] Interestingly, a systematic review and meta-analysis of 16 studies investigating the peripheral immune response failed to identify differences in pro-inflammatory cytokines including IL-6, TNF, IL-1β in OCD patients compared to controls.[126] More recently, a review of the literature of paediatric patients with OCD and movement disorders highlighted TNF, IL-1β and IL-17 to be altered in patients when compared to controls.[127] These findings are interesting, and spark the question of immune differences in OCD patients between a paediatric and adult cohort potentially having epigenetic influences in early life. In addition, comorbidities that may arise in life could alter the immune signature identified in early life, along with modifiers such as medications. Of interest, within the OCD meta-analysis described above, differences in IL-1β levels were only established in drug-naïve patients compared to controls.[126]

Within studies exploring the peripheral immune system in Tourette syndrome, a skewing towards pro-inflammatory findings is also present.[128] However, similar to findings from other NDDS such as ASD and OCD, there remains discrepancies on the direction of inflammation within Tourette syndrome studies. Serum concentrations of proinflammatory cytokines IL-12 and TNF were elevated in patients with Tourette syndrome when compared to controls in a prospective longitudinal study.[129] In addition, plasma levels of pro-inflammatory cytokines such as IL-6, IL-1β and IL-17 were increased in Tourette syndrome patients compared to controls.[130] A cross-sectional study attempted to investigate markers of inflammation in a cohort of children, adolescents, and adults with Tourette syndrome and identified increase levels of C-reactive protein (CRP) and neopterin, sensitive markers for inflammatory processes.[131] However in a further study, individuals with Tourette syndrome showed lower serum concentrations of TNF, soluble interleukin-1 receptor-antagonist (IL1-ra) and monocyte-derived soluble CD14, in addition to high levels of monocytes when

compared to controls. This section provided some evidence of inflammation and immune dysregulation reported within the literature of NDD syndromes, with a generally increased, but sometimes decreased, trend in cytokine levels.

1.3 Genetics and epigenetics in Neurodevelopmental disorders

1.3.1 Genetics

The complexity of neurodevelopmental disorders can be partly owed to the heterogenous aetiology within individuals. A systematic comprehension of NDD aetiology and comorbidity is yet to be understood. Despite the recognised hereditary contribution of various neurodevelopmental conditions, the genetics underlying these disorders are still unclear. While monogenic forms of neurodevelopmental disorders exist, they are often associated with genes highly vulnerable to disruptive variants.[32,33] Genetics play a crucial part in the aetiology of NDDs, with studies reporting higher rates of ASD in siblings of affected individuals, alongside high heritability rates in twins.[132,133] Analogous to ASD, the rates of Tourette syndrome in first-degree relatives are higher when compared to second-degree relatives or controls.[134] A meta-analysis of twin and familial risk OCD risk recently conducted revealed first-degree relatives of individuals with OCD were 7.18 times at risk of OCD compared to controls.[135]

Genome wide association studies (GWAS) aim to identify genes and alleles which contribute to the risk of developing complex traits. By searching the genome in an unbiased fashion, regions harbouring common genetic variants such as single nucleotide polymorphisms (SNPs) are tested for statistical significance to identify associations between genotype and phenotype. SNPs are substitutions of a nucleotide at a specific location within the genome, present in a large portion of the population.[136] The genotypes of individuals displaying a trait of interest, and controls that don't have the trait, are investigated using dense microarray sequencing to search for frequences of SNP markers. A meta-analysis of

ASD GWAS have identified a significant loci spanning genes such as *PITX3* (transcription factor), *CUEDC2* (regulates cytokine production), *HDAC4* (histone deacetylase) and *MACROD2* (deacetylase).[137] Through familial and GWAS, genes such as *SLITRK1*, *NTN4*, and *HDC*, which are involved in neurite growth and neurotransmission, have been implicated in Tourette syndrome.[138-140] A meta-analysis of two OCD GWAS studies failed to identify genome-wide significant findings, however genes such as *CASC8/CASC11* (long non-coding RNAs), *GRID2* (glutamate receptor), *KIT* (receptor tyrosine kinase) have been implicated.[141]

Despite multiple studies performed to try and understand the genetics of these complex disorders, no one gene is believed to cause NDDs. Monogenic forms of neurodevelopmental disorders account for 10% of cases (such as Rett syndrome and Kabuki syndrome). Instead, small dysregulations within multiple interacting genes and pathways combined with epigenetic and environmental effects is thought to be operating. It is critical to emphasise that the associated highly penetrant genes only elucidate a minority of patients with complex NDD such as ASD, Tourette syndrome and OCD. Environmental factors are also implicated in the aetiology of NDDs, with pre/perinatal conditions such as maternal psychosocial stress, as well as epigenetics being predominant considerations.

1.3.2 Epigenetics

Neurodevelopmental disorders are now considered to arise from a multifactorial interplay of genetic, epigenetic and environmental influences that result in expression of these symptoms. Distributions to neurodevelopment due to these various influences may have dire consequences on NDD expression.[74] The flow of biological information moves from DNA to RNA and is translated to proteins. Many cellular processes orchestrate the regulated transition of information to reach a functional protein, and epigenetic processes operate in this transition.[142] Neurodevelopment, where development of the central nervous system and

its many vital cell types is made possible by epigenetic mechanisms that allows proliferation or differentiation.[143]

The concept of epigenetics was first described by Conrad Waddington, where he showed the effects of environmental stimuli (such as temperature) can be observed phenotypically in offspring, and continue across generations, without the external stimuli.[144] Now, our definition of epigenetics refers to phenotypic heritable variations arising from environmental difference without changes in genomic sequence.[142,144,145] Epigenetics represent vital mechanisms whereby environmental effects leave their mark through profound changes in gene expression or function, without altering the genomic sequence.[146] Epigenetics is incredibly important for complex conditions such as neurodevelopmental disorders, where a link between environmental influences and genetic expression can be drawn. The focus of many epigenetic studies is DNA methylation, where integration of the microarray technology allowed for a first grasp of how this field can be explored in a high-throughput fashion.[147] Investigations of epigenetic signatures are tissue-specific,[148] a fact requiring substantial consideration within the field of NDDs.

Epigenetic mechanisms can also rely on changing chemical moieties in chromatin to exhibit genomic expression, through cross talk of DNA methylation, histone and chromatin modifications, micro-RNA expression, and protein post-translational modifications (Figure 1.2). Post-translational modifications can be in the form of methylation, phosphorylation, acetylation, ubiquitylation and sumoylation. Histone proteins are basic proteins acting as spools where ~ 147 base pairs of DNA wind around an octamer of histone proteins (two copies each of histones H2A, H2B, H3, H4), referred to as a nucleosome. Packed units of nucleosomes are known as chromatin, existing as euchromatin (lightly packed), or heterochromatin (densely packed). Tightness of DNA around histone proteins has impacts on accessibility of DNA to be transcribed.

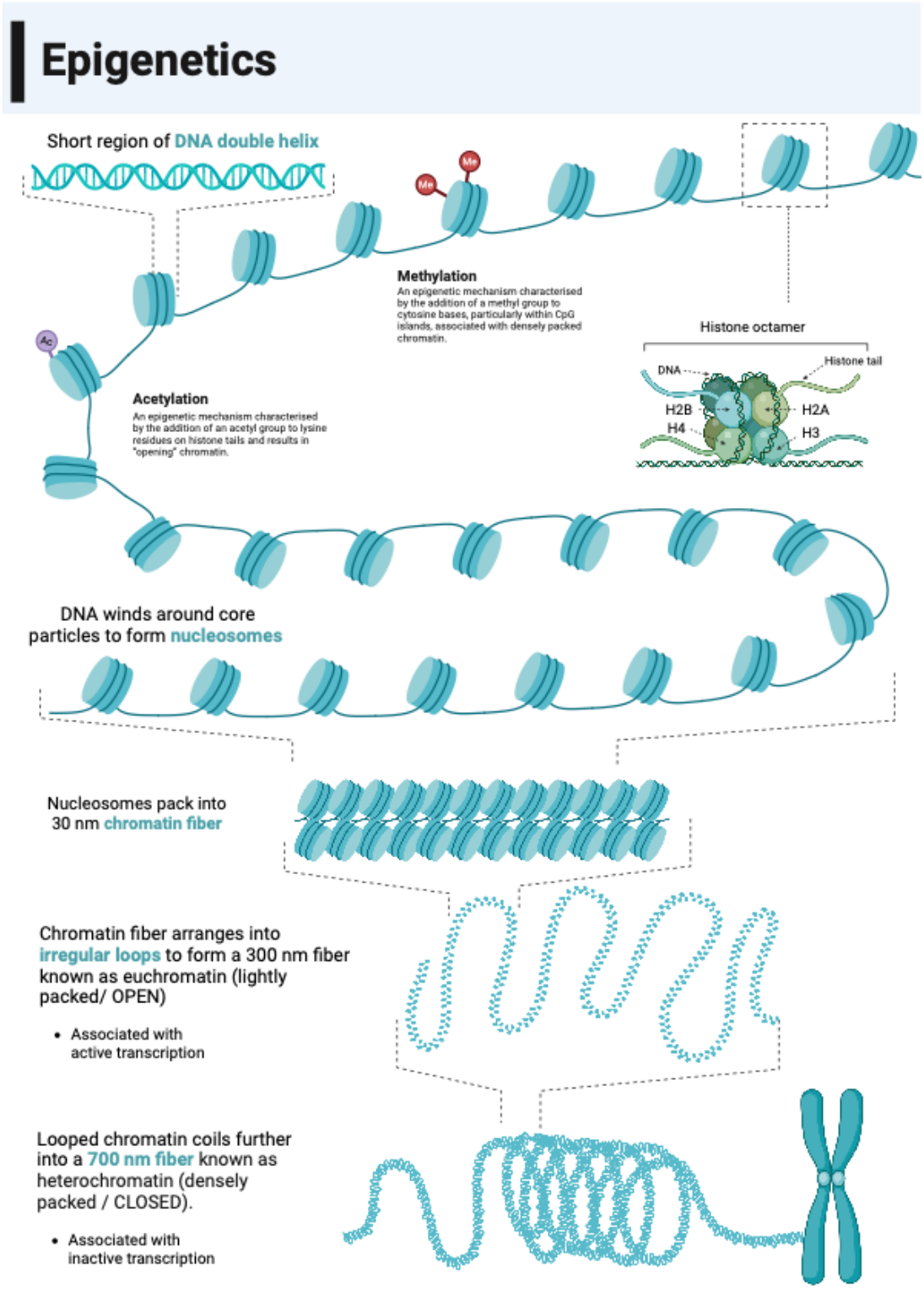


Figure 1.2 Epigenetic mechanisms
Epigenetic modifications are reversible and heritable gene expression changes that don't alter DNA sequences. The figure highlights a short region of DNA, which is wrapped around histones (histone octamer configuration is shown on the right). The addition of epigenetic modifications such as DNA methylation and acetylation are shown as markers attached to nucleosomes, associated with open and closed chromatin respectively. Chromatin units are known as nucleosome, which assemble into chromatin. Loosely packed chromatin is associated with active transcription, while densely packed chromatin is associated with inactive transcription. Figure generated using Biorender.

DNA methylation was the first discovered epigenetic mechanism and entails the addition of a methyl group to cytosine bases of DNA with the help of methyltransferase enzymes.[149] DNA methylation, particularly occurs within CpG islands (regions with repeats of cytosine followed by guanine).[150] Next, histone modification consists of post-translational modifications specifically within histone proteins, where they have a role in how tightly DNA is wrapped around histones, and thereby influence accessibility of DNA for transcription.[149] Another form of epigenetic modifications involves RNA, both coding and non-coding RNA (ncRNA). Within ncRNAs, microRNAs guide gene expression post-transcriptionally by binding to the untranslated regions of mRNAs and disrupt translation.[151] Finally, post-translational modifications to proteins following the process of translation have the ability to alter function, conformation and stability of proteins.[152]

Development of the CNS is a complex process requiring regulated gene expression, fulfilled by transcriptional factors and epigenetic processes.[153] Coordination of key epigenetic processes including DNA methylation and histone modifications throughout neurodevelopment, form the basis of epigenetic trajectories critical for cell maturation and subsequent cell fate[154,155] The effects of MIA extends past changes outlined in Section 1.3, where animal models have pointed long-lasting epigenetic alternations such as DNA methylation, histone modifications, and microRNA changes within offspring brains.[146,156] Within human studies, maternal risk factors of NDD in offspring outlined prior such as anxiety, depression and smoking during gestation have been associated with epigenetic modifications within the placenta, umbilical cord and peripheral blood of offspring.[157,158]

1.4 Impact of multi-Omic approaches in Neurodevelopmental disorders

The flow of genetic information is stored in the form of genes as DNA, which are then transcribed into RNA, and finally translated into proteins. Researchers have been investigating the various levels of biology for decades, however in-depth analyses of one or multiple of these levels was not possible until exploration of the genome started with The Human Genome Project in late 1980s.[159] Utilisation of high-throughput technologies such as RNA sequencing, and mass spectrometry have become a standard within current times, where there is increased instrument accessibility and improved protocol development which account for various models and samples.[160] Inclusion of the suffix "omics" to the end of a molecular term infers an inclusive and global assessment of a set of molecules.[161] The transcriptome examines the complete set of transcripts in a sample, including mRNAs, non-coding RNAs and small RNAs, with the aim of quantifying changes in expression levels of each transcript. The proteome involves the study of peptides in a sample, including the peptide's quantification, abundance, modification and interactions. Utilisation of complex explorations into various levels of biology is needed when investigating complex conditions such as neurodevelopmental disorders.

1.4.1 Investigating the transcriptome using RNA-sequencing

Researchers have investigated gene expression for a long time with the utilisation of molecular biology techniques such as polymerase chain reaction (PCR).[162] However, these explorations often involved single transcripts, and genes previously known to be implicated, leaving little room for novel discoveries. The introduction of high-throughput sequencing allowed for an in-depth global search into samples or organisms of interest. Transcriptomics is the study of all transcripts in a given sample, including mRNA, non-coding RNAs and small RNAs.[163] Initial work within the field saw the utilisation of hybridisation-based

microarray assays, followed by sequence based approaches, both of which bear many caveats and problems.[163,164] Development of next-generation sequencing technologies such as RNA sequencing revolutionised transcriptomics by providing in-depth sequencing of complementary DNA to explore gene expression on a global scale within samples.[165]

RNA sequencing involves the preparation of a sequencing library from complementary DNA isolated from RNA, which is then sequenced on a next generation sequencing platform (Figure 1.3).[163,165] As with other experiments utilising biological samples, careful planning and consideration of proper controls is required for optimal results and analysis while balancing time and resources. These include the use of biological and technical replicates, the depth of sequencing (total number of reads sequenced), and the transcriptome coverage (breadth of coverage based on depth) needed.[166]

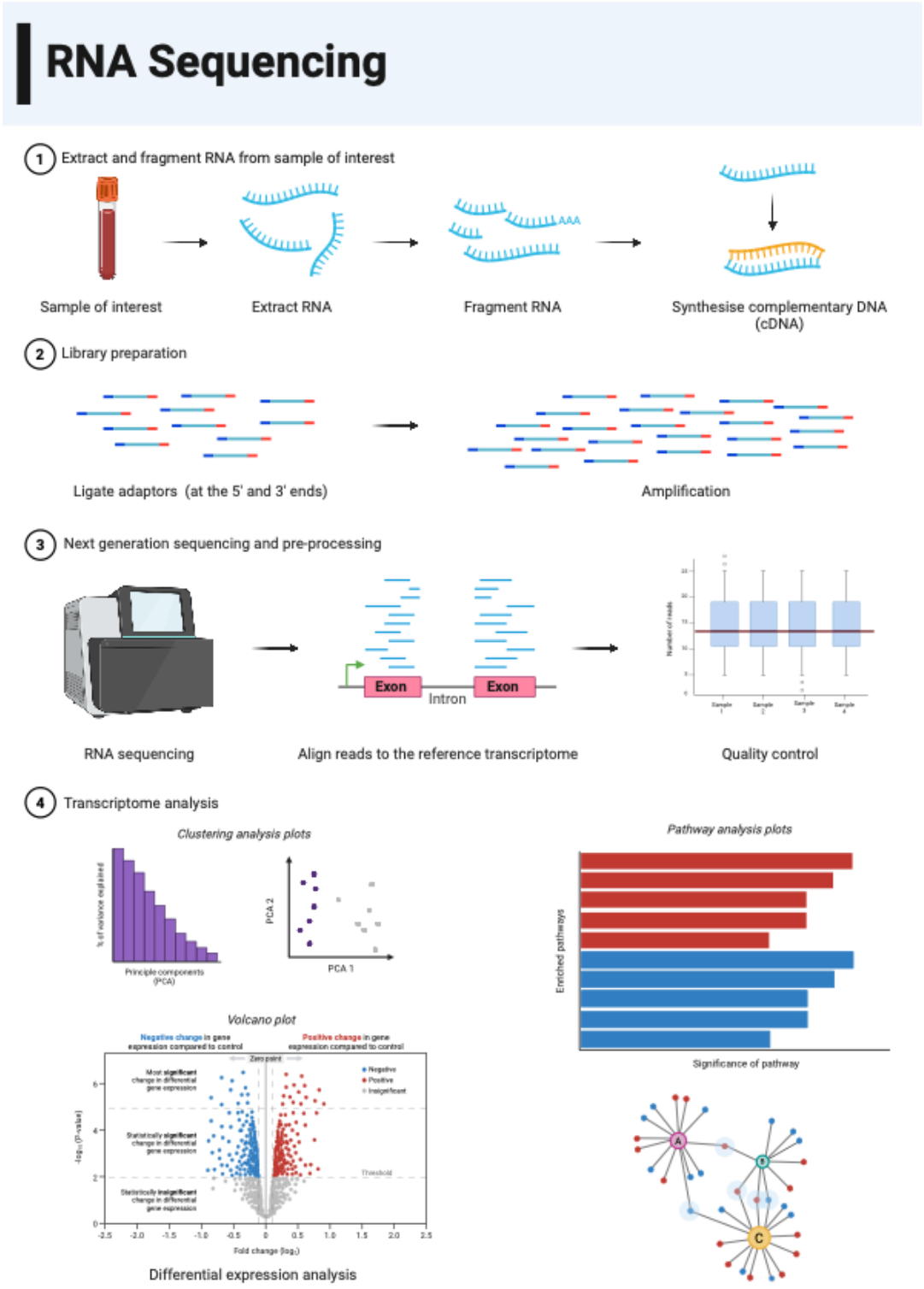


Figure 1.3 Transcriptomics by RNA sequencing
RNA sequencing protocol from sample preparation to transcriptome analysis figure examples. (1) The protocol is initiated with isolation of RNA from the sample of interest, fragmentation of RNA samples (of the different RNA types), and synthesis of complementary DNA (cDNA). (2) Following cDNA synthesis, the sequencing library is generated by synthesising adaptors to the 5' and 3' ends of cDNA and amplifying the sequences through polymerase chain reaction (PCR). (3) The generated library is then RNA sequenced on a next generation sequencing machine (eg, Illumina) and the reads are pre-processed by aligning to a reference transcriptome. The final section of this step involves quality control measurements of the samples. (4) Finally, the transcriptome is analysed, often starting with clustering analyses to identify trends within samples and differential expression analysis to identify genes with distinctive expression in group(s) of interest in comparison to controls. Finally, pathway analysis can be performed to identify biological pathways enriched in the differentially expressed genes. Figure generated using Biorender.

Transcriptomic analyses of neurodevelopmental disorders have provided a robust collection of inflammatory and immune evidence. Studies utilising post-mortem brain samples from individuals with neurodevelopmental disorders have provided an insight into brain transcriptomic changes, a vital and implicated region otherwise inaccessible. Transcriptomic studies utilising post-mortem brain samples of individuals with ASD have pointed towards an up-regulated immune pathways and activated microglial markers, along with a down-regulated synaptic and neuronal signals.[167] This signalling pattern of an up-regulated immune response and a down-regulated neuronal message has been repeatedly identified in subsequent studies investigating the ASD brain transcriptome.[168-171] In addition, He et al., performed an integrated transcriptomic analysis of ASD datasets including post-mortem brain samples, peripheral blood samples and patient cell lines[172]. The integration of multiple dataset types identified a larger up-regulated gene expression pattern in brain samples when compared to peripheral datasets of ASD, along with enriched pathways involved in inflammation, oxidative stress, and mitochondrial dysfunction. To add, a recent profiling of the ASD brain transcriptome – focusing on RNA sequencing studies, identified previously implicated pathways involved in the immune response and osteoclast differentiation, along with novel differentially expressed genes.[173] An RNA sequencing analysis performed on blood samples of adults with ASD identified an enriched immune signal when compared to controls, adding further to the immune evidence associated with NDDs.[174]

Similarly, transcriptomic analyses of OCD post-mortem brain samples revealed global synaptic signalling dysregulations and enrichment of neuronal genes.[175] Initially, Lisboa et al., explored three striatal areas including the putamen, caudate nucleus and accumbens nucleus in six cases and eight controls and identified commonly enriched synaptic signalling, along with distinct signatures within each region investigated. In addition, the authors identified an enriched immune response and inflammatory signature within the caudate

nucleus region only consisting of cell adhesion molecules and microglial enrichment.[175] The most recent transcriptomic analysis of OCD post-mortem brains attempted to explore the orbitofrontal cortex along with two striatal regions (caudate and nucleus accumbens) compared to controls. This analysis highlighted the majority of the differentially expressed signal to arise from the striatal region, and implicated pathways in OCD such as synaptic neurotransmission and glutamatergic synapses.[176] The only RNA sequencing study of individuals with Tourette syndrome utilised post-mortem brain samples, and pointed towards genes and pathways down-regulated interneuron signalling, and up-regulated immune response including microglial an astrocyte inflammation.[110] Lisboa et al., compared their OCD transcriptome analysis to the Tourette syndrome analysis, and identified that while similarities were present in the DEGs, the direction of expression was comparable in the caudate nucleus only, while lower levels were observed in the other brain areas.[175]

1.4.2 Investigating the proteome using mass spectrometry

Proteomics refers to large-scale investigations of all sets of proteins within a cell type or tissue. [177] Much like investigations of genes, initial studies of proteins involved explorations of single proteins by means of two-dimensional gel electrophoresis. The field of proteomics evolved from gel analyses to sequencing by Edman degradation and finally with the development of mass spectrometry (Figure 1.4). Utilisation of mass spectrometry allows for sensitive, accurate and quantitate protein information by detecting the presence and abundance of peptides. Analysing the proteome of a sample through mass spectrometry requires digestion of samples into peptides, and further ionisation depending on sample complexity.[178] Liquid chromatography (LC) is commonly utilised to charge and dry molecules in complex samples and deliver to the mass spectrometer, while matrix assisted laser desorption ionisation (MALDI) is utilised for simple peptide mixtures (<100 proteins) and involves subjecting samples to laser irradiation.[178,179]

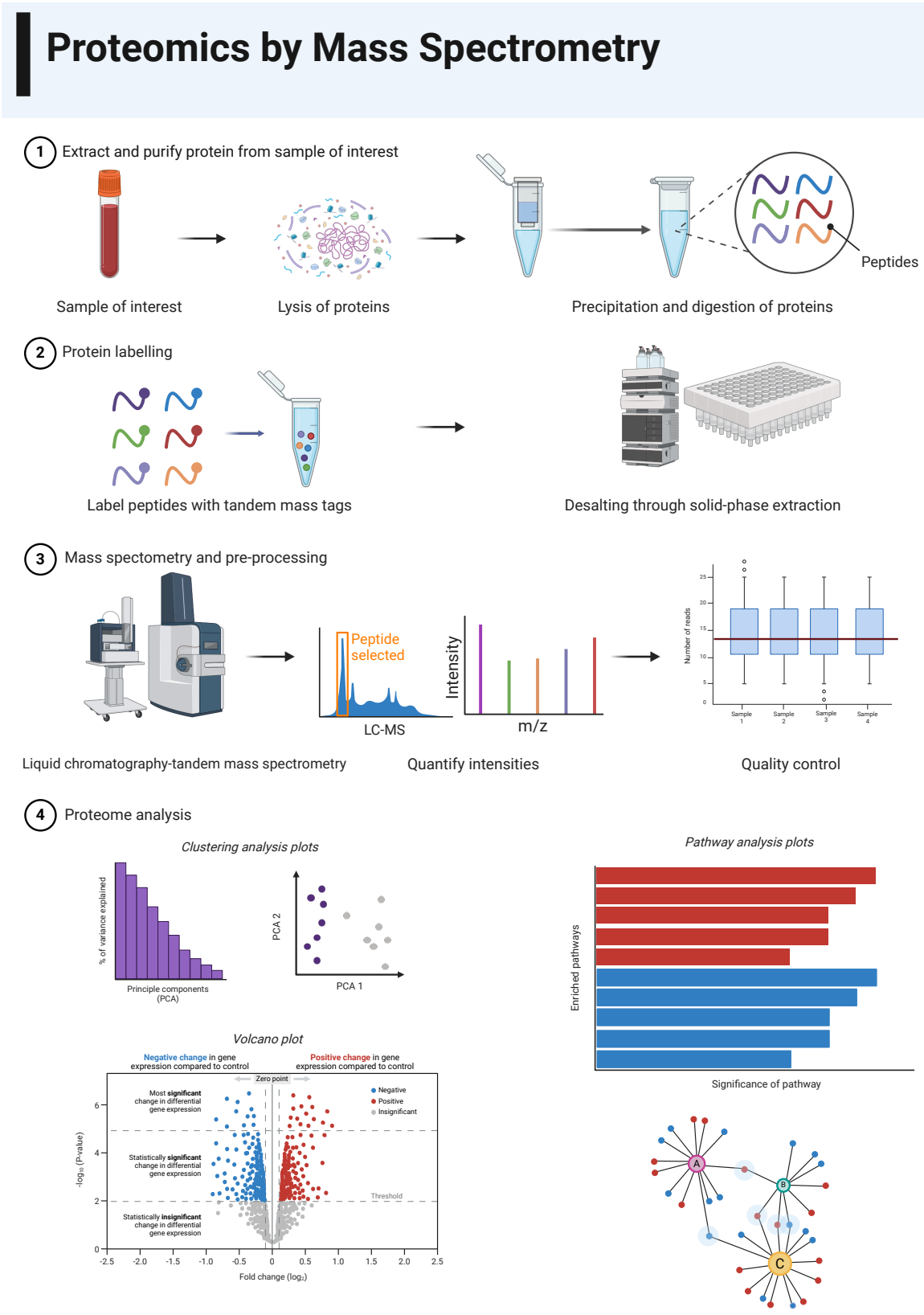


Figure 1.4 Proteomics by mass spectrometry
Proteomics protocol by mass spectrometry from sample preparation to proteome analysis. (1) The protocol is initiated with lysis of proteins from sample of interest, and the digestion of the sample into peptides. (2) Following this, the peptides are labelled for multiplexing by mass tandem tags, and undergo desalting to purify from impurities such as salts. (3) The purified sample is then passed through liquid chromatography mass spectrometry for analysis, the reading intensities are analysed and samples are passed through quality check measurements. (4) Finally, the proteome is analysed, often starting with clustering analyses to identify trends within samples and differential expression analysis to identify proteins with distinctive expression in group(s) of interest in comparison to controls. Finally, pathway analysis can be performed to identify biological pathways enriched in the differentially expressed proteins. Figure generated using Biorender.

Explorations of specific proteins in a simple protein mixture is best done through top-down proteomics, which allows for high coverage and characterisation of proteins. However, this technique lacks sensitivity and high-throughput capacity. In contrast, bottom-up (otherwise known as shot-gun) proteomics utilises liquid chromatography-mass spectrometry LC-MS/MS for a greater coverage of proteins within a sample, with the increased sensitivity required for a global analysis.[180] However, a disadvantage of the shot-gun approach is the favouring of more abundant proteins, with bias against low abundance proteins.

High-throughput experimental designs require careful consideration of sample numbers, as a method of reducing batch differences in multiple experiments. For this reason, multi-plexing was made possible by protein labels or tags to quantify and identify proteins, a more common choice in the field due to current instrumental errors and variability within runs (Figure 1.4B).[181] The utilisation of chemical or metabolic tags allows for a homogenous analysis of samples and quantification in a single experiment, reducing variability and batch effects. An example of commonly used tags is tandem mass tags (TMT) reagents, consisting of an amine-reactive group used for labelling and identifying each sample, attached with a mass normalization group and a reporter ion group. While the structure and mass of each TMT reagent is identical, the difference is in the distribution of five heavy isotopes within the reporter ion and normalisation groups, measured as the reported ion size during mass spectrometry.[182]

Proteomic investigations in the field of neurodevelopmental disorders are biased towards ASD, with a handful of studies examining OCD and no current study exist for Tourette syndrome. The majority of ASD proteomic studies have utilised plasma – while this sample type is challenging to use in mass spectrometry due to the large rage in protein expression, current development in technologies and workflows is able to circumvent these challenges.[183]

Proteomic analyses of ASD plasma samples has implicated inflammation and complement system, and the cytoskeletal system.[184-187] Serum provides another sample type to explore the proteome of individuals with ASD, where it reflects systemic changes occurring throughout the body at relatively stable levels.[188] Proteomic investigations in serum of ASD patients compared to controls have implicated the immune response including the complement system, and lipid metabolism. [189-191] Similarly, proteomic analyses of saliva samples from ASD patients enriched immune response and inflammatory signals,[192,193] and stratified patients based on severity.[194] The currently available proteomic explorations of OCD patients have utilised serum samples to implicate immune pathways including phagocytosis B cell receptor signalling.[195,196]

1.5 Program of research

So far, this thesis has established a framework of justification for investigation of inflammation and epigenetics within neurodevelopmental disorders. The current section will summarise the literature discussed above and provide an overview of the contents of this thesis.

Neurodevelopmental disorders are neurological conditions with high comorbidity rates of other neuropsychiatric conditions and NDDs.[2] An estimated 10% of children under the age of five are diagnosed with a NDD.[12] NDDs cause significant impairment to daily functioning, as well as personal, social and economic strain on families. In Australia, the diagnosis of ASD costs a family ~$35,000 annually.[19] A complex interplay of genetic, epigenetic and environmental influences contribute to NDD development.[29-31] Inflammation during the gestational period is thought to result in increased expression of these conditions.[35] A neurodevelopmental continuum has been proposed to better model the distinct disorders that exist, due to the high comorbidity and the shared genetic and environmental risk.[2,37] Neurodevelopment is a highly orchestrated active timeline of events,

beginning from the third gestational week, and extending postnatally throughout late adolescence.[38] Gene expression and epigenetic processes can be affected by environmental factors which impact the extremely organised development of the brain.[35]

Coordinated teamwork from the nervous, endocrine and the immune systems are vital for proper development of the offspring.[45] The immune system is fundamental for protection from a range of insults such as infections, playing a crucial role in the development of the central nervous system by maintaining homeostasis and promoting inflammation.[46,47] Development in utero is a critical period where the gestational environment can have major consequences on the developmental outcome of the offspring.[51] A large body of evidence, including pre-clinical and clinical studies, support the involvement of maternal immune dysregulation during gestation as a risk factor for neurodevelopmental disorders and neuropsychiatric conditions such as ASD.[52] Epidemiological data has linked various infections during pregnancy to an increased risk of neurodevelopmental disorders in the offspring. A chronic inflammatory state in pregnant mothers coupled with genetic and epigenetic risk factors, can result in cellular stress and further inflammation.[74] A plethora of animal models have been generated to investigate maternal immune activation and have contributed vastly to what we know understand regarding NDD risk, and an activated immune response during pregnancy. MIA is thought to act as a 'disease primer' for neuropsychiatric symptoms, increasing susceptibility to effects of other risk factors later in life.[74]

Paediatric Acute Neuropsychiatric Syndrome (PANS) is a clinical entity describing children who present with abrupt onset emotional changes and OCD symptoms. Bidirectional communication of the immune system and the central nervous system are recognised as vital processes contributing to normal brain development. As such, interactions of the two systems are implicated in the pathophysiology of neurodevelopmental disorders

such as ASD, Tourette syndrome, OCD, as well as PANS.[100] Genetics play a crucial part in the aetiology of NDDs, with studies reporting higher rates of ASD in siblings of affected individuals, alongside high heritability rates in twins.[132,133] While monogenic forms of neurodevelopmental disorders exist, they are often associated with significant disease risk, associated with genes highly vulnerable to disruptive variants.[32,33] Coordination of key epigenetic processes including DNA methylation and histone modifications throughout neurodevelopment, form the basis of epigenetic trajectories critical for cell maturation and specification subsequently.[154,155] Utilisation of high-throughput technologies such as RNA sequencing, and mass spectrometry have become a standard within current times, where there is increased instrument accessibility and improved protocol development which account for various models and samples.[160]

1.5.1 Aims of thesis

The program of research within this thesis aims to further investigate the role of inflammation and epigenetics within neurodevelopmental and neuropsychiatric disorders. The first aim is to utilise high throughput experimental techniques such as RNA Sequencing and Proteomics by mass spectrometry to explore biological pathways underlying the mechanism of disease within neurodevelopmental disorders. This is examined firstly in Chapter 2, where publicly available RNA Sequencing datasets utilising postmortem brain samples of individuals with ASD and Tourette syndrome are investigated. Firstly, the datasets are analysed separately to pinpoint dysregulated biological pathways, and further examined conjointly to identify a common signal within these comorbid conditions. In addition, Chapter 3 will focus on two cohorts of children with PANS and examine proteomic changes in peripheral blood mononuclear cells compared to cells from neurotypical controls. Recruitment of two cohorts of children with the PANS phenotype will allow for exploration of proteomic changes within peripheral cells of two NDD groups, in the hopes of replicating

a proteomic signal. Through utilising current high-throughput technologies such an RNA-sequencing and proteomic advances, biological samples from individuals with neurodevelopmental disorders are explored.

The second aim of the work presented in this thesis underly exploring the functional aspect of the immune dysregulations presented within Chapter 4. This exploration utilises peripheral blood mononuclear cells of children with the PANS phenotype, and applied molecular biology techniques to underpin the immune aberrations present within this cohort that differ from those in a control group. A functional assay is developed, which assess immune response through measurement of pro-inflammatory cytokines at the mRNA and protein level following a bacterial challenge. The novelty of the work described lies within utilising several timepoints of LPS stimulation in the cells of PANS patients and controls. Through assessment of key cytokine markers in functional analysis of the immune response in PANS patients compared to controls, a clearer landscape of the biology of PANS may be uncovered.

Chapter 2

Common targetable inflammatory pathways in brain transcriptome of autism spectrum disorders and Tourette syndrome

---

Alshammery, S, Patel, S, Jones, H. J., Han, V. X., Gloss, B. S., Gold, W. A., Dale, R. C. (2022). *Frontiers in Neuroscience*, doi: 10.3389/fnins.2022.999346

This publication has been adapted and reformatted for presentation in this thesis. The published version of this manuscript can be found in Chapter 7.1 Appendix.

2.1 Preamble

Amongst the consistently emergent literature of neurodevelopmental disorders, each new study within the field sparks more questions in our journey of understanding these complex conditions. As highlighted in Chapter 1, investigations utilising high-throughput sequencing data from precious tissues are a resource that can be tapped into using various tools to deepen our understanding of disease. The utilisation of these datasets, increasingly becoming publicly available, not only encourages reproducibility of results, but may enable novel discoveries when combined with other findings. The study presented in this chapter utilises RNA sequencing datasets of post-mortem brain tissues from individuals with ASD and Tourette syndrome, to explore dysregulated genes and pathways. While these individual studies in

ASD and Tourette syndrome exist as solitary studies, the focus of these studies was on the down-regulated neuronal message. In contrast, our investigation focuses on the up-regulated enriched immune findings.

Transcriptomic analyses of individuals with NDDs have pointed towards down-regulated dysregulations involving neuronal and synaptic function, and GABA neurotransmission.[110,170,171] On the other hand, the up-regulated findings generally consisted of inflammation and microglial dysregulation. The neuronal down-regulated signal is associated with the genetic variation of NDDs, while the up-regulated findings have been suggested to be secondary to environmental factors.[110,170,171] The study presented within this chapter will analyse publicly available RNA sequencing datasets from the PsychENCODE Consortium with the aim of identifying dysregulated genes and biological pathways. Identification of dysregulated pathways in ASD and Tourette syndrome can help shed light on the underlying pathophysiology on these CNS conditions. In addition, the shared genetic heterogeneity and high comorbidity rates of NDD conditions employed us to identify commonalities between the ASD and Tourette syndrome datasets.

Within this chapter, I performed all primary and secondary analyses as described below, using open source datasets from the PsychENCODE Consortium. The bioinformatic workflow along with all supplementary material can be found at https://github.com/sarahalshammery/ASDTS.

2.2 Abstract

Neurodevelopmental disorders (NDDs), including autism-spectrum disorders (ASD) and Tourette syndrome (TS) are common brain conditions which often co-exist, and have no approved treatments targeting disease mechanisms. Accumulating literature implicates the immune system in NDDs, and transcriptomics of post-mortem brain tissue has revealed an

inflammatory signal. We interrogated two RNA-sequencing datasets of ASD and TS and identified differentially expressed genes, to explore commonly enriched pathways through GO, KEGG and Reactome. The DEGs (*FDR* < 0.05) in the ASD dataset (n=248) and the TS dataset (n=156) enriched pathways involving inflammation, cytokines, signal transduction and cell signalling. Of the DEGs from the ASD and TS analyses, 23 were shared, all of which were up-regulated: interaction networks of the common protein-coding genes using STRING revealed 5 central up-regulated hub genes: *CCL2*, *ICAM1*, *HMOX1*, *MYC* and *SOCS3*. Applying KEGG and Reactome analysis to the 23 common genes identified pathways involving the innate immune response such as interleukin and interferon signalling pathways. These findings bring new evidence of shared immune signalling in ASD and TS brain transcriptome, to support the overlapping symptoms that individuals with these complex disorders experience.

2.3 Introduction

Neurodevelopmental disorders (NDDs), such as autism-spectrum disorders (ASD) and tic disorders including Tourette syndrome (TS), are neurological conditions which commonly co-exist and have shared genetic contributions.[197] ASD is characterised by social communication and language deficits, and repetitive stereotypical behaviour. Tics are repetitive stereotyped movements (motor tics) or vocalisations (vocal tics), and when present for more than 12 months, fulfill a diagnosis of TS. Tics are present in 11-22% of children with ASD, while ASD is present in 12% of children diagnosed with TS.[198-200] Limited disease specific treatments are currently available for NDDs, and management focuses on symptom mitigation and developmental support.[6,201]

The genetic aetiology of neurodevelopmental disorders is thought to be due to variants in multiple genes that converge on common pathways.[34,202] However genetic aetiologies in these disorders are unable to explain the wide phenotypic heterogeneity, instead, the interaction

between environmental and genetic factors are proposed to play an important role in pathogenesis of NDDs. In addition, immune dysregulation and inflammation have long been suggested to contribute to the pathophysiology, where early insults during gestation, such as maternal immune activation (MIA), can impact the development of the foetal brain.[36,132-134,137-140] MIA, encompassing maternal conditions such as infection, asthma, obesity, autoimmune disease, and psychosocial stress, are associated with increased incidence of NDDs in offspring, such as ASD and TS.[203-206] MIA is thought to act as a disease primer, which in addition to genetic predisposition, results in increased expression of neurodevelopmental disorders.[74] Studies have also shown dysregulation in proinflammatory cytokines such as IL-12, TNF, monocyte chemoattractant protein 2 (MCP-2), and IL-2 in the brains and peripheral blood of individuals with ASD and TS.[107,118,121,129]

Transcriptomic analyses (RNA sequencing) of post-mortem brains from individuals with ASD have shown upregulated genes involved in inflammation and microglial dysregulation.[170,171] Similarly, analysis of post-mortem brain striatum from individuals with TS identified up-regulated genes in immune and inflammatory pathways, and implicated microglial activation as a primary source of inflammation.[207] In both the ASD and TS brain transcriptome studies, the downregulated genes were enriched in pathways involved in synaptic function and GABA neurotransmission, aligning with the genetic variation found in these disorders.[110,170,171] By contrast, the upregulated inflammatory findings were considered more likely to be due to environmental factors or secondary.[110,170,171]

Given the shared genetic heterogeneity and comorbidity of NDDs, there is an increasing need to examine common disease pathways. As inflammation has been reported in brain transcriptomics in both ASD and TS, we examined for shared gene expression between ASD and TS in order to improve our understanding of the pathophysiology of NDDs and provide future potential therapeutic targets.[110,170,171]

2.4 Methods

2.4.1 Data availability, and open-source bioinformatic analysis

Human brain transcriptome data (RNA-seq) from two independent published studies were obtained with authors permission from synapse.org and analysed for differential gene expression and pathway enrichment analysis.[110,171] Unlike TS, where only one study interrogating the brain transcriptome exists, there are a number of studies investigating ASD brain transcriptome.[170-172,208,209] The current ASD dataset was chosen as it presented the largest cohort of samples.[170,171] The ASD data were downloaded from synapse.org (ID: syn8234507) as count files, and RNA-seq metadata of 42 ASD cases were matched with 43 neurotypical controls (NC).[171] The pre-frontal cortex (PFC) region was chosen for the ASD analysis given the large sample size with matched controls. The TS data was downloaded as BAM files from synapse.org (ID: syn3158906), which included putamen and the caudate nucleus regions from 9 TS cases and 9 normal controls.[110] The bioinformatic workflow, including all utilised code, quality control figures, and supplementary figures and tables can be found at https://github.com/sarahalshammery/ASDTS.

2.4.2 Demographic and clinical variables of cases and controls

2.4.2.1 Autism spectrum disorder

A total of 42 ASD cases and 43 normal control PFC samples were utilised in this analysis (Supplementary Table 1).[171] The ASD cohort selected (n=42) consisted of nine female cases (21.43%) and 33 male cases (78.57%), with mean age of 26.38, median of 22.5 and range of 2 – 67 years. The normal control cohort selected (n=43) comprised of nine females (20.93%) and 34 males (79.07%), with mean age of 28.63, median of 24, and range of 4 – 60 years. A Mann-Whitney test indicated no significant difference (U = 831, *P value* = 0.5295) between the ages of the ASD and normal control cohorts. The full demographic data can be accessed from https://doi.org/10.7303/syn12080241.

2.4.2.2 Tourette syndrome

A total of 9 TS cases and 9 normal control caudate nucleus and putamen samples were included (Supplementary Table 1).[110] The TS cohort (n=9) entailed four female cases (44.44%), and five male cases (55.56%) with mean age of 62.77, median of 52, and range of 29 – 84 years. The normal control (NC) cohort (n = 9) consisted of four (44.44%) females and five males (55.6%) with mean age of 58, median of 52, and range of 4 – 60 years. The full demographic data is in the supplementary material of the original study (See their Supplementary Table 2[110]). There was no statistical differences in the age of the TS cases in comparison to normal controls.[110]

2.4.3 Data quality control

The ASD dataset was prepared and sequenced as described (www.doi.rog/10.7303/syn4587615), reads were mapped against the Genome Reference Consortium Human Build 37 (GRCh37, otherwise known as hg19). The TS dataset were mapped against GRCh37 (hg19), and gene level counts for reference sequence (RefSeq) genes were assessed using HTSeq-count.[110] The raw counts for each dataset were converted to the counts per million (cpm) scale and filtered by expression using the *filerthyexp* function.[210] The data was normalised as per the EdgeR guide using Trimmed Mean of M-values (TMM) normalisation.[207]

2.4.4 Differential gene expression analysis

Genes with an *FDR* of < 0.05 following differential gene expression analysis of each dataset were considered differentially expressed genes (DEGs) in this investigation. The DEGs were identified by a quasi-likelihood (QL) negative binomial (NB) generalised log-linear model (glmQLF). Genes with a logFC >= 0 were considered to be up-regulated, and those below 0 were down-regulated. DEGs were visualised through a volcano plot using the ggplot 2 package.[211]

2.4.5 Pathway and Network enrichment analysis

Enrichments of the DEGs were identified through an over-representation analysis using Gene Ontology (GO) Biological Process, Reactome and the Kyoto Encyclopedia of Genes Genomes (KEGG), through the ClusterProfiler package (False Discovery Rate (*FDR*) < 0.05).[212-218] These are databases which allow genes to be grouped based on their relationships (GO), or the participation in pathways (Reactome and KEGG). For the main individual analyses, pathways enriched by less than 10 genes were excluded. Given the perceived more significant mechanistic insights of the Reactome results, they are presented in the main text, whereas GO and KEGG are presented in the supplementary material.

The protein-coding DEGs which were common to both the ASD and the TS DGE analyses, were visualised using a protein-protein interaction (PPI) network through the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; https://string-db.org/), with an interaction score > 0.4, and default active interaction sources.[218] The PPI network from the DEGs common to both ASD and TS datasets were further imported into Cytoscape.[219] CytoHubba, an app for Cytoscape was used to identify hub genes by ranking nodes by network features through the MCC method.[220] The expression of the hub genes in the disease cohorts compared to controls were visualised using the ggplot 2 package.[211] A Shapiro-Wilk test was utilised to test normality of the hub genes' counts.

2.5 Results

2.5.1 Transcriptional signatures

To identify relationships within the cases and their respective controls, we set out to explore differences based on transcriptome signatures. The ASD and TS cases were not observed to be transcriptionally distinct from their respective controls using hierarchal clustering analyses (Supplementary Figure 1 and Supplementary Figure 2; Github).

2.5.2 Differential gene expression

2.5.2.1 Autism spectrum disorders

The DEGs within the PFC of ASD cases compared to neurotypical controls consisted of 239 up-regulated genes and 9 down-regulated genes, represented through a volcano plot (Figure 2.1A). Results of the DGE analysis can be accessed in supplemental material (Supplementary Table 2A-B; GitHub).

Figure 2.1 Volcano plot of differentially expressed genes in ASD and TS
Differential gene expression analysis was performed on the transcripts of A) Autism spectrum disorders (ASD) cases and neurotypical controls and B) Tourette syndrome (TS) cases and neurotypical control samples. The y-axis represents statistical significance (-log10FDR) and the x-axis represents gene expression log fold change (logFC). The top 20 differentially expressed genes were labelled for ease of viewing, all of which had an up-regulated expression in both the ASD and TS datasets.

2.5.3 Tourette syndrome

The DEGs within the striatum of individuals with TS compared to neurotypical controls consisted of 143 up-regulated genes and 13 down-regulated genes, as shown in the volcano

plot (Figure 2.1B). Results of the DGE analysis can be accessed in supplemental material (Supplementary Table 3A-B; Github).

2.5.4 Immune pathways are enriched in ASD and TS brain transcriptome

2.5.4.1 Autism spectrum disorder

To explore enriched terms and pathways in the ASD DEGs, over-representation pathway analyses were conducted through three databases (FDR <0.05). The GO analysis revealed 337 terms, consisting mainly of up-regulated DEGs, and involved many immune response and inflammatory signalling, along with epigenetic terms (Supplementary Table 2C, Supplementary Figure 3; Github). The top 3 GO terms were "humoral immune response", "leukocyte mediated immunity" and "lymphocyte mediated immunity". Over-representation analysis using KEGG revealed 9 pathways, majority of which were enriched by up-regulated genes (Supplementary Figure 4; Github). The top 3 KEGG pathways (based on FDR) were "Systemic lupus erythematosus", "Neutrophil extracellular trap formation" , and "Staphylococcus aureus infection" (Supplementary Table 2D; Github). Enrichment of the DEGs using Reactome revealed 9 pathways, mostly enriched by up-regulated DEGs (Figure 2.2A, C). Of the 9 pathways, the top 3 Reactome pathways (based on FDR and count) were "Interleukin-4 and Interleukin-13 signalling", "Signalling by interleukins", and "Interferon signalling". Overall, 4/9 Reactome pathways were involved in the immune response consisting of cytokine signalling, innate and adaptive immune response pathways, 2/9 pathways were involved in signal transduction, 2/9 pathways were disease related, and 1/9 pathway belonged to gene expression and transcription. A full list of pathways from the three databases can be found in supplemental material (Supplementary Table 2Cf-E; Github).

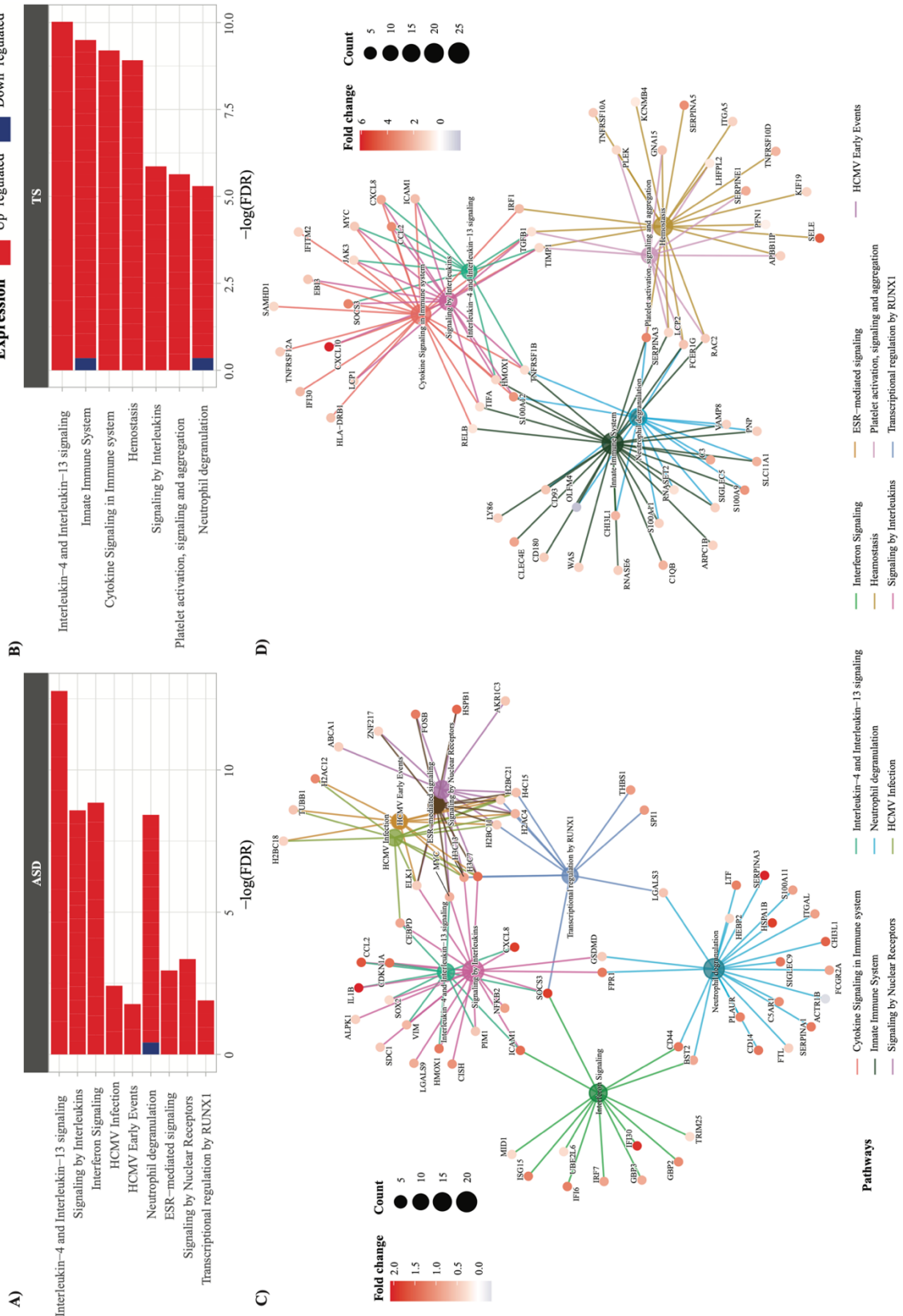


Figure 2.2 Pathway enrichment analysis in autism spectrum disorders (ASD) and Tourette syndrome (TS).
Reactome enrichment analysis (*FDR* < 0.05) of the differentially expressed genes (DEGs) in the brain transcriptome of individuals with ASD compared with controls (A,C) and the DEGs of individuals with TS compared with controls (B,D). Panels (A,B): ASD enrichment results (A) and TS enrichment results (B) presented as bar plots of the top 10 pathways (y-axis), with the statistical significance of the pathways presented by the x-axis. Panels (C,D): Connectivity network (CNET) plots of the top 5 enriched pathways and the interactions of genes that make up the pathways, represented by each gene's fold change. The enriched pathways in ASD (C) and TS (D) are represented by a colour.

2.5.4.2 Tourette syndrome

The DEGs within the TS analysis enriched several terms and pathways from the three databases (*FDR* <0.05). GO over-representation analysis revealed 135 terms, majority of which were enriched by up-regulated genes (Supplementary Table 3C, Supplementary Figure 5; Github). The top 3 enriched GO terms were "immune response", "cell activation" and "leukocyte activation". Over-representation analysis using KEGG did not enrich any pathways. Enrichment of the DEGs using Reactome revealed 7 pathways, most of which were enriched by up-regulated DEGs (Figure 2.2B, D). Of the 7 pathways, the top 3 Reactome pathways (sorted by *FDR*) were "Interleukin-4 and Interleukin-13 signalling", " Innate Immune System" " Cytokine Signalling in Immune system" . Overall, 5/7 Reactome pathways were involved in the immune response consisting of cytokine signalling, innate and adaptive immune response pathways, and 2/7 pathways were involved in the homeostasis pathway. The full list of pathways can be found in supplemental material (Supplementary Table 3C-D; Github).

2.5.5 Differentially expressed genes common to ASD and TS

Of the DEGs from the ASD analysis, and the DEGs from the TS analysis, 23 DEGs were found to be shared. In both the ASD and TS datasets, 23/23 of the common genes had an up-regulated expression. The common protein-coding DEGs were mapped into a PPI network, and their expression in the ASD and TS cohorts was visualised (Figure 2.3). From this network, we identified the top five hub genes using Cytoscape and CytoHubba, which consisted of C-C Motif Chemokine Ligand 2 (*CCL2*) , Intercellular Adhesion Molecule 1 (*ICAM1*), Heme Oxygenase 1 (*HMOX1*), MYC Proto-Oncogene (*MYC*), and Suppressor Of Cytokine Signalling 3 (*SOCS3*; Table 2.1).[219,220] The raw data are presented in log scale for the five hub genes in cases compared to controls, shown for ASD and TS (Figure 2.3B). A full list of the common DEGs can be found in supplemental material (Supplementary Table 4; Github).

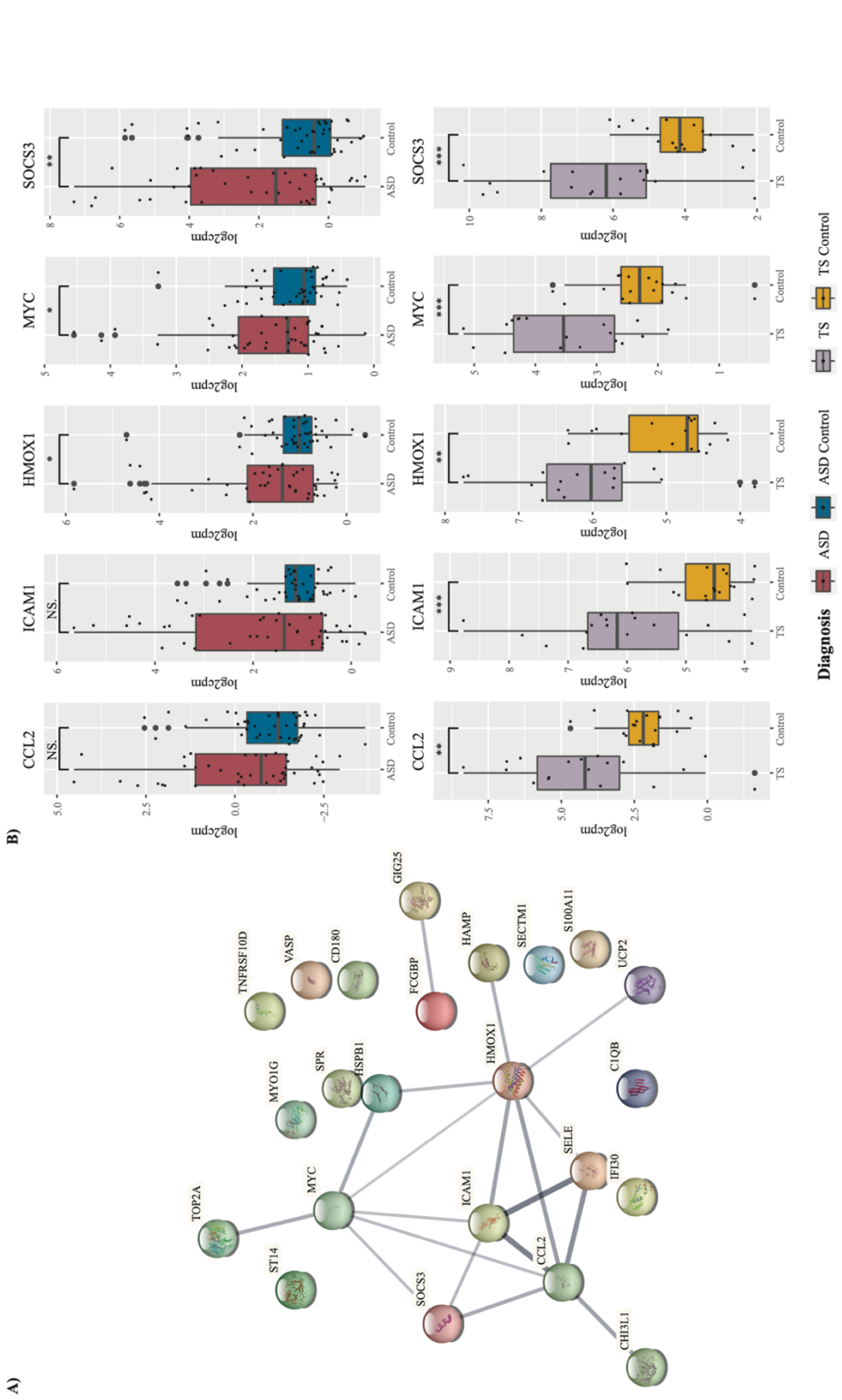


Figure 2.3 Protein network of common differentially expressed genes and expression of hub genes in autism spectrum disorders (ASD and Tourette syndrome (TS).

(A) Protein-protein-interaction (PPI) network of genes found to be commonly differentially expressed in ASD and TS. The network consists of nodes (circles) and edges (lines) representative of predicted functional associations of the common protein-coding genes. Edge thickness is indicative of the strength of predicted evidence. (B) Hub genes central to the network were identified using Cytoscape, and the expression (log2cpm; y-axis) of the five hub genes in cases and neurotypical control (NC) cohorts. Expression of the five hub genes in ASD cases (n = 42; red) against neurotypical controls (n = 43; blue) and TS cases (n = 9; purple) and neurotypical controls (n = 9; yellow). The median is shown as the black line in each group's box, with the small black dots representing each sample. The whiskers on either side of the boxes represent the minimum (Q1-1.5*IQR) and maximum (Q3 + 1.5*IQR) log2cpm excluding outliers. The big black dots represent potential outliers (***p < 0.001, **p < 0.01, *p < 0.05, NS, non-significant, Mann–Whitney–Wilcoxon Test). Network generated using STRING.

Table 2.1 Up-regulated hub genes in ASD and TS

Up-regulated hub genes in autism spectrum disorders (ASD) and Tourette syndrome (TS).

Gene	*Gene Name*	*Type of protein*	*Protein Function*	*Reference*
CCL2 / MCP-1	C-C Motif Chemokine Ligand 2 / Monocyte Chemotactic and Activating Factor 1	Chemotactic cytokine.	Produced by microglia, neurons, astrocytes and mononuclear phagocytes, CCL2 recruits monocytes to the site of infection during inflammatory events.	118,221
ICAM1	Intercellular Adhesion Molecule 1	Immunoglobulin-like transmembrane glycoprotein expressed in the endothelial lumen.	Injury to the blood brain barrier results in microglia and astrocytes surrounding the capillary endothelial cells, where release of ICAM1 is responsible for eliminating antigens.	222
HMOX1	Heme oxygenase 1	Rate limiting enzymes that catalyses degradation of heme into biliverdin, ferrous ion, and carbon monoxide.	As a by-product of catabolising heme, HMOX1 has protective effects in vascular inflammation.	223
MYC	Myelocytomatosis Proto-Oncogene	Transcription factor, binds DNA in a non-specific manner.	Involved in the regulation of immune checkpoints such as CD47 and PD-L1, and regulates expression of cells within the innate and adaptive immune responses.	224,225
SOCS3	Suppressor Of Cytokine Signalling 3	Suppressor Of cytokine signalling family, part of a negative feedback system	Regulates cytokine signal transduction through STAT3 activation, using the gp130 receptor.	226

2.5.6 Common differentially expressed genes in ASD and TS enrich immune pathways

As many of the enriched dysregulated pathways in ASD and TS overlapped, we set out to explore enriched pathways from the 23 DEGs common to both disorders, using overrepresentation analyses through Reactome. The Reactome analysis revealed up-regulated genes enriched in 6 pathways in ASD and 6 pathways in TS, with the top three common pathways involved in "Interleukin-4 and Interleukin-13 signalling", "Interferon gamma signalling" and "Signalling by Interleukins" (Figure 2.4). The full list of pathways can be found in the supplementary material (Supplementary Table 4).

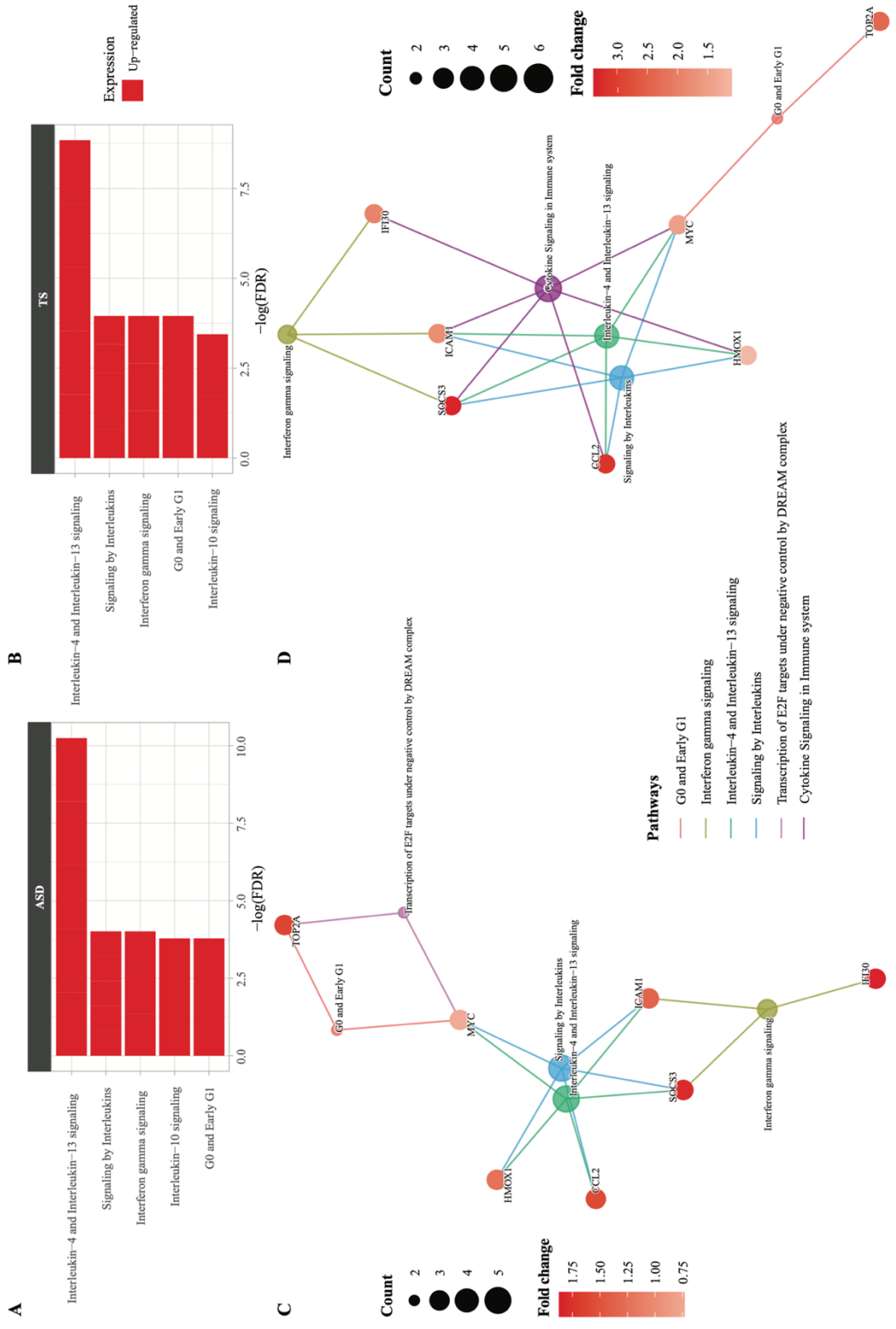


Figure 2.4 Overrepresented Reactome pathways common to autism spectrum disorders (ASD) and Tourette syndrome (TS).
Reactome enrichment analysis (*FDR* < 0.05) of the common differentially expressed genes (DEGs) in the brain transcriptome of individuals with ASD compared with controls (A,C) and individuals with TS) compared with controls (B,D). Panels (A,B): ASD enrichment results (A) and TS enrichment results (B) presented as bar plots of the top 10 pathways (y-axis), with the statistical significance of the pathways presented by the x-axis. Panels (C,D): Connectivity network (CNET) plots of the top 5 enriched pathways and the interactions of genes that make up the pathways, represented by each gene's fold change. The enriched pathways in ASD (C) and TS (D) are represented by a colour.

2.6 Discussion

In this study we investigated enriched immune and inflammatory pathways in post-mortem brain tissue of individuals with ASD and TS, as well as pathways common to both disorders. As the focus of our hypothesis was to explore the immune response present in the seminal datasets, the paper's focal point will be the inflammatory findings. Differential gene expression of the PFC region in ASD revealed that the majority (239 genes) of the 248 DEGs were up-regulated compared to normal controls. Analogous to this, in the striatum of TS, the majority (143 genes) of the identified 156 DEGs were also up-regulated compared to controls. This analysis validates the previous studies of up-regulated genes in post-mortem brains of individuals with ASD and TS.[110,167]

The identified dominant signal of immune response and inflammation from the ASD GO enrichment analysis aligns with studies investigating brain transcriptome and pathology of individuals with ASD, and supports the involvement of astrocytes and activated microglia.[167,171,227] Of interest, the top 3 GO terms (by *FDR*) involved the humoral immune response and leukocyte mediated immunity. These terms were enriched by genes including *IL1b*, *TLR8*, complement genes (*C1QB*, *C1R*,*C2*), and chemokines (*CXCL5*, *CXCL8*) – all of which are involved in inflammation.

The enriched pathways established by the KEGG and Reactome analyses in the ASD cases identified major cellular pathways with therapeutic potential. The differential expression of central immune genes comprising cytokines, and CD cell markers (such as *IL1B*, *CD14*, *CD44*), support the reports of dysregulated cytokine levels in brains of individuals with ASD.[107,228] Next, involvement of complement genes vital in phagocytosis (*C1QA*, *C1QB*, *C1QC*, *C1R*), which play a central role in immunity, response to infection, as well as synaptic pruning, further implicate the involvement of the immune system in ASD.[229-231] In addition, the enrichment of histone subunits fundamental to gene expression and

epigenetic regulation (*H3C13*, *H3C7*, *H2BC11*, *H2BC3*), supports the concept of potential association between epigenetic regulation and inflammation.[232]

Analysis of the TS differentially expressed genes using GO identified numerous enriched immune response and inflammatory signalling terms. The enriched pathways highlighted by the Reactome analysis in TS identified up-regulated DEGs involved in the immune response such as cytokine signalling (*CXCL8*, *CXCL10*, *CCL2*).[118] In addition, pathways involving genes within major histocompatibility complexes II (i.e., *ICAM1*, *HLA-DRB1*) and the S100 family (*S100A9*, *S100A11*, *S100A12*) were enriched. These findings were similarly observed in the original analysis of these TS cases.[207]

Given the substantial comorbidity and overlap between NDDs, we identified genes and pathways common to both ASD and TS. We identified 23 common DEGs, all of which were up-regulated in both disorders. From the 23 common genes, five were determined hub genes: *CCL2*, *ICAM1, HMOX1, MYC,* and *SOCS3,* all of which are involved in the immune response.

Our investigation has confirmed immune and inflammatory pathways are commonly enriched by up-regulated genes in ASD and TS. To further explore these intersecting findings, the 23 genes common to ASD and TS were analysed separately, which repeatedly identified enriched inflammatory pathways involving interleukin and interferon signalling. These pathways were enriched by the hub genes, which have a role in the immune response. We utilised this approach as it allowed for comparison of the same genes within both disorders, while employing the distinct *FDRs* from each analysis, offering insight into the strength of each disorder's signal.

Our current study identified commonly enriched inflammatory pathways, however, several questions regarding the involvement of the immune response in ASD and TS remain unanswered. The cause of the identified inflammatory signals is still ambiguous, in addition

to its nature. Research investigating the source of inflammation in NDDs has suggested it is an environmental or secondary component, rather than genetic[110,167]. In particular, the influence of MIA, which could create a neuroinflammatory environment in offspring, may alter immune signalling pathways and epigenetic control of cell function during the critical periods of development.[36] In addition, the identified inflammatory signal might be casual and pathogenic, or alternatively reactive or protective in origin, which cannot be deduced from the current investigation. Further functional and mechanistic explorations of tissue from individuals with NDDs might elucidate the nature of this inflammation.

Despite our findings, this study has several caveats. Firstly, our analysis involved different brain regions from the two disorders, prefrontal cortex for ASD, and caudate and putamen for TS, as corresponding brain region data was not available for the two disorders at the time of analysis. Secondly, the majority of the samples within the two datasets were not children, as cohorts of paediatric post-mortem brain samples are scarce. Therefore, our analysis represents late-stage disease, and it is unclear if the findings will be reflected in younger cohorts. It is not known whether the inflammatory signal seen in ASD and TS accumulates over the course of life or is present in childhood. Inflammation and the involvement of a dysregulated immune response is present in brain transcriptome data of both ASD and TS. Although classified as clinically distinct disorders, ASD and TS have common genetic aetiologies, along with overlaps in symptoms and comorbidities. We provide biological evidence that there is shared dysregulation of immune response and inflammatory signalling pathways in NDDs. Further studies to understand the cause and potential gene-environmental contribution to this inflammatory signal in these complex disorders is warranted.

Chapter 3

Investigating the proteome: peripheral blood mononuclear cells of children with Paediatric Acute Neuropsychiatric Syndrome

3.1 Preamble

Chapter 2 of this thesis utilised publicly available RNA sequencing datasets of post-mortem brain samples from individuals with ASD and Tourette syndrome. The analysis performed yielded an up-regulated inflammatory signal when the two datasets were analysed separately as well as together. From these findings, our exploration of the immune response within individuals with NDDs, particularly children with Paediatric Acute Neuropsychiatric Syndrome (PANS) was kickstarted using high-throughput technologies. PANS is a neurodevelopmental disorder phenotype, dominated by OCD and often presents with ASD and tic symptoms. The hallmark of PANS is an infection-provoked, abrupt onset of symptoms. Our group utilised high-throughput bulk and single cell RNA sequencing within peripheral blood mononuclear cells of children with NDDs and identified an immune response signature, coupled with translation defects when compared to controls. Our group's hypothesis is that PANS is an epigenetic neuro-immune disorder, and therefore was selected for our investigation. Thus, the natural next step in exploring neurodevelopmental disorder biology was to investigate the proteome of children with PANS compared to that of controls.

We recruited two cohorts of PANS to identify differentially expressed proteins in peripheral blood cells and the corresponding enriched biological pathways, in the hope of improving our understanding of children with PANS.

3.2 Abstract

Neurodevelopmental disorders (NDDs) are heterogenous brain conditions characterised by impaired development of the central nervous system, and include Autism spectrum disorders (ASD), Attention-Deficit/Hyperactivity Disorder (ADHD), obsessive-compulsive disorder (OCD) and Tourette syndrome. These disorders often co-exist within individuals and have been shown to exhibit high clinical variability in symptom presentation. One subset of these disorders is termed Paediatric Acute Neuropsychiatric Syndrome (PANS), a clinical diagnosis given to children presenting with abrupt neuropsychiatric symptoms including obsessive-compulsive disorder and eating restriction. PANS is a condition which is less studied than NDDs such as ASD or OCD. Little is known about the cause of this disorder, but it is thought to be triggered by infections, metabolic disturbances and other inflammatory reactions. While highly regulated gene expression is important to the intricate processes of neurodevelopment, such as control at transcriptional and translational levels, proteins represent a molecular intermediary, providing vital insight into the complex processes within our cells by linking phenotype and underlying mechanisms. With modern high-throughput proteomic technologies, clinical investigations in complex conditions such as PANS are now able to identify proteomic changes in patient samples compared to controls. Proteomic investigations focusing on idiopathic neurodevelopmental disorders are lacking, with those currently available mostly within the field of ASD. The aim of this chapter was to investigate the proteome of peripheral blood mononuclear cells of children with PANS and identify differentially expressed proteins to aid our understanding of this complex neurodevelopmental disorder. To the best of our knowledge, this this is the first study to

investigate the proteome of children with PANS. In this study we identified three key dysregulated pathways in the blood proteome of children with PANS, namely translational (ribosomal) dysregulation, metabolic processes and the immune response. The work within this chapter highlights the dysregulations within the blood proteome of children with PANS, opening the doors for future studies to explore these pathways and to identify biomarkers and potential therapeutic targets for this condition.

3.3 Neurodevelopmental disorders

Neurodevelopmental disorders are heterogenous brain conditions characterised by impaired brain development, with complex interplay of genetic, environmental, and epigenetic regulators.[2,29-31] Neurodevelopmental disorders include a range of conditions such as intellectual disability, communication disorders, Autism spectrum disorders (ASD), Attention-Deficit/Hyperactivity Disorder (ADHD), tic disorders such as Tourette syndrome, and other neuropsychiatric conditions such as Obsessive-compulsive disorder (OCD). These conditions often co-exist within individuals and have been shown to exhibit high clinical variability.[2] An estimated 10% of children under the age of five are diagnosed with a neurodevelopmental disorder.[12] A minority of children with neurodevelopmental disorders harbour a rare pathogenic DNA variant. However, for the majority of children with neurodevelopmental disorders, there are no biomarkers,[233] potentially owed to the wide heterogeneity within individuals and syndromes, coupled with a lack of mechanistic understanding.

While there is a genetic component for neurodevelopmental disorders evidenced from twin, familial and genome-wide association studies, only a minority of cases exhibit highly penetrant monogenic aetiologies.[33] Instead, susceptibility is more likely to arise from vulnerability alleles with low penetrance. For example, the Simons Foundation Autism

Research Initiative (SFARI) database lists ~1000 genes linked to ASD, which include translation components, regulators and genes involved in translational control.[234]

A condition termed Paediatric Acute Neuropsychiatric Syndrome (PANS) described a subset of children present with abrupt onset emotional changes such as obsessive-compulsive disorder and eating restriction, sometimes in the context of other neurodevelopmental symptoms, or pre-existing autism.[99] The literature on this subgroup of children with neurodevelopmental disorders is divided on whether to classify PANS as a separate autoimmune condition, a "discrete biological entity", or a clinical phenotype within the 'neurodevelopmental disorder' spectrum. Our group hypothesises that PANS is not a 'discrete autoimmune entity' but instead a 'clinical phenotype', demonstrating the ongoing interaction between the immune response and central nervous system in some children with neurodevelopmental disorders.[79] We hypothesise that the complex interplay of genetic, epigenetic and environmental influences linked to neurodevelopmental disorders are believed to converge on common molecular pathways.[34,202]

3.4 Introduction to the field of proteomics

Proteins represent a critical link between translated genetic code and cellular activities, providing vital insight into the complex processes within our biology. Proteomics is the study of dynamic protein products in a cell at a given time which collectively dictate cellular function, by use of high-throughput technologies.[235] Preliminary introductions of the term emphasised the static nature of the genome, and focused on the expectation that the proteome is dynamic due to the ability of cells to respond to perturbations.[236] The field initially utilised two-dimensional gel electrophoresis, further coupled to mass spectrometry to assess changes in protein. However, accurate, quantitative, and reproducible measurement of protein abundance, along with variations and modifications can be performed by utilising high-throughput technologies such as shot-gun mass spectrometry.

Mass spectrometry involves the digestion of samples into peptides, which are then analysed depending on their complexity: liquid chromatography (LC) is used for complex samples, and matrix assisted laser desorption ionisation (MALDI) is utilised for simple peptide mixtures (<100 proteins).[178] When wanting to explore specific proteins in a simple protein mixture, top-down proteomics allows for high coverage and characterisation of proteins within a given sample. However, this technique lacks sensitivity and high-throughput capacity. In contrast, bottom-up(shot-gun) proteomics utilising liquid chromatography-mass spectrometry LC-MS/MS allows for a greater coverage of proteins within a sample, with the increased sensitivity required for a global analysis.[180] One disadvantage of utilising the shot-gun approach is the favouring of more abundant proteins, with bias against low abundance proteins.

Clinical investigations into complex conditions such as NDDs require comparisons between a number of different patients and samples types as dysregulations of individual genes or proteins are often small compared to controls, adding an element of difficulty to early proteomic explorations later solved by multi-plexing.[178] Multi-plexing was made possible by introducing the use of protein labels to quantify and identify proteins, a more common choice in the field due to current instrumental errors and variability within runs.[181] The utilisation of chemical or metabolic tags allows for a homogenous analysis of samples and quantification in a single experiment, reducing variability and batch effects. An example of commonly used tags is tandem mass tags (TMT) reagents, composed of an amine-reactive group employed for labelling and identifying each sample, attached with a mass normalization group and a reporter ion group. While the structure and mass of each TMT reagent is identical, the difference is in the distribution of five heavy isotopes within the reporter ion and normalisation groups, measured as the reported ion size during mass spectrometry.[182]

3.5 Translational dysregulations in neurodevelopmental disorders

As discussed in Chapter 1.2, neurodevelopment is a highly orchestrated active timeline of events, beginning form the third gestational week, and extending postnatally throughout late adolescence.[38] Highly regulated gene expression is important to the intricate processes of neurodevelopment, controlled at transcriptional and translational levels.[237]

The first step in gene expression is transcription, initiated by unravelling the double helix of DNA. This exposes the base strands that are utilised as a template to generate the mRNA molecule, where a protein is then translated from the mRNA. The principal components of protein synthesis are ribosomes, small dense particles of ribonucleoproteins consisting of 80 ribosomal proteins (RP) and 4 ribosomal RNAs (rRNAs) arranged into a small (40S) and a large (60S) subunit.[237,238] These are collectively referred to as the 80S in eukaryotes. Assembly of the ribosomal complex at the nucleolus, where copies of the 47S pre-rRNA gene (rDNA) are transcribed by RNA-Polymerase-1 (Pol1), initiate the process of ribosomal production (Figure 3.1). Within the majority of cells, only half of these rDNA genes are transcriptionally active, playing a structural role within chromatin organization and genomic integrity preservation.[239] The newly transcribed 8S, 5.8S, and 28S rRNA are acquired into the nucleus along with the required ribosomal proteins, where the 60S subunit is formed, while the 40S ribosome subunit is formed from the 18S rRNA and ribosomal proteins.[237,238] Following on, the two pre-cursor ribosomal subunits are exported from the nucleus to the cytoplasm for final assembly.

Translation &
Ribosomal biogenesis

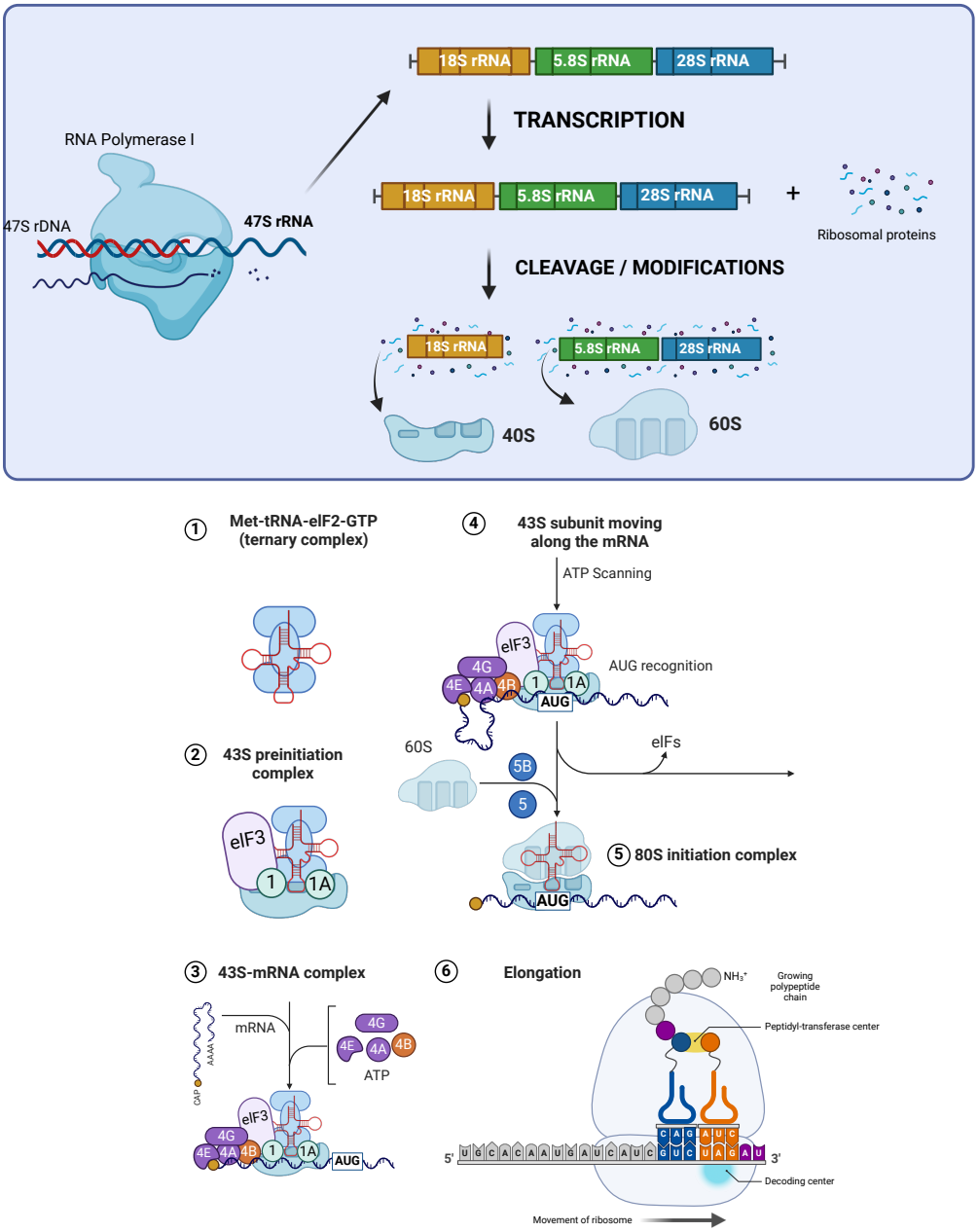


Figure 3.1 Translation and ribosomal biogenesis
Figure outlines Translation and ribosomal biogenesis. In brief, the outlined box illustrates the process of generating the principal components of protein synthesis, the ribosomal subunit. The 47S pre-rRNA gene (rDNA) is transcribed by RNA-Polymerase-1, initiating the process of ribosomal production. The transcribed 8S, 5.8S, and 28S rRNA are acquired to form the 40S and 60S subunits. Next, the numbered processes illustrate the process of translation. This begins with formation of the 43S preinitiation complex, where initiator Met-tRNA (Met-tRNAiMet) is delivered to the 40S subunit by eukaryotic initiation factor(eIF)2-GTP (#1 and #2). Recognition of the 5'-cap (m7Gppp) of mRNA molecules by the eIF4F complex starts initiation (#3), where 43S subunit moves along an mRNA molecule to find the AUG start codon (#4), where the 60S unit then joins to form the elongation-competent 80S ribosome (#5). Elongation is commenced by the ribosome (bound to the Met-tRNA) moving down the mRNA in the 5' to 3' direction to form a polypeptide chain (#6). Figure generated using Biorender.

Protein synthesis, otherwise known as translation, is comprised of initiation, elongation, and termination.[240,241] The process of canonical translation is commenced by formation of the 43S preinitiation complex, where initiator Met-tRNA (Met-tRNAiMet) is delivered to the 40S subunit by eukaryotic initiation factor(eIF)2-GTP (Figure 3.1B). Initiation is commenced by recognition of the 5'-cap (m7Gppp) of mRNA molecules by the eIF4F complex, consisting of eIF4E, eIF4G, and eIF4A. A vital and rate-limiting component, eIF4E recruits the 43S preinitiation complex once binding of the cap end of mRNA is initiated. During the process of protein synthesis, the 43S subunit moves along an mRNA molecule to find the AUG start codon, where the 60S unit then joins to form the elongation-competent 80S ribosome. The process of elongation is commenced by the ribosome (bound to the Met-tRNA) moving down the mRNA in the 5' to 3' direction to form a polypeptide chain. The order of which tRNA bind to the nucleotide codons is determined by the sequence of the mRNA strand, where each three nucleotides constitute a specific peptide. Upon recognition of a stop codon (UAG, UGA, or UAA) the termination process is initiated, and the polypeptide chain is released.

Translation is a highly regulated process, where dysregulations in proteins' expression or quality may disrupt normal cellular processes, and contribute to disease pathogenesis.[240] Disturbances to this process have been implicated in neurodevelopmental disorders, where dysregulation of the mTOR pathway was correlated with severity of ASD in children.[242] In addition, syndromic forms of neurodevelopmental disorders have been associated with mutations in translation factors such as eIFs, and ribosomal proteins.[237,243] Furthermore, environmental disruptions to ribosomal biogenesis can impact translational processes, where they are vital for neurodevelopmental processes such as morphogenesis and synapse formation.[237]

3.6 Clinical investigations of the proteome

As mentioned in Chapter 1.5, the field of proteomics allows for large-scale explorations of protein expression within various cell types and tissues. Similar to other high-throughput methods, proteomics utilising mass spectrometry allows for a thorough investigation of the cellular protein composition to enable a deeper understandings into disorders such as PANS. Our hypothesis is that PANS is a disorder of immune dysregulation, and thus utilising peripheral blood mononuclear cells is of great value to uncovering immune dysregulation.

The field of neurology and neuroscience have utilised a plethora of animal models to investigate the proteome.[177] However, specific animal investigations focusing on idiopathic neurodevelopmental disorders such as PANS are lacking, with those currently available only within the field of autism. One study investigated the brain proteomes of five mouse models by utilising known genetic syndromes within these conditions, and identified dysregulated pathways involved in synaptic signalling, cell transport, and epigenetic terms such as phosphorylation. Although this study focused on genetic models of autism, the terms and pathways identified have been implicated within the clinical literature of ASD in various levels of biology.[244]

The clinical literature on proteomic investigations within neurodevelopmental disorders is scarce, with the majority of studies conducted within autism spectrum disorders (Table 3.1). Within the literature investigating the peripheral proteome of individuals with NDDs, a common theme involving inflammation and the immune response was present, including elevated inflammatory proteins (immunoglobulins, complement, SERPINE), along with kinases, transcription factors and heat shock proteins. Proteomic investigations of autism spectrum disorders utilising peripheral samples have pointed towards an involvement of the immune response and inflammation, corroborating with previous findings within the transcriptome (See Chapter 2 of this thesis). Multiple sample types have

been utilised to investigate the proteome of individuals with these neurodevelopmental conditions. Corbett *et al*, identified differentially expressed proteins in the plasma of children with autism spectrum disorders; the proteins were involved within the complement system and apolipoproteins, further reinforcing the inflammatory evidence present within these conditions.[189] In addition, a number of studies (majority in ASD) have utilised other peripheral sample types such as plasma, urine, saliva and peripheral blood cells.

To add, post-mortem brain tissue analysis has provided insight into synaptic signalling and scaffolding within two different brain regions in autism spectrum disorders and pointed towards dysregulations within protein expression.[245] However, it is important to question the state of proteome stability in these post-mortem samples considering the dynamic range of the proteome along with the brain and its structures. A recent Mendelian randomisation analysis tested the association of plasma protein and neurodevelopmental disorders in children, causally associating higher levels of MAPKAPK3 (involved in kinase signalling) and MRPL33 (involved in ribosomal function) to an increased risk of ASD.[246] The literature of ASD implicates the major pathways that these two mentioned proteins are involved in. While the authors of the above study associated an increase of MANBA (lysosomal protein linked to NDD abnormalities) levels to a lower risk of ADHD, no proteins of risk were identified for Tourette syndrome. To the best of my knowledge, there is no study to have investigated the proteome in children with PANS, providing a gap within our understanding of this complex subgroup of neurodevelopmental disorders.

Table 3.1 Mass spectrometry proteomic studies in periphery of individuals with neurodevelopmental disorders

Reference	*Condition*	*Method*	*Results*
Corbett et al.,[189]	ASD	LC-MS/MS performed on the serum of 69 ASD and 35 controls	Identified ↑FHR1, ↑C1Q, ↑FN1, ↓APOB-100
Castagnola et al.,[247]	ASD	LC-MS/MS performed on the saliva of 27 ASD and 24 controls	Identified STATH, HTN1, PRP
Taurines et al.,[248]	ASD	MALDI-TOF MS on serum of 16 ASD and 16 controls (males)	Identified peaks which differentiated the ASD group from controls, and linked their findings to dysregulations in serotonin, norepinephrine and neurotrophic factor pathways.
Momeni et al., [249]	ASD	MALDI-TOF MS on plasma from 32 ASD and 31 controls	Identified peptide fragments of the C3 complement protein differentially expressed in the ASD group compared to controls.
Ngounou Wetie et al.,[190]		LC-MS/MS on serum of 7 ASD and 7 controls (males)	↑APOA1, APOA4, PON2
Steeb et al.,[191]	ASD	LC-MS/MS on serum from 30 ASD and 29 controls	Differentially expressed proteins generated by splitting by gender. Enriched pathways involved lipid metabolism, cell growth and inflammation.
Ngounou Wetie et al.,[192]	ASD	LC-MS/MS performed on saliva of 6 ASD and 6 controls (Males)	↑FRAT1, KIF14, ITGA6, and ↓AMY1A, CREBBP, TF, and enriched pathways involve oxidative stress, lipid metabolism, immune response and inflammation
Ngounou Wetie et al.,[193]	ASD	LC-MS/MS performed on saliva of 6 ASD and 6 controls (Males)	↑PIP, LTF, IGKC, IGHG1, and ↓ HTN1, STATH, PRH, and enriched pathways involve the immune response and inflammation
Suganya et al.,[250]	ASD	MALDI-TOF MS performed on urine samples from 30 ASD and 30 controls	↑KNG1, IGHG1 and MASP2
Cortelazzo et al.,[184]	ASD	LC-MS/MS on plasma from 30 ASD and 30 controls	↑A2M, SERPINA3, HP, and ↓TF, TTR, RBP4, and enriched pathways involving the acute phase response
Feng et al.,[185]	ASD	MALDI-TOF following other experimental techniques on 15 ASD plasma samples and 15 controls	↑C8A and IGKC, and enriched pathways involving the immune response
Shen et al.,[186]	ASD	LC-MS/MS performed on pooled plasma of 30 ASD and 30 controls.	Differential expression of complement proteins. Enriched pathways involved the complement system, the cytoskeleton cell migration and synaptogenesis.
Shen et al.,[251]	ASD	LC-MS/MS performed on pooled samples from peripheral blood mononuclear cells of 30 ASD and 30 controls.	↑ HSPD1, HSPA5, ACO2, and ↓ANXA11, UGCRC2, STOM, and enriched pathways involved within mitochondrial activation, metabolic processes, endoplasmic reticulum stress and the immune response.
Mota et al.,[194]	ASD	LC-MS/MS performed on saliva from 34 ASD and 41 controls.	Stratified analysis based on degree of severity.
Zhang et al.,[187]	ASD	Data independent acquisition LC-MS on plasma from 45 ASD children and age and gender-matched controls.	45 differentially expressed proteins involved in the complement cascade, extracellular matrix organisation and inflammation, amongst others. Machine learning techniques identified early diagnostic biomarkers of ASD in the proteins BTD and CA1.
Zamanian-Azodi et al.,[195]	OCD	MALDI-TOF performed on serum from 35 women with OCD washing subtype undertaking Fluoxetine therapy and 20 healthy women.	Decreased IG kapa chain C region (IGKC), enriched pathways involved in phagocytosis and B cell receptor signalling.
Zamanian-Azodi et al.,[196]	OCD	MALD-TOF on serum from 12 OCD washing subtype, 12 OCD with fluoxetine and 20 healthy controls (all females)	Differential proteins include GC, HP, HPX and IGKC, gene ontology performed on genes of interest, of which is inflammation.

ASD = Autism spectrum disorder, OCD = Obsessive-compulsive disorder, LC-MS/MS = Liquid chromatography mass spectrometry, MALDI-TOF = Matrix assisted laser desorption/ionisation.

3.6.1 Investigating the proteome of children with PANS

Chapter 2 of this thesis involved analysing publicly available RNA sequencing datasets of post-mortem brain samples of individuals with ASD and Tourette syndrome. The analysis performed yielded an up-regulated inflammatory signal, which was further explored by the group in the context of neurodevelopmental disorders. Our group also utilised high-throughput bulk and single cell RNA sequencing performed on peripheral blood mononuclear cells of children with neurodevelopmental disorders and identified an immune response signature, coupled with translation defects when compared to controls.

The central dogma of biology explains the flow of genetic information in a species from DNA to RNA to protein. Therefore, perturbations at each step may have detrimental effects and consequences on the one after it.[252] Proteomic expression levels can be determined by multiple factors of which are translation rates, protein synthesis delay and transport of proteins. Assessment of correlation between mRNA and protein levels is typically done by two methods: (1) across gene-correlations, where concentrations of several proteins are measured under the same condition, and (2) within gene-correlations, where one protein can be correlated with the variation of its coding mRNA across different conditions, tissues, and individuals.[253] From our group's work, the natural next step in exploring neurodevelopmental disorder biology is to investigate the proteome. While transcriptomic analysis provides insights into transcriptional regulation of individual genes, genes may give rise to multiple protein isoforms as a result of alternate splicing, further aiding the exploration of the proteome within these conditions. The multi-factorial origin of neurodevelopmental disorders, along with the results described above pointed towards a need to explore the proteome within these conditions. Thus, the goal of this chapter was to develop a pipeline of analysing the proteome of peripheral blood mononuclear cells of children with PANS, to

identify differentially expressed proteins when compared to controls. The design of the study is highlighted in Figure 3.2 below.

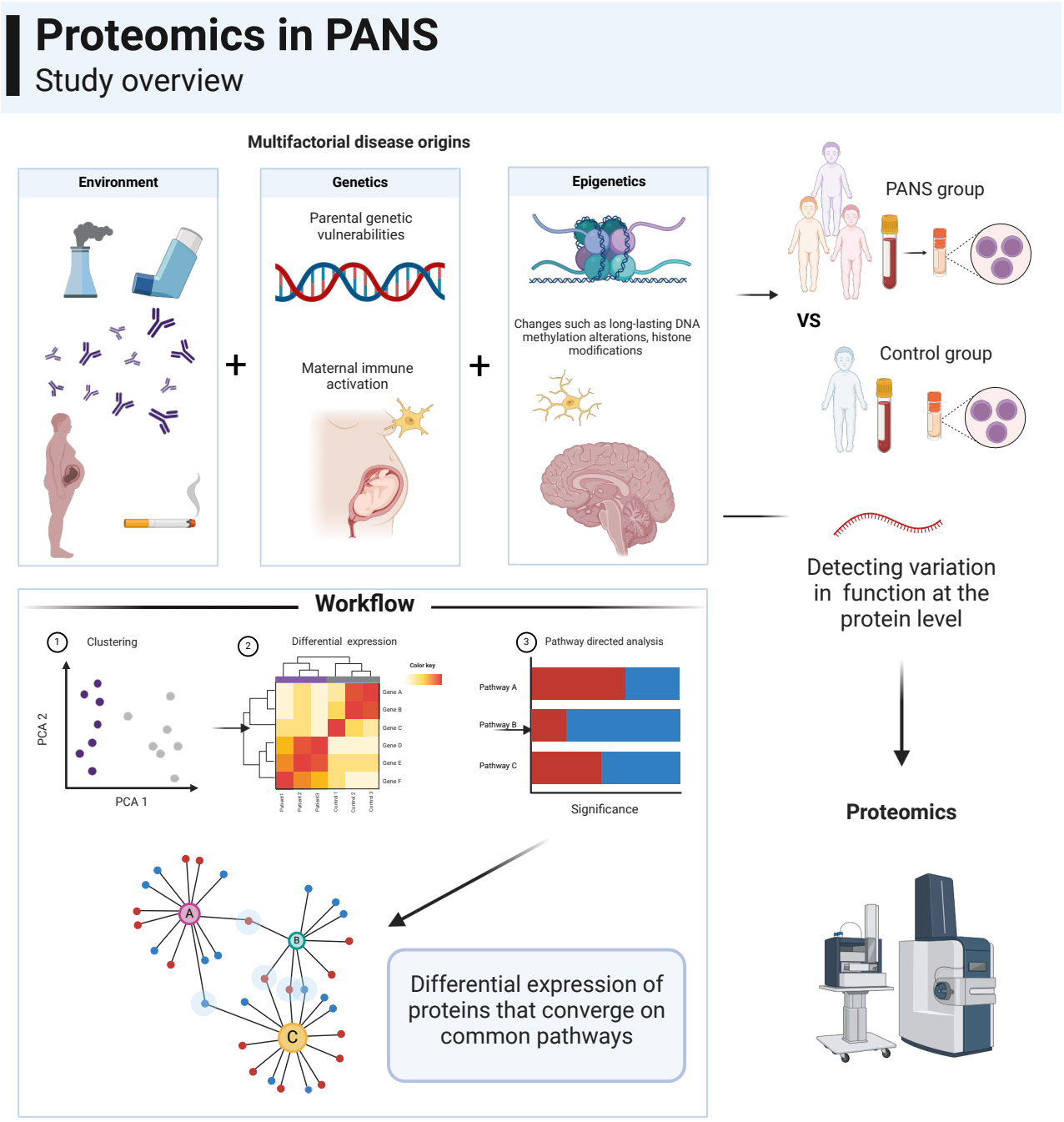


Figure 3.2 Study overview
Study overview of the proteomic investigation utilising peripheral blood mononuclear cells of children with Paediatric Acute Neuropsychiatric Syndrome (PANS) compared to a control group. The disease origin of neurodevelopmental disorders includes environmental, genetic and epigenetic factors, which contribute to disease pathogenesis. The aim of this study involves investigating variation in protein level through mass spectrometry. The workflow involves utilising bioinformatics to investigate protein reads to perform clustering analysis, differential expression, and pathway analysis. Out group's hypothesis is that differentially expressed proteins will enrich biological pathways which will have commonality in patients with neurodevelopmental disorders. Figure generated using Biorender.

The overall hypothesis of this study focuses on a dysregulated innate immune response present in children with PANS. In Chapter 2, a transcriptome inflammatory signal was identified in post-mortem brain samples of individuals with ASD and Tourette syndrome. Therefore, we wanted to focus our next investigation to investigate the proteome of NDDs, and assess the immune response at the protein level. The inaccessibility of the brain necessitates the application of peripheral cells in understanding the pathophysiology of neurodevelopmental disorders. As the hypothesis involves an abnormal immune system, our group has utilised peripheral blood cells to investigate this signature as a valid sample type. Therefore, the aim of this chapter was to investigate the proteome of peripheral blood mononuclear cells of children with PANS to identify differentially expressed proteins. From this, sample enrichment of dysregulated pathways between patients and matched controls will aid our understanding of these complex neurodevelopmental disorders.

3.7 Method

3.7.1 Cohorts

Children clinically diagnosed with PANS with atypical, abrupt-onset symptoms relating to these conditions and aged and sex matched controls were recruited from a specialist neurodevelopmental disorders clinic at the Children's Hospital at Westmead.

Two cohorts (each n=4 per cohort) of children with PANS were recruited along with age and sex matched controls. Children with PANS fulfilled criteria as per section 1.2.6. Briefly, PANS criteria are fulfilled (i) abrupt, dramatic onset of OCD or severely restricted food intake and (ii) concurrent presence of additional neuropsychiatric symptoms, (with similarly severe and acute onset). The samples were collected during the chronic phase of the disorder, rather than the acute phase, and no case was experiencing a flare up. In addition, patients and controls were screened to ensure they had no infections in the two weeks preceding sample collection. Each cohort's patient and control demographics are shown in tables

below. Within cohort 2, a patient sample (PANS3) failed to reach QC and was therefore excluded from the analysis. The demographics and clinical variables of the patients in Cohort 1 and Cohort 2 are presented in sections 3.7.1.1 and 3.7.1.2, respectively.

3.7.1.1 Cohort 1

ID	PANS1	PANS2	PANS3	PANS4
GENDER	M	M	M	F
FAMILY HISTORY NDD OR PSYCHIATRY	Nil	Anxiety, depression (mat)	Anxiety, depression (mat)	ASD (pat)
FAMILY HISTORY AUTOIMMUNE OTHER	Encephalitis (bro)	Hashimoto thyroiditis (Mat)	-	Hashimoto thyroiditis (Mat)
PRECEDING NDD NP	Tics	Mild separation anxiety	ASD	-
AGE AT FIRST PANS	10	10.5	3	12
TRIGGER OF FIRST EVENT	URTI (one week)	URTI (one week)	Infection, mouth ulcer	-
PREDOMINANT PANS SYMPTOM	OCD	OCD (confessional), separation anxiety	OCD, emotional dysregulation, rage	Separation anxiety, eating restriction, OCD, repetitive movements
DURATION FIRST PANS EVENT	1 month	3 months	1 month	4 months
TIMING OF SAMPLE RELATIVE TO FIRST EVENT	2 years	2 years	6 years	8 months
DURATION FOLLOWUP	5 years	4yr	10 years	4 years
FURTHER NO. PANS EVENTS	4/year, any infection	3/year, infected skin triggers	2-3 per year	1 per year
CURRENT NDD NP DIAGNOSTICS	Tourette, OCD, depression	OCD, separation anxiety, ADHD	ASD, Tourette, OCD, rage,	OCD, anxiety
CONVENTIONAL TX	Sertraline, fluoxetine, quetiapine, lithium, ECT	Sertraline, quetiapine, clonidine, Ritalin LA	Ritalin, fluoxetine, quetiapine, aripiprazole	Guanfacine, fluvoxamine
IMMUNE TX	IVIg	IVIg, azithromycin	IVIg, ruxolitinib	IVIg

ADHD = Attention deficit hyperactivity disorder. ASD = Autism spectrum disorders. **ECT** = . IVIg = Intravenous immunoglobulin. NDD = Neurodevelopmental disorders. NP = Neuropsychiatric. OCD = Obsessive compulsive disorder. Tx = Treatment. URTI = Upper respiratory tract infection.

3.7.1.2 Cohort 2

ID	PANS1	PANS2	PANS3	PANS4
GENDER	F	M	F	M
FAMILY HISTORY NDD OR PSYCHIATRY	Schizophrenia (pat)	-	-	Anxiety, ADHD (Mat)
FAMILY HISTORY AUTOIMMUNE OTHER	Natural killer fertility (Mat)	CVID (Mat)	Vitiligo and Hashimoto thyroiditis (mat)	-
PRECEDING NDD NP	-	Mild speech delay	-	-
AGE AT FIRST PANS	4.5	4.5	3.8	4.5
TRIGGER OF FIRST EVENT	Gastro then tonsillitis, then URTI,	Viral infection	Vaccine MR and tonsilitis	Perianal strep infection
PREDOMINANT PANS SYMPTOM	Regression, confusion, OCD, aggression, tics, cognitive decline, anxiety	Separation anxiety, OCD	OCD, sensory issues	OCD
DURATION FIRST PANS EVENT	4 months		2 months	2 months
TIMING OF SAMPLE RELATIVE TO FIRST EVENT	2 years	8 years	6 years	4 years
DURATION FOLLOWUP	5 years	5 years	7 years	7 years
FURTHER NO PANS EVENTS	2 per year, infection provoked	3 per year, all infections	4 per year, infection provoked	2-3 per year, infection provoked
CURRENT NDD NP DIAGNOSTICS	ASD, mild ID, OCD, aggression, tics,	Word finding, ADHD, OCD, depression	OCD, sensory issues	ADHD, OCD
CONVENTIONAL TX	Lamotrigine, aripiprazole	-	Sertraline	Sertraline
IMMUNE TX	IVIg	IVIg	IVIg, ruxolitinib	IVIg

ADHD = Attention deficit hyperactivity disorder. CVID = combined variable immune deficiency. NDD = Neurodevelopmental disorders. ID = Intellectual Disability. IVIg = Intravenous immunoglobulin. MR = Mumps Rubella. NP = Neuropsychiatric. OCD = Obsessive compulsive disorder. Tx = Treatment. URTI = Upper respiratory tract infection.

3.7.2 Sample collection

The objective of this investigation was to measure the chronic immune profile of peripheral blood mononuclear cells (PBMCs); thus, sample collection was conducted from patients in the chronic state and not acute phase (on average, sample collection was performed 2 years after a PANS diagnosis). Fresh peripheral blood was drawn into sodium citrate tubes, and peripheral blood mononuclear cells (PBMCs) were processed within 6 hours of sample collection. PBMC isolation was performed using density gradient centrifugation. Firstly, blood was diluted 1:1 with wash buffer (phosphate buffered saline and 10% fetal bovine serum) and layered on 4.5ml of ficoll paque (Merck) in 15ml SepMate tubes (Stemcell). The blood was then centrifuged at 1200g for 15 minutes, and careful pipetting of the clear band (which contains the PBMC faction) was extracted into a separate tube. This cell population was washed twice with wash buffer and counted, before resuspending in 200ul of 1x lysis buffer (contents in Box 1). Lysed proteome samples was then vortexed briefly and heated at 85C for 10 minutes before storing in -80C.

BOX 1 - Lysis buffer used to lyse peripheral blood mononuclear cells.

REAGENT	PURPOSE
0.8% Triton X-100	Detergent utilised to break open the cell and expose cellular components.
50mM HEPEs 7.5 pH, with NaOH	To maintain pH.
2mM phenylmethylsulfonyl (PMSF)	Serine protease inhibitor which prevents the degradation of proteins during cell lysis from proteases.
EDTA free protease inhibitor	Chelating agent utilised to prevent protein degradation.
PhoSTOP	Phosphatase inhibitor cocktail, stops the de-phosphorylation of proteins during cell lysis.

3.7.3 Sample processing

This section describes the processing of the lysed PBMC proteomic samples at the Proteomics facility at the Children's Medical Research Institute. Sample handling, and mass spectrometry were performed by Dr Mark Graham at the proteomic facility. A summary of the protocol is seen in Figure 3.3. Secondary analysis following the normalisation steps were performed by me (the student)

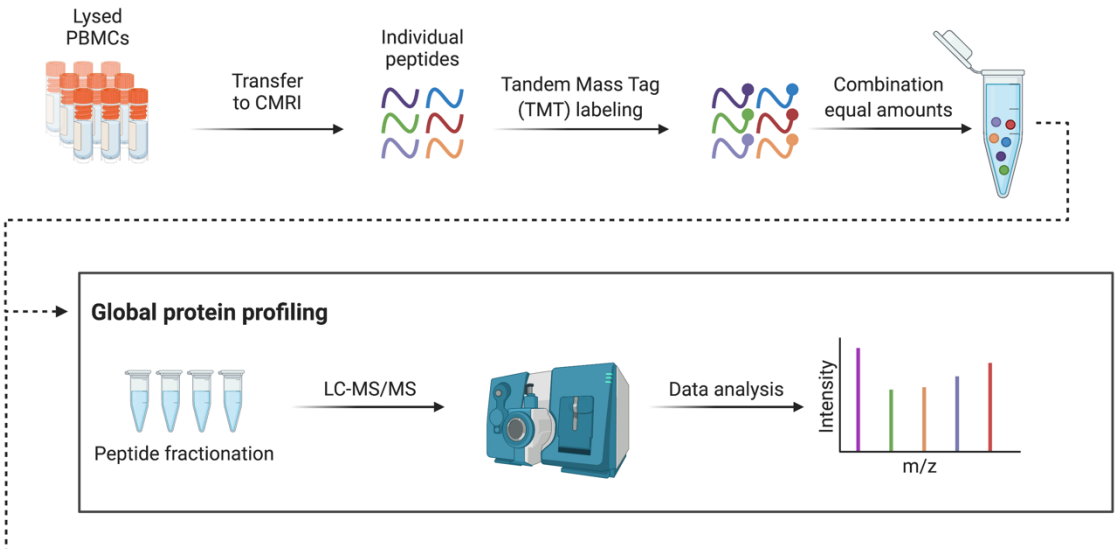


Figure 3.3 Method overview
An overview of the methods utilised for the proteome analysis performed. Generated using Biorender.

The lysed proteomic fraction from peripheral blood mononuclear cell samples (performed by myself) were sent to the proteomic facility at CMRI once each cohort was completed. Firstly, nucleic acids within the samples were degraded by thawing at 37 °C for 30 min, followed by the addition of 10 units of benzonase. Next, Sodium dodecyl sulphate (SDS) was added to each sample to make up to 2% concentration, along with 10mM of tris(2-carboxyethyl)phosphine to denature proteins and disrupt proteins bonds. A second incubation at 85 °C for 10 min to aid with protein denaturation was performed, followed by cooling of each sample and addition of iodoacetamide (20mM) for 30 minutes at room

temperature. This step allowed for extra protein stability and prevented the re-formation of protein bonds. Next, precipitation of the samples was performed following the chloroform-methanol method, whereby proteins were concentrated, resulting in a pellet for each sample. From there, pellets were dried at 37 °C for 1.5 h to ensure all solvent was evaporated. Protein digestion was initiated by the addition of 20 μL of 7.8M Urea with 100 mM HEPES (8 pH) solution, as well as 3ug of LysC for 12 hours at 28 °C. As multiple enzyme digestions are recommended in proteomic analyses, the samples were subjected to another two steps of digestion using 3 μg of trypsin each, for 8 hours at 28 °C. Next, to measure peptides within each sample, UV absorption was read at 280nm.

While there are many methods to the measurement of protein abundance within a given sample using quantitative techniques, this study utilised the tandem mass tags (TMTpro) 16plex reagents for each of the two cohorts described.[182] There are 16 tags composed of an amine-reactive group utilised for labelling and identifying each sample, coupled with a mass normalization group and a reporter ion group. These tags differ in distribution of five heavy isotopes found within the reporter ion and normalisation groups, responsible for the reported ion size during mass spectrometry. As such, these reagents allow for simultaneous quantifications across multiple samples in a single experiment. For clinical studies such as the one conducted here, this is crucial to reduce variability and batch effects.

The TMT-labelled samples were then prepared for down-stream analyses by desalting through utilising solid-phase extraction (Sep-Pak 3cc tC18 cartridges). This is a crucial step within the protocol, whereby removing salts, detergents, and other impurities can have a major impact on the quality of down-stream analysis. Next, separation of compounds within samples was performed by hydrophilic interaction liquid chromatography separation using a Dionex UltiMate 3000 HPLC system. Collection of the fractions was done in a 96-well plate at 30 second intervals using a Probot. The fractions were then combined

and dried to remove any remaining solvent and reconstituted in 0.1% formic acid to ensure peptide solubility and ionisation.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis is a technique whereby analytes of interest (such as peptides, metabolites) are separated from each other within samples. This technique first utilises liquid chromatography, where separation of analytes is done based on strength of interaction with the mobile and stationary phase, reliant on chemical properties such as the size and charge of the protein. To start separating the samples, the analytes first interact with the stationary phase. When the interaction of analytes is stronger with the mobile phase (the solvent flowing through the chromatographic column), they are eluted.[254] The samples and fractions were analysed using a data-dependent acquisition LC-MS/MS. This is where the mass spectrometers then selects ions to fragment dependent on abundance within the precursor ion scan. The following parameters were utilised for each MS/MS scan:

Table 3.2 Data dependent acquisition
Parameters set for the data-dependent acquisition LC-MS/MS.

PARAMETER	SETTING
Resolution of MS/MS scans	35,000
Automatic gain control	200,000
Max ion time	115 ms
Loop count	12
Isolation window	1.1 m/z
First mass	Fixed at m/z 120
Normalised collision energy	30
Dynamic exclusion	35 s
Ion charge exclusion rate	8

Data Pre-processing Processing of raw LC-MS/MS data for each batch was performed at the proteomic facility using the MaxQuant software (version 1.6.7.0). The parameters utilised are in the table below (Table 3.3), with those not mentioned left as default.

Table 3.3 Proteomics MaxQuant parameters
The parameters used in version 1.6.7.0 of MaxQuant, where the raw proteomics data was processed.

PARAMETER	SETTING
Variable modifications	Oxidation (M), acetyl (protein N-terminus), deamidation (NQ), phospho (STY)
Fixed modifications	Carbamidomethyl (C)
Digestion	Trypsin/P (Max of 3 missed cleavages)
TMTpro Correction Factors	Lot Vj313476
Minimum Reporter Peptide Ion	0.6
Reference Proteome	Homo Sapiens, 78,120 Entries And 20,600 Genes (Downloaded 19/07/2021)
Minimum Peptide Length	6 Da
Maximum Peptide Length	6000 Da
Second peptide search	Enabled
Dependent peptides search	Enabled
Peptide Spectrum matching	1%
Protein discovery rate	1%
Minimum score for modified peptides	40

Normalisation The raw data was imported into the R environment, where a log 2 transformation was performed. Next, as an initial normalisation step, the scaled normalisation function from the limma package was utilised between the samples. While the prominence of data normalisation is acknowledged, there is no standardised technique or gold standard. In order to remove batch effects and other biologically irrelevant artifacts between samples, the remove unwanted variation (*RUV*) R package was utilised.[255] The RUV III method employed in this analysis applies a list of control or "housekeeping" proteins, those with minimal change in abundance between the samples analysed, in this case the cases and controls.[256] This is done by having a design matrix that lists the case and control samples, as well as the list of control proteins.

Differential expression Similar to Chapter 2, where differential expression was performed on transcriptomic data, we employed similar methods to identify differentially expressed proteins from proteomic analyses. Differential expression analysis of proteins was performed using linear modelling, where the cases of each cohort were compared to their matched controls. The limma R package was utilised, with the linear model for comparison

performed by the *lmFit* function and the p-values calculated using the empirical Bayes method *eBayes* function.[257] As with utilising high-throughput data, many statistical tests would result in false-positives. This is why a false discovery rate (FDR) was utilised, by accounting for false discoveries when testing thousands of proteins, known as multiple-hypothesis testing, we can be more confident of each p value generated. The false discovery rate correction was applied to the moderated p-values by calculating the q-values (also known as adjusted p values).[258] Differentially expressed proteins were identified by filtering for proteins with FDR < 0.05.

3.7.4 Data processing

This section and those after were performed by me (the student). The unfiltered and filtered counts were imported into the R environment to explore the data and visually represent the effect of normalisation and filtering. A box-and-whisker plot was generated on the unfiltered and filtered data as using the ggplot package.[259]

3.7.5 Data exploration

Data exploration is a vital part of analysing high-throughput studies, as it allows for methods of quantifying variances or differences within samples analysed. Data analysis in this thesis were performed in the R environment (version 3.5.1, https://www.r-project.org/) with RStudio for ease of viewing.[260,261] Clustering analysis such as hierarchal dendrogram was utilised to explore the data and identify variations between cases and controls. The dendrogram was generated using the *hclust* function,[264] using euclidean distances, which are mathematical distances between two points, within a multidimensional space that explain how similar or dissimilar points are. In the case of interpreting these distances, the shorter the distance is, the more similar two samples are.

In addition, another form of data exploration utilised is dimensionality reduction, a technique that simplifies high-throughput data into the form of principal component

analysis. The protein expression values (counts) of each dataset were utilised to calculate principal components, which allow groupings of samples to be easily identified. Principal component analysis was performed on the logged normalised counts of each dataset, by utilising the *prcomp* function. First, a variance histogram was generated using the ggplot2 package, which identified the percentage of variance explained by each principal component.[211] Next, a scatter plot was generated, plotting the samples against the two components with the highest variability. The dendrogram and principal component analysis plots were combined using the *ggarrange* function from the *ggpubr* package.[262]

3.7.6 Pathway analysis

Pathway analysis was a vital next step in the workflow, as the goal of this study was to explore dysregulated interactions of proteins between cases and controls, which may give novel insight into complex connections in neurodevelopmental disorders. The pathway analysis results have the potential to aid our understanding of these complex disorders, as well as elucidate future mechanisms to focus therapeutics on. The following cutoff threshold were applied in each pathway analysis performed: A minimum of 10 proteins per enriched pathway, a maximum of 500 proteins per enriched pathway, and an FDR threshold of < 0.05. The first cutoff ensures that pathways with <10 proteins are not included in the result, whereby their presence may not provide enough biological information. In addition, the cutoff of <500 proteins per pathway similarly ensures exclusion of common (broad) biological themes which are not meaningful to the results, such as the process of metabolism. Two types of pathway analysis methods were utilised.

Firstly, an over-representation test (ORA) was performed; An over-representation analysis utilises a list of differentially expressed proteins (which are significant after FDR) to identify themes and pathways over-represented within a dataset, compared to what would be expected to by chance. For that reason, the list of differentially expressed proteins is utilised

against a list of all proteins included in the analysis. ORA utilises Fisher's exact test to assess if lists of proteins are significantly enriched within pathways from the databases tested. To identify enriched pathways from the differentially expressed proteins in each cohort, the first over-representation analyses using Gene Ontology (GO) and Reactome through the ClusterProfiler package were performed (FDR) <0.05.[212-214,218] These are databases which allow genes to be grouped based on their relationships (GO), or the participation in pathways (Reactome). The GO database further divides into three subsections: Biological processes, Cellular compartment and Molecular function. The ClusterProfiler functions *enrichGO* and *enrichPathway* were utilised for the GO and Reactome analyses respectively.

The second type of pathway analysis performed was gene set enrichment analysis (GSEA), conducted for all three cohorts to identify enriched pathways. This type of pathway analysis identifies sets of proteins distributed together based on ranking. This study utilised a summary statistic to rank proteins in a descending manner comprised of the expression direction of the fold change (FC) ***** - log10(FDR) of each protein. This statistic is then used for the analysis, with the expectation that the weight of proteins correlates with the phenotype, yielding an enrichment score for each pathway. The enrichment score is based on the cumulative weights of the ranked proteins within a list; proteins clustered at top of the ranked list (i.e. those most differentially expressed) signal an up-regulated expression, and those at the bottom of the list (least differentially expressed) signal a down-regulated expression. A normalised enrichment score is utilised to account for variability within size of each pathway's set list of proteins. The ClusterProfiler functions *gseGO* and *gsePathway* were utilised for the GO and Reactome analyses.

3.8 Results

This section will describe the findings from proteomic investigations of two PANS cohorts. The aim of this research project was to identify dysregulated proteins and biological pathways between children with PANS compared to age and sex matched controls. We utilised two cohorts for this investigation in the aim of validating and replicating our findings, in anticipation of the heterogenous nature of NDD presentation in children.

3.8.1 Quality control and normalisation

Filtering and normalisation are central steps in analysing high-throughput experiments such as proteomic analyses, by accounting for external factors that don't have biological relevance on the resultant expression levels.

3.8.1.1 Cohort 1

The effect of filtration and normalisation can be observed in Figure 3.4, where box-and-whisker plots compare the counts before and after normalisation . The first part of the figure shows the counts of each sample prior to filtering and normalisation, where the second part highlights the counts post filtering and normalisation steps. As part B illustrates, the counts of all samples have similar means (white dots), centring around the median of all samples (black line). From this figure, we can be confident that the data can be carried down for further analysis.

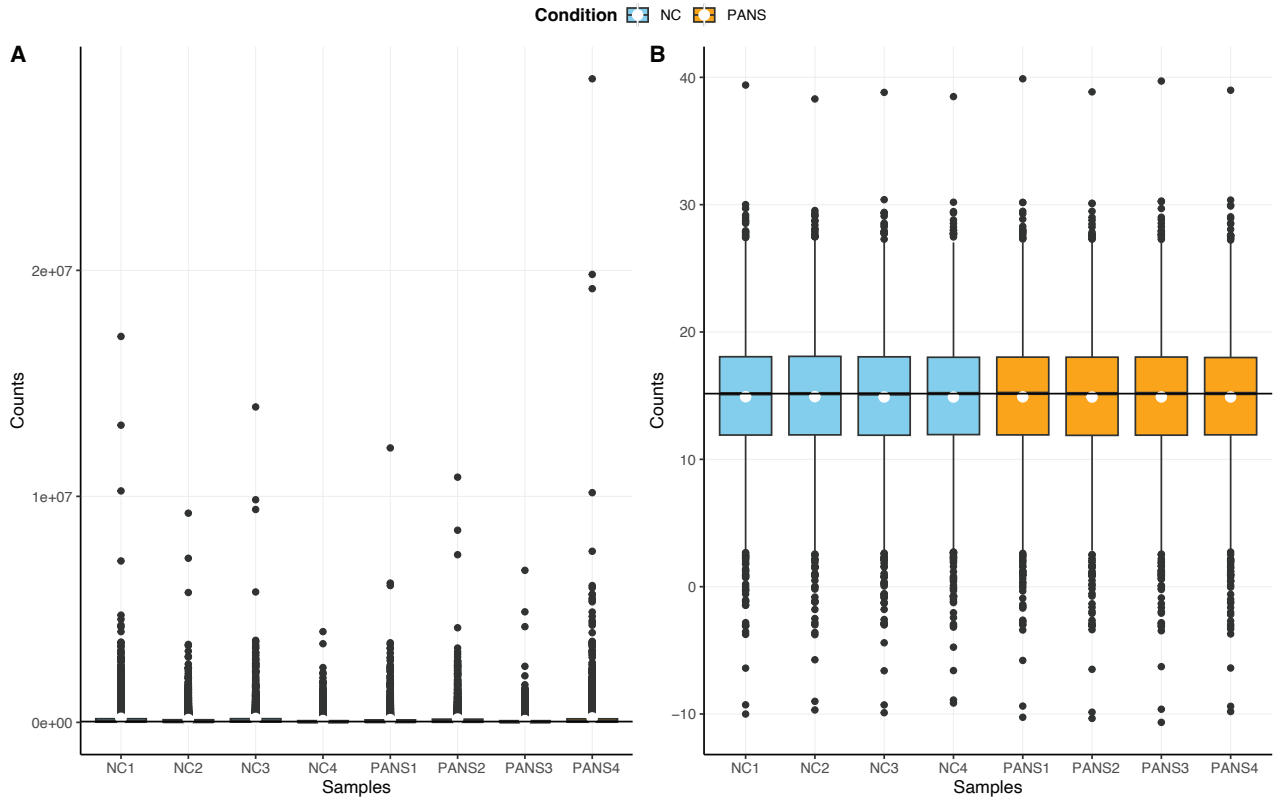


Figure 3.4 Cohort 1 Count comparison pre and post quality control
Quality control visualising the proteome counts from the first cohort of children with Paediatric Acute Neuropsychiatric Syndrome (PANS; Orange) vs neurotypical controls (NC; Blue). Boxplot of the counts (y-axis), against the samples (x-axis). The y-axis scale differs between the two panels due to data skewing following normalisation and filtering. A) Counts prior to filtering and RUV III normalisation. B) Counts after filtering and RUV III normalisation. The black line represents the median of all samples, while the white dots represent the mean of each sample.

3.8.1.2 Cohort 2

Similarly, effect of filtration and normalisation on cohort 2's data can be observed in Figure 3.5, where box-and-whisker plots compare the counts before and after pre-analysis steps. The effect of normalisation is observed by all samples having similar means and medians, which allows us confidence to utilise the data for further analysis.

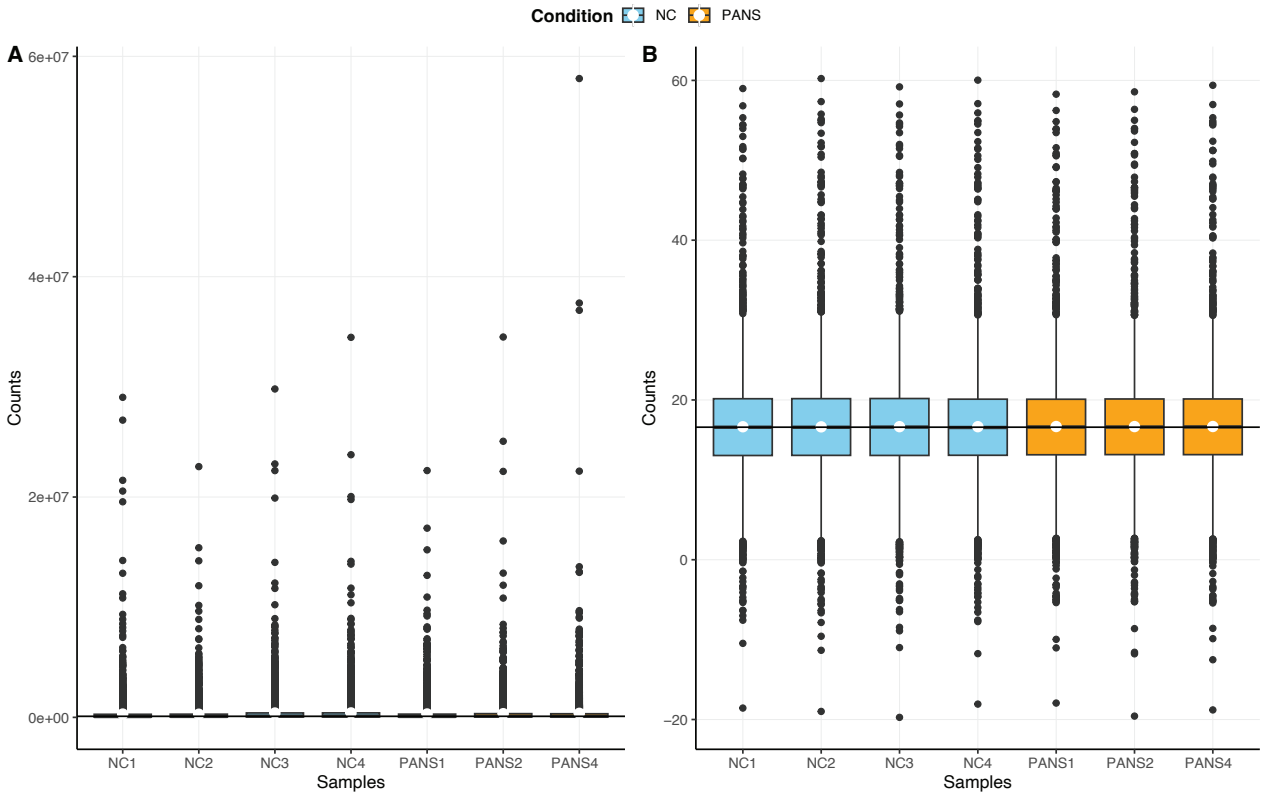


Figure 3.5 Cohort 2 Count comparison pre and post quality control
Quality control visualising the proteome counts from the second cohort of children with Paediatric Acute Neuropsychiatric Syndrome (PANS; Orange) vs neurotypical controls (NC; Blue).. Boxplot of the counts (y-axis), against the samples (x-axis). The y-axis scale differs between the two panels due to data skewing following normalisation and filtering. A) Counts prior to filtering and RUV III normalisation. B) Counts after filtering and RUV III normalisation. The black line represents the median of all samples, while the white dots represent the mean of each sample

3.8.2 Exploratory data analysis

Investigating a dataset using exploratory methods allows for an idea of what to expect prior to performing statistical investigations such as differential expression and pathway analyses. To explore relationships within the cases and their respective controls based on proteomic output, I utilised exploratory data analysis techniques. The first technique explored was a

hierarchal clustering dendrogram, which plots the samples of an analysis and is read from the bottom up. Next, a principal component analysis was performed as another step to explore the data, therefore generating two figures (see Figure 3.6 and Figure 3.7 below). This method visualises the data by reducing the dimensionality, expressed as principal components that account for variations within the data. The variance histogram graphically represents how much of the variation between samples can be explained by each principal component, when is then shown as a dot pot of the samples against the two highest principal components.

3.8.2.1 Cohort 1

To investigate how the samples in Cohort 1 differ based on proteomic readings, I employed data exploration methods such as a hierarchal cluster dendrogram and a principal component analysis (Figure 3.6). First, the dendrogram (upper part of the figure) highlighted great distinction between the patients and controls where a clear separation is seen within the two groups. Samples PANS2 and PANS3 were most similar to each other, followed by PANS1 and PANS4. Similarly, NC1 and NC3 were most similar to each other, followed by NC4 and NC2. The variance histogram indicated that principal components PC1 and PC2 accounted for the highest variability within the cohort, reporting for 71.6% and 13.83% of the variance, respectively. These components were then utilised to generate a scatter plot of the first cohort, where the samples in cases segregated clearly from the controls.

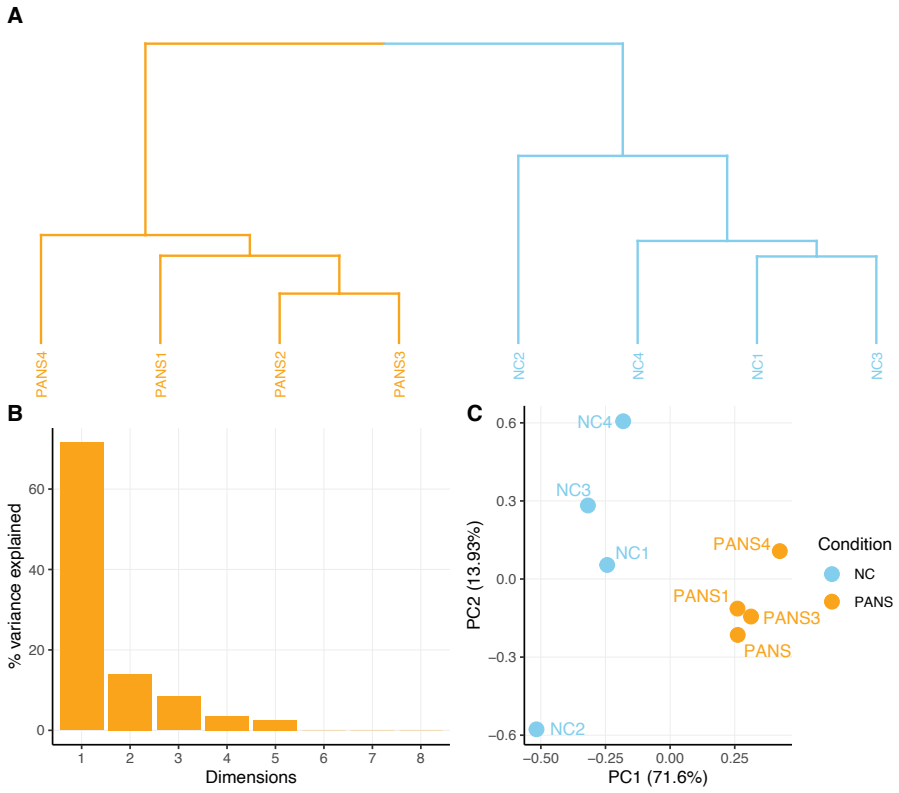


Figure 3.6 Cohort 1 Exploratory data analysis
Exploratory data analysis of the first cohort generated to identify how the children with Paediatric Acute Neuropsychiatric Syndrome (PANS; Orange) vs neurotypical controls (NC; Blue) cluster relative to each other based on the counts. A) Hierarchal clustering dendrogram. B) Variance histogram that identifies how much of the variance within the data can be explained by multiple dimensions. C) Principal component analysis of samples clustering relative to each other in the first (PC1; x axis) and second (PC2; y axis) components. There is clear separation of the PANS samples from the NC samples.

3.8.2.2 Cohort 2

Similar to the steps employed above, clustering analyses such as hierarchal clustering and principal component analysis were utilised to explore the relationship between samples of Cohort 2 (Figure 3.7). From cohort 2, one patient samples (PANS3) failed quality control criteria, and thus there are only three samples utilised in this cohort's analysis. The dendrogram showed segregation of the PANS cases from their respective controls. From the figure, it can be observed that PANS4 and PANS1 were most alike, followed by PANS2. For the controls, NC1 and NC2 were the most correlated, followed by NC3 and NC4. Next, the variance histogram showed that principal component PC1 and PC 2 accounted for the majority of variability within Cohort 2. The principal component analysis plot emphasised

the separation between the cases and controls particularly within the first dimension as expected.

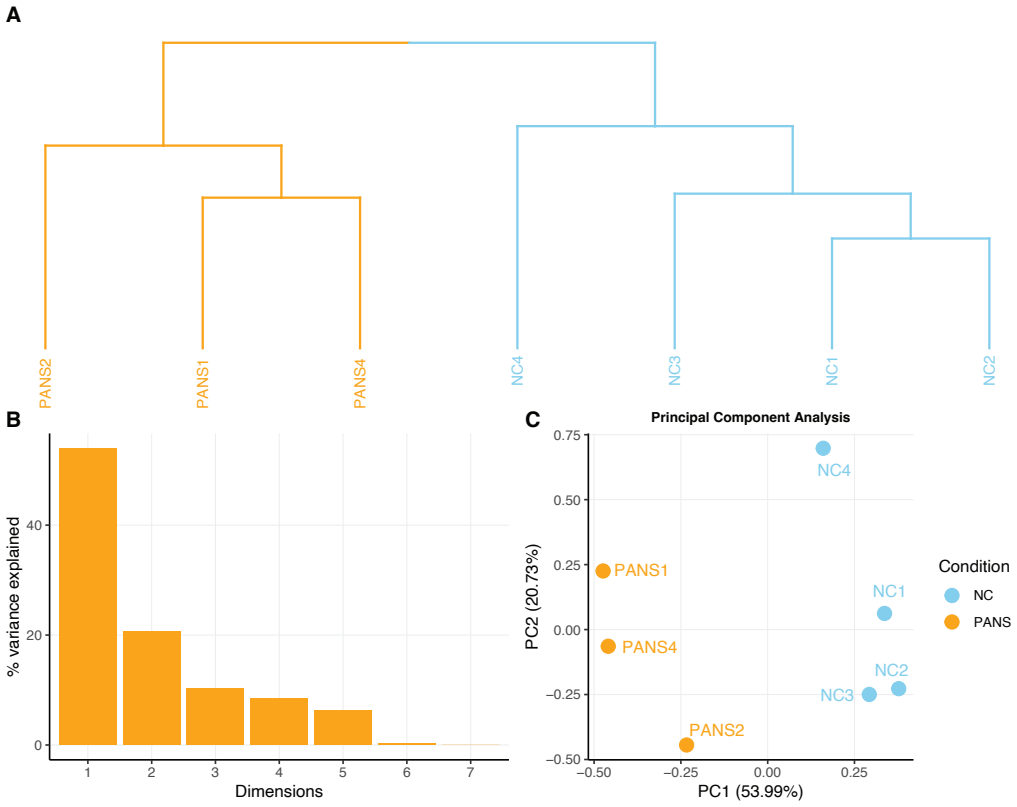


Figure 3.7 Cohort 2 Exploratory data analysis
Exploratory data analysis of the first cohort generated to identify how the children with Paediatric Acute Neuropsychiatric Syndrome (PANS; Orange) vs neurotypical controls (NC; Blue) cluster relative to each other based on the counts. A) Hierarchal clustering dendrogram. B) Variance histogram that identifies how much of the variance within the data can be explained by multiple dimensions. C) Principal component analysis of samples clustering relative to each other in the first (PC1; x axis) and second (PC2; y axis) components. There is clear separation of the PANS samples from the NC samples.

3.8.3 Differential expression

3.8.3.1 Cohort 1

Differential expression to identify significant proteins was performed as per Section 3.7.3 of methods. The analysis identified 4,591 proteins, 2973 of which were recognised as differentially expressed proteins at FDR < 0.05. From this, 1,450 proteins had an up-regulated expressions and 1523 proteins had a down-regulated expression. A volcano plot shows the distribution in Figure 3.8. The top up-regulated proteins included a heat shock protein (HSPB1), a tissue inhibitor of Metalloproteinases (TIMP3) and a glycosyl

phosphatidylinositol-linked glycoprotein (CD109). The down-regulated proteins included a major histocompatibility complex (HLA-DRB1), a histone (H4C1), and a NADH:ubiquinone oxidoreductase (NDUFAF4).

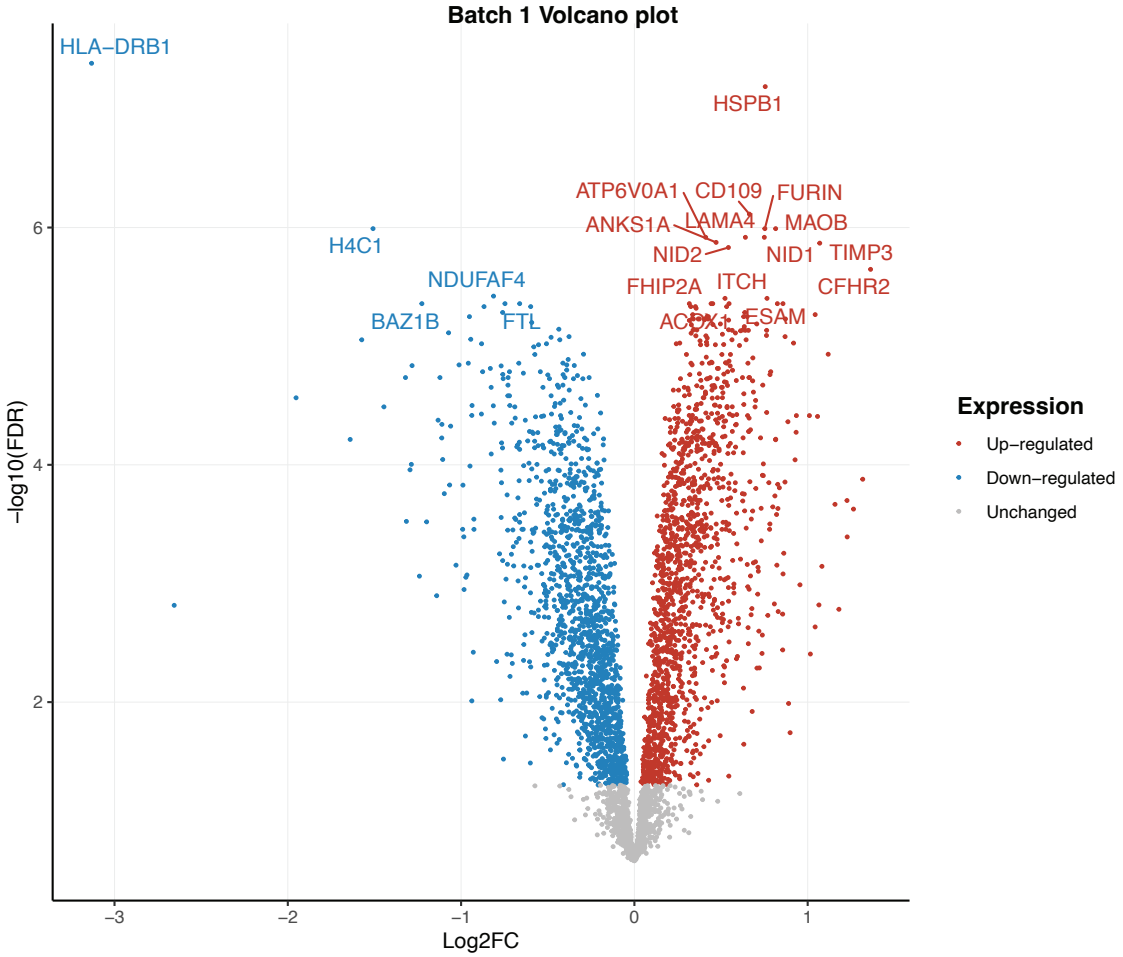


Figure 3.8 Cohort 1 Volcano plot
Volcano plot of differentially expressed proteins in the first cohort. Differential expression analysis (FDR < 0.05) was performed on the proteins of children with Paediatric Acute Neuropsychiatric Syndrome (PANS) and neurotypical controls. The y-axis represents statistical significance (−log10FDR) and the x-axis represents gene expression log fold change (logFC). Differentially expressed proteins with up-regulated expressions are shown in red, while those with down-regulated expression are in blue. The top 20 differentially expressed proteins were labelled for ease of viewing.

3.8.3.2 Cohort 2

Out of the 5,708 proteins identified in the second Cohort, 2380 were recognised as differentially expressed proteins at FDR < 0.05. From this, 1,416 proteins had an up-regulated expressions and 964 proteins had a down-regulated expression. A volcano plot shows the distribution in Figure 3.9. The top up-regulated genes included myosin proteins

(MYH9, MYL12A, MYL12B, MYL6 and MYH14), a cytoskeletal protein (VCL) and a kinase (DOCK7). The top down-regulated proteins included a lipoprotein (APOC3), a histone (H2BC14), and an RNA polymerase (POLR2L).

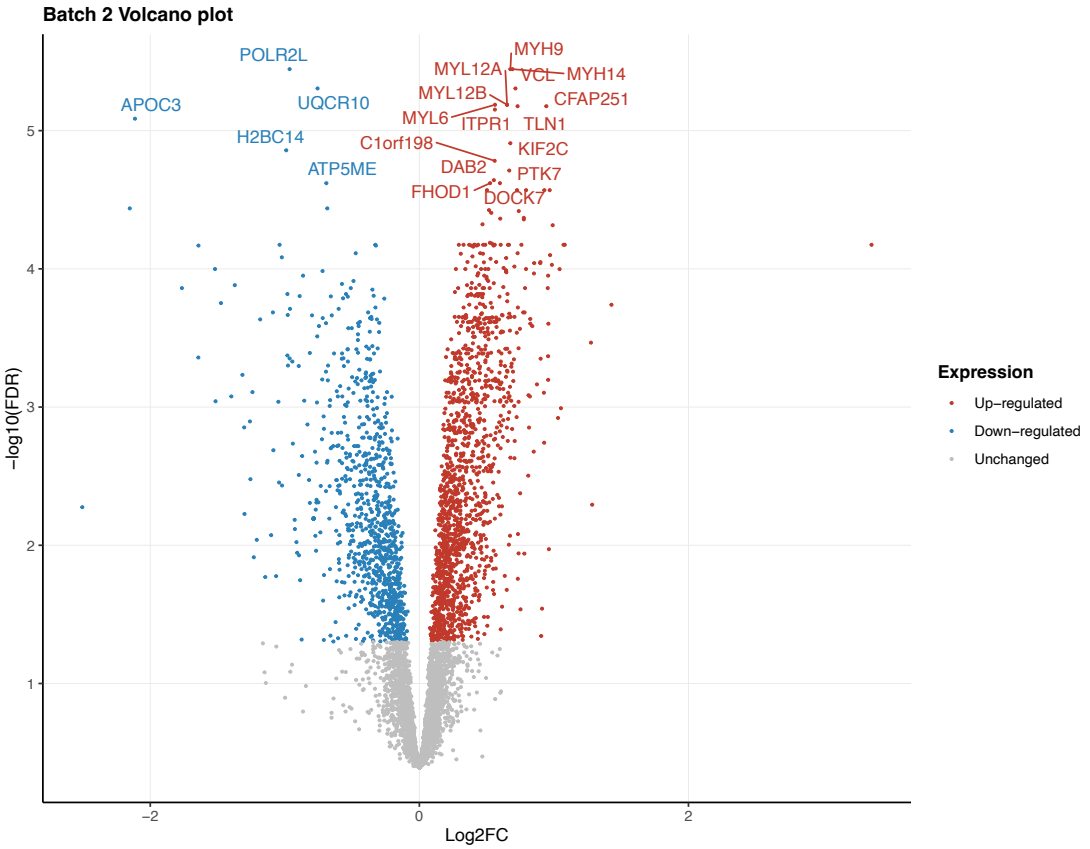


Figure 3.9 Cohort 2 Volcano plot
Volcano plot of differentially expressed proteins in the second cohort. Differential expression analysis (FDR < 0.05) was performed on the proteins of children with Paediatric Acute Neuropsychiatric Syndrome (PANS) and neurotypical controls. The y-axis represents statistical significance (−log10FDR) and the x-axis represents gene expression log fold change (logFC). Differentially expressed proteins with up-regulated expressions are shown in red, while those with down-regulated expression are in blue. The top 20 differentially expressed proteins were labelled for ease of viewing.

3.8.4 Pathway analysis

Although the focus thus far has relied on individual proteins and their regulation, we know that genes or proteins don't work alone. Particularly within the context of such complex conditions such as neurodevelopmental and neuropsychiatric disorders, where the presumed culprit is a complex interplay of proteins and genes. This is why a pathway analysis approach

is crucial to uncover and understand the mechanisms of these disorders in peripheral blood mononuclear cells of children with PANS.

As mentioned above in Section 3.7.6, the first type of pathway analysis performed involved utilises over-representation analysis for the Gene Ontology database and the Reactome database. Following from this, a Gene Set Enrichment Analysis was performed using the two databases. Figures will show the top 10 up-regulated and top 10 down-regulated pathways.

3.8.4.1 Cohort 1

3.8.4.1.1 Over-representation analysis

Gene Ontology Enrichments of the 2,973 proteins identified 60 up-regulated, and 30 down-regulated terms through utilising an over-representation analysis of the gene ontology database (Figure 3.10). Within the gene ontology database, there are three ontologies or sections which genes and proteins are grouped into: biological processes, cellular compartment, and molecular function. The biological processes ontology focuses on the biological routes genes and proteins contribute to. The cellular compartment ontology aims to describe the locations of where genes and proteins are active within the cell. Finally, the molecular function ontology encompasses the biochemical activity of a gene or protein at a molecular level. The top up-regulated terms were "blood coagulation" in biological processes, "platelet alpha granule" in cellular compartment and "signalling receptor binding" in molecular function. The top down-regulated terms were "RNA processing" in biological process, "ribonucleoprotein complex" in cellular compartment and "DNA binding" in molecular function.

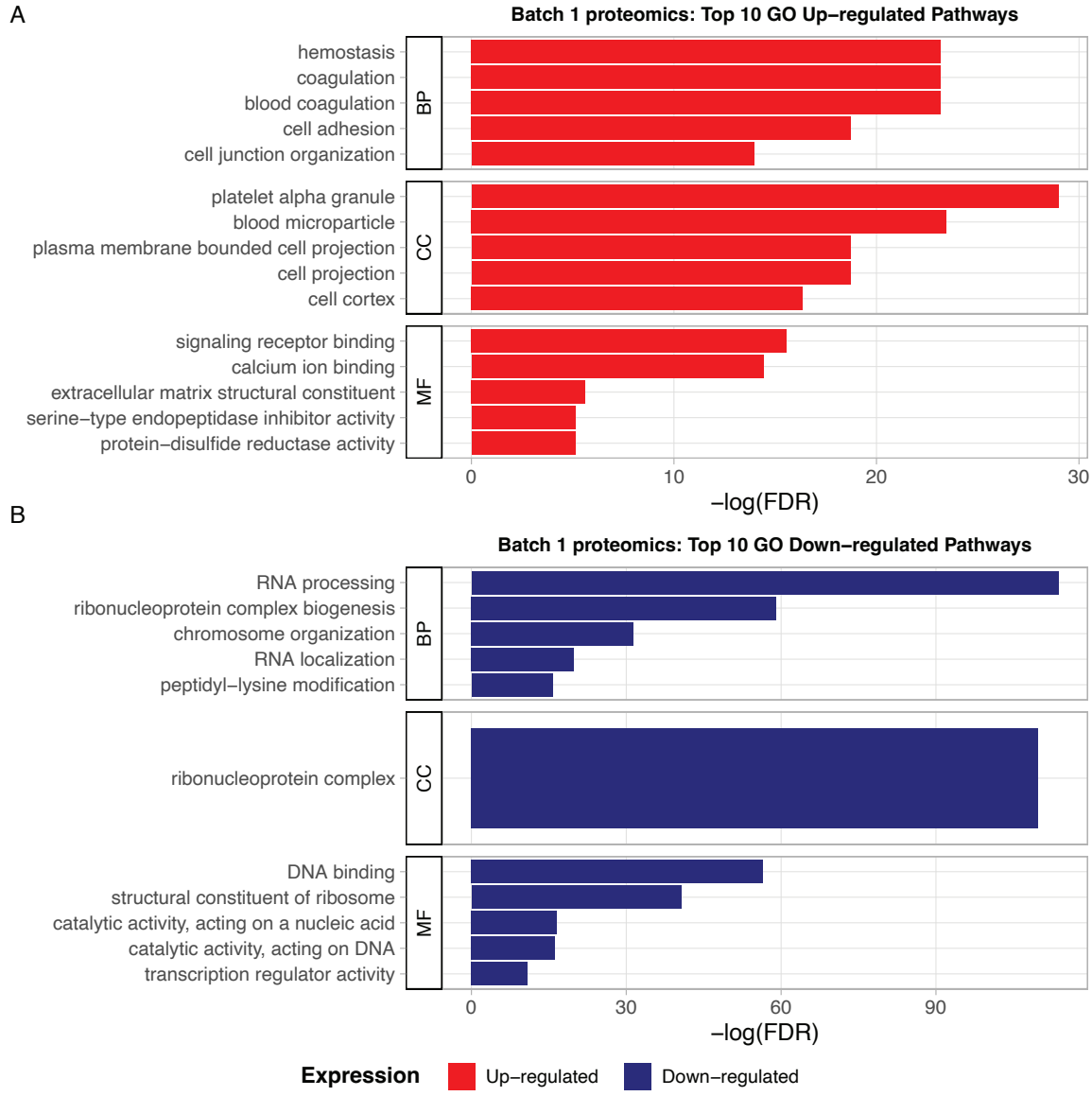


Figure 3.10 Cohort 1 Over-representation analysis: Gene Ontology
The differentially expressed proteins (FDR <0.05) were utilised to perform over-representation analysis using the Gene Ontology database. In the bar charts, the y axis depicts the top five enriched pathways split by ontology. The x axis shows the -log10(false discovery rate) of each pathway. The ontology results are presented on each row: the top row is biological processes (BP), the middle row is cellular compartment (CC), and the bottom row is molecular function (MF). A) Enrichments of the up-regulated proteins. B) Enrichments of the down-regulated proteins. Expression (Red = Up-regulated, blue = down-regulated).

Reactome Next, I utilised the Reactome database to also perform an over-representation analysis and identified 92 up-regulated and 181 down-regulated pathways (Figure 3.11). The top up-regulated pathways were involved in haemostasis, with the top pathway being "Haemostasis". The top down-regulated pathways were involved in RNA processing, with the top pathway being "Metabolism of RNA".

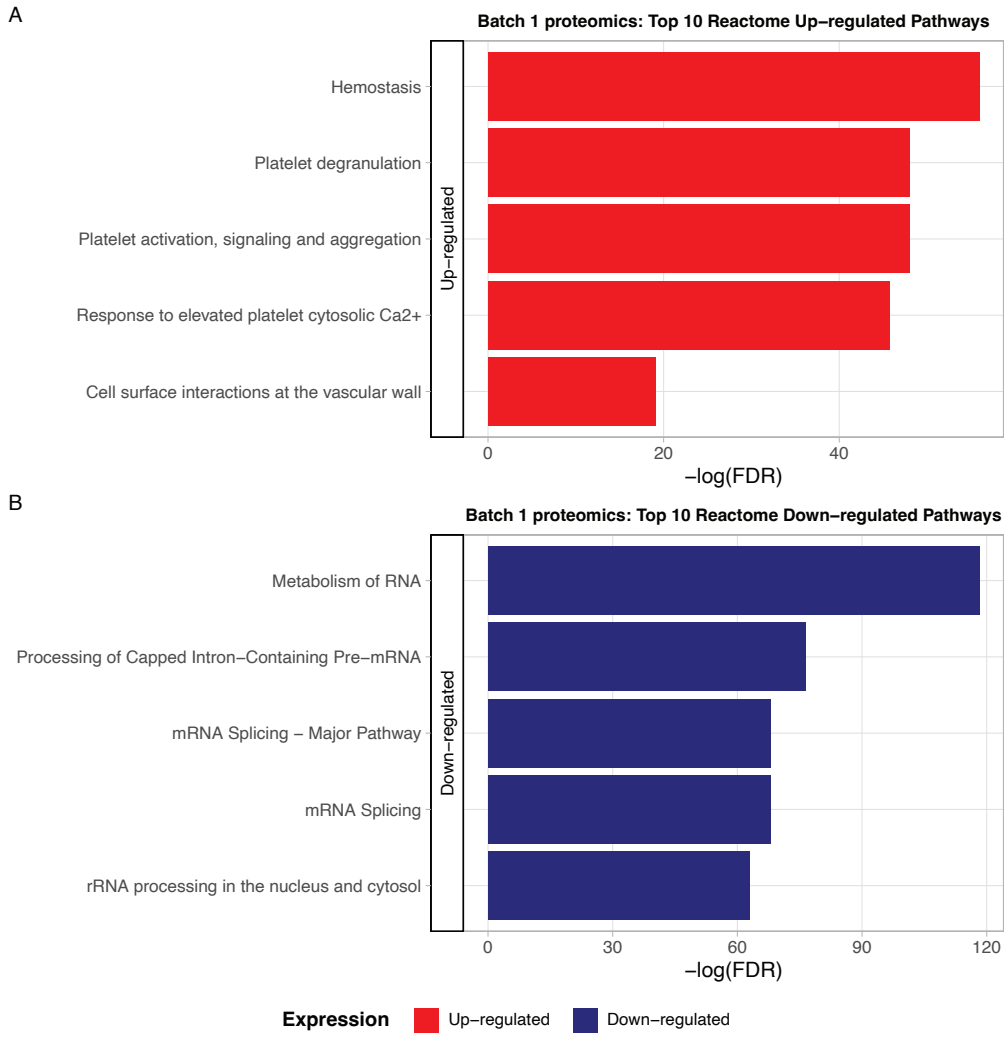


Figure 3.11 Cohort 1 Over-representation analysis: Reactome
The differentially expressed proteins (FDR <0.05) were utilised to perform over-representation analysis using the Reactome database. In the bar charts, the y axis depicts the top ten enriched pathways split. The x axis shows the -log10(false discovery rate) of each pathway. A) Enrichments of the up-regulated proteins. B) Enrichments of the down-regulated proteins. Expression (Red = Up-regulated, blue = down-regulated).

3.8.4.1.2 Gene Set Enrichment Analysis

Gene Ontology GSEA utilises a ranking approach to identify enriched pathways, where the most differentially expressed proteins carry more weight based on the direction of change. Analysis of cohort 1's proteins using the GSEA technique identified 42 up-regulated and 27 down-regulated enriched terms (Figure 3.12). Of the biological processes enrichments, the top up-regulated term was "coagulation" and the top down-regulated term was "RNA processing". The cellular compartment ontology identified "blood microparticle" as the top up-regulated term, while "ribonuclear protein complex" was the top down-

regulated terms. Of the up-regulated molecular function enrichments, the top up-regulated term was "calcium ion binding" while "structural constituent of ribosome" was the top down-regulated term.

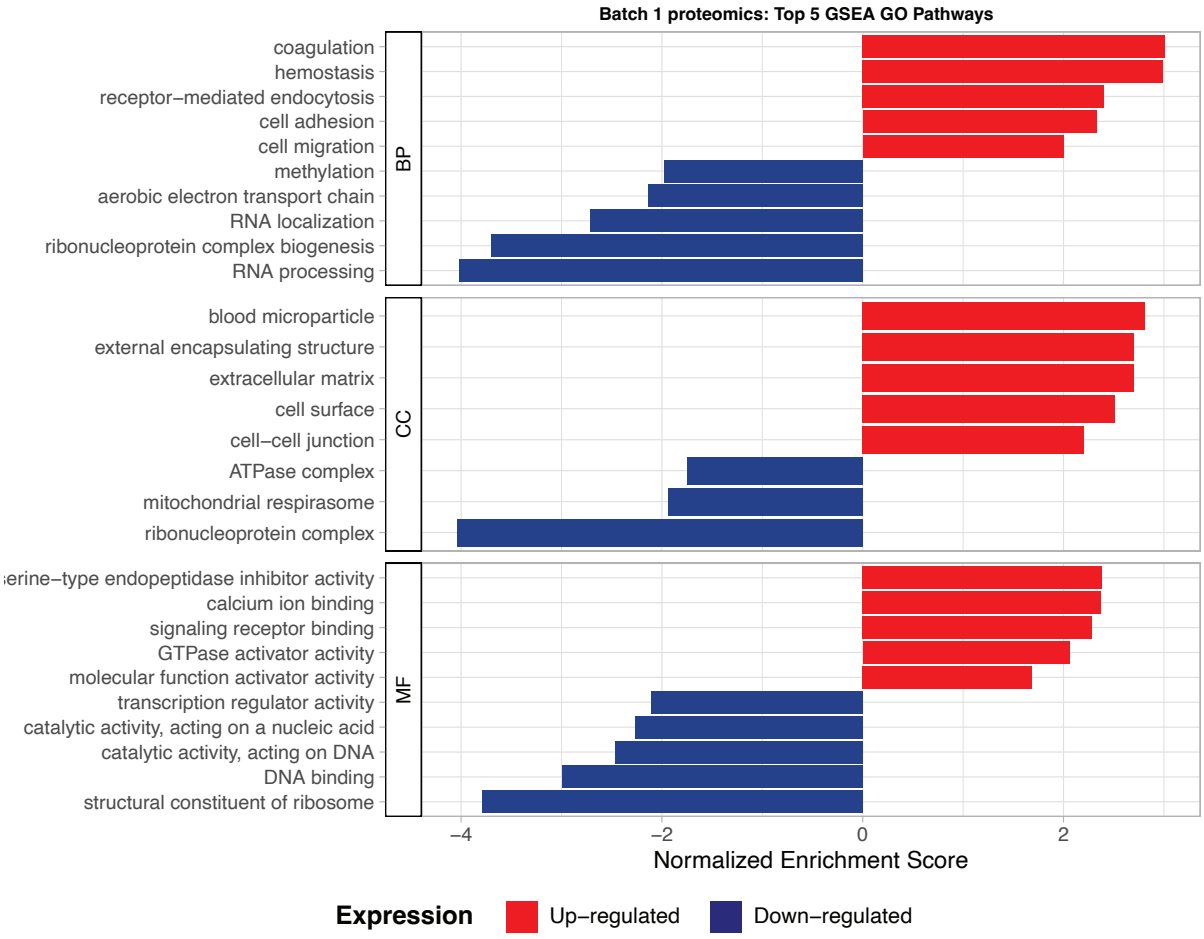


Figure 3.12 Cohort 1 Gene Set Enrichment Analysis: Gene Ontology
The differentially expressed proteins, ranked by signed logfc x log(false discovery rate) were used to perform Gene Set Enrichment Analysis (GSEA) using the Gene Ontology database. In this bar chart, the y axis depicts the top five enriched pathways split by ontology. The ontology results are presented on each row: the top row is biological processes (BP), the middle row is cellular compartment (CC) and the bottom row is molecular function (MF).The x axis shows the normalised enrichment score, with a positive number shows inclination of genes within the pathway to be found at the top of the inputted list (Red = Up-regulated), while a negative number shows that genes within the pathway can be found at the bottom of the list (Blue = Down-regulated). The up-regulated and down-regulated proteins are illustrated together due to the nature of the GSEA analysis, where all proteins are analysed as one combined list based on the ranking.

As the findings from the ORA and GSEA aligned, a connectivity network plot (CNET) was generated to highlight proteins within the enriched pathways from the GSEA. Within Figure 3.13, the top biological processes terms are observed in panel A (up-regulated) and panel B (down-regulated). The up-regulated proteins involved within coagulation

included protein kinases (PRKG1, PRKCQ), serine protease inhibitors (the SERPINE family), and heat shock proteins (HSBP1). The down-regulated proteins within RNA processing included methyltransferases (METTL16), ribosomal proteins (RPS14, RPS6), and the deadbox protein family of RNA helicases (DDX1, DDX47) .

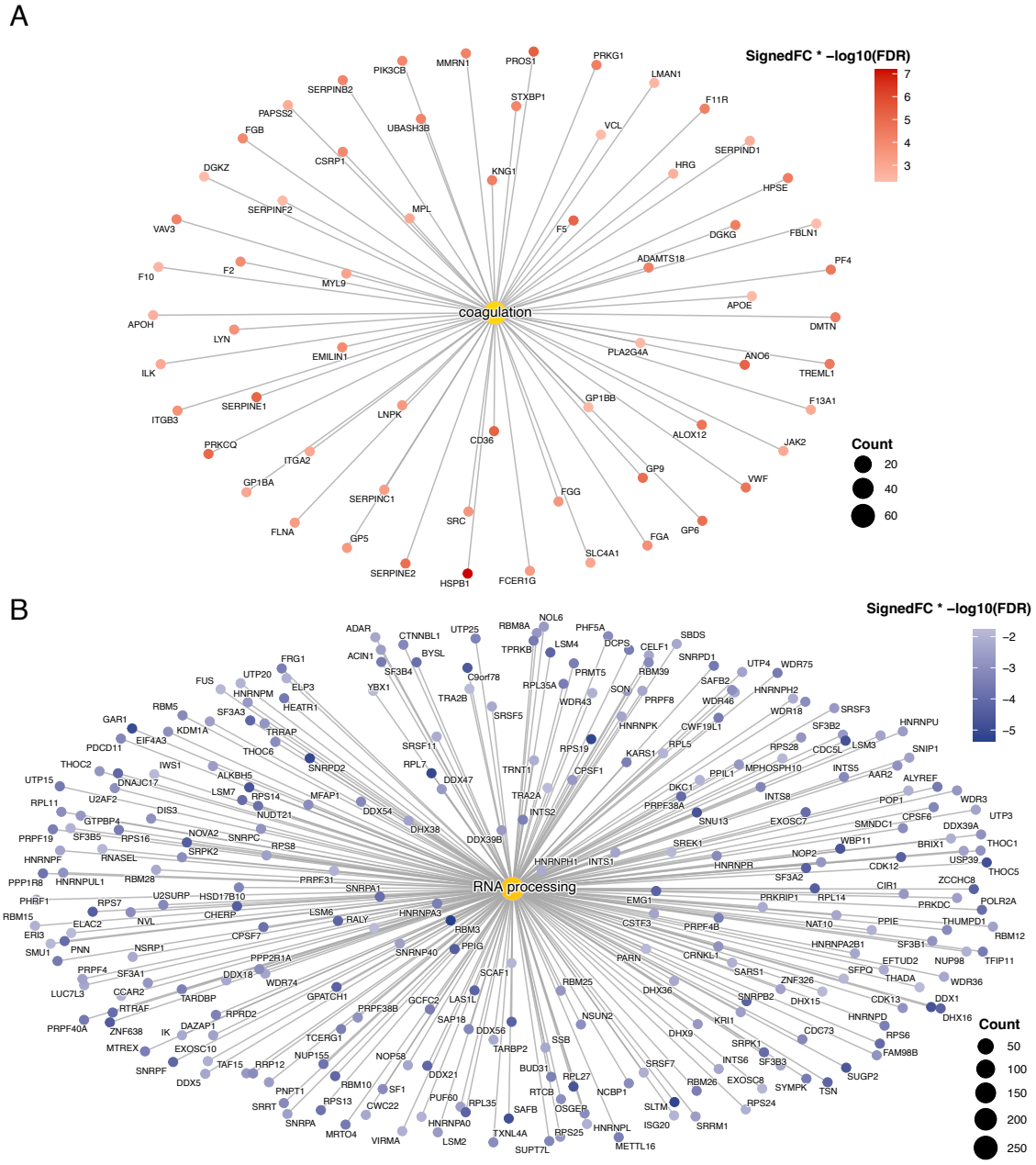


Figure 3.13 Cohort 1 Gene Set Enrichment Analysis: Gene Ontology CNET
Connectivity network (CNET) plots of the top up-regulated (A) and down-regulated (B) enriched pathways from the Gene Ontology database through Gene Set Enrichment Analysis (GSEA). The GSEA analysis was performed on a list of proteins from an analysis of children with Paediatric Acute Neuropsychiatric Syndrome (PANS) compared to controls. The CNET plots highlight the interactions of proteins that make up the pathways, represented by each proteins rank [signed logfc x log(false discovery rate)].

Reactome Similarly, I utilised a gene set enrichment analysis method using the Reactome database for cohort 1. The analysis identified 124 up-regulated and 203 down-regulated pathways (Figure 3.14). The top up-regulated pathways were involved in haemostasis, with the top pathway being "Platelet degranulation". The top down-regulated pathways were involved in RNA processing, with the top pathway being "Metabolism of RNA".

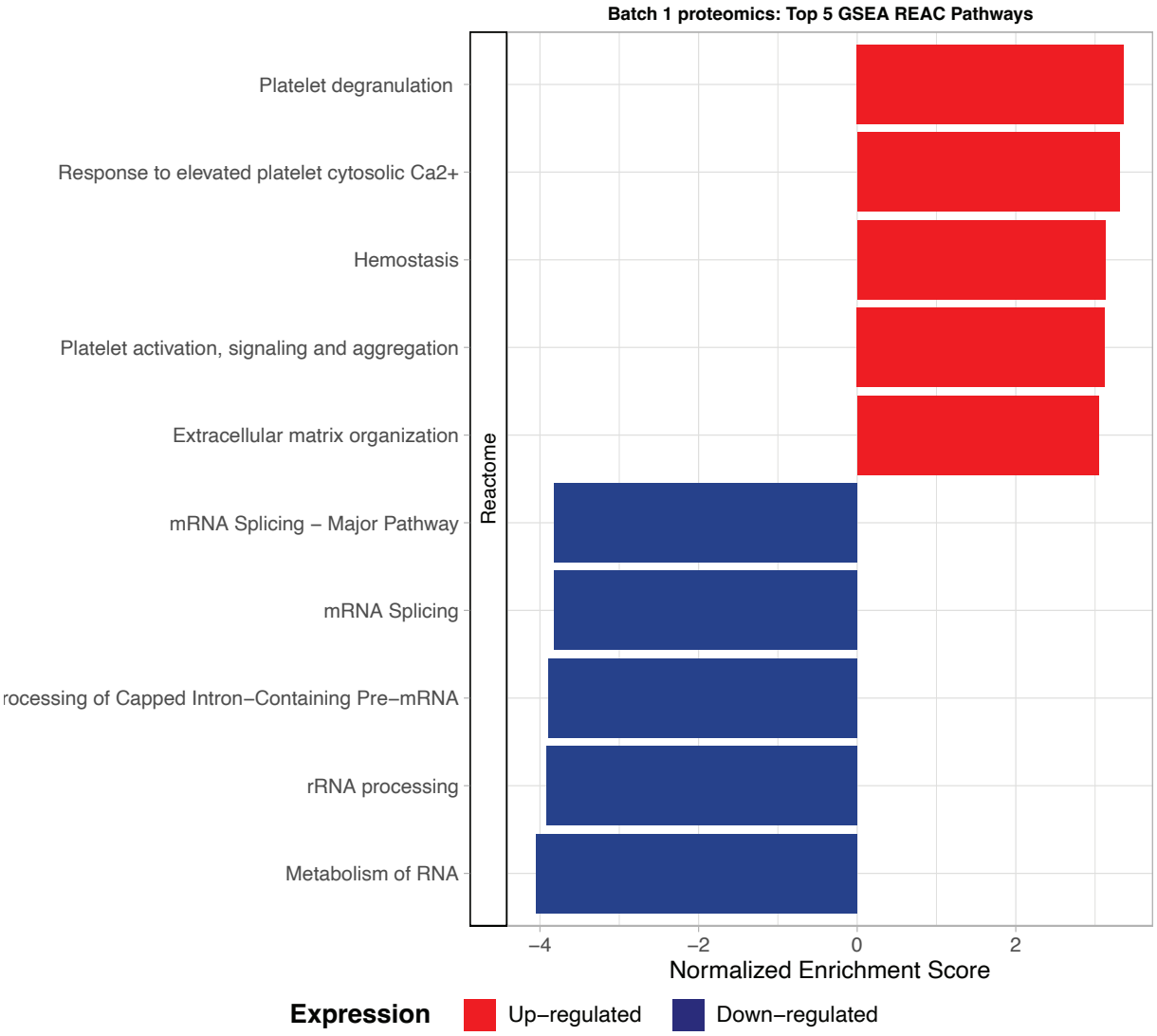


Figure 3.14 Cohort 1 Gene Set Enrichment Analysis: Reactome
The differentially expressed proteins, ranked by signed logfc x log(false discovery rate) were used to perform Gene Set Enrichment Analysis (GSEA) using the Reactome database. In this bar chart, the y axis depicts the top five enriched pathways. The x axis shows the normalised enrichment score, with a positive number shows inclination of genes within the pathway to be found at the top of the inputted gene list (Red = Up-regulated), while a negative number shows that genes within the pathway can be found at the bottom of the gene list (Blue = Down-regulated). The up-regulated and down-regulated proteins are illustrated together due to the nature of the GSEA analysis, where all proteins are analysed as one combined list based on the ranking.

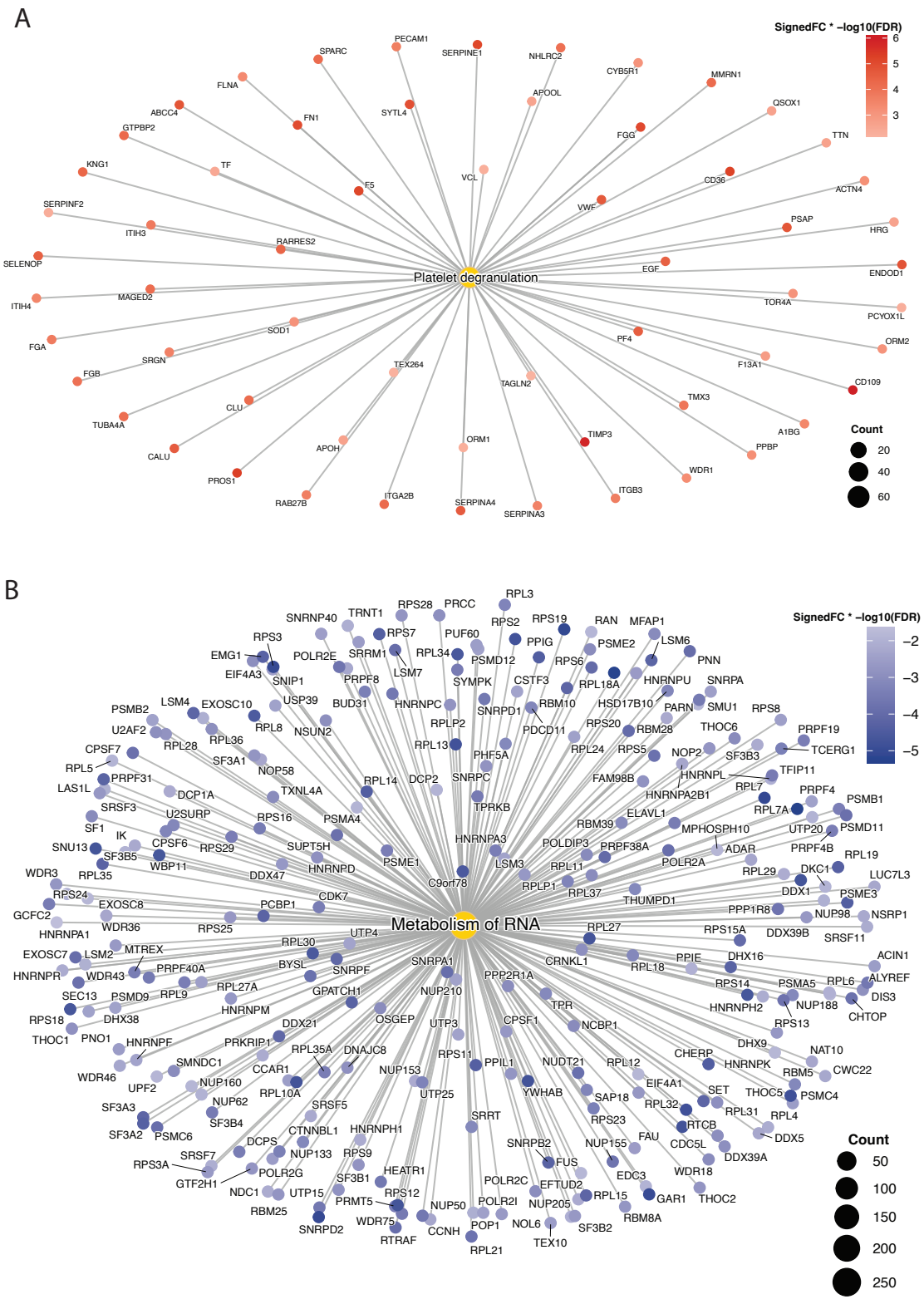


Figure 3.15 Cohort 1 Gene Set Enrichment Analysis: Reactome CNET
Connectivity network (CNET) plots of the top up-regulated (A) and down-regulated (B) enriched pathways from the Reactome database through Gene Set Enrichment Analysis (GSEA). The GSEA analysis was performed on a list of proteins from an analysis of children with Paediatric Acute Neuropsychiatric Syndrome (PANS) compared to controls. The CNET plots highlight the interactions of proteins that make up the pathways, represented by each proteins rank [signed logfc x log(false discovery rate)].

A connectivity plot highlighting the proteins within the enriched pathways is observed in Figure 3.15, where the top pathways are observed in panel A (up-regulated) and panel B (down-regulated). The up-regulated enrichments of platelet degranulation involved proteins such as serine protease inhibitors (the SERPINE family), cell adhesion molecules (PECAM1,CD36) and matrix metalloproteinases (TIMP3) . The down-regulated enrichments of metabolism of RNA involved proteins such as ribosomal proteins (RPL30, RPS8), small nuclear ribonucleoproteins (SNRPA, SNU13), and eukaryotic translation initiation factors (EIF4A1,EIF4A3).

3.8.4.1.3 Summary of pathway results

The pathway analysis of cohort 1 utilised two techniques, over-representation analysis and gene set enrichment analysis. These two methods yielded similar findings, where the up-regulated enrichments pointed towards haemostasias, immune and cell signalling influenced by serine protease inhibitors, protein kinases, and cell adhesion molecules. To contrast, the down-regulated enrichments were involved in RNA processing and splicing with dominant involvement of ribosomal proteins, methyltransferases and demethylases.

3.8.4.2 Cohort 2

3.8.4.2.1 Over-representation analysis

Gene Ontology As with the first cohort, commencement of pathway analysis will be through utilising an ORA approach. Firstly, the differentially expressed proteins were utilised to enrich over-represented terms from the three ontologies. Following filtering, 22 up-regulated biological processes terms were identified, along with nine cellular compartment terms, and 15 molecular function terms (Figure 3.16 highlights the top five pathways). The top up-regulated terms were "actin filament-based process" in biological processes, "actin cytoskeleton" in cellular compartment and "cytoskeletal protein binding" in molecular function. Following from that, we explored enrichments of the 964 down-regulated proteins.

The top down-regulated terms were "proteolysis involved in protein catabolic process" in biological processes, "proteosome complex" in cellular compartment, and "structural constituent of a ribosome" in molecular function.

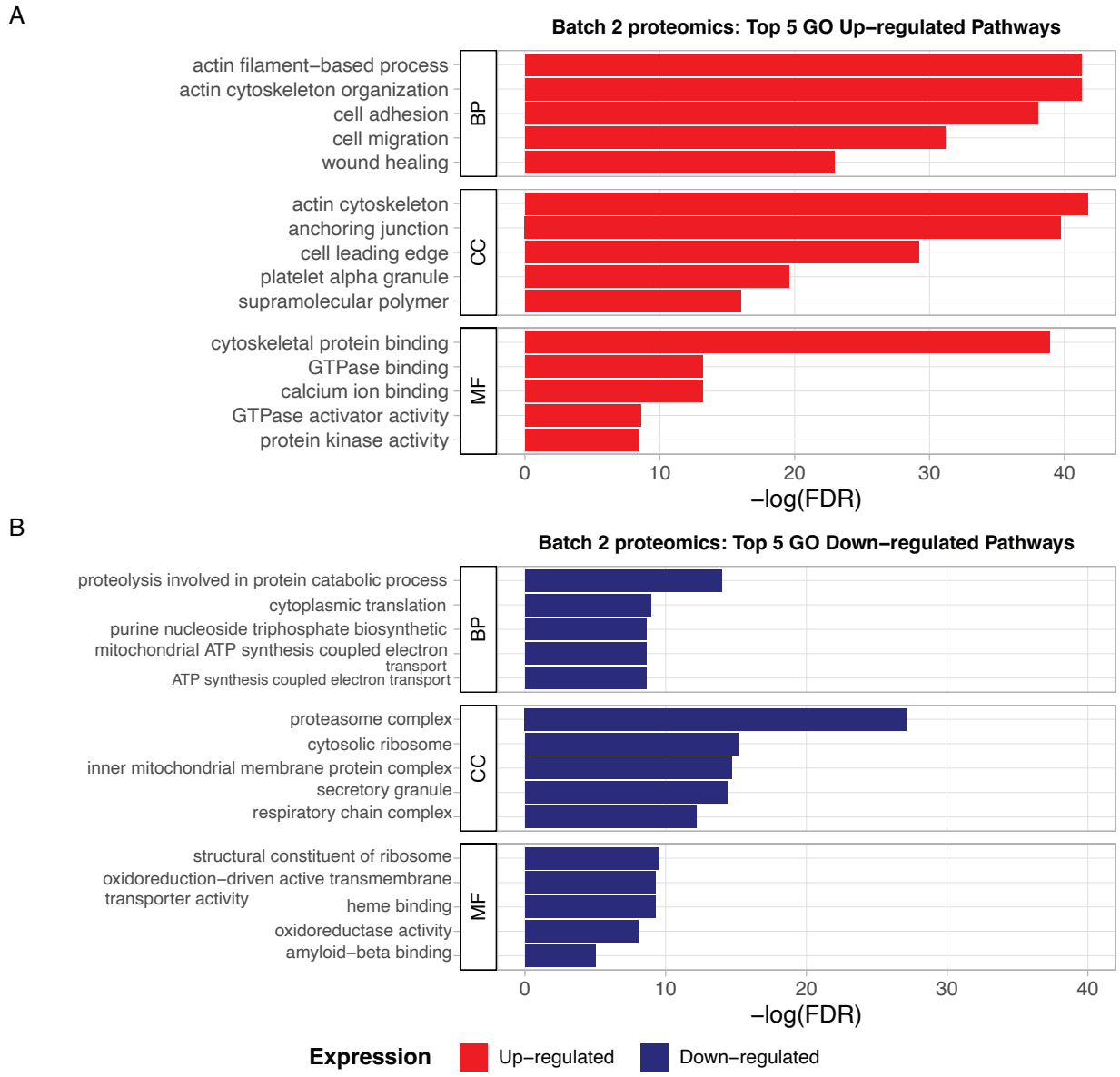


Figure 3.16 Cohort 2 Over-representation analysis: Gene Ontology
The differentially expressed proteins (FDR <0.05) were utilised to perform over-representation analysis using the Gene Ontology database. In the bar charts, the y axis depicts the top ten enriched pathways split by ontology. The x axis shows the -log10(false discovery rate) of each pathway. A) Enrichments of the up-regulated proteins. B) Enrichments of the down-regulated proteins. Expression (Red = Up-regulated, blue = down-regulated).

Reactome Similarly to the steps taken prior, the 1,416 up-regulated proteins were applied to enrich 137 pathways within the Reactome database (Figure 3.17). The up-regulated pathways were involved in haemostasis and cell signalling, where the top pathway was "Hemostasis". Enrichment of the down-regulated proteins were involved in DNA replication and chromatin, where the top pathway was Assembly of the re-replicative complex".

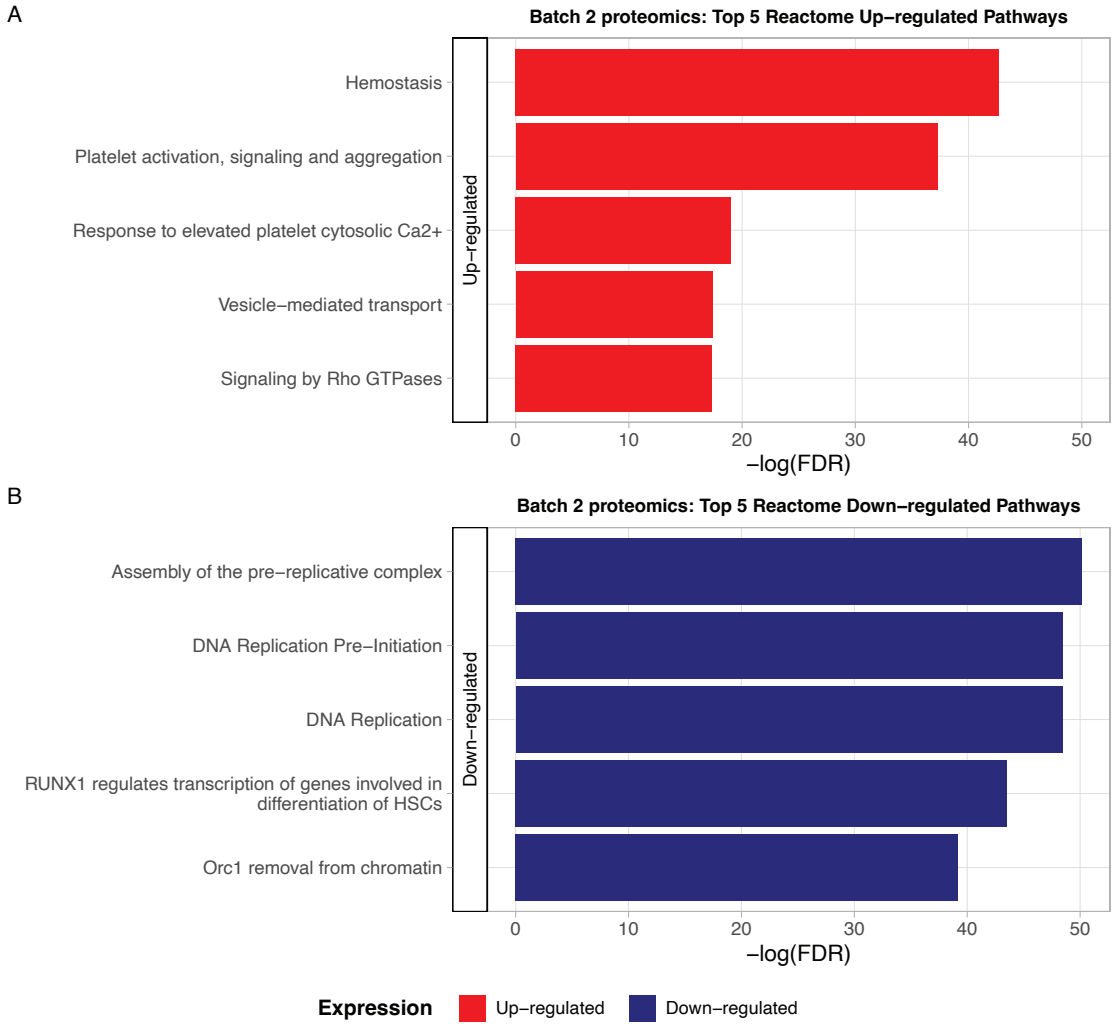


Figure 3.17 Cohort 2 Over-representation analysis: Reactome
The differentially expressed proteins (FDR <0.05) were utilised to perform over-representation analysis using the Reactome database. In the bar charts, the y axis depicts the top ten enriched pathways split. The x axis shows the -log10(false discovery rate) of each pathway. A) Enrichments of the up-regulated proteins. B) Enrichments of the down-regulated proteins. Expression (Red = Up-regulated, blue = down-regulated).

3.8.4.2.2 Gene Set Enrichment Analysis

Gene Ontology To assess enrichments of the same set of proteins using the GSEA method, I then performed a second set of pathway analysis for Cohort 2. This is depicted in Figure 3.18, where the top five enrichments within each ontology is visualised. The biological processes ontology identified 25 up-regulated terms and 17 down-regulated terms. The cellular compartment ontology identifed 7 up-regulated, and 6 down-regulated terms. The molecular function ontology enriched 17 up-rgulated and 10 down-regulated terms The top up-regulated enrichments were "actin filament-based process" in biological processes, "actin cytoskeleton" in cellular compartment and "cytoskeletal protein binding" in molecular function. The top down-regulated enrichments were "cytoplasmic translation" in biological processes, "peptidase complex" in cellular compartment and "structural constituent of ribosome" in molecular function.

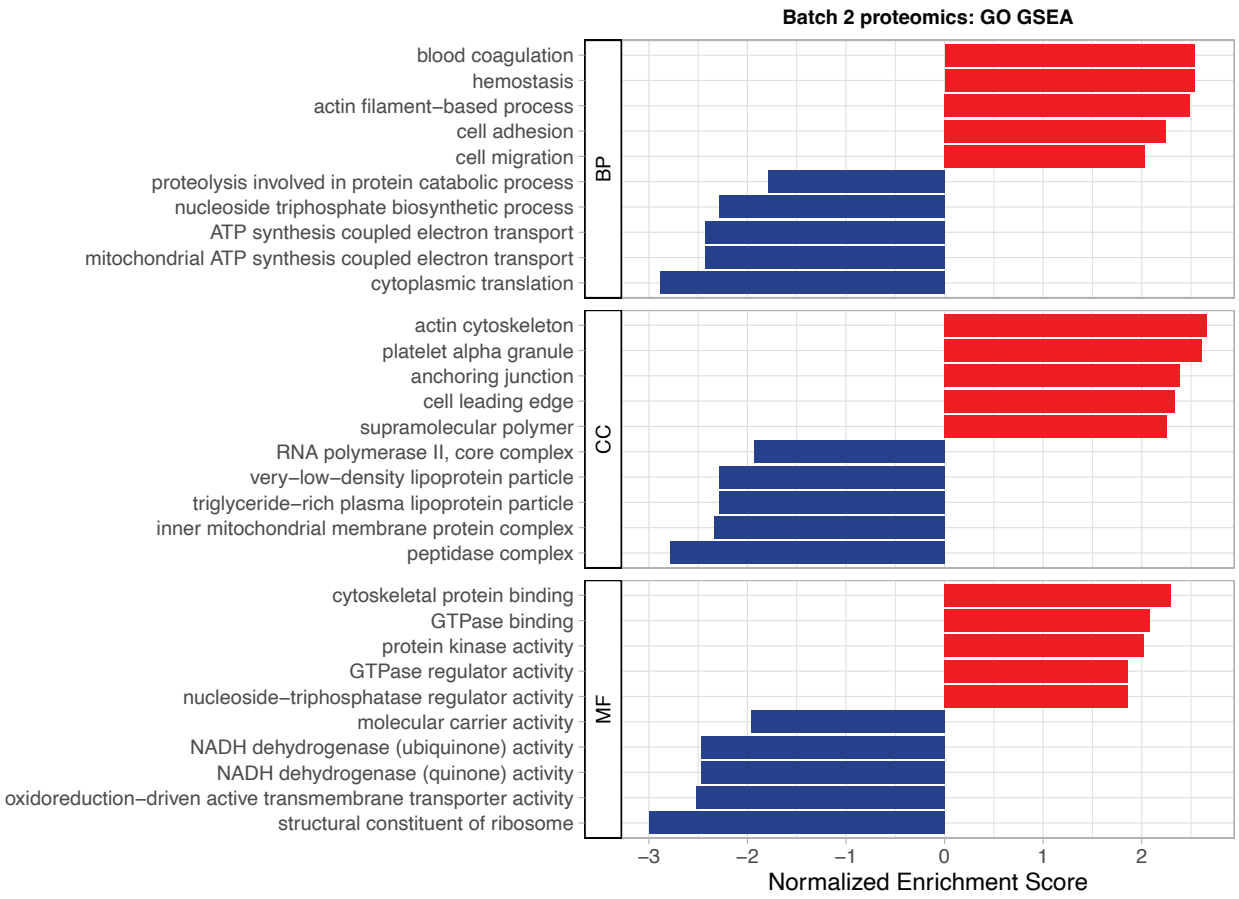


Figure 3.18 Cohort 2 Gene Set Enrichment Analysis: Gene Ontology
The differentially expressed proteins, ranked by signed logfc x log(false discovery rate) were used to perform Gene Set Enrichment Analysis (GSEA) using the Gene Ontology database. In this bar chart, the y axis depicts the top five enriched pathways split by ontology. The ontology results are presented on each row: the top row is biological processes (BP), the middle row is cellular compartment (CC) and the bottom row is molecular function (MF).The x axis shows the normalised enrichment score, with a positive number shows inclination of genes within the pathway to be found at the top of the inputted list (Red = Up-regulated), while a negative number shows that genes within the pathway can be found at the bottom of the list (Blue = Down-regulated). The up-regulated and down-regulated proteins are illustrated together due to the nature of the GSEA analysis, where all proteins are analysed as one combined list based on the ranking.

Similar to the steps undertaken for the first cohort, a connectivity plot was utilised for the GSEA section to shed light onto the proteins driving the enriched pathways. In Figure 3.19 the top biological processes terms are observed in panel A (up-regulated) and panel B (down-regulated). From panel A, the up-regulated enrichments are driven by protein kinases (PLEK, PIK3CB), Integrins (ITGB3a) , serine protease inhibitors (the SERPINE family), and myosin (the MYH and MYL families, TLN1). For the down-regulated enrichments, the CNET plot shows that the signal is driven by ribosomal proteins (RPS29, RPS20, RPL35A),

methyltransferases (METTL3), and eukaryotic translation initiation factors (EIF3M, EIF4A1, EIF2D).

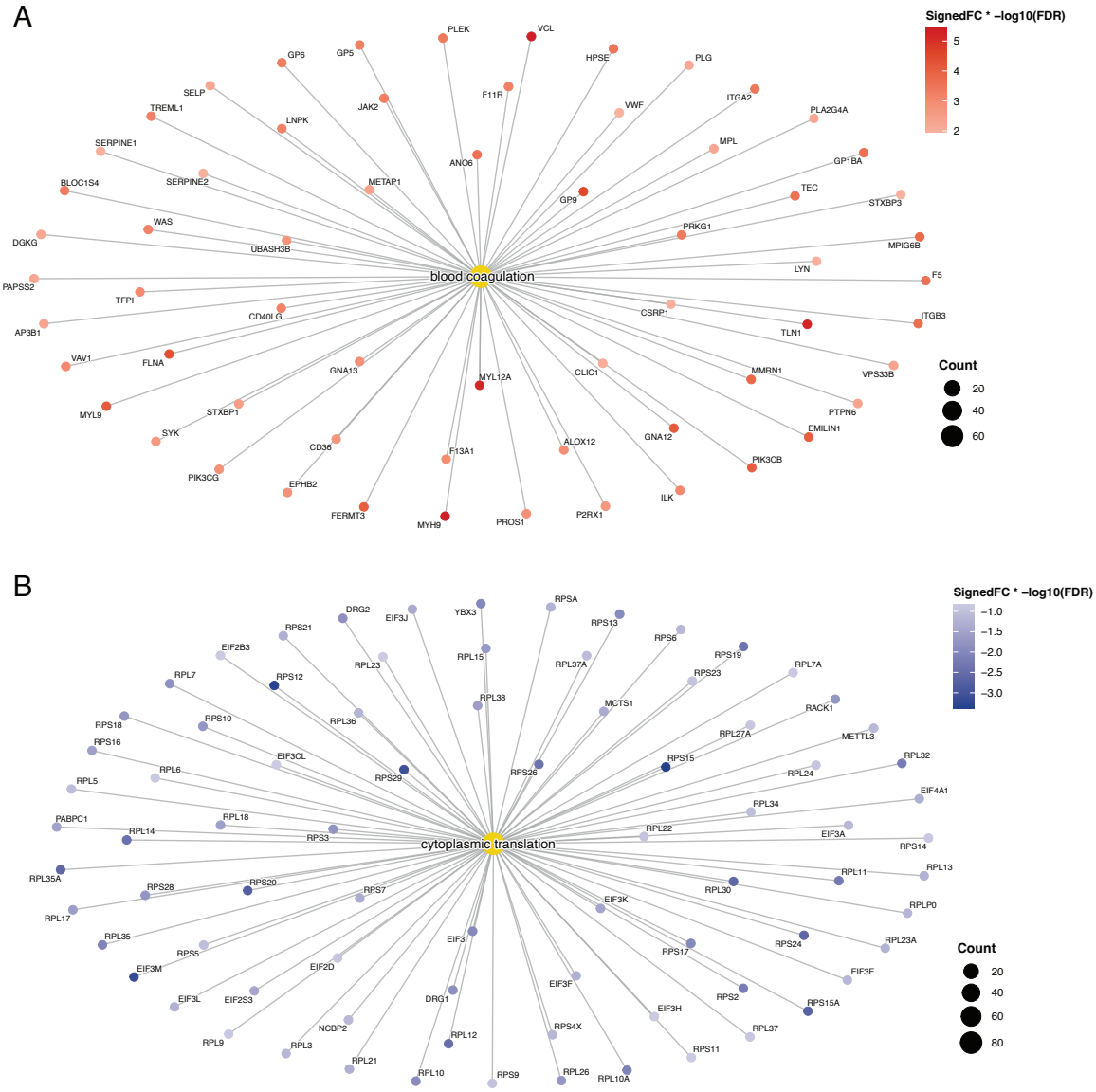


Figure 3.19 Cohort 2 Gene Set Enrichment Analysis: Gene Ontology CNET
Connectivity network (CNET) plots of the top up-regulated (A) and down-regulated (B) enriched pathways from the Gene Ontology database through Gene Set Enrichment Analysis (GSEA). The GSEA analysis was performed on a list of proteins from an analysis of children with Paediatric Acute Neuropsychiatric Syndrome (PANS) compared to controls. The CNET plots highlight the interactions of proteins that make up the pathways, represented by each proteins rank [signed logfc x log(false discovery rate)].

Reactome Further, I explored enrichments of the differentially expressed proteins using the Reactome database (Figure 3.20). The gene set enrichment analysis identified 226 up-regulated and 303 down-regulated pathways. The up-regulated pathways were involved

in haemostasis, with the top pathway being "Platelet activation, signalling and aggregation". The down-regulated pathways were involved in translation and DNA replication, with the top pathway being "Assembly of the pre-replicative complex" .

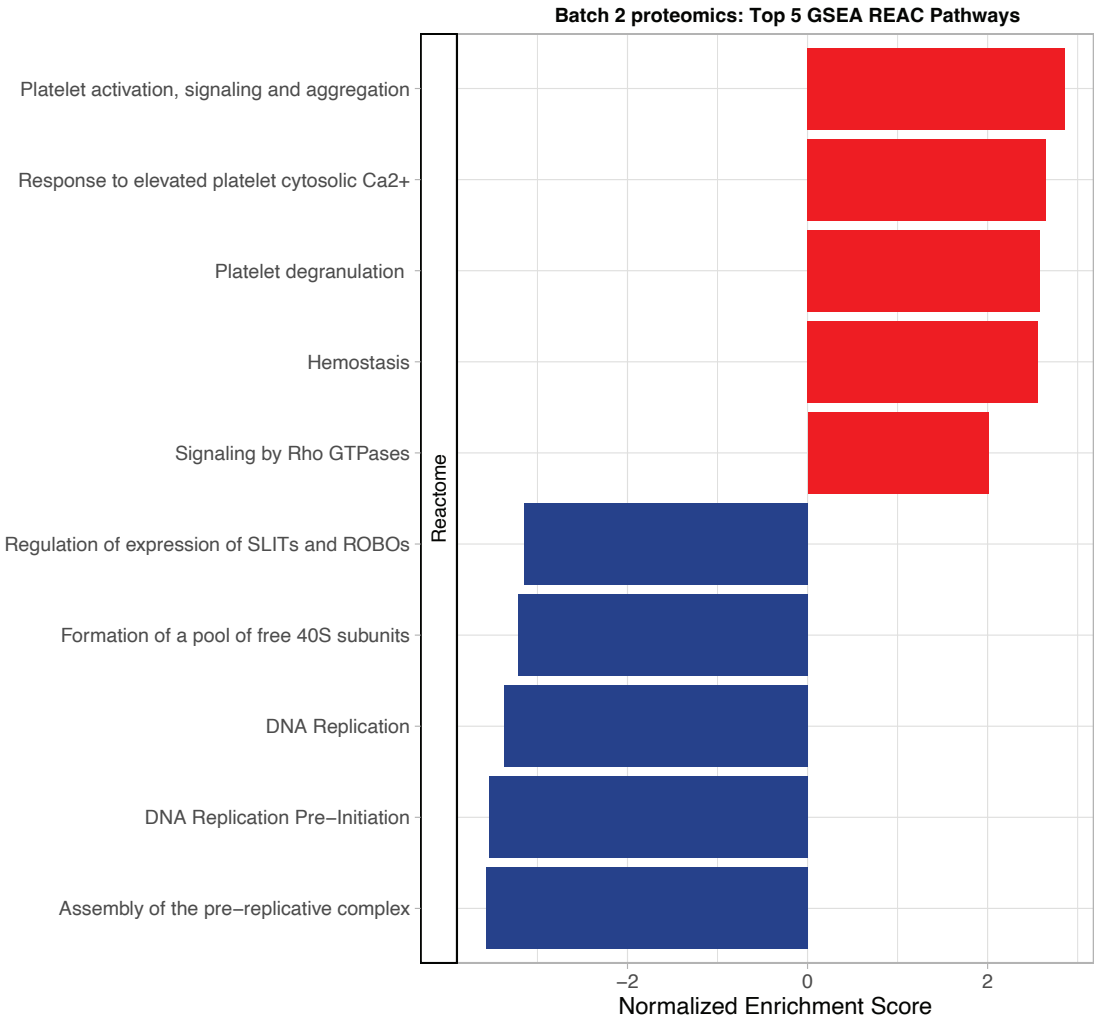


Figure 3.20 Cohort 2 Gene Set Enrichment Analysis: Reactome
The differentially expressed proteins, ranked by signed logfc x log(false discovery rate) were used to perform Gene Set Enrichment Analysis (GSEA) using the Reactome database. In this bar chart, the y axis depicts the top five enriched pathways. The x axis shows the normalised enrichment score, with a positive number shows inclination of genes within the pathway to be found at the top of the inputted gene list (Red = Up-regulated), while a negative number shows that genes within the pathway can be found at the bottom of the gene list (Blue = Down-regulated). The up-regulated and down-regulated proteins are illustrated together due to the nature of the GSEA analysis, where all proteins are analysed as one combined list based on the ranking.

A connectivity plot highlighting the proteins within the enriched pathways of cohort 2's Reactome GSEA is observed in Figure 3.21 the top pathways are observed in panel A (up-regulated) and panel B (down-regulated). The up-regulated enrichments were driven by kinases (PIK3CB,PIK3R1), glycoproteins (GP6, GP9), and serine protease inhibitors (the

SERPINE family). The down-regulated enrichments consisted of proteins such as histones (H4C5,H2BC14), proteasome components (PSMD12, PSMB7), and mini-chromosome maintenance proteins (MCM4, MCM7).

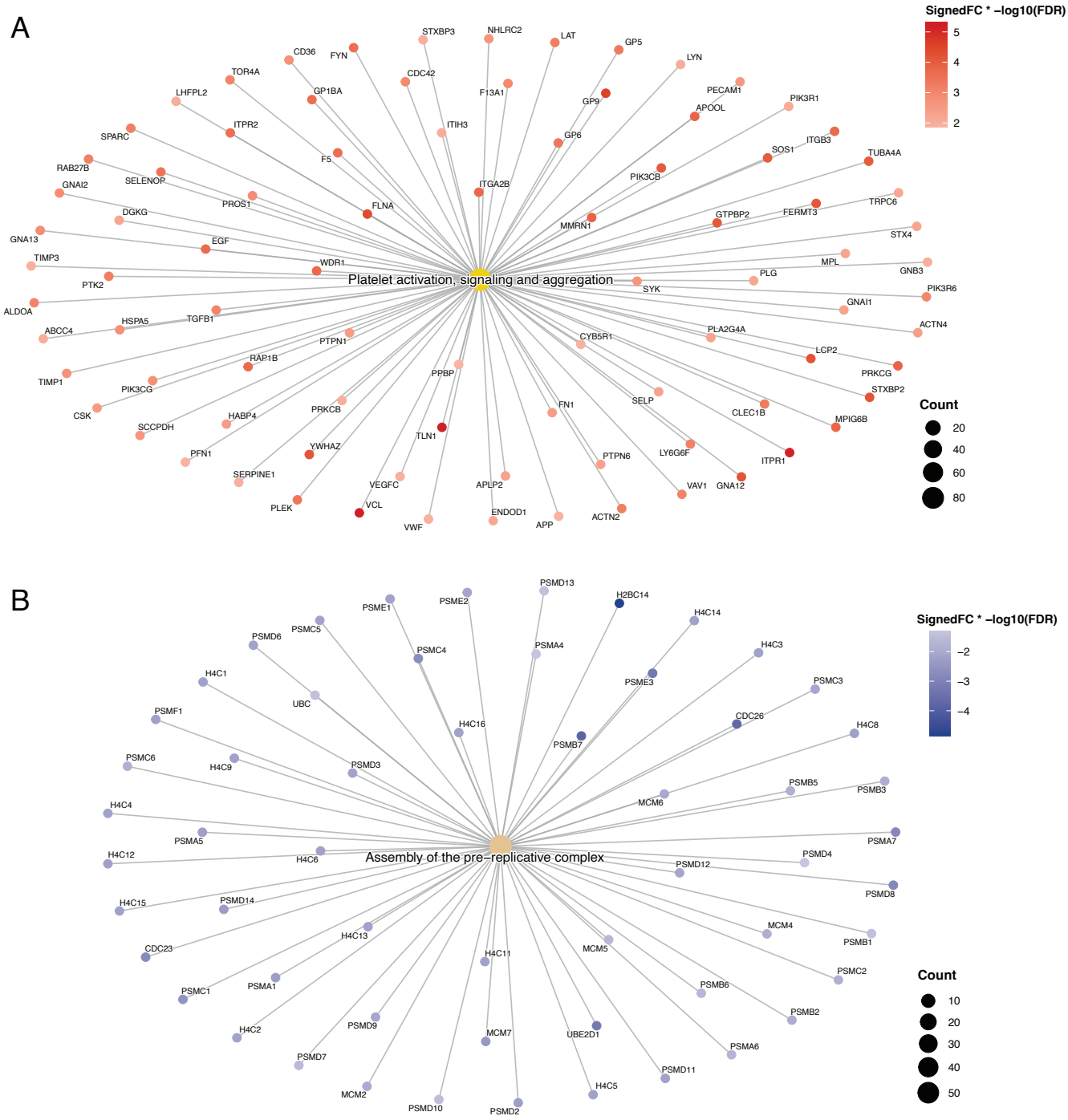


Figure 3.21 Cohort 2 Cohort 2 Gene Set Enrichment Analysis: Reactome CNET
Connectivity network (CNET) plots of the top up-regulated (A) and down-regulated (B) enriched pathways from the Reactome database through Gene Set Enrichment Analysis (GSEA). The GSEA analysis was performed on a list of proteins from an analysis of children with Paediatric Acute Neuropsychiatric Syndrome (PANS) compared to controls. The CNET plots highlight the interactions of proteins that make up the pathways, represented by each proteins rank [signed logfc x log(false discovery rate)].

3.8.4.2.3 Summary

The pathway analysis of cohort 2 utilised two techniques, over-representation analysis and gene set enrichment analysis. These two methods yielded similar findings, where the up-regulated enrichments pointed towards haemostasias, immune and cell signalling influenced by serine protease inhibitors, protein kinases, and myosin molecules. To contrast, the down-regulated enrichments were involved in RNA processing and splicing with dominant involvement of ribosomal proteins, and eukaryotic translation initiation factors, methyltransferases and histones.

3.9 Discussion

The work within this chapter investigated the proteome of peripheral blood mononuclear cells within two PANS cohorts compared to controls. These parameters were utilised in this investigation to assess dysregulated proteins and pathways within peripheral blood mononuclear cells, and further our understanding of neurodevelopmental disorders at a proteome level. Investigations of the transcriptome within the brain of individuals with neurodevelopmental disorders in Chapter 2 of this thesis, combined with bulk and single cell RNA sequencing efforts by our group prompted the exploration of the proteome within this subgroup of NDDs as the natural next step. Previous findings of inflammation, translational machinery, and epigenetics within PANS from the subgroup meant for a need to explore proteins within these conditions.

Peripheral blood mononuclear cells from children with PANS were subjected to mass spectrometry to identify differential proteins compared to controls. Following from data exploration within each cohort, which pointed towards clear distinction between the proteome of the PANS subgroup when compared to controls, differential expression of proteins was performed. Within cohort 1, the top differentially expressed proteins included a heat shock protein (↑HSPB1), a tissue inhibitor of Metalloproteinases (↑TIMP3) along with

a major histocompatibility complex (↓HLA-DRB1), and a histone (↓H4C1). From cohort 2, the top differentially expressed proteins included myosin proteins (↑MYH9, MYL12A, MYL12B, MYL6 and MYH14), a cytoskeletal protein (↑VCL), along with a lipoprotein (↓APOC3), a histone (↓H2BC14), and an RNA polymerase (↓POLR2L). Taken together, these findings suggest immune molecules, histones and RNA processing are involved. However, choosing the most abnormal molecules is one way, our group's hypothesis is that a "pathway-driven" approach is arguably more informative of the whole picture of dysregulated proteome, rather than a small number of the most significant proteins.

Due to the complexity and multifactorial origins of these neurodevelopmental disorders, the main results within this chapter lie within the enriched dysregulated pathways that resulted from the identifications of differential proteins in the cases compared to the controls. Within cohort 1, an up-regulated signal was identified involved within haemostasis and blood coagulation, along with cell signalling, driven by proteins such as protein kinases and cell adhesion molecules. In addition, the down-regulated enrichments involved RNA processing, and translation, influenced by proteins such as methyltransferases, ribosomal proteins and eukaryotic translation initiation factors. A similar signal was observed in the enriched pathways from cohort 2, where gene ontology and Reactome investigations identified an up-regulated signal within haemostasis, the cytoskeleton, and cell signalling involving proteins such as protein kinases, and myosin molecules. To add, the enriched down-regulated pathways involved mitochondrial, cell activity and translational themes driven by ribosomal proteins, eukaryotic translation initiation factors and histones.

These similar findings within the two cohort are a great foundation step to uncovering proteomic dysregulations within PBMCs of children with PANS compared to controls. To start, the findings within the two cohorts presented corroborate with previous reports within the literature of neurodevelopmental proteomics in the periphery (Table 3.1

above). However, it is important to mention the bulk of this previous literature is within ASD, and only two studies were found to be published in OCD. A proteome analysis of peripheral blood mononuclear cells from ASD cases identified enriched mitochondrial and ER stress pathway findings, and associated these enrichments to an inflammatory response, further implicating inflammation found within the proteome in ASD pathogenesis.[251] It is important to note that the literature on proteomics within these psychiatric conditions is lacking, and that reduces our ability to compare our findings. Our group has consistently identified an immune response dysregulation within peripheral blood mononuclear cells of children with NDDs. The enriched blood coagulation terms and pathways may be an indication of a systemically activated immune response within the cells of these children. Inflammation and haemostasis rely on an interdependent relationship, whereby thrombo-inflammatory processes are linked to an imbalanced coagulation system and excess platelet activation.[263] In addition, the enriched findings might be a result of crosstalk between the CNS and the peripheral immune response, where cytokine release within the CNS can aggravate haemostasis pathways within the periphery. As a result of this, peripheral immune cells can respond to inflammation through platelet activation,[264] a pathway which consistently appeared in our results. Finally, investigation into a range of sample types from these children including plasma or saliva might yield diverse findings or confirm the results identified within this work.

Dysregulation within translational machinery have also been implicated within neurodevelopmental disorders across various biological levels such the transcriptome and proteome. For example, highly penetrant variants in ribosomal proteins (RP) and eukaryotic translation initiation factors (EIF) genes can cause NDD.[237] However, most individuals with neurodevelopmental disorders do not have highly penetrant DNA variants.[32,33] To add, external factors such as stress may impact translational processes through disruption of

ribosomal biogenesis.[265] The ribosome is a highly energy-intensive organelle, and its control is vital particularly when cells are under stress. These notions taken together with the dysregulated ribosomal proteins in the PANS cohort, point towards ribosomal and translational control acting as a marker of cellular stress.

To add, cohort 1 has enriched pathways involving RNA polymerase II, one of three enzymes responsible for transcribing all protein-coding genes, as well as many noncoding RNAs within eukaryotes.[266] Cytoskeletal organisation was a reoccurring theme within the two cohorts within this study. While actin is a major component of the cytoskeleton and is involved in cell motility and transport, the literature points towards a regulatory role within gene transcription. Nuclear actin is vital for all RNA polymerases, of interest however is its role in the pre-initiation complexes of RNA polymerase II, where it's been found to play a role within active transcription.[267] In addition, actin has been implicated in chromatin remodelling complexes, a vital class of nuclear enzymatic complexes with consequence on DNA accessibility that influence transcription, replication and repair.[268,269]

Furthermore, current findings of metabolic dysregulation reported within the blood of children with these complex conditions corroborate with a brain proteomic study of ASD, where proteins involved in energy metabolism along with synaptic function, and myelination were observed.[270] A recent metabolomic study investigated the plasma metabolic profile within children with PANS and identified changes within metabolic pathways induced by inflammation and dysregulation within energy metabolism – consistent with results identified in this thesis.[271] This is relevant to the groups work as recent metabolic investigations have similarly identified inflammation and metabolic dysregulations.

To the best of our knowledge, this this is the first study to investigate the proteome of children with PANS. The results presented within this chapter are generated from two separate batches of proteomic investigations, performed on cells from children with

neurodevelopmental disorders, and in particular PANS diagnoses. Of interest within these two findings is the commonality within the results identified, acting as confirmation of the signatures identified. The work within this chapter is vital as it points to dysregulations within the blood proteome of children with PANS, opening the doors for future studies to explore ribosomal dysregulations, metabolic processes and the cell signalling as biomarkers and potential therapeutic targets within these conditions.

Although this work adds a plethora of knowledge into our understanding of complex neurodevelopmental disorders, there are many caveats that need to be mentioned. Firstly, the same size within each cohort was small (n=4), future work investigating complex neuropsychiatric conditions such as PANS should utilise larger sample sizes where available for a comprehensive investigation. To add, the patients included in this work were receiving intravenous immunoglobulin therapy on a regular basis as treatment for psychiatric symptoms resulting from recurrent infections. The proteomic signature of children with PANS not receiving treatment may have a different effect when compared to those on this therapeutic. In addition, future studies investigating PANS should explore if PANS as an entity is different to OCD and Tourette syndrome. Currently, the proteomic investigations presented within this chapter have been individually analysed. Future efforts into understanding the biology of these complex conditions should focus on integrating multiple batches of children with neurodevelopmental disorders to cover the heterogeneity presentation within these conditions. Furthermore, integration of multiple types of high-throughput experimentations such as RNA sequencing and proteomic data of the same patients through utilising data integration techniques such as the MixOmics package, may allow for a deeper dive into the biology of these complex disorders. MixOmics is a collaborative project allowing a holistic view of a system through combination of omic analyses and utilising a multivariate method.[272]

Chapter 4

Characterising the innate immune response in peripheral blood mononuclear cells of children with Paediatric Acute Neuropsychiatric Syndrome through the toll-like receptor pathway

4.1 Preamble

The immune response plays a crucial role in regulating the highly orchestrated strides of neurodevelopment, and thus has been implicated in neurodevelopmental conditions such as ASD, Tourette syndrome and OCD. The literature has pointed towards immune morphological dysregulations, as well as the presence of aberrant inflammatory markers in individuals with these disorders.[100,105,107,110,123-125,129,273] The recurrent infections, immune dysregulation and developmental regression in children with the PANS phenotype potentially suggests immune dysregulation peripherally and within the CNS, that warrants further exploration. Work within the group, including results described in Chapter 3 of this thesis, have identified a dysregulated immune signature within the transcriptome and the proteome of peripheral blood of children with PANS.[78,79,274] Thus, to validate the functional immunological dysregulations, I developed a functional stimulation assay to characterise the innate immune response in peripheral blood mononuclear cells of children with PANS compared to neurotypical controls following LPS stimulation. Together with the previous

two chapters analysing the transcriptome and proteome of neurodevelopmental disorders, this assay will enable us to better understand the immune dysregulation in children with PANS and neurodevelopmental disorders on a molecular and cellular level.

4.2 Abstract

The association of inflammation and the immune response has been proposed to be involved in neurodevelopmental disorders for a long time. The activation of Toll-like receptors has been implicated in these conditions through preclinical, *ex vivo* and *in vitro* studies, mainly within autism spectrum disorders. The aim of this chapter was to develop a stimulation assay to assess optimal time and dosage of stimulation using the TLR4 agonist, lipopolysaccharide, in peripheral blood mononuclear cells, with the overall goal of utilising this assay to assess immune difference between children with Paediatric Acute Neuropsychiatric Syndrome and neurotypical matched controls. The assay was developed by culture of peripheral blood mononuclear cells were from healthy donors in 96-well U bottom plates and treated with LPS at 500ng/ml and 1000ng/ml for 30 minutes, 3 hours, 6 hours, and 24 hours. The main objective was measurement of proinflammatory cytokines Interleukin-6 and tumour necrosis factor, by utilising reverse transcriptase quantitative polymerase chain reaction and enzyme linked immune assays. I observed that the 500ng/ml dose yielded optimal stimulation of the TLR pathway for the 30 minute, 3 hour and 24 hour durations. Specifically, a peak response at 3-hours was observed for *IL-6* mRNA, which declined over the 24 hour period. In contrast, *TNF* mRNA levels were highest at 30 minutes post stimulation and declined over the subsequent timepoints tested. Next, I utilised the optimised assay on a cohort of children with PANS compared to controls, where peripheral blood mononuclear cells were stimulated with 500ng/ml of LPS for 30 minutes, 3 hours, and 24 hours. Investigation of gene expression identified a repressed immune response in PANS compared to controls, marked by lower expression of the proinflammatory cytokines *IL-6* and *TNF* at an RNA level. In

addition, analysing the secreted cytokines following cell stimulations similarly yielded an aberrant immune response, where lower levels of the proinflammatory cytokines IL-6 and TNF (measured by ELISA) were observed in patients when compared to controls. The work described in this chapter is the first to assess the immune response of peripheral blood mononuclear cells from children with PANS following a bacterial challenge, compared to controls at various timepoints. The assay provides a valuable platform to investigate the effect of LPS stimulation on cells of children with PANS at the specified dose and timings. In addition, there is potential for extension of this work to include a range of cytokines and inflammatory molecules activated downstream, as a result of LPS stimulation. Finally, characterisation of the functional immune response in children with PANS through this assay can lead to further efforts to improve our understanding of other neurodevelopmental conditions.

4.3 Introduction

Neurodevelopment is a highly orchestrated active timeline of events, where cells and neural structures within the central nervous system are formed through processes such as neurulation and neurogenesis.[38] The developmental period from gestation throughout the early years of life is critical due to the rapid development of the central nervous system – influenced by genetic, epigenetic, and environmental factors.[35] The developing brain is vulnerable to environmental influences which impact genetically determined developmental processes. Neurodevelopmental and neuropsychiatric conditions such as autism spectrum disorders, and schizophrenia have been linked to disturbances of early CNS such as maternal infection.[35,275]

Neurodevelopmental disorders is an over-arching term which describes a group of heterogenous conditions with onset in the critical developmental period.[2] These are complex neurological conditions, encompassing autism spectrum disorders (ASD), Tourette

syndrome, obsessive compulsive disorder (OCD), intellectual disabilities and learning disorders. Currently, 10% of children under the age of five are affected by one or more neurodevelopmental disorder, often presenting with overlapping symptoms and diagnoses.[12] A plethora of genetic, epigenetic, and environmental influences are now recognised to contribute to the development of these conditions[29-31]. The involvement of genetics within these neurodevelopmental conditions is attributed to common vulnerability genes with low penetrance, where only a minority of cases have rare highly penetrant genetic variants.[33] The current lack of understanding of neurodevelopmental disorders' biology, coupled with the complex presentations of these heterogenous conditions, presents challenges for patient diagnosis and treatment.

4.3.1 The immune system in neurodevelopmental disorders

The biological course of neurodevelopment is dynamic and dependent on synaptogenesis, where synapses are formed from mid-gestation throughout the early years life, and pruned up to 21 years of life by the resident immune cells of the CNS, the microglia.[276] While the immune system is vital for protection from infections and other insults, it also plays a fundamental role in CNS development by maintaining homeostasis and promoting inflammation.[46,47] The involvement of the immune response in neurodevelopmental disorders is increasingly recognised, with reports of inflammation within the brain and the periphery of individuals with these conditions.[105,107,118,123-125,129,175,207] Consistent with the literature,[277-279] work within our group has shown that children with neurodevelopmental disorders exhibit higher rates of infections (throat infections, ear infections and mouth ulcers) in the first five years of life, and the last 12 reported months when compared to typically developing controls (Under review, Appendix 7.2).

Inflammation during the gestational period is thought to affect early central nervous system formation, and result in increased expression of neurodevelopmental vulnerability

genes.[35] Our group reviewed the current clinical evidence associating maternal inflammatory states such as infection during pregnancy and the association with childhood neurodevelopmental disorders.[36] The link between an activated immune response during gestation and offspring autism spectrum disorders and schizophrenia, was recognised in mothers infected by the rubella virus, and further validated by additional outbreaks.[67] Other than infections, additional maternal conditions linked to chronic inflammation including obesity, gestational diabetes, pre-eclampsia, depression, autoimmune disease, have been shown to increase the risk of offspring neurodevelopmental outcomes such as ASD, ADHD and Tourette syndrome.[78] These risk factors result in cellular stress and further inflammation within the pregnant mother, and are associated with an increased risk of neurodevelopmental disorders within the foetus. Furthermore, our group highlighted that cumulative maternal inflammatory factors were associated with increased risk of neurodevelopmental disorders in offspring.[280] To illustrate this, a single autoimmune disease in the mother had an adjusted odds ratio of 1.25 in the child developing autism spectrum disorder by age nine. When this maternal risk factor is combined with childhood infection, the risk of ASD in the child is increased to 1.72.[280] To add, previous work by the group has similarly identified that mothers of children with obsessive compulsive disorder and tics have higher rates of maternal autoimmune conditions when compared to mothers of controls.[79] Interestingly, hospital admissions due to maternal viral infection (within the first trimester), and bacterial infections (within the second trimester), have shown association with offspring autism diagnosis.[281] Prospective birth cohort studies offer great insight into an otherwise inaccessible area of research – An increased risk of autism in offspring was associated with elevated levels of inflammatory cytokines such as interferon γ (IFN- γ), Interleukins 4 and 5, and TNF in maternal serum and amniotic fluid.[76,282] In addition, maternal C-Reactive protein (CRP) levels, a marker of systemic inflammation, was associated with an increased risk of offspring autism spectrum disorder within a Finnish birth cohort study.[283]

Paediatric Acute Neuropsychiatric Syndrome Environmental influences such as infections during the postnatal period have been linked to worsening of neuropsychiatric symptoms within children.[96] As discussed in Chapter 1.2.6, a subset of children present with abrupt onset emotional changes such as obsessive-compulsive disorder and eating restriction, a condition termed Paediatric Acute Neuropsychiatric Syndrome (PANS).[99] This condition is often referred to as a "diagnosis of exclusion" whereby other neurological conditions must be eliminated beforehand.[99] Currently, the literature is divided on whether to classify PANS as a "discrete biological (autoimmune) entity", or a clinical phenotype which is part of the neurodevelopmental disorder spectrum. It is our groups' hypothesis, that PANS is not a 'discrete autoimmune entity' but instead a 'clinical phenotype' that demonstrates the ongoing interaction between the immune response and central nervous system within individuals with neurodevelopmental disorders.[79] This controversy behind PANS is partly due to the unknown aetiology and pathogenesis, and lack of specific biomarkers. Numerous reports within the literature, along with studies produced by our group, have documented an autoimmune maternal history in PANS cases,[78,79,203,205,284,285] supporting the concept of a heritable or environmental form of immune dysregulation.

4.3.2 Clinical studies

The literature of NDDs has demonstrated immune abnormalities within the CNS and periphery of affected individuals compared to controls, through assessment of inflammatory cytokines, morphological and functional abnormalities. Live brain tissue is difficult to access for research, studies utilising post-mortem brain samples offer great insight into complex neuroanatomical disease, and biological insights in to people suffering from neurodevelopmental disorders. Reports employing post-mortem brain have identified morphological abnormalities consistent with activation within the CNS resident immune cells, the microglia, in those with neurodevelopmental disorders compared to controls using

cytokine measurements and high-throughput transcriptomic sequencing.[100,107,110,273] In addition, studies have observed increased expression of inflammatory factors within the brains of individuals with autism, OCD and Tourette syndrome.[118]

Dysregulated cytokine and interferon levels have been detected in the periphery (blood, serum, and plasma) of individuals with neurodevelopmental disorders, further implicating an aberrant immune response in these conditions.[105,123-125,129] A review focused on evidence of immune dysregulation within the autism spectrum disorder literature, covering cytokines and their potential use as biomarkers:[286] Of interest, increased cytokine expression including IL-1β and IL-6 within the plasma of children with autism was found to be associated with symptom severity and impaired communication.[121] Although these findings provide support for the role of inflammation in neurodevelopmental disorders, there is significant heterogeneity in findings and methodological variables.[286]

4.3.3 Toll-like receptor pathway

Toll-like receptors (TLRs) are one of many pattern recognition receptors utilised by the innate immune system to detect microbes.[287] As discussed above, animal models of neurodevelopmental disorders modelling maternal immune activation utilise TLR ligands to induce an innate immune response. These animal models demonstrate that activation of the TLR pathway induces neurobehavioural perturbations in offspring akin to neuropsychiatric symptoms. Work from our group identified toll-like receptor activation as a common feature to many maternal inflammatory risk factors associated with neurodevelopmental disorder development in offspring.[288]

The TLR family is comprised of 10 transmembrane proteins (1-10) in humans, which can be found on the cell surface or within intra-cellular compartments such as endosomes. The structure of TLRs consists of three main components (Figure 4.1). Firstly, at the N-terminus, a folded structure comprising of 19-25 leucine-rich repeats (LRRs) recognise both

exogenous and endogenous molecular signals. The middle section involves a single transmembrane domain essential for anchoring on the cell surface. Finally, the endodomain is the site of interaction for adaptor molecules leading to signal transduction. Upon ligand binding, TLRs dimerise to form hetero- or homodimers, with the addition of accessory molecules such as CD14 and MD2.[287,289-291]

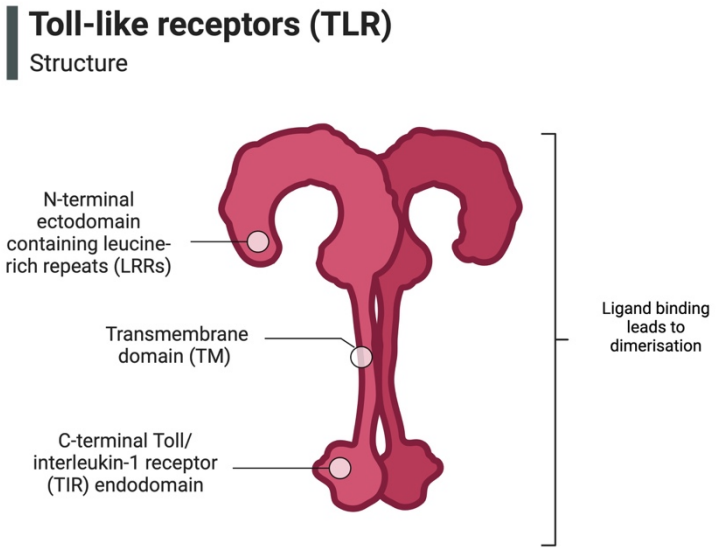


Figure 4.1 Structure of a toll-like receptor
Figure highlights the structure of a toll-like receptor. Generated using Biorender.

TLRs detect pathogens by recognition of molecular signatures specific to microbes known as pathogen-associated molecular patterns (PAMPs), as well as signals produced as a result of cell death, known as damage-associated molecules patterns (DAMPs). Stimulation of these pathways results in signalling cascades that lead to the production of cytokines and interferons.

Peripheral blood mononuclear cells express a wide range of TLRs. Peripheral blood mononuclear cells are consist of lymphocytes (70-90%), monocytes (10-20%), and dendritic cells (1-2%).[292] The monocyte subset is important in this research due to (1) their high expression of TLR expression, (2) monocytes and microglia being from the same cell lineage, and (3) the ability of monocytes to travel from the periphery and infiltrate the CNS, with the

goal of differentiating into a microglia-like subset in response to inflammation and injury.[57,293,294] Of interest, Ashwood *et al.,* have demonstrated that a subset of children with autism have discordant numbers of immune cell types when compared to controls, where they highlighted 20-25% higher levels of B cells in cases, which may have a role in explaining the involvement of the immune response within neurodevelopmental disorders.[295] In addition, children with OCD have also been identified to have enrichment of monocytes when compared to controls.[296]

Activation of toll-like receptors by agonist such as lipopolysaccharide (LPS) results in signalling cascades which produce pro-inflammatory cytokines. LPS stimulation in PBMCs increases mRNA expression of interleukins *IL-1β*, *IL8*, *IL-15*, interferons *IFN-β* and *IFN-γ*, *TNF* and *IL-6*.[297] Figure 4.2 highlights the toll-like receptor pathway, where activation by LPS induces TLR4 homo-dimerisation, and the resultant initiation of the two parts of the pathway: The MyD88-dependent and/or the TRIF-dependent pathway(s).

Myeloid differentiation primary-response protein 88 (MyD88) is a signalling adaptor recruited to the cytosol by TIRAP following TLR4 activation.[287,298,299] This is referred to as the myddosome, the formation of which leads to the autophosphorylation of IRAK 1, 2 and 4. The recruitment of TRAF6, resulting in the activation of TAK1 is crucial for initiation of the Iκ kinase (IKK) complex. A cascade of ubiquitination events then occurs, rendering the nuclear factor - κβ (NF-κβ) complex active. This activation results in the translocation of NF-κβ to the nucleus, where it is able to promote gene expression of pro-inflammatory cytokines such as IL-6, TNF and IL-1β.

On the other hand, the TRIF-dependent pathway is triggered by either TLR3 activation, or by the delayed activation of TLR4 with the assistance of the adaptor molecule TRAM.[289,300] First, TRIF is recruited to the cytosol following dimerisation of the toll like receptor. Following translocation of TRIF, it binds to the adaptor protein TRAF3, which

results in TBK and IKK activation, crucial kinases for IRF3 phosphorylation. Phosphorylation of IRF3 then results in its translocation to the nucleus, where its able to promote gene expression of type I interferons such as INF-α and INF-β.

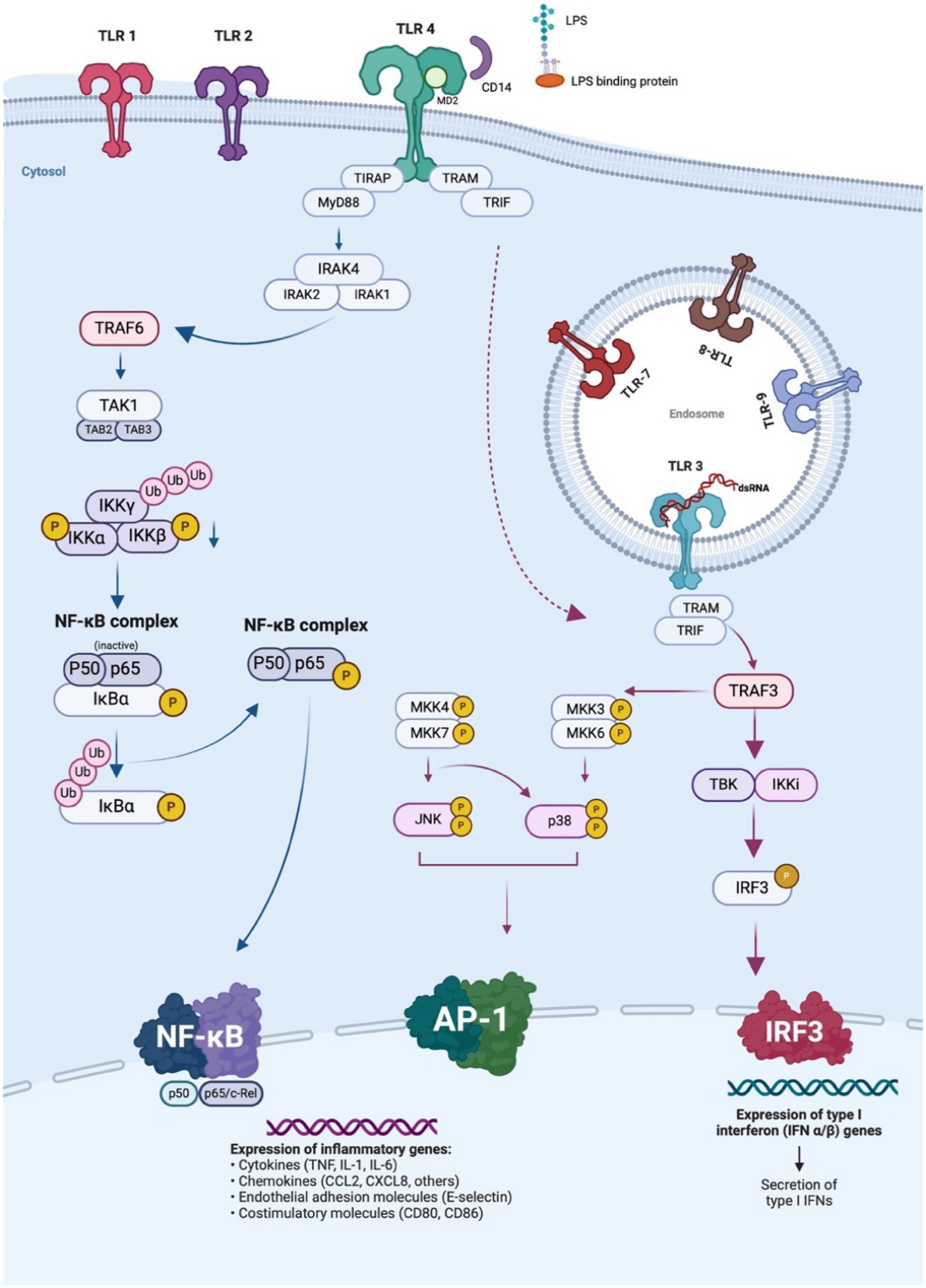


Figure 4.2 Toll-like receptor pathway
Toll-like receptor signalling cascade. Figure directs into two main pathways, the MYD88-dependent and the TRIF-dependent pathway to translocate transcription factors into the nucleus. The results response is increased gene expression of inflammatory genes and type I interferons. Figure generated using Biorender.

4.3.4 Functional TLR assay in neurodevelopmental disorders

The inaccessibility of the brain means utilisation of the peripheral tissue (blood) becomes of utmost importance in understanding the pathophysiology of neurodevelopmental disorders. The cell type most equipped for this task is monocytes, which have the ability to recognise PAMPs and differentiate into macrophages to further sustain the immune response. This recognition can then lead to proinflammatory cytokine production, which as discussed above, can alter developmental trajectories. Another reason for utilising peripheral blood mononuclear cells, is that LPS greatly regulates intracellular and secreted cytokine production in monocytes when compared to T lymphocytes, even at very low concentrations.[301]

To date, there have been no studies to assess the optimal dose and time of LPS stimulation on peripheral blood mononuclear cells, and utilise an LPS stimulation assay in neurodevelopmental disorders, and in particular, children with a PANS phenotype. Table 4.1 below summarises the literature of peripheral blood mononuclear cells or monocytes stimulated with LPS in individuals with neurodevelopmental disorders. These studies reported an increased pro-inflammatory cytokine expression in stimulated patient cells compared to controls. To note, the majority of the studies utilised samples from individuals with autism spectrum disorders. Jyonouchi *et al.,* initially tested 3 LPS concentrations on a subset of their cohort, however no timepoints were mentioned.[302]

Table 4.1 Literature of Toll-like receptor PBMC stimulation in neurodevelopmental disorders
Note: Presence of * refers to information omitted from this table as it is not relevant to the current work.

Reference	*Cohort*	*Methodology*	*Findings*
Juonouchi, et al.[302]	Children (n=59) with ASD and developmental regression, compared to typically developing siblings (n=23) and controls (n=17)	Stimulated PBMCS with 0.1 ug/ml LPS. Measured the supernatant for cytokine release of TNF, IL-6 and IL-1B along with other regulatory markers.	Increased cytokine expression of TNF, IL-6 and IL-B.
Enstrom, et al.[303]	Children (n= 17) with ASD and typically developing controls	Stimulated monocytes isolated from PBMCS with 10 ng/μL LPS and other ligands. Cytokines were measured in supernatants using Luminex assays.	TLR4 stimulation yielded increased cytokine expression of TNF, IL-6 and IL-B.
Jyonouchi, et al.[304]	Children (N=19 with ASD, and n= 19/26 with ASD + recurrent infections, and controls (19 amongst other groups.	Stimulated PBMCS with 0.1 ug/ml LPS overnight. Pre-treatment was also performed with LPS overnight as another condition. Cytokine measurements were performed by ELISA kits.	IL-23 expression was increased in patients compared to controls following LPS stimulation. LPS pre-treatment revealed lower IL-1B production in patients compared to controls.
Jyonouchi, et al.[305]	Children (n= 24) with ASD, children with PANS (n=16), controls (n=16) along with food allergy cohorts. *	Stimulated monocytes isolated from PBMCS with 0.1 ug/ml LPS along with other TLR agonists. Levels of secreted pro-inflammatory cytokines were measured ELISA kits.	Increased IL-6 and IL-1B production in patients compared to controls.
Hughes, et al.[306]	Children (N=26) with ASD or pervasive developmental disorder and typically developing controls (n=22)	Stimulated monocytes isolated from PBMCS with 1ug/ml LPS and a TLR2 agonist for 24 hours. Gene expression was measured using RNA sequencing	Activation of TLR genes, and up-regulation of pathways involved in the immune response using KEGG and GO.

ASD = Autism spectrum disorders. ELISA = enzyme-linked immunosorbent assay. GO = Gene Ontology. IL = Interleukin. KEGG = Kyoto Encyclopedia of Genes and Genomes. LPS = Lipopolysaccharide. PANS = Paediatric acute neuropsychiatric disorders. PBMCS = Peripheral blood mononuclear cells. RNA = Ribonucleic acid. TLR = Toll-like receptor. TNF = Tumour necrosis factor.

Previous chapters of this thesis have highlighted cellular dysregulation in children with neurodevelopmental disorders, chiefly within the PANS phenotype. In particular, Chapter 3 describes a proteomic investigation of a PANS cohort, where we found dysregulation of translation (ribosomal) and immune signalling pathways. To add, work within the group utilising single-cell RNA Sequencing technologies using the same cohort of patients has similarly recognised immune and translational (ribosomal) findings, along with other dysregulations within mitochondrial and endocytosis signalling pathways. In addition, these children have been shown to have higher rates of childhood infections when compared to neurotypical controls.

The recurrent infections in children with PANS suggests an aberrant immune response, the cause of which is still unclear. In addition, the literature on functional assays in PANS is scarce. To address this abnormality, I developed an assay to measure production of vital pro-inflammatory cytokines in peripheral blood mononuclear cells after stimulation of the toll like receptor pathway. Original studies exploring the immune response using a TLR agonists have historically utilised Poly:IC to mimic viral infection, and LPS for bacterial insults. In my assay, LPS was chosen as the TLR agonist of choice due to its ability to recruit the MyD88 and TRIF pathways within the TLR signalling cascades. Currently, there is no consensus on the optimal dosage and treatment time of LPS to induce activation of vital proinflammatory cytokines such as IL-6 and TNF. Nor is there previous work that has measured down-stream effects of TLR stimulation at various timepoints. Thus, an assay measuring the expression levels of vital cytokine levels in stimulated cells is important. In addition, this chapter adds to the literature of peripheral blood mononuclear cells by detailing methods of working with a minimal number of cells – a consequence of paediatric clinical samples.

Therefore, the first aim of the current study is to develop a protocol to culture peripheral blood mononuclear cells and assess the TLR response following stimulation with LPS at two different doses at various timepoints. Furthermore, the second aim was to utilise the developed assay to evaluate gene expression and cytokine production of IL-6 and TNF in peripheral blood mononuclear cells of children with PANS compared to controls. Thus, the hypothesis of this chapter is that stimulation of peripheral blood mononuclear cells with lipopolysaccharide will be time and dose dependent, and we hypothesise there will be a dysregulated immune response in the cells of children with PANS when compared to controls.

4.4 Assay development and optimisation

This section defines the steps taken to develop the TLR assay described in this chapter, and the optimisation steps performed prior to using patient samples. 4.4 details the development of best methods for culturing peripheral blood mononuclear cells previously cryopreserved, as well as working out the best dosage and time points for the TLR stimulant used to showcase a change in expression of genes of interest. A graphical summary is presented in Figure 4.3.

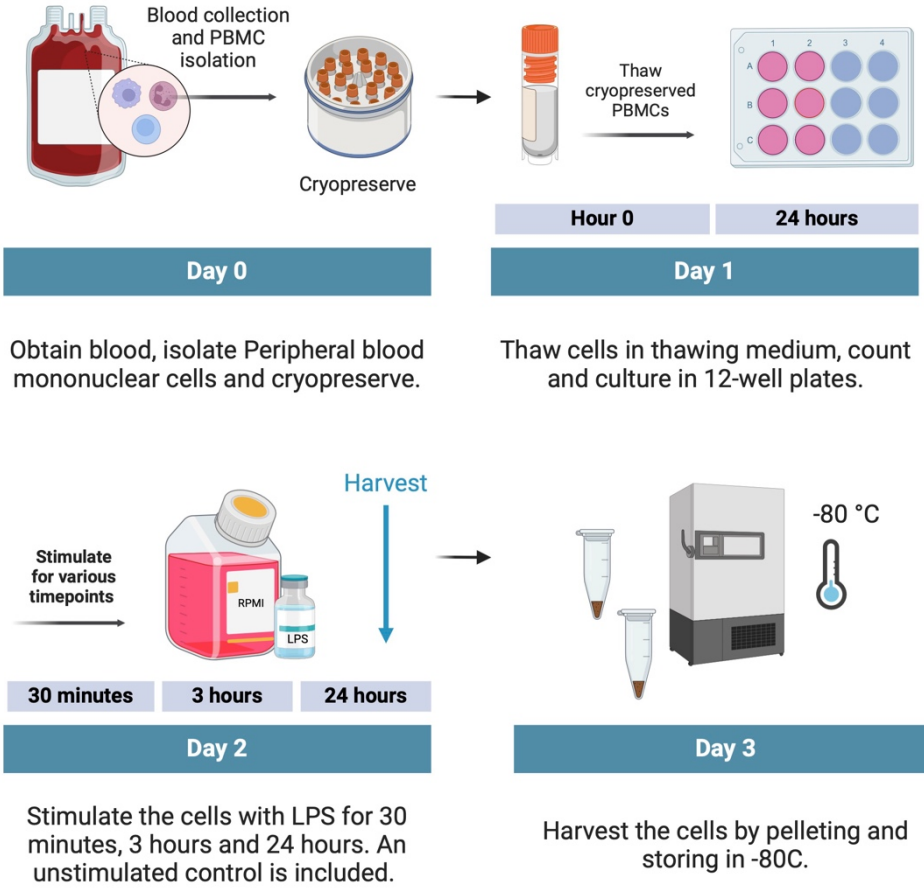
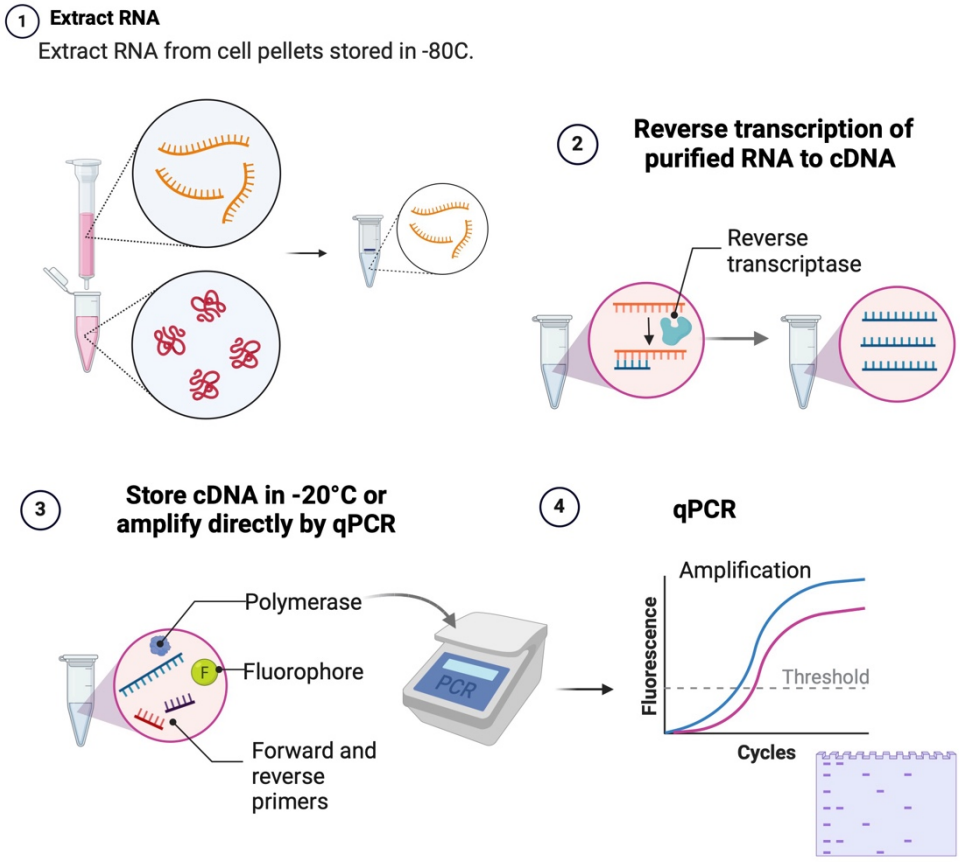


Figure 4.3 Method development graphical summary Figure is a graphical summary of the Toll-like receptor assay. Figure generated using Biorender.



4.4.1 Isolation and culture of control peripheral blood mononuclear cell

Method Human buffy coat, obtained from healthy donors (Red Cross Australia, Alexandria), was used to isolate peripheral blood mononuclear cells for assay development. Buffy coat was diluted 1:1 with wash buffer (phosphate buffered saline supplemented with 2% fetal bovine serum; FBS - Cat SH30084.03, Cytiva), and layered on 12 mL of Ficoll-Paque (Cat #GE17-5446-52, Merck) in 50mL SepMate tubes (Cat #85450, Stemcell). Next, the blood was centrifuged at 1200g for 15 minutes, and the PBMC fraction was extracted. This cell population was washed twice with wash buffer and counted, before aliquots were resuspended in dimethyl sulfoxide (DMSO) supplemented with 10% FBS. Aliquots were frozen in a Mr. Frosty™ Freezing Container (Cat #5100-001, Thermo Fisher) and kept in -80C for 24 hours, and transferred to liquid nitrogen for long term storage. Utilising the freezing container allows for optimal cell preservation as it cools cells -1°C/minute.

Culture of control peripheral blood mononuclear cells Cryopreserved control PBMC aliquots were thawed by gentle immersion in a water bath until cells appeared to resemble a slurry-like solution. The thawed PBMCs were added to 10mL of pre-warmed culture media (RPMI, supplemented with 10% FBS and 1x Glutamax; Cat #35050061, Thermo Fisher) and centrifuged at 250g for 10 minutes. PBMCs were then resuspended in media and incubated with DNase I for 15 minutes RT. This treatment step was included in the protocol to combat the cell clumping that arise due to low temperature storage in FBS. Environmental stressors and handling can impact cells and result in cell death, which results in the release of DNA that clumps cells together. Cells were counted, and 8mL of media was added for another spin at 1200g for 10 minutes. Cells were then resuspended in appropriate volume of media for culturing, based on cell count. Sterile 12-well plates were prepared by adding 1mL of media prior to the addition of $1x10^6$ of cells. All experiments utilised $1x10^6$

cells per well to standardise across samples and conditions. Cells were allowed to rest overnight in 37 °C, 5% CO_2 set incubators prior to any further handling.

Challenge The current practice in the lab was utilising a centrifuge speed of 250g for cell pelleting. When thawing previously cryopreserved cells, I observed that approximately only 2/3 of the initial starting cell count remained for culture, which means a significant number of cells were lost. This is vital within the current samples required for the assay, as there is a limited number of starting cells that can be obtained from paediatric cases and controls.

Troubleshooting An experiment with the help of Dr Brooke Keating and Dr Hiroya Nishida was performed to assess the effect of centrifuge speed on cell yield following thawing steps described above. Two cryopreserved aliquots of peripheral blood mononuclear cells from the same control individual were thawed according to the steps describe above. Centrifuge speed was changed at each wash step, and cell counts were performed following each centrifugation step (Figure 4.4).

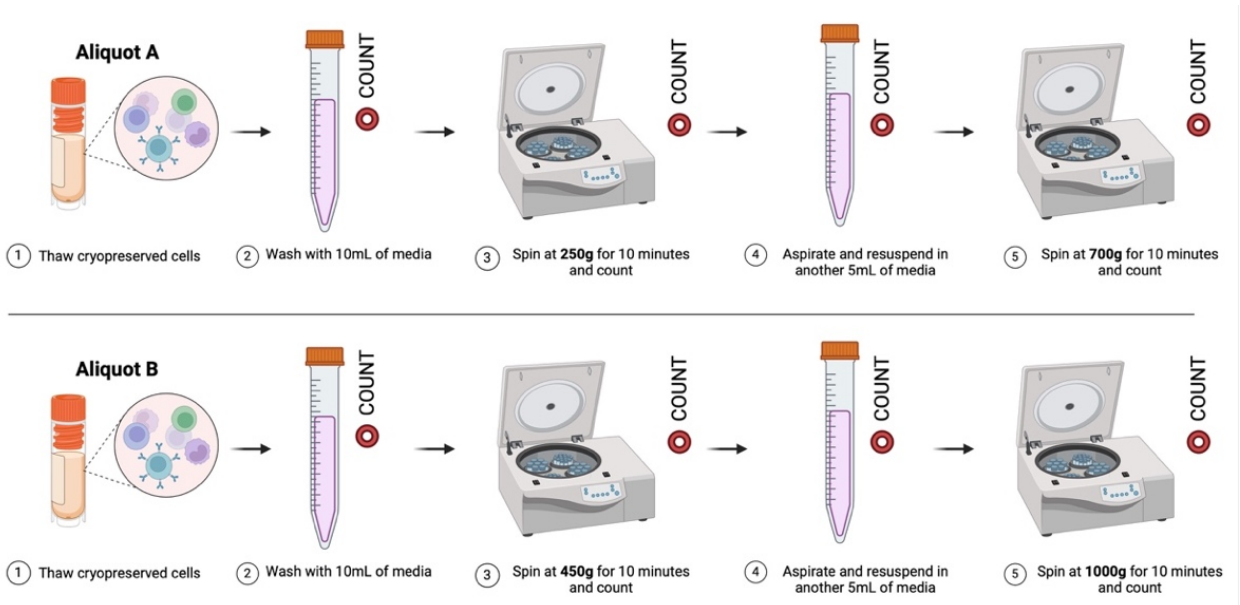


Figure 4.4 Troubleshooting centrifugation speed
Figure generated using Biorender.

Results Initial cell counts of both Aliquot A and Aliquot B were recorded at 10 x10^6 cells (Table 4.2). Following thawing, I observed that Aliquot A had more cells than Aliquot B. For the first step in this experiment, Aliquot A was centrifuged at 250g, and Aliquot B was centrifuged at 450g. Following from this first centrifuging, Aliquot A lost 26.97% of cells, while Aliquot B lost 36.18% of cells. The second centrifugation step saw a 25.22% decrease in cells in Aliquot A, compared to the 13.4% decrease in cells of Aliquot B. While both aliquots had a similar number of cells after the second centrifugation step, it can be observed that more cells were lost in the supernatant of Aliquot A, when compared to Aliquot B.

Table 4.2 Cell count following peripheral blood mononuclear cell washes at various centrifuge speeds

	Aliquot A		*Aliquot B*	
	Sample	**Supernatant**	**Sample**	**Supernatant**
Starting cell count	10 x10^6 cells	-	10 x10^6 cells	-
Count after thawing	7.6 x10^6 cells	-	7 x10^6 cells	-
Count after first spin	5.55 x10^6 cells	1.2 x10^6 cells	4.85 x10^6 cells	200,00 cells
Count after second spin	4.15 x10^6 cells	50,000	4.2 x10^6 cells	0

From this experiment, a low centrifuge speed can be observed to result in a large number of cells lost. Thus, an increase in centrifuge speed was applied to all future protocols, from the initial 250g to 700g for the two wash steps. This centrifuge speed increase allowed for optimal cell count within the pellets of samples, and minimised loss in the supernatant following washes.

4.4.2 RNA extraction of cryopreserved control peripheral blood mononuclear cells

Method RNA extraction of control PBMC pellets was performed using multiple spin-column kits, according to manufacturer's instructions (Figure 4.5). These kits included the RNeasy Mini Kit (Cat # 74106, Qiagen), as well as two Zymo Research kits, Direct-zol mini-prep and Quick-RNA mini-prep kits (Cat # R2051, Cat # R1054). Sample pellets were resuspended in 350ul of TRL buffer (Qiagen) or 800ul Trizol (Zymo Research) and homogenised 5-10 times using a 22g syringe before 70% ethanol was added to each sample. Complete homogenisation of samples prior to extraction is crucial to allow RNA release following disruption of cell membranes. Next, the samples were subjected to multiple washes and spins to purify the RNA in the silica spin column. The purpose of the multiple washes is to reduce contaminants (salts, proteins) within samples, which are spun down in the flow-through and discarded, with the RNA remaining bound to the column membrane. A DNase I treatment was then performed for 15 minutes (RT) to eliminate genomic DNA contamination from each sample. This is crucial for PBMCs as I wanted to guarantee the highest quality of RNA, as well as removing any impurities that may interfere with down-stream gene expression. RNA concentrations were measured using a Nanodrop spectrophotometer (Cat #ND2000, Thermo Fisher).

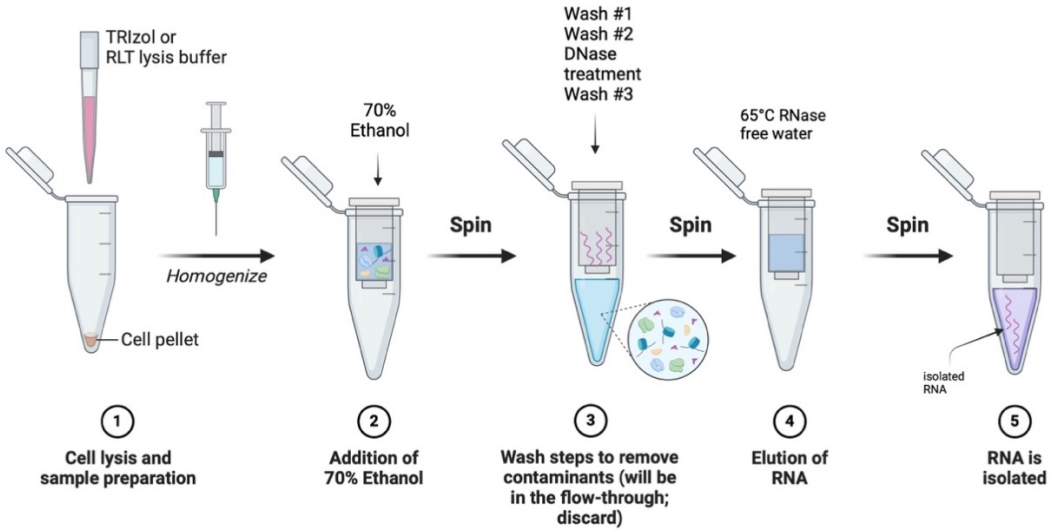


Figure 4.5 RNA extraction protocol summary
Graphical summary of the steps involved in an RNA extraction. Figure generated using Biorender.

Challenges Optimisation of this assay utilised peripheral blood mononuclear cells previously cryopreserved by the team. Initial experiments handling PBMCs proved some difficulties in obtaining an RNA yield that could be utilised for down-stream experiments. Multiple kits were trialled to assess yield, quality and reproducibility of RNA extracted. Extractions were performed as per protocol, and manufacturer was contacted for extra recommendations to improve results.

Troubleshooting The manufacturers (Qiagen and Zymo Research) were contacted for tips on improving the concentration of RNA extracted and were implemented within the protocol. The first improvement utilised was heating of the RNAse free water to 65C and double eluting the sample, i.e. the water was added to the spin column, the sample was centrifuged, and the step was repeated once more. The effect of heating the water to high temperatures prior to elution allowed the RNA bound to the membrane to be released more readily. Next, utilisation of a smaller needle gauge for homogenisation of samples resulted in a more efficiently homogenised sample, which allowed for increased yield of RNA and higher quality.

Results The top three extractions from each kit are shown in Table 4.3. As it can be observed, the kits had a wide range of RNA concentrations observed from similar starting material. Although the RNA concentrations were low, the integrity and purity of the samples as shown in the 260/280 ratio was not up to standard. Within the literature, it is agreed that for the 260/280 ratio, a value between 1.8-2.00 constitutes an acceptable RNA integrity, free from protein contaminants.[307] The table highlights that the highest and most reproducible yield was produced by the Qiagen RNeasy Mini kit. Due to its great reproducibility, the kit was utilised for further experiments.

Table 4.3 RNA extraction comparison
Three different RNA extraction kits were trialled to assess RNA yield and quality of cryopreserved control peripheral blood mononuclear cells. The Rneasy mini kit provided the most consistent yield (260/280) of RNA from the samples measured (n=3), and was utilised for further experiments.

Kit	*Cell count*	*Eluted volume*	*Concentration*	*260/280*
Direct-zol	4.4 x 10^6	25ul	54.3 ng/µL	1.74
Direct-zol	4.75 x10^6	25ul	304.8ng/µL	1.63
Direct-zol	4.75 x10^6	25ul	58.6 ng/µL	2.06
Quick-zol	4.75 x10^6	50ul	8.3 ng/µL	2.27
Quick-zol	4.75 x10^6	25ul	13.1 ng/µL	2.50
Rneasy mini	4.4 x 10^6	25ul	220.6ng/µL	2.00
Rneasy mini	5 x 10^6	20ul	59.3ng/µL	2.04
Rneasy mini	5 x 10^6	20ul	138.9ng/µL	1.88

4.4.3 cDNA and RT-qPCR of peripheral blood mononuclear cells

cDNA synthesis RNA with a 260/280 ratio of >1.7 and a concentration >10ng/µL were reverse transcribed (RT) to synthesise complementary deoxyribonucleic acid (cDNA) using the SuperScript™ IV Reverse Transcriptase (Cat #18090010, Thermo Fisher) according to manufacturer's instructions. Where possible, samples were performed in batches, each containing unstimulated (control) samples, with a negative RT included for each. All samples were reverse transcribed to include the same amount of starting material. Depending on the concentrations of RNA in each experiment, cDNA was synthesised at 35-200ng. Briefly, 50uM of random decamers along with a mix of 10mM deoxynucleotide triphosphates (dNTP; Cat #R0181, Thermo Fisher) were added to each sample. The samples were heated at 65C for 5 minutes and placed on ice for one minute to allow the denatured RNA to stabilise, as well as prevent RNA strands from reannealing to each other. To initiate the final reverse transcription reaction, a mix containing a buffer, an RNase inhibitor, dNTPs

and DTT was made up and split into two tubes. Tubes containing the superscript enzyme for the positive RT samples are referred to as RT-positive samples, while tubes with water for control instead of the enzyme are referred to as RT-negative samples. The RT-negative sample is crucial, as any amplification present is an indication of contamination – as there was no enzyme added, RNA would be present and not converted into cDNA. After the appropriate mix was added to each sample, an incubation step for 10 minutes at RT was performed, followed by 50C for 10 minutes, and 80C for 10 minutes. The samples then were stored in -20C until the PCR was performed.

Primer design. The primers used for this assay are in Table 4.4 The housekeeping primer Beta-2-microglobulin was designed using NCBI's Primer-BLAST. This gene was utilised as a house-keeping gene in this experiment, instead of the most applied *GAPDH* due to its stable expression in PBMCs of both healthy and diseased populations.[308] Primer parameters included <20 base pairs long, melting temperature of 60C, with a GC content of 40-60%. The primer was designed as exon-spanning, in order to avoid amplification of intronic DNA.

Table 4.4 RT-qPCR primers

Gene	*Forward*	*Reverse*	*Size*	*Source*
Beta-2-Microglobulin (B2M)	GCTCGCGCTACTCT CTCTTT	TCTGAATGCTCCAC TTTTTCAA	191 bp	Designed
Interleukin-6 (IL-6)	ACAGCCACTCACCT CTTC	AAGTCTCCTCATTG AATCCA	122bp	Nielsen *et al.,*[309]
Tumour necrosis factor (TNF)	CTTCTCCTTCCTGAT CGTGG	GCTGGTTATCTCTC AGCTCCA	108bp	Nielsen *et al.,*[309]

Method Next, reverse transcribed quantitative polymerase chain reaction (RT-qPCR) was performed using a QuantStudio 6Pro (Thermo Fisher) in 384-well plates. A pre-made master mix was purchased, the PowerUp SYBR Green mastermix (Cat #A25776, Thermo Fisher). The manufacturer's guide described a template input of 1-10ng cDNA, with the mastermix at 2x. Therefore, the contents of each 20ul PCR reaction are shown in Table 4.5 below. The PCR cycle steps included a hot-start activation step at 95°for 10 min, followed by 40 cycles amplification cycles of 95°for 15 seconds, and 60C for 1 minute. A melt curve was also included in each run, consisting of 95 for 15 seconds, and 60C for 1 minute and 95C for 1 second. Each sample contained a minimum of two RT-positive wells, one RT-negative and one no template control (NTC). The NTC is a commonly included control in PCR reactions, where water is added to the well, to assess if any contamination is present within the mastermix.

Table 4.5 Initial qPCR 10uL reaction optimisation

Reagent	*Initial concentration*	*Concentration in reaction*	*In each reaction*
PowerUp Master Mix	2X	1X	10uL
Forward + Reverse primer	100 μM	1μM	2uL
Template cDNA	25ng	6.25ng	2.5uL

Data analysis The quantitation cycle (C_q) values denote each sample's cycle number where the reaction curve intersects the threshold. The C_q were imported into Microsoft Excel, where the delta-delta comparative threshold ($2^{-\Delta\Delta C_t}$) method was employed.[310] Each PCR contained a housekeeping reference gene for all samples; Beta-2-microglobulin, which was used as the reference in all calculations. The Delta Comparative threshold (ΔC_t) was calculated by subtracting the C_q values of the gene of interest from the house-keeping gene's

C_q for each replicate. Next, an average of control (unstimulated) sample's house-keeping C_t replicates was calculated to be used as a reference. From there, The $\Delta\Delta C_t$ was calculated by subtracting the average of controls from the delta C_q of each sample. Next, fold changes were calculated by $Fold\ change = 2^{-(\Delta\Delta Ct)}$. An average of control (unstimulated) fold changes was calculated, which was then subtracted from each sample's fold change. This allowed for a normalised fold change to be calculated, considering the expression of samples in relation to the unstimulated control condition. The fold changes then were imported into GraphPad Prism, where all statistical analyses were conducted. The fold changes were tested for normality using a Shapiro-Wilks test and analysed using a Mann-Whitney test (due to non-parametric data distribution).

Results A qPCR run was performed using the concentrations in Table 4.5, along with manufacturer's cycle settings on the primers of *IL-6*, *TNF* and the housekeeping gene *B2M* (Figure 4.6). The first part of the figure shows the amplification and melt plots, where single peaks can be observed for RT-positive samples, and no amplification of RT-negative samples indicating no contamination. The house-keeping gene *B2M* (in red) showed a good profile (amplified ~ cycle 15), while for genes of interest *IL-6* and *TNF*, a slightly longer time was required to amplify a signal. The second part of the figure details gel electrophoresis of amplified products to confirm RT-qPCR results are at the correct band sizes. All samples were utilised, including the RT-positive, RT-negative, and NTC. With the presence of single bands at the RT-positive samples, and no bands present for the negative samples, I confirmed that primers were amplified correctly, and ready to be utilised for the assay.

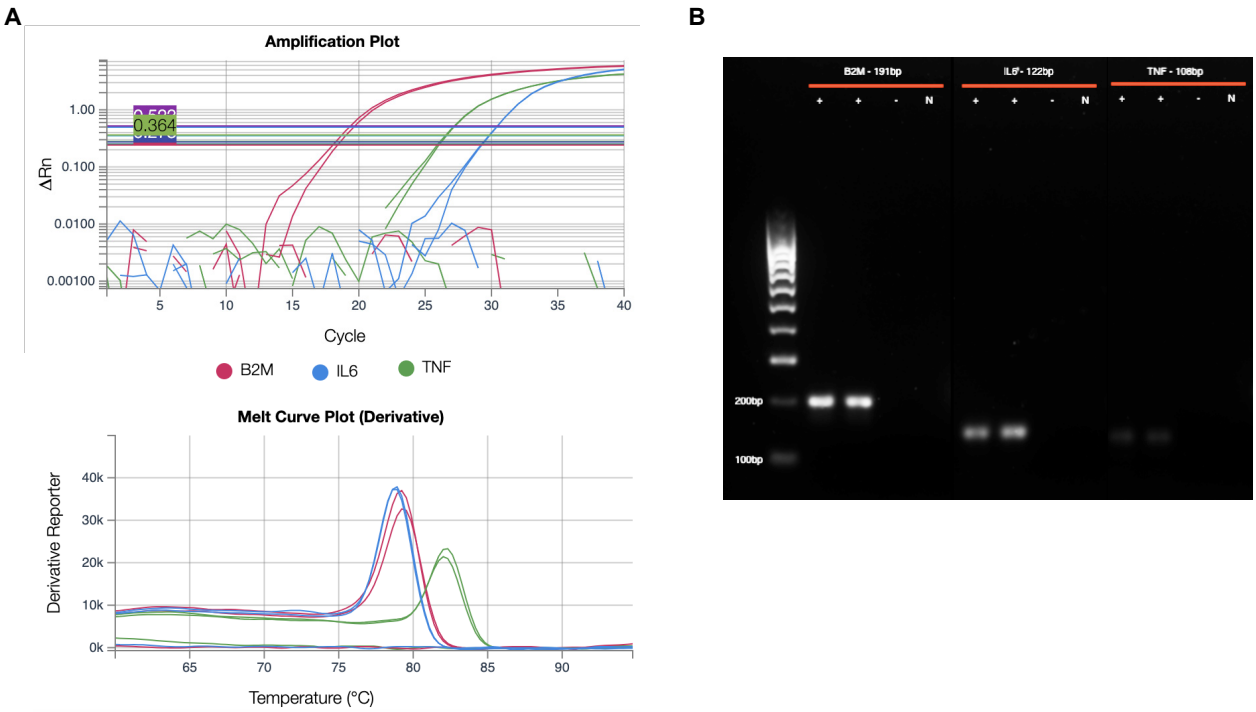


Figure 4.6 Master mix optimisation
The PowerUp™ SYBR™ Green Master Mix was trialled in a polymerase chain reaction (PCR) using the primers most of interest; Interleukin 6 (IL-6) and Tumour necrosis factor (TNF) along with the housekeeping gene Beta-2-Microglobulin (B2M). A) Amplification and melt curve plots of the PCR run for all three primers. B) Agarose gel electrophoresis of qPCR products visualised on a 1.5% agarose gel stained with GelRed. Lane 1: DNA ladder. Lanes 2-5: qPCR products for house-keeping gene B2M. Lanes 6-9: qPCR products for IL-6. Lanes 10-13: qPCR products for TNF. Cycling conditions were 95C 10mins, 95C 15s, 60C 1 mins and a melt step. As bands of genes of interest (IL-6, TNF) appeared faint, and higher concentrations of primers was utilised for future experiments.

From this step, assessment of manufacturer's cycle settings were confirmed to amplify primers of interest. Additionally, it provided a threshold of expectations for what cycle these primers should be rising above detection levels in unstimulated samples. As the *TNF* bands appear weak in signal (Figure 4.6B), future experiments were noted to utilise higher concentrations of primers in the final reaction.

4.4.4 Time-dose response

Following from above, the next part of the assay involved optimising the time-dose response within control peripheral blood mononuclear cells to further develop the protocol.

Method Control peripheral blood mononuclear cells allowed to rest overnight were stimulated using Lipopolysaccharide (LPS, from Escherichia coli 0111:B4; Cat #tlrl-3pelps,

InvivoGen) at concentrations of 500ng/mL, and 1000ng/mL for 30 minutes, 3 hours, 6 hours, and 24 hours. An unstimulated (control) condition was included, where warm PBS was added to control wells. To increase validity of the assay, triplicates of unstimulated and stimulated wells were included for each sample. Cells were harvested by adding PBS and scraping using a cell lifter (Cat #3008, Corning/Life Sciences) before pelleting by centrifuging at 500g for 5 minutes. Pellets were stored in -80C until processed.

Result After optimising the assay's protocol to fit within the constraints of working with PBMCs, the effect of dose and time of LPS stimulation on PBMCs was explored. Initial experimentations included a timeline of 0 (Unstimulated), 30 minutes, 3 hours, 6 hours, and 24 hours of LPS stimulation at concentrations of 500ng/mL and 1000ng/mL. Gene expression levels of *IL-6* and *TNF* (relative to house-keeping gene *B2M*) are shown in Figure 4.7 below. Treatment of control PBMCs with 500ng/ml of LPS showed an increase in *IL-6* expression at the 3-hour mark, which declined over the 6-hour timepoint (Figure 4.7A). Stimulating the cells with 1000ng/mL of LPS highlighted a similar trend of *IL-6* expression at the 3-hour mark, yet relatively lower in relative expression. Interestingly, the peak response in *IL-6* expression using 1000ng/mL LPS stimulation was observed at the 6-hour mark. A similar trend of declining levels of *IL-6* expression were observed in both dosages at the 24-hour mark. Treatment of lifeblood PBMCs with 500ng/mL and 1000ng/mL of LPS allowed us to observe a rapid increase in *TNF* expression at 30-minutes compared to baseline (Figure 4.7B). At the 500ng/mL dose, there is a transient decline of *TNF* expression at 3-hours compared to 6-hour timepoints. Stimulating with 1000ng/mL showed a return of *TNF* expression to baseline at the 3-hour mark. Surprisingly, a secondary increase in *TNF* expression was then observed at the 6-hour timepoint, however this diminished at the 24-hour mark. These findings were reproduced in repeat experiments. Based on the presented results, the 500ng/mL LPS dose was utilised for further experiments, with the 6-hour timepoint dropped. This decision was taken due to the 500ng/ml stimulation providing

higher magnitude of change when compared to the higher concentration, in addition to technical restraints such as feasibility of experimental timeframe.

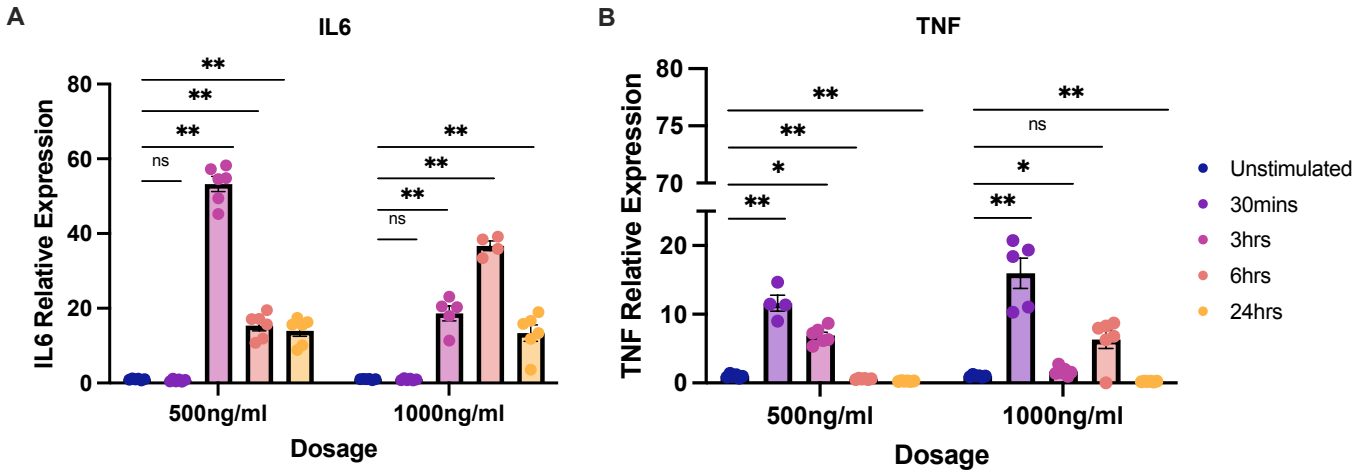


Figure 4.7 LPS time-dose response assay
Method development: relative gene expression levels of IL-6 (A) and TNF (B) in control PBMCs following LPS stimulation. Gene expression was measured following stimulation at 500 ng/mL and 1000 ng/mL concentrations at 0 hours, 30 minutes, 3 hours, 6 hours, and 24 hours. Results are presented as fold change compared to unstimulated PBMCs, calculated using the 2^(-ΔΔCt) method normalised to Beta 2 Microglobulin. Error bars represent the standard error of the mean (SEM) from triplicate measurements. Statistical significance was determined using a Mann-Whitney test, *P < 0·05, **P < 0·01, ***P < 0·001.

4.4.5 Trial run

As I embarked on performing this important assay, a decision was made to run through the entire protocol using non-precious samples previously stored. This was performed to assess any complications that may arise from thawing patient and control cells previously cryopreserved. These samples slightly differ from the control PBMCs used in assay development in that they were (1) obtained using blood tubes rather than from buffy coats, and (2) cryopreserved with a lower cell yield.

The first change conducted in this experiment was the utilisation of 96-well U-bottom plates, which allow the monocyte cell population to adhere to the bottom of the plate, while the lymphocytes float in media. This change was implemented as it allows the lysing of cells directly in the well following plate spinning and aspiration of the supernatant. By reducing steps of transferring the cell pellet, there is the potential of (1) minimising sample

loss, (2) reducing contamination such as those that may degrade RNA, and (3) more efficiently breaking down cells and lysing the cell contents. Sterile 96-well U-bottom plates (Cat #650 180, Greiner Bio-one, Cellstar) supplemented with 100uL of PBMC media were used to culture 500,000 cells/per well.

Method PBMC aliquots stored in liquid nitrogen were thawed by gently immersing in a water bath (37C) until a slurry-like mixture remained. These aliquots were then transferred to 10mls of warm PBMC media and centrifuged at 250-750g for 10 minutes. An increase in centrifuge speeds were associated with improved cell yields (Section 4.4.1) – this change was utilised during the study for all PBMC handling within the group. The centrifuge speed differed for some samples as I performed an experiment to highlight higher centrifuge speed result in improved cell yield – which was performed halfway through my experimental work. A second spin was performed with extra 5mls of PBMC media to completely wash the cells from DMSO, which is toxic at room temperature. An aliquot was taken before the second spin to record the number of cells post-thawing. Following from this, the cells were resuspended in appropriate volume of PBMC media, such that there were 500,000 cells per 100uL. Where possible, cells were cultured in duplicates for each timepoint measured (Figure 4.8).

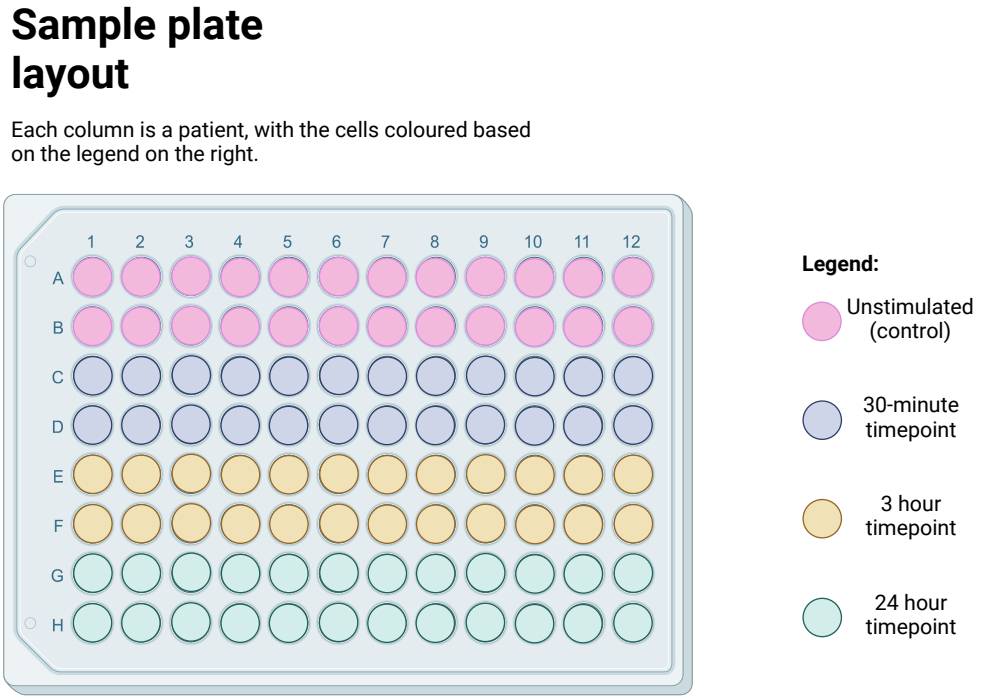


Figure 4.8 Sample plate layout
A graphical representation of how patient and control samples were plated in a 96-well U bottom plate. Each column represents a patient or control sample, with every two rows representing a stimulation time point (colour-coded, legend on the right). Figure generated using Biorender.

Cells were allowed to rest for 3 hours in the incubator prior to any stimulation. To harvest the samples, 50uL of PBS was added to each well followed by centrifuging the plate at 450g for 5 minutes. The plate was then placed on ice and moved to an RNAse-free hood. From each well, the supernatant was aspirated and moved into Eppendorf tubes, and moved to -80C. The well (which then only contained the pellet) was then flooded with 350uL of RLT buffer supplemented with B-mercaptoethanol (as per manufacturer's instructions) and stored in Eppendorf tubes. The RLT-lysed samples were then stored in -80C until RNA was extracted as per section 4.4.2 above.

Initial measurement of RNA yield and quality was performed with a Nanodrop, however the results from this trial run showed low concentrations and contamination of salts and proteins as evident by the high 260/280 ratios.

This led to questioning if the current method for measuring RNA is the best. This is an important step in the protocol due to the obtained low RNA concentrations, which make these precious samples even more valuable. A decision was made to assess the samples using a more sensitive technique such as the Tapestation. The Tapestation is an instrument used to measure and assess nucleic acid size, integrity, and quality through electrophoresis. This method of quantifying RNA quantity and quality allowed for accurate measurements when compared to the nanodrop previously utilised. Where the Nanodrop provides quick reading of the absorbance of RNA samples at different wavelengths, from which RNA concentrations and quality can then be inferred, it is not the most accurate at providing the RNA integrity. The integrity of RNA is important when utilising for down-stream applications such as RT-qPCR, where degradation can impact on the interpretation of results. This is why the Tapestation, an instrument that not only provides the concentration of RNA reads, but also the RIN which measures the level of degradation of RNA, was preferred. I measured samples using both the Nanodrop and Tapestation to compare the readings from the two instruments, shown in Table 4.6 below. The concentration of RNA measured using the nanodrop and the Tapestation showed similar readouts, however, the quality of RNA differed. Where the nanodrop displayed high 260/280 ratios indicating high contaminants, the RIN showed high quality RNA suitable for use in down-stream applications. A RIN is a measurement reflecting the quality of RNA samples measured, with a higher number correlating intact RNA.[311] It is accepted that a RIN value of >8 passes quality control.

Table 4.6 Comparison of RNA readings
RNA samples were eluted in 25ul of water and measured using both the Nanodrop and Tapestation.

	NANODROP		*TAPESTATION*	
Sample	**Concentration**	**260/280**	**Concentration**	**RIN**
1	11.5 ng/µL	2.31	10.7 ng/µL	8.5
2	7.8 ng/µL	2.5	3.99 ng/µL	8.5
3	10.4 ng/µL	2.36	4.97 ng/µL	8.7
4	8.9 ng/µL	2.33	7.1 ng/µL	8.9
5	10.6 ng/µL	2.08	22.3 ng/µL	9.3
6	10.9 ng/µL	2.22	9.62 ng/µL	8.9
7	9.2 ng/µL	2.85	5.67 ng/µL	8.9
8	9.9 ng/µL	2.57	7.23 ng/µL	9
9	9.3 ng/µL	2.51	7.34 ng/µL	9
10	8.1 ng/µL	2.68	5.52 ng/µL	8.9

From this experiment, we confirmed the quality of RNA samples produced pass quality control using the Tapestation. However, the concentration of these samples is still relatively low and would require careful handling and use for the assay. For accuracy in reading the concentration of RNA samples, final measurements were performed using a TapeStation at the Westmead Scientific Platforms, which are supported by the Westmead Research Hub, the Cancer Institute New South Wales, the National Health and Medical Research Council and the Ian Potter Foundation.

4.4.6 Summary of assay parameters

To summarise this section, development of a toll-like receptor stimulation assay provided many obstacles that were optimised. First, culture of peripheral blood mononuclear cells was conducted in 96-well U bottom plates, instead of flat wells to allow for efficient handling of cells for the down-stream application of RT-qPCR. The method of RNA extraction was decided by comparison of three different spin-column kits, where the Qiagen RNeasy mini kit proved a reliable and consistent yield of RNA that would serve for the purpose of this

assay. Measurement of extracted RNA was also optimised, where the Tapestation system provided a more accurate reading of samples' quantity, as well as integrity. This decision was important to implement due to the low yields of RNA obtained. Next, the concentrations of input material and primers were tested, where primers of interest were confirmed to amplify efficiently in both unstimulated and stimulated cells. Finally, an optimal dosage of LPS stimulation of 500ng/ml and three timepoints (30 minutes, 3 hours and 24 hours) were decided as they allowed for prime gene expression response for the genes of interest, *IL-6* and *TNF*.

4.5 Assessment of the innate immune response by stimulating the toll-like receptor pathway in peripheral blood mononuclear cells of children with Paediatric Acute Neuropsychiatric Syndrome versus controls

A graphical summary of the steps taken in this section is outlined in the figure below:

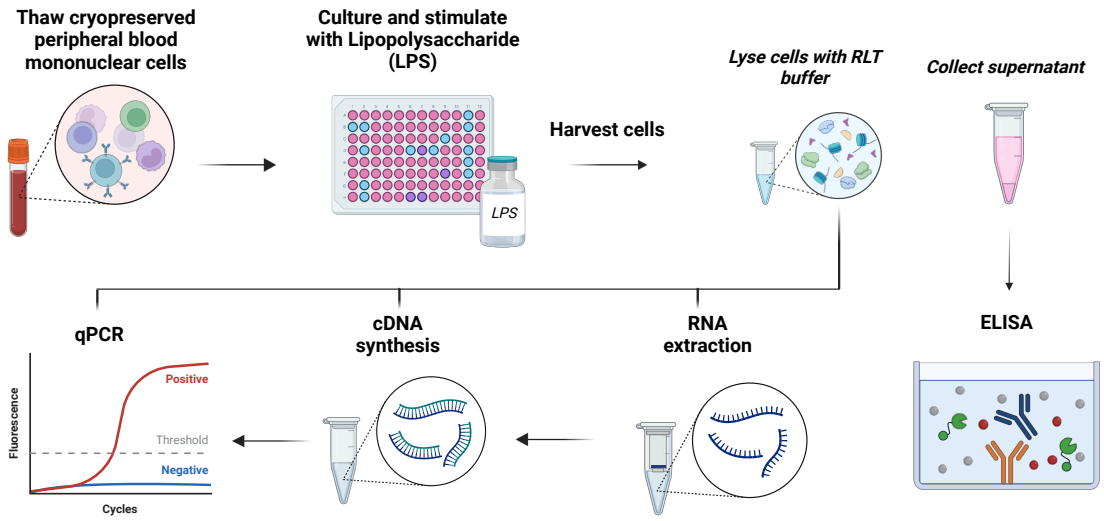


Figure 4.9 Assay method summary
A graphical summary of steps taken to perform the toll-like receptor assay following optimisation of methods. Peripheral blood mononuclear cells were thawed, cultured in 96-well u bottom plates, and stimulated with lipopolysaccharide for multiple timepoints. The cells were harvested by centrifugation, where supernatant was collected for Enzyme-linked immunosorbent assay (ELISA). The pelleted cells were lysed, and RNA was extracted. Complementary DNA (cDNA) was synthesised for quantitative polymerase chain reaction (PCR). Figure was generated using Biorender.

4.5.1 Patient cohort: Paediatric Active Neuropsychiatric Syndrome (PANS)

The above sections of this chapter described the journey of optimising the TLR stimulation assay, with the ultimate goal of assessing the response of LPS on the PBMCs of children with PANS, along with age and sex matched neurotypical controls. In Section 4.5, I performed RT-qPCR and ELISA's on PBMCs of children and controls using a bacterial challenge as per section 4.4 above.

4.5.2 Demographics and clinical variables of cases and controls

A total of seven children fulfilling the Chang *et al.*,[99] criteria for a diagnosis of Paediatric Active Neuropsychiatric Syndrome (PANS) were recruited for this assay. The PANS patients were in the chronic phase of disease (remained symptomatic) and had no infection in the 2 weeks preceding sample collection. Each patient was age and sex matched with neurotypical controls (no neurological disorders) who also had no infection in the 2 weeks preceding sample collection. The patient cohort consisted of three females (42.85%) and four male cases (57.14%), with a mean age of 10.57, median of 9, and range of 5-15 years. The control cohort also comprised three females (42.85%) and four males (57.14%), with mean age of 11.86, median of 10, and range of 8-16 years. A Mann–Whitney test indicated no significant difference ($U = 16.5$, P-value = 0.95) between the ages of the PANS and control cohorts. A table of the PANS patients can be found below (Table 4.7).

Routine blood testing for the patients included in this assay was performed to assess the proportion of immune cells in the blood. In addition, this was corroborated with testing blood from the controls utilised in this assay. The proportions of immune cells (monocytes and lymphocytes) were comparable in patients compared to controls (Appendix, 7.2.1)

Table 4.7 Demographics of the PANS cases

ID	PANS1	PANS2	PANS3	PANS4	PANS5	PANS6	PANS7
GENDER	M	M	F	F	M	F	M
FAMILY HISTORY NDD OR PSYCHIATRY	ADHD (mat)	Nil	Depression (pat, mat)	Anxiety (mat)	Anxiety, depression (mat)	Nil	ASD, ADHD (siblings)
FAMILY HISTORY AUTOIMMUNE OTHER	Graves' disease (mat)	Multiple sclerosis, Graves' disease (mat, Crohn's disease (pat)	Vitiligo (mat)	C-ANCA (mat)	Hashimoto thyroiditis, coeliac disease, psoriasis (mat)	Nil	Nil
PRECEDING NDD NP	Mild developmental delay	Nil	Nil	Nil	Nil	Mild developmental delay	Nil
AGE AT FIRST PANS	5	4	3	10	1.5	1.8	2
TRIGGER OF FIRST EVENT	Strep throat	Unclear	Vaccine and infection	Salmonella enteritis	Croup	Staph scaled skin syndrome	Trauma (adoption)
PREDOMINANT PANS SYMPTOM	OCD, agitation	OCD, processing	Inattention, OCD, emotional dysregulation	OCD, anxiety, urinary frequency	Autistic regression, OCD, irritability, sensory issues	Separation anxiety	Autistic regression, OCD, irritability, repetitive movements
DURATION FIRST PANS EVENT	2 months	1 month	6 months		4 months	2 months	6 months
TIMING OF SAMPLE RELATIVE TO FIRST EVENT	2 years	2 years	6 years	2 years	4 years	3 years	5 years
DURATION FOLLOWUP	8 years	7 years	8 years	3 years	4 years	4 years	4 years
FURTHER NO PANS EVENTS	2-3 per year	1-3 per year	3 per year	Nil	2 per year (any infection)	5 per year	3 per year
CURRENT NDD NP DIAGNOSTICS	ASD, OCD, anxiety, ADHD	OCD, depression	Tourette, OCD, depression	OCD, anxiety	ASD, OCD, ID (mod), SIB	ADHD, odd, OCD, tics	ASD, OCD, anxiety, aggression, tics
CONVENTIONAL TX	Ritalin, Risperidone, Aripiprazole, Fluoxetine	Fluoxetine	Aripiprazole	Sertraline	Ritalin, Risperidone, Guanfacine	Aripiprazole, Ritalin, Fluoxetine	Vyvanse, Risperidone, Fluoxetine

ADHD = Attention Deficit Hyperactivity Disorder. ASD = Autism spectrum disorders. C-ANCA = antineutrophil cytoplasmic antibodies. ID = Intellectual disability. OCD = Obsessive-compulsive disorder. Pat = Paternal. Mat = Maternal.

4.5.3 Sample collection

Fresh peripheral blood from patients and controls was drawn into sodium citrate tubes, and peripheral blood mononuclear cells were processed within 6 hours of sample collection. PBMC isolation was performed using density gradient centrifugation. Firstly, blood was diluted 1:1 with wash buffer and layered on 4.5ml of Ficoll-Paque in 15mL SepMate tubes. The blood was then centrifuged at 1200g for 15 minutes, and the PBMC faction was extracted. This cell population was washed twice with wash buffer and counted, before resuspending in freezing media.

4.5.4 Cell culture and stimulation

Previously cryopreserved cohort PBMCs were thawed according to section 4.4 above. Cells were cultured in warm PBMC media within 96-well U bottom plates at a density of 500,000 cells/per well. Cells were stimulated with 500ng/mL LPS for 30 minutes, 3 hours, and 24 hours in staggered timing. An unstimulated condition was included in duplicates for each sample by adding warm PBS (-/-) as a control.

4.5.5 Sample harvesting

As described above, sample harvesting was performed by adding 50uL of PBS to each well followed by centrifuging the plate at 450g for 5 minutes. The plate was then placed on ice and moved to an RNAse-free hood. From each well, the supernatant was aspirated and moved into Eppendorf tubes, and moved to -80C. The well was then flooded with 350uL of RLT buffer (supplemented with B-mercaptoethanol) and stored in Eppendorf tubes. The RLT-lysed samples were then stored in -80C until RNA was extracted as per section 4.4.2 above. RNA samples were measurements using a TapeStation at the Westmead Scientific Platforms, within the Westmead Research Hub.

4.5.6 RT-qPCR of cohort samples

cDNA was prepared from each sample as per section 4.4.3 above. The cut-off values for assessing the quality of RNA to be transcribed is an RNA Integrity (RIN) > 8. RT-qPCR was performed as per section 4.4.3 above. Each reaction contained 0.28ng of cDNA in a 10 µl volume made up of 5uL PowerUp Mastermix and 0.75uM primers.

4.5.7 Data analysis

RT-qPCR data analysis was performed using the QuantStudio software to assess the quality of each qPCR run and export the data to Microsoft Excel. From there, all analyses were as previously outlined.

4.5.8 Enzyme-linked immunosorbent assay (ELISA)

Cells release cytokines within the culture medium, following centrifugation the cells are pooled into a pellet and the remaining liquid is known as the supernatant. Measurement of proteins within this supernatant following LPS stimulation at various timepoints allows for a profile of protein concentrations in the current cohort. Too often, the supernatant cannot be utilised directly and needs to be diluted to fit within the detection ranges of kits and reader machines. An initial experiment using an IL-6 kit (Cat #EH2IL6, Thermo Fisher) was performed utilising different dilutions to assess the optimal dilution factor for the cell supernatant. Samples were observed to be best detected at a dilution of 1:20. Final soluble cytokines were measured in the supernatant of samples using human ELISA kits for IL-6 and TNF (Cat #EH2IL6, and #KAC1751, Thermo Fisher) according to manufacturer's instructions. The assays were performed with a standard curve (according to manufacturer's instructions) to warrant reliability of results with a cut-off of 0.99 implemented for the R-squared value (Figure 4.10).

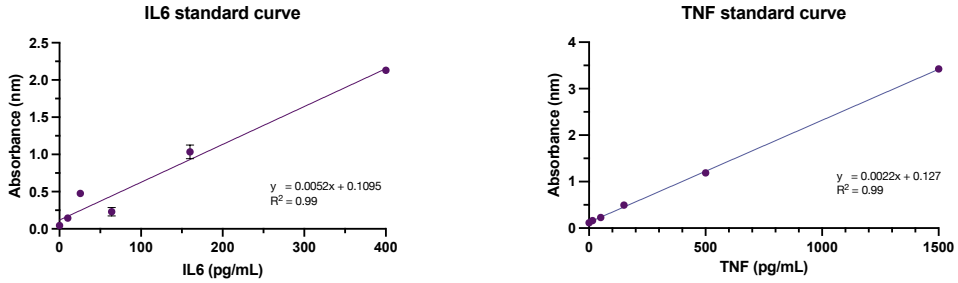


Figure 4.10 ELISA standard curves
Standard curves from ELISA experiments measuring cytokine production of lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells (PBMCs). Standard curves were performed according to manufacturer's instructions.

4.5.9 Results

4.5.9.1 PBMCs of children with PANS show lower expression of pro-inflammatory genes

Gene expression levels of *IL-6* in control cells were increasing in a time dependant matter until peaking at the 3-hour mark and declining over the 24-hour period. Levels of *IL-6* in the patient's cells showed a repressed response at the 30-minute timepoint when compared to the controls (*p value* < 0.05; Figure 4.11A), as well as relatively lower expression levels at the 3 hour and 24-hour timepoints. Expression of *TNF* in both control and patient cells showed an increase at 30 minutes which diminished over the timepoints measured (Figure 4.11B). The patient cells showed a relatively higher expression at the 30-minute mark when compared to the controls (*IL-6* 30-minutes), however this declined at the 3 hours and 24-hour timepoints – neither *IL-6* nor *TNF* were statistically significant.

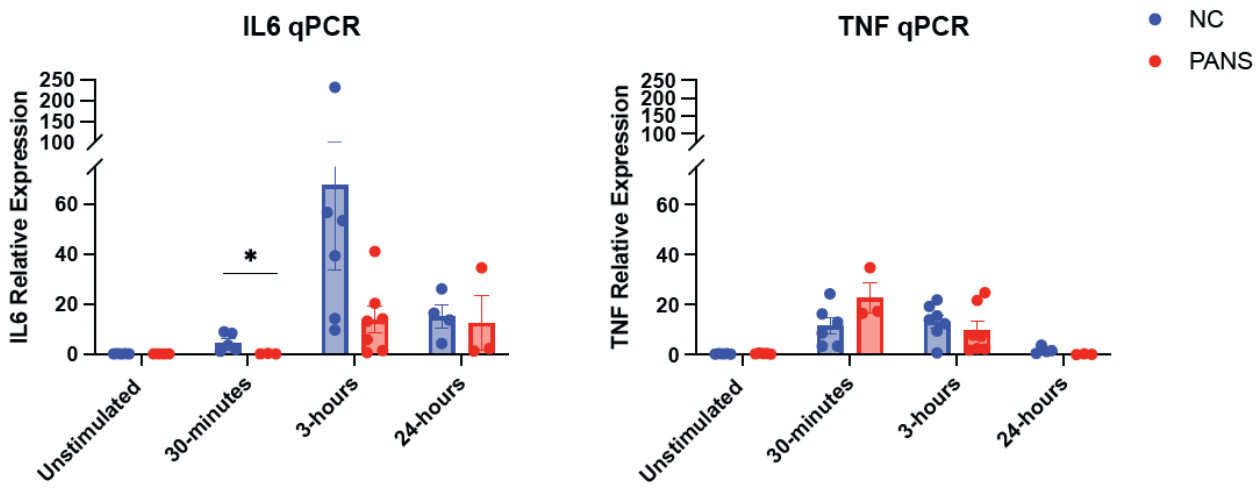


Figure 4.11 Gene expression in PBMCs of children with PANS
Gene expression and cytokine production of lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells (PBMCs). PBMCs of children with Paediatric Active Neuropsychiatric Syndrome (PANS) and neurotypical controls (NC) were stimulated with 500ng/mL LPS for 30 minutes, 3 hours, and 24 hours. Gene expression of Interleukin 6 (IL-6), and tumour necrosis factor (TNF) were measured using quantitative reverse transcription polymerase chain reaction. Target gene expression was normalised to household gene, Beta-2-Microglobulin. Bars and whiskers represent median with interquartile range; n = 7. Mann-Whitney test, *P < 0·05, **P < 0·01, ***P < 0·001.

4.5.9.2 Repressed pro-inflammatory cytokine production in PBMCs of children with PANS as shown by enzyme-linked immunosorbent assay (ELISA)

To assess cytokine production of common pro-inflammatory cytokines such as IL-6 and TNF in the cohort's PBMCs challenged with LPS at 30 minutes, 3 hours and 24 hours and an unstimulated control, ELISAs were performed as per section 4.5.8. A standard curve for each kit to warrant reliability of results (R-squared value cut-off 0.99) was performed. Unlike the RNA findings shown above which peaked at the 30 minute and 3 hour timepoints, the protein concentrations were observed to increase over time with the highest values evident at 24 hours. At 3 hours, the IL-6 concentrations were significantly lower in the PANS patients when compared to controls (*p value* < 0.01; Figure 4.12). At the 24 hour timepoint, the PANS patients still had lower IL-6 values, however this did not reach statistical significance. For TNF, a lower concentration was observed in the PANS patients compared to controls at the 30 minute timepoint (*p value* < 0.01; Figure 4.12). At the 24 hour timepoint,

the PANS patients still had lower levels of TNF when compared to controls, however this did not reach statistical significance.

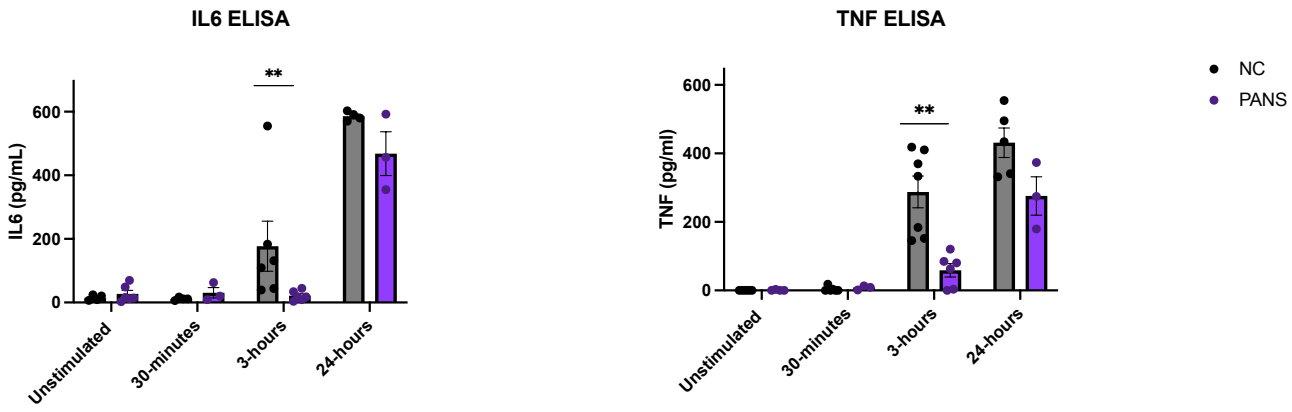


Figure 4.12 ELISA of IL-6 and TNF in PBMCs of children with PANS
Cytokine production of lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells (PBMCs). PBMCs of children with Paediatric Active Neuropsychiatric Syndrome (PANS) and neurotypical controls (NC) were stimulated with 500ng/mL LPS for 30 minutes, 3 hours, and 24 hours. Release of IL-6 and TNF into the cell culture media was measured using ELISA. Bars and whiskers represent median with interquartile range; n = 7. Mann-Whitney test, *P < 0·05, **P < 0·01, ***P < 0·001.

4.6 Discussion

The first aim of this chapter was to optimise a TLR stimulation assay using PBMC to identify the optimal LPS time and dosage to activate the innate immune response. These parameters were carried forward to assess the functional innate immune response of children with PANS compared to controls. This was achieved through utilising gene expression and cytokine assays for IL-6 and TNF, major pro-inflammatory cytokines in this signalling cascade.

The optimisation of a functional assay to assess optimal time and dose of LPS stimulation on PBMCs was performed using two dosages. For IL-6, the 500ng/ml dose showed an increase in expression at the 3-hour mark, which reduced over the 6-hour and 24-hour timepoints. In comparison, the 1000ng/ml dose presented an increase in IL-6 expression that continued from the 3-hour to the 6-hour timepoints, declining over the 24-hour period. At both the 500ng/ml and 1000ng/ml, TNF expression was observed to peak at the 30-minute timepoint. At 500ng/ml, TNF levels declined over the 3 hour, 6 hour and

24 hour periods. At the 1000ng/ml, TNF levels decreased at 3 hours of LPS stimulated, increased again at the 6-hour timepoint, and decreased at 24 hours. I observed that the 500ng/ml treatment of LPS was adequate to elicit an immune response of IL-6 and TNF readouts in control PBMCs. I decided that due to technical constraints such as feasibility of experimental time frame and cell handling, that the 500ng/ml dose would be carried forward into the analysis, where it provided adequate magnitude of change. In addition, the 6-hour timepoint was dropped as it did not provide insights into these cytokine's expression that the 3-hour and 24-hour mark did not.

The main results presented in this chapter highlight a repressed immune response in PBMCs of children with PANS when compared to those from neurotypical controls at both the mRNA and protein level. To add, the protein profile presented in the ELISA are consistent with established biological frameworks, where a peak mRNA signal was observed at the earlier timepoints for *IL-6* (3 hours) and *TNF* (30-minutes), while the protein signal displayed a more linear pattern of increase over the 24 hour period of testing. The control samples displayed this described increasing profile, while the PANS patients appeared to have a repressed response. These findings may describe an aberrant immune system in response to TLR stimulation, reflecting an inability of children with NDDs to regulate cytokine expression, and further extending the narrative of a dysregulated innate immune system in these children. The literature describing cytokine levels within NDD cohorts has implicated an increase of IL-6, TNF, along with other proinflammatory cytokines when compared to controls.[312] Currently, there are only a few studies that investigated the effect of LPS stimulation in PBMCs of children with NDDs, where dysregulation of cytokine responses were described.[302-304] Our study described in this chapter is one of the first to highlight a repressed immune response following a bacterial challenge in PBMCs of children with PANS compared to controls.

Inflammation in NDDs is commonly described notion, with studies reporting findings within the brain and the periphery of affected individuals. The TLR pathway was explored in this assay due to its importance in initiating an immune response within the brain and the periphery. Other options to investigate the immune response within these conditions include utilising cell programming techniques such as induced microglia-like cells from PBMCs to explore morphology and assess functions such as phagocytosis. The resident immune cells within the CNS have vital contributions in inflammation, however, are inaccessible. Induced microglia-like cells are monocyte-derived cells that offer an alternative to exploring the immune cells of the brain, with the added bonus of recapitulating a patient's architectural pathology including epigenetics.[313] This contrasts with utilising induced pluripotent stem cells, where despite generating functional microglial cells, the patient's epigenetic markers would be "wiped" in the lengthy generation process. The TLR stimulation assay worked for the purpose of this thesis due to the limited cell numbers yielded, and the timeframe when taking into account other projects. Moreover, the bacterial ligand LPS was utilised due to its ability to activate the two arms of the toll like receptor pathway. LPS is a potent stimulant of TLR4, where it binds to accessory molecules to induce TLR dimerisation and results in subsequent signalling cascades. Other ligands such as Poly:IC could have been explored, as they provided specific mechanisms in stimulating the TLR3 pathway and thus induce interferon expression directly.

This chapter described the journey of generating a stimulation assay to measure the immune response of PBMCs following a bacterial challenge. Although the portrayed findings address a gap within the literature, there are many limitations to the work. Firstly, this assay assessed the effect of two LPS dosages only. In addition, while the time points tested covered a wide range, an improvement could be made by the addition of a 12 hour timepoint to allow a full spectrum of measurement. However as mentioned above, there is a lack of uniformity

in stimulation studies in relation to the LPS dosage and treatment time. The literature in NDDs describes a range of dosages and stimulation times depending on the measured outcome. Future work utilising this assay could employ a wider range than what was utilised here.

Next, due to time constraints, I only tested the effects of the assay on the mRNA and protein of two main cytokines within the TLR pathway. Future work implementing this assay should utilise the ability of LPS to activate the two arms of the TLR pathway and assess production of interferons and other cytokines. Stimulation of PBMCs with LPS activates the two arms of the toll-like receptor pathway, and therefore presents a plethora of cytokines and other inflammatory molecules that could have been measured. However, as a functional assay was employed, this would have been difficult at the measurement stage.

The assay described in this section aimed to compare two dosages and provide a stepping stone into identifying functional immune difference in children with PANS compared to controls. Future work could focus on improving the type of assay by utilising technologically advanced kits such as the Luminex xMAP INTELLIFLEX System (Cat #APX2020, ThermoFisher), which allow for the simultaneous measurement of mRNA and protein expression levels. This is an alternative to high-throughput experimentations such as RNA-sequencing and proteomic analyses which still allow for measurement of multiple genes and proteins of interest.

In addition, a flow cytometry-based assay could have allowed for exploration of immune markers, as well as have offered the added benefit of cell type specific readouts. Within this work, we utilised peripheral blood mononuclear cells – which contain monocytes and lymphocytes. While the main cell type stimulated by LPS within this assay is monocytes, the lymphocyte subset of PBMCs are also affected by LPS (directly and indirectly).

Therefore, a future goal is to utilise flow cytometry to assay a variety of cell types following LPS stimulation.

To add, utilising of other techniques such as a western blot could have provided additional findings particularly around the phosphorylation of key transcription factors such as NF-κβ. These suggestions were not employed in the original assay due to practicality of the cell type, where post-thawing I had limited numbers of cells to work with, along with the many samples required for each timepoint making it not feasible to utilise these techniques. In addition, the amount of blood collected from paediatric patients for research purposes following routine investigations is limited. The blood collected from these children was performed at different times with a required processing window of 6 hours, which would have made performing a live assay difficult.

From this chapter, I have provided a rationale that children with NDD such as PANS have immune dysregulation evidenced through a functional assay. Measurement of key pro-inflammatory cytokines (IL-6 and TNF) at the mRNA and protein level highlighted a repressed immune response in children with PANS compared to controls. While this assay and the findings presented are preliminary, targeting immune function may serve as a potential therapeutic for neurodevelopmental and neuropsychiatric conditions. Integrating functional assays into routine investigations may give insight into complex presentations, and lead into personalised management plans.

Chapter 5

Insights towards a deeper understanding of complex neurodevelopmental conditions: From bioinformatics to therapeutic potential

---

Complex neurodevelopmental and neuropsychiatric conditions such as ASD, Tourette syndrome, and OCD affect brain development and function between early gestation and early adulthood . An estimated 10% of children under the age of five are diagnosed with a NDD.[12] The program of research undertaken within this thesis aimed to investigate dysregulated biological pathways which may underly mechanisms of disease in NDD. Chapter 1 introduced the concept of NDDs and outlined the literature, which implicated a complex interplay of genetic, epigenetic and environmental influences.[29-31] While monogenic forms of NDDs exist, a large proportion of NDDs do not have high penetrating pathogenic mutations, and are instead due to common gene variants.[32,33] A large body of evidence supports the involvement of environmental factors such as maternal immune dysregulation during gestation as a risk factor for NDDs.[52] The pathophysiology and aetiology of NDDs are therefore complex, involving polygenic and environmental influences. It is likely that there is an interplay between both genes and environmental factors, possibly via epigenetic regulation.

To understand overlaps in dysregulated genes and biological pathways in NDDs, such as ASD and Tourette syndrome, I investigated publicly available high-throughput datasets from post-mortem brain tissue. The utilisation of these datasets, increasingly becoming publicly available, not only encourages reproducibility of results, but may enable novel discoveries when combined with other techniques and findings. The first aim of this thesis was firstly explored in Chapter 2, where I utilised bioinformatic techniques to analyse post-mortem RNA sequencing datasets from individuals with ASD and Tourette syndrome to identify dysregulated genes and biological pathways. While the two datasets have been analysed within their respective studies, I focused on replicating the original findings, and additionally identifying a common signal between the two datasets. This approach was valid due to the high comorbidity rates of NDDs, where in particular tics are present in 11-22% of children with ASD, while ASD is present in 12% of children diagnosed with Tourette syndrome.[198-200] The up-regulated inflammatory findings from this study reaffirms the immune literature within NDDs, and provides more evidence for a common signal between these conditions.

To continue investigating the first aim of this thesis, the second high-throughput technique utilised to explore biological pathways underpinning NDDs is explored within Chapter 3. Building on the findings from Chapter 2, where a clear inflammatory signal was observed to be up-regulated within the RNA of brain samples of individuals with NDDs, our group aimed to explore this immune evidence in children with NDDs, particularly those that satisfy criteria for PANS. It is unclear if PANS is a 'distinct entity' of a clinical phenotype within the spectrum of NDD. The literature utilising proteomic approaches to analyse peripheral (blood, saliva) samples from patients with NDDs is limited, with majority of studies focusing on ASD. The findings from proteomics investigations in NDDs studies implicate inflammation and the complement pathway.[184-187,189-191] PANS is a clinical entity of

NDDs, characterised by abrupt-onset neuropsychiatric symptoms coupled with an activated immune response. We recruited two cohorts of children with PANS (each n =4) during the chronic phase, with no infection in the two weeks preceding blood collection. As we hypothesised that PANS is a disorder of immune dysregulation, utilising peripheral blood mononuclear cells is of great value to exploring the presence or absence of immune dysregulation. From the two cohorts, proteome analysis identified an involvement of the immune response, translation (ribosomal) and epigenetics in PANS. This study is the first to investigate the proteome of children with PANS, allowing for a beginning understanding of the functional profile of this condition. Utilising two cohorts allowed for a validation of the findings and assurance that the replication of methods yields a consistent result in children with PANS despite the heterogenous presentations of the condition.

A functional immune assay to complement our findings from previous brain and blood results was developed in Chapter 4. Chapters 2 and 3 utilised high-throughput sequencing and bioinformatic tools to investigate dysregulated biological pathways at the RNA and protein level. An aberrant immune response was identified in the RNA of individuals with ASD and Tourette syndrome, as well as the proteome of children with the PANS phenotype. Building on these two chapters, the final aim of this thesis was to investigate the functional immune response in peripheral blood mononuclear cells of children with PANS compared to controls. Firstly, a TLR stimulation assay was developed to identify the optimal LPS timing and dosage to elicit an inflammatory response measured by proinflammatory cytokines at the mRNA and protein levels. Subsequently, the assay was utilised on a PANS cohort (n=7) and a control cohort (n=7), where gene expression and secreted cytokines investigations of the proinflammatory cytokines IL-6 and TNF identified a repressed immune response in PANS compared to controls. The work described in this chapter is the first to assess the immune response of peripheral blood mononuclear cells from children with PANS following a bacterial challenge, compared to controls at various

timepoints. The assay provides a valuable platform to investigate the effect of LPS stimulation on cells of children with PANS at the specified dose and timings. In addition, there is potential for extension to include a range of cytokines and inflammatory molecules activated downstream as a result of LPS stimulation.

Taken together, the body of research presented within this thesis highlights the importance of exploring dysregulated pathways in NDD pathogenesis. The findings add to growing evidence that a dysregulated immune response is associated with NDDs, which might be related to epigenetic regulation and translational (ribosomal) mechanisms. Overall, this body of work contributed to furthering our understanding of the biological underpinnings of NDDs through developing bioinformatic tools and molecular biology techniques. Utilisation of high-throughput data, both publicly available and group generated, allowed for a deep exploration of the RNA and protein of individuals with complex neuropsychiatric conditions. Building on from the results of the two investigations (Chapters 2 and 3) paved the way for a functional assay to be developed to assess the immune response, where a novel repressed immune signature was identified in peripheral cells of children with PANS (Chapter 4). The main findings from this thesis highlight the immune and inflammatory component observed in the brain and periphery of individuals with NDDs, which leads us one step closer to understanding mechanisms with modifier potential. Genetics has paved the way for high-throughput investigations in complex conditions, particularly within the neuropsychiatric realm. However, our understanding of complex neurodevelopmental conditions such as ASD, Tourette syndrome, OCD and PANS is not well developed. This body of work recognised the literature detailing interplay of genetics, epigenetic and environmental factors which influence NDD expression, and has contributed towards furthering this evidence. Firstly, this thesis contributed towards establishing a bioinformatic foundation to analysing high-throughput data within the group and assisted in

being a stepping stone for other technologies to be analysed using the R language. One of which, is the proteomic datasets analysed in Chapter 3. This thesis can act as a resource and stepping stone for analysing complex datasets for other NDD conditions such as cerebral palsy. To add, a defined time-profile of the effect of LPS stimulation on peripheral blood mononuclear cells is lacking within the literature, which proved a struggle when designing the assay. There is no gold standard dosage or timing for LPS stimulation, what this thesis has provided a profile of what is to be expected of two dosages and four timepoints. Finally, this work identified a consistently dysregulated immune response signal within brain tissue and blood cells of individuals with complex neurodevelopmental disorders, adding on to the literature of these conditions.

While this body of work has contributed to furthering our knowledge of complex neurodevelopmental and neuropsychiatric conditions, there are many limitations. From the three main result chapters presented (Chapters 2, 3 and 4), I have outlined limitations pertaining to each study. To begin, within Chapter 2 the main limitation is the utilisation of publicly available datasets in a range of ages of individuals with ASD and Tourette syndrome, which couldn't be avoided due to the scarcity of the brain tissue utilised. To add, Chapter 3 utilised bulk proteomic analysis of peripheral blood mononuclear cells of children with PANS. The technology utilised to sequence the samples (brain tissue and PBMCs) wasn't cell type specific. Current technologies such as single cell RNA sequencing and proteomics methodologies offer the advantage of high-throughput sequencing of RNA molecules, with the specificity of cell-type specific analysis. Future work investigating complex conditions such as NDDs may utilise these technologies to identify gene expression and protein signatures specific to each cell type.

Another caveat in this research is the small sample size utilised for the cohorts, in particular those recruited by our group in Chapters 3 and 4. While this could not have been

avoided for the current study due to time constraints, limited amount of blood samples obtained, and low cell yield, we employed a second cohort in Chapter 3 to act a validator of the results identified in the initial proteomic investigation. Future work investigating these complex conditions should aim to increase sample size, and concurrently recruit validation cohorts as was done here. In addition, the PANS cohort had heterogenous clinical presentations, and were in the chronic phase of disease. Future investigations should shed light on the biology of children during the acute phase and investigate drug naïve signatures. To add, future investigations should aim to investigate PANS and non-PANS NDD presentations to better capitulate neurodevelopmental conditions spectrums. It is entirely possible that the LPS response observed in PANS patients may be different if measured in patients with ASD or Tourette syndrome. Next, within Chapter 3, the two cohorts underwent individual analyses following a proteomic bioinformatic pipeline. While the findings were similar, the two datasets could have also been analysed concurrently. Within Chapter 4, many caveats exist in the assay designed, such as utilising only two dosages of LPS, and using only one stimulating agent. This was due to time constraints of the project, as well as technical availability. To add, I only measured two pro-inflammatory cytokines in the PANS cohort, a result of time constraints and cell yield shortcomings.

Finally, a major consideration is the link between peripheral inflammation and neuroinflammation. Chapter 1 investigated a transcriptomic signature of up-regulated inflammation in post-mortem brains of individuals with ASD and Tourette syndrome. On the other hand, Chapters 3 and 4 identified a dysregulated immune response in peripheral blood mononuclear cells of children with PANS. Within the PANS proteome, we observed an up-regulated immune response compared to controls. However, stimulation with LPS identified a cytokine hypo-response in peripheral cell of children with PANS compared to controls at the mRNA and protein levels of IL-6 and TNF. This observed immune

dysregulation of complex conditions such as PANS necessitates animal models where both the blood and brain are investigated concurrently. Utilisation of omic technologies examining peripheral blood and brain tissue can aid in increasing our understanding of these conditions, at various levels of biology (genome, transcriptome, proteome, epigenetics). These explorations can allow for clear understanding of the mechanisms underlying complex conditions, as paediatric brain tissue is precious and hard to access. Work expanding on pre-clinical findings can bring us one step closer to developing therapies which target disease mechanisms.

Following on from the notion of blood versus brain, a deficient immune response within the periphery could result in an inflammatory response centrally. Our group's hypothesis is that the periphery is representative of the central nervous system, with environmental factors influencing this bidirectional relationship. Our findings highlight an aberrant immune response in peripheral blood cells of children with PANS. What we are lacking is an understanding of the mechanisms within the CNS, particularly brain cells such as microglia. It is plausible that microglial cells of individuals with PANS are impaired due to peripheral dysregulations. To add, cytokines play a major role within an aberrant peripheral immune response, where they may be able to cross the blood brain barrier and result in neuroinflammation led by microglial cells.

There remains many unanswered question in the journey of uncovering the complexity of NDDs. The field needs studies with larger cohorts that cover the wide range of symptoms presented by those with complex NDDs, in particular PANS. To add, advancements of more sophisticated approaches to treatment than the current methods utilised are required, such as developing precision medicine plans, along with utilising the concept of drug repurposing. This technique has already gained traction with the appearance of the COVID-19 pandemic, where cancer therapies were utilised as antiviral treatments,

either alone or as adjuvant therapies.[314] Finally, combining omic datasets with computational techniques including machine learning offers a plethora of possibilities, one example of this is the LINCS L1000 data project. The L1000 project is a collection of gene expression profiles combining perturbagens (small drug molecules) at many time-points, dosages and cell lines.[315] This publicly available gene expression data was combined with a computational drug repurposing pipeline to identify better therapeutics for COVID-19.[316] Utilising high-throughput experimentation techniques coupled with functional understanding of patient samples to identify dysregulated pathway, as was done in this body of work, will move the field towards a better understanding of the biological underpinnings of complex conditions. From further research developing this strategy, we'll be able to address the identified pathways in a more targeted approach to help individuals with NDDs.

Chapter 6

References

1 American Psychiatric Association. *Diagnostic and statistical manual of mental disorders*. 5 edn, (American Psychiatric Association, 2013).

2 Morris-Rosendahl, D. J. & Crocq, M. A. Neurodevelopmental disorders-the history and future of a diagnostic concept . *Dialogues Clin. Neurosci.* **22**, 65-72 (2020). https://doi.org:10.31887/DCNS.2020.22.1/macrocq

3 Homberg, J. R. *et al.* Understanding autism and other neurodevelopmental disorders through experimental translational neurobehavioral models. *Neurosci. Biobehav. Rev.* **65**, 292-312 (2016). https://doi.org:10.1016/j.neubiorev.2016.03.013

4 Estes, A. *et al.* Behavioral, cognitive, and adaptive development in infants with autism spectrum disorder in the first 2 years of life. *J. Neurodev. Disord.* **7**, 24 (2015). https://doi.org:10.1186/s11689-015-9117-6

5 Loomes, R., Hull, L. & Mandy, W. P. L. What Is the Male-to-Female Ratio in Autism Spectrum Disorder? A Systematic Review and Meta-Analysis. *J. Am. Acad. Child Adolesc. Psychiatry* **56**, 466-474 (2017). https://doi.org:10.1016/j.jaac.2017.03.013

6 Mittal, S. O. Tics and Tourette's syndrome. *Drugs Context* **9**, 2019-2012-2012 (2020). https://doi.org:10.7573/dic.2019-12-2

7 Quezada, J. & Coffman, K. A. Current Approaches and New Developments in the Pharmacological Management of Tourette Syndrome. *CNS Drugs* **32**, 33-45 (2018). https://doi.org:10.1007/s40263-017-0486-0

8 Robertson, M. M. Movement disorders: Tourette syndrome--beyond swearing and sex? *Nat Rev Neurol* **10**, 6-8 (2014). https://doi.org:10.1038/nrneurol.2013.248

9 Knight, T. *et al.* Prevalence of Tic Disorders: A Systematic Review and Meta-Analysis. *Pediatric Neurology* **47**, 77-90 (2012). https://doi.org:https://doi.org/10.1016/j.pediatrneurol.2012.05.002

10 Lin, H. *et al.* Psychosocial stress predicts future symptom severities in children and adolescents with Tourette syndrome and/or obsessive-compulsive disorder. *J Child Psychol Psychiatry* **48**, 157-166 (2007). https://doi.org:10.1111/j.1469-7610.2006.01687.x

11 Taylor, S. Early versus late onset obsessive-compulsive disorder: evidence for distinct subtypes. *Clin. Psychol. Rev.* **31**, 1083-1100 (2011). https://doi.org:10.1016/j.cpr.2011.06.007

12 Collaborators, G. R. o. D. D. Developmental disabilities among children younger than 5 years in 195 countries and territories, 1990-2016: a systematic analysis for the

Global Burden of Disease Study 2016. *Lancet Glob Health* **6**, e1100-e1121 (2018). https://doi.org:10.1016/s2214-109x(18)30309-7

13 Australian Bereau of Statistics. (Canberra: ABS, 2018).

14 Nielsen, T. C., Nassar, N., Boulton, K. A., Guastella, A. J. & Lain, S. J. Estimating the Prevalence of Autism Spectrum Disorder in New South Wales, Australia: A Data Linkage Study of Three Routinely Collected Datasets. *J. Autism Dev. Disord.* **54**, 1558-1566 (2024). https://doi.org:10.1007/s10803-022-05887-3

15 May, T. & Williams, K. Brief Report: Gender and Age of Diagnosis Time Trends in Children with Autism Using Australian Medicare Data. *J. Autism Dev. Disord.* **48**, 4056-4062 (2018). https://doi.org:10.1007/s10803-018-3609-7

16 May, T., Brignell, A. & Williams, K. Autism Spectrum Disorder Prevalence in Children Aged 12-13 Years From the Longitudinal Study of Australian Children. *Autism Res.* **13**, 821-827 (2020). https://doi.org:10.1002/aur.2286

17 Robertson, M. M. A personal 35 year perspective on Gilles de la Tourette syndrome: prevalence, phenomenology, comorbidities, and coexistent psychopathologies. *Lancet Psychiatry* **2**, 68-87 (2015). https://doi.org:10.1016/s2215-0366(14)00132-1

18 Statistics., A. B. o. (Canberra: ABS, 2020-21).

19 Horlin, C., Falkmer, M., Parsons, R., Albrecht, M. A. & Falkmer, T. The cost of autism spectrum disorders. *PLoS One* **9**, e106552 (2014). https://doi.org:10.1371/journal.pone.0106552

20 Network, M. N. Review of the burden of disease for neurological, mental health and substance use disorders in Australia. (2019).

21 Dyason, K. M. *et al.* Hospital admission characteristics for children and adolescents with OCD in Sydney, Australia. *Gen. Hosp. Psychiatry* **85**, 236-238 (2023). https://doi.org:https://doi.org/10.1016/j.genhosppsych.2023.09.008

22 Hirschtritt, M. E. *et al.* Lifetime prevalence, age of risk, and genetic relationships of comorbid psychiatric disorders in Tourette syndrome. *JAMA Psychiatry* **72**, 325-333 (2015). https://doi.org:10.1001/jamapsychiatry.2014.2650

23 Hours, C., Recasens, C. & Baleyte, J. M. ASD and ADHD Comorbidity: What Are We Talking About? *Front Psychiatry* **13**, 837424 (2022). https://doi.org:10.3389/fpsyt.2022.837424

24 Vish, N. L. & Stolfi, A. Relationship of Children's Emotional and Behavioral Disorders With Health Care Utilization and Missed School. *Acad. Pediatr.* **20**, 687-695 (2020). https://doi.org:10.1016/j.acap.2020.02.017

25 Reynolds, K. C., Patriquin, M., Alfano, C. A., Loveland, K. A. & Pearson, D. A. Parent-Reported Problematic Sleep Behaviors in Children with Comorbid Autism

Spectrum Disorder and Attention-Deficit/Hyperactivity Disorder. *Res. Autism Spectr. Disord.* **39**, 20-32 (2017). https://doi.org:10.1016/j.rasd.2017.04.003

26 Wiggs, L. & Stores, G. Sleep patterns and sleep disorders in children with autistic spectrum disorders: insights using parent report and actigraphy. *Dev. Med. Child Neurol.* **46**, 372-380 (2004). https://doi.org:10.1017/s0012162204000611

27 Liu, X., Hubbard, J. A., Fabes, R. A. & Adam, J. B. Sleep disturbances and correlates of children with autism spectrum disorders. *Child Psychiatry Hum. Dev.* **37**, 179-191 (2006). https://doi.org:10.1007/s10578-006-0028-3

28 Virolainen, S. J., VonHandorf, A., Viel, K., Weirauch, M. T. & Kottyan, L. C. Gene-environment interactions and their impact on human health. *Genes Immun.* **24**, 1-11 (2023). https://doi.org:10.1038/s41435-022-00192-6

29 Anttila, V. *et al.* Analysis of shared heritability in common disorders of the brain. *Science* **360** (2018). https://doi.org:10.1126/science.aap8757

30 Bale, T. L. *et al.* Early life programming and neurodevelopmental disorders. *Biol. Psychiatry* **68**, 314-319 (2010). https://doi.org:10.1016/j.biopsych.2010.05.028

31 Bale, T. L. Epigenetic and transgenerational reprogramming of brain development. *Nat. Rev. Neurosci.* **16**, 332-344 (2015). https://doi.org:10.1038/nrn3818

32 Parenti, I., Rabaneda, L. G., Schoen, H. & Novarino, G. Neurodevelopmental Disorders: From Genetics to Functional Pathways. *Trends Neurosci.* **43**, 608-621 (2020). https://doi.org:10.1016/j.tins.2020.05.004

33 Smoller, J. W. *et al.* Psychiatric genetics and the structure of psychopathology. *Mol. Psychiatry* **24**, 409-420 (2019). https://doi.org:10.1038/s41380-017-0010-4

34 Cross-Disorder Group of the Psychiatric Genomics Consortium. Genomic relationships, novel loci, and pleiotropic mechanisms across eight psychiatric disorders. *Cell* **179**, 1469-1482.e1411 (2019). https://doi.org:10.1016/j.cell.2019.11.020

35 Knuesel, I. *et al.* Maternal immune activation and abnormal brain development across CNS disorders. *Nat. Rev. Neurol.* **10**, 643-660 (2014). https://doi.org:10.1038/nrneurol.2014.187

36 Han, V. X., Patel, S., Jones, H. F. & Dale, R. C. Maternal immune activation and neuroinflammation in human neurodevelopmental disorders. *Nature Reviews Neurology*, 564–579 (2021). https://doi.org:10.1038/s41582-021-00530-8

37 Owen, M. J. & O'Donovan, M. C. Schizophrenia and the neurodevelopmental continuum:evidence from genomics. *World Psychiatry* **16**, 227-235 (2017). https://doi.org:10.1002/wps.20440

38 Stiles, J. & Jernigan, T. L. The basics of brain development. *Neuropsychol. Rev.* **20**, 327-348 (2010). https://doi.org:10.1007/s11065-010-9148-4

39 Azevedo, F. A. C. *et al.* Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *J. Comp. Neurol.* **513**, 532-541 (2009). https://doi.org:https://doi.org/10.1002/cne.21974

40 Tierney, A. L. & Nelson, C. A., 3rd. Brain Development and the Role of Experience in the Early Years. *Zero Three* **30**, 9-13 (2009).

41 Zengeler, K. E. & Lukens, J. R. Innate immunity at the crossroads of healthy brain maturation and neurodevelopmental disorders. *Nat. Rev. Immunol.* **21**, 454-468 (2021). https://doi.org:10.1038/s41577-020-00487-7

42 Stoner, R. *et al.* Patches of disorganization in the neocortex of children with autism. *N. Engl. J. Med.* **370**, 1209-1219 (2014). https://doi.org:10.1056/NEJMoa1307491

43 Fang, W. Q. *et al.* Overproduction of upper-layer neurons in the neocortex leads to autism-like features in mice. *Cell Rep.* **9**, 1635-1643 (2014). https://doi.org:10.1016/j.celrep.2014.11.003

44 Gomez Perdiguero, E. *et al.* Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature* **518**, 547-551 (2015). https://doi.org:10.1038/nature13989

45 Mancini, V. O. *et al.* Associations between the human immune system and gut microbiome with neurodevelopment in the first 5 years of life: A systematic scoping review. *Dev. Psychobiol.* **65**, e22360 (2023). https://doi.org:10.1002/dev.22360

46 Prins, J. R., Eskandar, S., Eggen, B. J. L. & Scherjon, S. A. Microglia, the missing link in maternal immune activation and fetal neurodevelopment; and a possible link in preeclampsia and disturbed neurodevelopment? *J. Reprod. Immunol.* **126**, 18-22 (2018). https://doi.org:10.1016/j.jri.2018.01.004

47 Morimoto, K. & Nakajima, K. Role of the Immune System in the Development of the Central Nervous System. *Front. Neurosci.* **13** (2019). https://doi.org:10.3389/fnins.2019.00916

48 Carson, M. J., Doose, J. M., Melchior, B., Schmid, C. D. & Ploix, C. C. CNS immune privilege: hiding in plain sight. *Immunol. Rev.* **213**, 48-65 (2006). https://doi.org:10.1111/j.1600-065X.2006.00441.x

49 Pan, W. & Kastin, A. J. Penetration of neurotrophins and cytokines across the blood-brain/blood-spinal cord barrier. *Adv Drug Deliv Rev* **36**, 291-298 (1999). https://doi.org:10.1016/s0169-409x(98)00086-6

50 Yarlagadda, A., Alfson, E. & Clayton, A. H. The blood brain barrier and the role of cytokines in neuropsychiatry. *Psychiatry (Edgmont)* **6**, 18-22 (2009).

51 Estes, M. L. & McAllister, A. K. Immune mediators in the brain and peripheral tissues in autism spectrum disorder. *Nat. Rev. Neurosci.* **16**, 469-486 (2015). https://doi.org:10.1038/nrn3978

52 Meltzer, A. & Van de Water, J. The role of the immune system in autism spectrum disorder. *Neuropsychopharmacology* **42**, 284-298 (2017). https://doi.org:10.1038/npp.2016.158

53 Bilbo, S. D. & Schwarz, J. M. Early-life programming of later-life brain and behavior: a critical role for the immune system. *Front. Behav. Neurosci.* **3**, 14 (2009). https://doi.org:10.3389/neuro.08.014.2009

54 Giulian, D., Young, D. G., Woodward, J., Brown, D. C. & Lachman, L. B. Interleukin-1 is an astroglial growth factor in the developing brain. *J. Neurosci.* **8**, 709-714 (1988). https://doi.org:10.1523/jneurosci.08-02-00709.1988

55 Schmitz, T. & Chew, L. J. Cytokines and myelination in the central nervous system. *ScientificWorldJournal* **8**, 1119-1147 (2008). https://doi.org:10.1100/tsw.2008.140

56 Barak, B., Feldman, N. & Okun, E. Toll-like receptors as developmental tools that regulate neurogenesis during development: an update. *Front. Neurosci.* **8** (2014). https://doi.org:10.3389/fnins.2014.00272

57 Prinz, M. & Priller, J. Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. *Nature Reviews Neuroscience* **15**, 300-312 (2014). https://doi.org:10.1038/nrn3722

58 Cowan, M. & Petri, W. A., Jr. Microglia: Immune Regulators of Neurodevelopment. *Front. Immunol.* **9**, 2576 (2018). https://doi.org:10.3389/fimmu.2018.02576

59 Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma in Vivo. *Science* **308**, 1314-1318 (2005). https://doi.org:doi:10.1126/science.1110647

60 Béchade, C., Cantaut-Belarif, Y. & Bessis, A. Microglial control of neuronal activity. *Front. Cell. Neurosci.* **7**, 32 (2013). https://doi.org:10.3389/fncel.2013.00032

61 Lim, S. H. *et al.* Neuronal synapse formation induced by microglia and interleukin 10. *PLoS One* **8**, e81218 (2013). https://doi.org:10.1371/journal.pone.0081218

62 Liao, H., Huang, W., Niu, R., Sun, L. & Zhang, L. Cross-talk between the epidermal growth factor-like repeats/fibronectin 6–8 repeats domains of Tenascin-R and microglia modulates neural stem/progenitor cell proliferation and differentiation. *J. Neurosci. Res.* **86**, 27-34 (2008). https://doi.org:https://doi.org/10.1002/jnr.21454

63 Stevens, B. *et al.* The classical complement cascade mediates CNS synapse elimination. *Cell* **131**, 1164-1178 (2007). https://doi.org:10.1016/j.cell.2007.10.036

64 Schafer, D. P., Lehrman, E. K. & Stevens, B. The "quad-partite" synapse: microglia-synapse interactions in the developing and mature CNS. *Glia* **61**, 24-36 (2013). https://doi.org:10.1002/glia.22389

65 Watabe, K., Osborne, D. & Kim, S. U. Phagocytic activity of human adult astrocytes and oligodendrocytes in culture. *J. Neuropathol. Exp. Neurol.* **48**, 499-506 (1989). https://doi.org:10.1097/00005072-198909000-00001

66 Galloway, D. A., Phillips, A. E. M., Owen, D. R. J. & Moore, C. S. Phagocytosis in the Brain: Homeostasis and Disease. *Front. Immunol.* **10** (2019). https://doi.org:10.3389/fimmu.2019.00790

67 Chess, S. Autism in children with congenital rubella. *J. Autism Child. Schizophr.* **1**, 33-47 (1971). https://doi.org:10.1007/bf01537741

68 Chess, S., Fernandez, P. & Korn, S. Behavioral consequences of congenital rubella. *J. Pediatr.* **93**, 699-703 (1978). https://doi.org:10.1016/s0022-3476(78)80921-4

69 Werenberg Dreier, J. *et al.* Fever and infections in pregnancy and risk of attention deficit/hyperactivity disorder in the offspring. *J. Child Psychol. Psychiatry* **57**, 540-548 (2016). https://doi.org:10.1111/jcpp.12480

70 Zerbo, O. *et al.* Maternal infection during pregnancy and autism spectrum disorders. *J. Autism Dev. Disord.* **45**, 4015-4025 (2015). https://doi.org:10.1007/s10803-013-2016-3

71 Jiang, H. Y. *et al.* Maternal infection during pregnancy and risk of autism spectrum disorders: a systematic review and meta-analysis. *Brain. Behav. Immun.* **58**, 165-172 (2016). https://doi.org:10.1016/j.bbi.2016.06.005

72 Holingue, C. *et al.* Interaction between Maternal Immune Activation and Antibiotic Use during Pregnancy and Child Risk of Autism Spectrum Disorder. *Autism Res.* **13**, 2230-2241 (2020). https://doi.org:10.1002/aur.2411

73 Tioleco, N. *et al.* Prenatal maternal infection and risk for autism in offspring: A meta-analysis. *Autism Res.* **14**, 1296-1316 (2021). https://doi.org:10.1002/aur.2499

74 Estes, M. L. & McAllister, A. K. Maternal immune activation: Implications for neuropsychiatric disorders. *Science* **353**, 772-777 (2016). https://doi.org:10.1126/science.aag3194

75 Hsiao, E. Y. & Patterson, P. H. Placental regulation of maternal-fetal interactions and brain development. *Dev. Neurobiol.* **72**, 1317-1326 (2012). https://doi.org:10.1002/dneu.22045

76 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). https://doi.org:10.1186/2040-2392-2-13

77 Patel, S. *et al.* Social impairments in autism spectrum disorder are related to maternal immune history profile. *Mol. Psychiatry* **23**, 1794-1797 (2018). https://doi.org:10.1038/mp.2017.201

78 Han, V. X. *et al.* Maternal acute and chronic inflammation in pregnancy is associated with common neurodevelopmental disorders: a systematic review. *Translational Psychiatry* **11** (2021). https://doi.org:10.1038/s41398-021-01198-w

79 Jones, H. F. *et al.* Maternal autoimmunity and inflammation are associated with childhood tics and obsessive-compulsive disorder: transcriptomic data show common enriched innate immune pathways. *Brain. Behav. Immun.* (2021). https://doi.org:10.1016/j.bbi.2020.12.035

80 Solek, C. M., Farooqi, N., Verly, M., Lim, T. K. & Ruthazer, E. S. Maternal immune activation in neurodevelopmental disorders. *Dev. Dyn.* **247**, 588-619 (2018). https://doi.org:https://doi.org/10.1002/dvdy.24612

81 Meyer, U. *et al.* The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology. *J. Neurosci.* **26**, 4752-4762 (2006). https://doi.org:10.1523/JNEUROSCI.0099-06.2006

82 Kentner, A. C. *et al.* Maternal immune activation: reporting guidelines to improve the rigor, reproducibility, and transparency of the model. *Neuropsychopharmacology* **44**, 245-258 (2019). https://doi.org:10.1038/s41386-018-0185-7

83 Ballendine, S. A. *et al.* Behavioral alterations in rat offspring following maternal immune activation and ELR-CXC chemokine receptor antagonism during pregnancy: implications for neurodevelopmental psychiatric disorders. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **57**, 155-165 (2015). https://doi.org:10.1016/j.pnpbp.2014.11.002

84 Careaga, M., Murai, T. & Bauman, M. D. Maternal immune activation and autism spectrum disorder: from rodents to nonhuman and human primates. *Biol. Psychiatry* **81**, 391-401 (2017). https://doi.org:10.1016/j.biopsych.2016.10.020

85 Smith, S. E., Li, J., Garbett, K., Mirnics, K. & Patterson, P. H. Maternal immune activation alters fetal brain development through interleukin-6. *J. Neurosci.* **27**, 10695-10702 (2007). https://doi.org:10.1523/JNEUROSCI.2178-07.2007

86 Choi, G. B. *et al.* The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science* **351**, 933-939 (2016). https://doi.org:doi:10.1126/science.aad0314

87 Bucknor, M. C., Gururajan, A., Dale, R. C. & Hofer, M. J. A comprehensive approach to modeling maternal immune activation in rodents. *Front. Neurosci.* **16**, 1071976 (2022). https://doi.org:10.3389/fnins.2022.1071976

88 Hall, M. B., Willis, D. E., Rodriguez, E. L. & Schwarz, J. M. Maternal immune activation as an epidemiological risk factor for neurodevelopmental disorders: Considerations of timing, severity, individual differences, and sex in human and rodent studies. *Front. Neurosci.* **17**, 1135559 (2023). https://doi.org:10.3389/fnins.2023.1135559

89 Guma, E., Plitman, E. & Chakravarty, M. M. The role of maternal immune activation in altering the neurodevelopmental trajectories of offspring: A translational review of neuroimaging studies with implications for autism spectrum disorder and schizophrenia. *Neurosci. Biobehav. Rev.* **104**, 141-157 (2019). https://doi.org:10.1016/j.neubiorev.2019.06.020

90 Bao, M., Hofsink, N. & Plösch, T. LPS versus Poly I:C model: comparison of long-term effects of bacterial and viral maternal immune activation on the offspring. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **322**, R99-r111 (2022). https://doi.org:10.1152/ajpregu.00087.2021

91 Boksa, P. Effects of prenatal infection on brain development and behavior: a review of findings from animal models. *Brain. Behav. Immun.* **24**, 881-897 (2010). https://doi.org:10.1016/j.bbi.2010.03.005

92 Wendeln, A. C. *et al.* Innate immune memory in the brain shapes neurological disease hallmarks. *Nature* **556**, 332-338 (2018). https://doi.org:10.1038/s41586-018-0023-4

93 Richetto, J., Calabrese, F., Riva, M. A. & Meyer, U. Prenatal immune activation induces maturation-dependent alterations in the prefrontal GABAergic transcriptome. *Schizophr. Bull.* **40**, 351-361 (2014). https://doi.org:10.1093/schbul/sbs195

94 Weber-Stadlbauer, U. *et al.* Transgenerational transmission and modification of pathological traits induced by prenatal immune activation. *Mol. Psychiatry* **22**, 102-112 (2017). https://doi.org:10.1038/mp.2016.41

95 Ronovsky, M. *et al.* Maternal immune activation transgenerationally modulates maternal care and offspring depression-like behavior. *Brain. Behav. Immun.* **63**, 127-136 (2017). https://doi.org:https://doi.org/10.1016/j.bbi.2016.10.016

96 Karlsson, H., Sjöqvist, H., Brynge, M., Gardner, R. & Dalman, C. Childhood infections and autism spectrum disorders and/or intellectual disability: a register-based cohort study. *J. Neurodev. Disord.* **14**, 12 (2022). https://doi.org:10.1186/s11689-022-09422-4

97 Swedo, S. E. *et al.* Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections: clinical description of the first 50 cases. *Am. J. Psychiatry* **155**, 264-271 (1998). https://doi.org:10.1176/ajp.155.2.264

98 Swedo, S. E. *et al.* Clinical presentation of pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections in research and community settings. *J. Child Adolesc. Psychopharmacol.* **25**, 26-30 (2015). https://doi.org:10.1089/cap.2014.0073

99 Chang, K. *et al.* Clinical Evaluation of Youth with Pediatric Acute-Onset Neuropsychiatric Syndrome (PANS): Recommendations from the 2013 PANS Consensus Conference. *J. Child Adolesc. Psychopharmacol.* **25**, 3-13 (2014). https://doi.org:10.1089/cap.2014.0084

100 Frick, L. & Pittenger, C. Microglial Dysregulation in OCD, Tourette Syndrome, and PANDAS. *Journal of Immunology Research* **2016**, 8606057 (2016). https://doi.org:10.1155/2016/8606057

101 Tian, L., Ma, L., Kaarela, T. & Li, Z. Neuroimmune crosstalk in the central nervous system and its significance for neurological diseases. *J. Neuroinflammation* **9**, 155 (2012). https://doi.org:10.1186/1742-2094-9-155

102 Dantzer, R. Neuroimmune Interactions: From the Brain to the Immune System and Vice Versa. *Physiol. Rev.* **98**, 477-504 (2018). https://doi.org:10.1152/physrev.00039.2016

103 Wohleb, E. S. & Delpech, J. C. Dynamic cross-talk between microglia and peripheral monocytes underlies stress-induced neuroinflammation and behavioral consequences. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **79**, 40-48 (2017). https://doi.org:10.1016/j.pnpbp.2016.04.013

104 Lehmann, M. L. *et al.* Decoding microglia responses to psychosocial stress reveals blood-brain barrier breakdown that may drive stress susceptibility. *Sci. Rep.* **8**, 11240 (2018). https://doi.org:10.1038/s41598-018-28737-8

105 Gray, S. M. & Bloch, M. H. Systematic review of proinflammatory cytokines in obsessive-compulsive disorder. *Curr Psychiatry Rep* **14**, 220-228 (2012). https://doi.org:10.1007/s11920-012-0272-0

106 Bilbo, S. & Stevens, B. Microglia: The Brain's First Responders. *Cerebrum* **2017** (2017).

107 Vargas, D. L., Nascimbene, C., Krishnan, C., Zimmerman, A. W. & Pardo, C. A. Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann. Neurol.* **57**, 67-81 (2005). https://doi.org:10.1002/ana.20315

108 Morgan, J. T. *et al.* Microglial activation and increased microglial density observed in the dorsolateral prefrontal cortex in autism. *Biol. Psychiatry* **68**, 368-376 (2010). https://doi.org:10.1016/j.biopsych.2010.05.024

109 Morgan, J. T. *et al.* Abnormal microglial-neuronal spatial organization in the dorsolateral prefrontal cortex in autism. *Brain Res.* **1456**, 72-81 (2012). https://doi.org:10.1016/j.brainres.2012.03.036

110 Lennington, J. B. *et al.* Transcriptome analysis of the human striatum in Tourette syndrome. *Biol. Psychiatry* **79**, 372-382 (2016). https://doi.org:10.1016/j.biopsych.2014.07.018

111 Suzuki, K. *et al.* Microglial activation in young adults with autism spectrum disorder. *JAMA Psychiatry* **70**, 49-58 (2013). https://doi.org:10.1001/jamapsychiatry.2013.272

112 Kumar, A., Williams, M. T. & Chugani, H. T. Evaluation of basal ganglia and thalamic inflammation in children with pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection and tourette syndrome: a positron emission tomographic (PET) study using 11C-[R]-PK11195. *J. Child Neurol.* **30**, 749-756 (2015). https://doi.org:10.1177/0883073814543303

113 Hughes, H. K., R.J.Moreno & Ashwood, P. Innate immune dysfunction and neuroinflammation in autism spectrum disorder (ASD). *Brain. Behav. Immun.* **108**, 245-254 (2023). https://doi.org:https://doi.org/10.1016/j.bbi.2022.12.001

114 Li, X. *et al.* Elevated immune response in the brain of autistic patients. *J. Neuroimmunol.* **207**, 111-116 (2009). https://doi.org:10.1016/j.jneuroim.2008.12.002

115 Xu, N., Li, X. & Zhong, Y. Inflammatory cytokines: potential biomarkers of immunologic dysfunction in autism spectrum disorders. *Mediators Inflamm.* **2015**, 531518 (2015). https://doi.org:10.1155/2015/531518

116 Wei, H. *et al.* IL-6 is increased in the cerebellum of autistic brain and alters neural cell adhesion, migration and synaptic formation. *J. Neuroinflammation* **8**, 52 (2011). https://doi.org:10.1186/1742-2094-8-52

117 Young, A. M., Campbell, E., Lynch, S., Suckling, J. & Powis, S. J. Aberrant NF-kappaB expression in autism spectrum condition: a mechanism for neuroinflammation. *Front Psychiatry* **2**, 27 (2011). https://doi.org:10.3389/fpsyt.2011.00027

118 Morer, A. *et al.* Elevated expression of MCP-1, IL-2 and PTPR-N in basal ganglia of Tourette syndrome cases. *Brain. Behav. Immun.* **24**, 1069-1073 (2010). https://doi.org:10.1016/j.bbi.2010.02.007

119 Hong, J. J. *et al.* Microarray analysis in Tourette syndrome postmortem putamen. *J. Neurol. Sci.* **225**, 57-64 (2004). https://doi.org:10.1016/j.jns.2004.06.019

120 Careaga, M. *et al.* Immune endophenotypes in children with autism spectrum disorder. *Biol. Psychiatry* **81**, 434-441 (2017). https://doi.org:10.1016/j.biopsych.2015.08.036

121 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**, 40-45 (2011). https://doi.org:10.1016/j.bbi.2010.08.003

122 Saghazadeh, A. *et al.* A meta-analysis of pro-inflammatory cytokines in autism spectrum disorders: Effects of age, gender, and latitude. *J. Psychiatr. Res.* **115**, 90-102 (2019). https://doi.org:https://doi.org/10.1016/j.jpsychires.2019.05.019

123 Konuk, N. *et al.* Plasma levels of tumor necrosis factor-alpha and interleukin-6 in obsessive compulsive disorder. *Mediators Inflamm* **2007**, 65704 (2007). https://doi.org:10.1155/2007/65704

124 Karaguzel, E. O. *et al.* Blood levels of interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha and cognitive functions in patients with obsessive compulsive disorder. *Compr Psychiatry* **89**, 61-66 (2019). https://doi.org:10.1016/j.comppsych.2018.11.013

125 Rao, N. P. *et al.* Plasma cytokine abnormalities in drug-naive, comorbidity-free obsessive-compulsive disorder. *Psychiatry Res* **229**, 949-952 (2015). https://doi.org:10.1016/j.psychres.2015.07.009

126 Cosco, T. D. *et al.* Immune Aberrations in Obsessive-Compulsive Disorder: a Systematic Review and Meta-analysis. *Mol. Neurobiol.* **56**, 4751-4759 (2019). https://doi.org:10.1007/s12035-018-1409-x

127 Fabricius, R. A., Sørensen, C. B., Skov, L. & Debes, N. M. Cytokine profile of pediatric patients with obsessive-compulsive and/or movement disorder symptoms: A review. *Front. Pediatr.* **10** (2022). https://doi.org:10.3389/fped.2022.893815

128 Martino, D., Johnson, I. & Leckman, J. F. What Does Immunology Have to Do With Normal Brain Development and the Pathophysiology Underlying Tourette Syndrome and Related Neuropsychiatric Disorders? *Front. Neurol.* **11** (2020). https://doi.org:10.3389/fneur.2020.567407

129 Leckman, J. F. *et al.* Increased serum levels of interleukin-12 and tumor necrosis factor-alpha in Tourette's syndrome. *Biol. Psychiatry* **57**, 667-673 (2005). https://doi.org:10.1016/j.biopsych.2004.12.004

130 Cheng, Y.-h. *et al.* Detection of Autoantibodies and Increased Concentrations of Interleukins in Plasma from Patients with Tourette's Syndrome. *J. Mol. Neurosci.* **48**, 219-224 (2012). https://doi.org:10.1007/s12031-012-9811-8

131 Matz, J. *et al.* Altered monocyte activation markers in Tourette's syndrome: a case–control study. *BMC Psychiatry* **12**, 29 (2012). https://doi.org:10.1186/1471-244X-12-29

132 Tick, B., Bolton, P., Happe, F., Rutter, M. & Rijsdijk, F. Heritability of autism spectrum disorders: a meta-analysis of twin studies. *J. Child Psychol. Psychiatry* **57**, 585-595 (2016). https://doi.org:10.1111/jcpp.12499

133 Sandin, S. *et al.* The familial risk of autism. *JAMA* **311**, 1770-1777 (2014). https://doi.org:10.1001/jama.2014.4144

134 Mataix-Cols, D. *et al.* Familial Risks of Tourette Syndrome and Chronic Tic Disorders. A Population-Based Cohort Study. *JAMA Psychiatry* **72**, 787-793 (2015). https://doi.org:10.1001/jamapsychiatry.2015.0627

135 Blanco-Vieira, T. *et al.* The genetic epidemiology of obsessive-compulsive disorder: a systematic review and meta-analysis. *Translational Psychiatry* **13**, 230 (2023). https://doi.org:10.1038/s41398-023-02433-2

136 Chen, X. & Sullivan, P. F. Single nucleotide polymorphism genotyping: biochemistry, protocol, cost and throughput. *Pharmacogenomics J.* **3**, 77-96 (2003). https://doi.org:10.1038/sj.tpj.6500167

137 Autism Spectrum Disorders Working Group of The Psychiatric Genomics, C. Meta-analysis of GWAS of over 16,000 individuals with autism spectrum disorder highlights a novel locus at 10q24.32 and a significant overlap with schizophrenia. *Mol. Autism* **8**, 21 (2017). https://doi.org:10.1186/s13229-017-0137-9

138 Scharf, J. M. *et al.* Genome-wide association study of Tourette's syndrome. *Mol. Psychiatry* **18**, 721-728 (2013). https://doi.org:10.1038/mp.2012.69

139 Paschou, P. *et al.* Genetic association signal near NTN4 in Tourette syndrome. *Ann. Neurol.* **76**, 310-315 (2014). https://doi.org:10.1002/ana.24215

140 Frick, L., Rapanelli, M., Abbasi, E., Ohtsu, H. & Pittenger, C. Histamine regulation of microglia: Gene-environment interaction in the regulation of central nervous system inflammation. *Brain. Behav. Immun.* **57**, 326-337 (2016). https://doi.org:10.1016/j.bbi.2016.07.002

141 Arnold, P. D. *et al.* Revealing the complex genetic architecture of obsessive–compulsive disorder using meta-analysis. *Mol. Psychiatry* **23**, 1181-1188 (2018). https://doi.org:10.1038/mp.2017.154

142 Noble, D. Conrad Waddington and the origin of epigenetics. *J. Exp. Biol.* **218**, 816-818 (2015). https://doi.org:10.1242/jeb.120071

143 Salinas, R. D., Connolly, D. R. & Song, H. Invited Review: Epigenetics in neurodevelopment. *Neuropathol. Appl. Neurobiol.* **46**, 6-27 (2020). https://doi.org:https://doi.org/10.1111/nan.12608

144 Waddington, C. H. Towards a Theoretical Biology. *Nature* **218**, 525-527 (1968). https://doi.org:10.1038/218525a0

145 Stotz, K. & Griffiths, P. Epigenetics: ambiguities and implications. *History and Philosophy of the Life Sciences* **38**, 22 (2016). https://doi.org:10.1007/s40656-016-0121-2

146 Nardone, S. & Elliott, E. The interaction between the immune system and epigenetics in the etiology of autism spectrum disorders. *Front. Neurosci.* **10**, 329 (2016). https://doi.org:10.3389/fnins.2016.00329

147 Mattei, A. L., Bailly, N. & Meissner, A. DNA methylation: a historical perspective. *Trends Genet.* **38**, 676-707 (2022). https://doi.org:10.1016/j.tig.2022.03.010

148 Nestor, C. E. *et al.* Tissue type is a major modifier of the 5-hydroxymethylcytosine content of human genes. *Genome Res.* **22**, 467-477 (2012). https://doi.org:10.1101/gr.126417.111

149 Acharjee, S., Chauhan, S., Pal, R. & Tomar, R. S. in *Prog. Mol. Biol. Transl. Sci.* Vol. 197 (eds Vijai Singh & Indra Mani) 51-92 (Academic Press, 2023).

150 Razin, A. CpG methylation, chromatin structure and gene silencing-a three-way connection. *EMBO J.* **17**, 4905-4908 (1998). https://doi.org:10.1093/emboj/17.17.4905

151 Yao, Q., Chen, Y. & Zhou, X. The roles of microRNAs in epigenetic regulation. *Curr. Opin. Chem. Biol.* **51**, 11-17 (2019). https://doi.org:https://doi.org/10.1016/j.cbpa.2019.01.024

152 Zhong, Q. *et al.* Protein posttranslational modifications in health and diseases: Functions, regulatory mechanisms, and therapeutic implications. *MedComm (2020)* **4**, e261 (2023). https://doi.org:10.1002/mco2.261

153 Reichard, J. & Zimmer-Bensch, G. The Epigenome in Neurodevelopmental Disorders. *Front. Neurosci.* **15** (2021).

154 Cristancho, A. G. & Marsh, E. D. Epigenetics modifiers: potential hub for understanding and treating neurodevelopmental disorders from hypoxic injury. *J. Neurodev. Disord.* **12**, 37 (2020). https://doi.org:10.1186/s11689-020-09344-z

155 Yao, B. & Jin, P. Unlocking epigenetic codes in neurogenesis. *Genes Dev.* **28**, 1253-1271 (2014). https://doi.org:10.1101/gad.241547.114

156 Basil, P. *et al.* Prenatal maternal immune activation causes epigenetic differences in adolescent mouse brain. *Translational Psychiatry* **4**, e434 (2014). https://doi.org:10.1038/tp.2014.80

157 Banik, A. *et al.* Maternal factors that induce epigenetic changes contribute to neurological disorders in offspring. *Genes* **8**, 150 (2017). https://doi.org:10.3390/genes8060150

158 Hodyl, N. A., Roberts, C. T. & Bianco-Miotto, T. Cord Blood DNA Methylation Biomarkers for Predicting Neurodevelopmental Outcomes. *Genes (Basel)* **7** (2016). https://doi.org:10.3390/genes7120117

159 Collins, F. S., Morgan, M. & Patrinos, A. The Human Genome Project: lessons from large-scale biology. *Science* **300**, 286-290 (2003). https://doi.org:10.1126/science.1084564

160 Chen, C. *et al.* Applications of multi-omics analysis in human diseases. *MedComm (2020)* **4**, e315 (2023). https://doi.org:10.1002/mco2.315

161 Hasin, Y., Seldin, M. & Lusis, A. Multi-omics approaches to disease. *Genome Biol.* **18**, 83 (2017). https://doi.org:10.1186/s13059-017-1215-1

162 Deepak, S. *et al.* Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes. *Curr Genomics* **8**, 234-251 (2007). https://doi.org:10.2174/138920207781386960

163 Kukurba, K. R. & Montgomery, S. B. RNA Sequencing and Analysis. *Cold Spring Harb Protoc* **2015**, 951-969 (2015). https://doi.org:10.1101/pdb.top084970

164 Shendure, J. The beginning of the end for microarrays? *Nature Methods* **5**, 585-587 (2008). https://doi.org:10.1038/nmeth0708-585

165 Wang, Z., Gerstein, M. & Snyder, M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10**, 57-63 (2009). https://doi.org:10.1038/nrg2484

166 Sims, D., Sudbery, I., Ilott, N. E., Heger, A. & Ponting, C. P. Sequencing depth and coverage: key considerations in genomic analyses. *Nat. Rev. Genet.* **15**, 121-132 (2014). https://doi.org:10.1038/nrg3642

167 Voineagu, I. *et al.* Transcriptomic analysis of autistic brain reveals convergent molecular pathology. *Nature* **474**, 380-384 (2011). https://doi.org:10.1038/nature10110

168 Gupta, S. *et al.* Transcriptome analysis reveals dysregulation of innate immune response genes and neuronal activity-dependent genes in autism. *Nat Commun* **5**, 5748 (2014). https://doi.org:10.1038/ncomms6748

169 Parikshak, N. N. *et al.* Genome-wide changes in lncRNA, splicing, and regional gene expression patterns in autism. *Nature* **540**, 423-427 (2016). https://doi.org:10.1038/nature20612

170 Gandal, M. J. *et al.* Shared molecular neuropathology across major psychiatric disorders parallels polygenic overlap. *Science* **359**, 693-697 (2018). https://doi.org:10.1126/science.aad6469

171 Gandal, M. J. *et al.* Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. *Science* **362**, p. eaat8127 (2018). https://doi.org:10.1126/science.aat8127

172 He, Y., Zhou, Y., Ma, W. & Wang, J. An integrated transcriptomic analysis of autism spectrum disorder. *Sci. Rep.* **9** (2019). https://doi.org:10.1038/s41598-019-48160-x

173 Rahman, M. R. *et al.* Comprehensive Analysis of RNA-Seq Gene Expression Profiling of Brain Transcriptomes Reveals Novel Genes, Regulators, and Pathways in Autism Spectrum Disorder. *Brain Sci* **10** (2020). https://doi.org:10.3390/brainsci10100747

174 Horiuchi, F. *et al.* Identification of aberrant innate and adaptive immunity based on changes in global gene expression in the blood of adults with autism spectrum disorder. *J. Neuroinflammation* **18**, 102 (2021). https://doi.org:10.1186/s12974-021-02154-7

175 Lisboa, B. C. G. *et al.* Initial findings of striatum tripartite model in OCD brain samples based on transcriptome analysis. *Sci. Rep.* **9**, 3086 (2019). https://doi.org:10.1038/s41598-019-38965-1

176 Piantadosi, S. C. *et al.* Transcriptome alterations are enriched for synapse-associated genes in the striatum of subjects with obsessive-compulsive disorder. *Translational Psychiatry* **11**, 171 (2021). https://doi.org:10.1038/s41398-021-01290-1

177 Xu, Y. *et al.* Proteomic insights into synaptic signaling in the brain: the past, present and future. *Mol. Brain* **14**, 37 (2021). https://doi.org:10.1186/s13041-021-00750-5

178 Murtaza, N., Uy, J. & Singh, K. K. Emerging proteomic approaches to identify the underlying pathophysiology of neurodevelopmental and neurodegenerative disorders. *Mol. Autism* **11**, 27 (2020). https://doi.org:10.1186/s13229-020-00334-5

179 Graves, P. R. & Haystead, T. A. Molecular biologist's guide to proteomics. *Microbiol. Mol. Biol. Rev.* **66**, 39-63; table of contents (2002). https://doi.org:10.1128/mmbr.66.1.39-63.2002

180 Glish, G. L. & Vachet, R. W. The basics of mass spectrometry in the twenty-first century. *Nature Reviews Drug Discovery* **2**, 140-150 (2003). https://doi.org:10.1038/nrd1011

181 Pappireddi, N., Martin, L. & Wühr, M. A Review on Quantitative Multiplexed Proteomics. *Chembiochem* **20**, 1210-1224 (2019). https://doi.org:10.1002/cbic.201800650

182 Li, J. *et al.* TMTpro reagents: a set of isobaric labeling mass tags enables simultaneous proteome-wide measurements across 16 samples. *Nature Methods* **17**, 399-404 (2020). https://doi.org:10.1038/s41592-020-0781-4

183 He, B., Huang, Z., Huang, C. & Nice, E. C. Clinical applications of plasma proteomics and peptidomics: Towards precision medicine. *PROTEOMICS – Clinical Applications* **16**, 2100097 (2022). https://doi.org:https://doi.org/10.1002/prca.202100097

184 Cortelazzo, A. *et al.* Expression and oxidative modifications of plasma proteins in autism spectrum disorders: Interplay between inflammatory response and lipid peroxidation. *Proteomics Clin. Appl.* **10**, 1103-1112 (2016). https://doi.org:10.1002/prca.201500076

185 Feng, C. *et al.* Redox proteomic identification of carbonylated proteins in autism plasma: insight into oxidative stress and its related biomarkers in autism. *Clin. Proteomics* **14**, 2 (2017). https://doi.org:10.1186/s12014-017-9138-0

186 Shen, L. *et al.* iTRAQ-Based Proteomic Analysis Reveals Protein Profile in Plasma from Children with Autism. *PROTEOMICS – Clinical Applications* **12**, 1700085 (2018). https://doi.org:https://doi.org/10.1002/prca.201700085

187 Zhang, H. *et al.* The use of data independent acquisition based proteomic analysis and machine learning to reveal potential biomarkers for autism spectrum disorder. *J. Proteomics* **278**, 104872 (2023). https://doi.org:https://doi.org/10.1016/j.jprot.2023.104872

188 Li, C. *et al.* Clinical application of serum-based proteomics technology in human tumor research. *Anal. Biochem.* **663**, 115031 (2023). https://doi.org:https://doi.org/10.1016/j.ab.2022.115031

189 Corbett, B. A. *et al.* A proteomic study of serum from children with autism showing differential expression of apolipoproteins and complement proteins. *Mol. Psychiatry* **12**, 292-306 (2007). https://doi.org:10.1038/sj.mp.4001943

190 Ngounou Wetie, A. G., Shipp, D. A. & Darie, C. C. Bottlenecks in proteomics. *Adv. Exp. Med. Biol.* **806**, 581-593 (2014). https://doi.org:10.1007/978-3-319-06068-2_29

191 Steeb, H. *et al.* Serum proteomic analysis identifies sex-specific differences in lipid metabolism and inflammation profiles in adults diagnosed with Asperger syndrome. *Mol. Autism* **5**, 4 (2014). https://doi.org:10.1186/2040-2392-5-4

192 Ngounou Wetie, A. G. *et al.* Comparative two-dimensional polyacrylamide gel electrophoresis of the salivary proteome of children with autism spectrum disorder. *J. Cell. Mol. Med.* **19**, 2664-2678 (2015). https://doi.org:https://doi.org/10.1111/jcmm.12658

193 Ngounou Wetie, A. G. *et al.* A Pilot Proteomic Analysis of Salivary Biomarkers in Autism Spectrum Disorder. *Autism Res.* **8**, 338-350 (2015). https://doi.org:https://doi.org/10.1002/aur.1450

194 Mota, F. S. B. *et al.* Potential protein markers in children with Autistic Spectrum Disorder (ASD) revealed by salivary proteomics. *Int. J. Biol. Macromol.* **199**, 243-251 (2022). https://doi.org:10.1016/j.ijbiomac.2022.01.011

195 Zamanian Azodi, M. *et al.* Fluoxetine Regulates Ig Kappa Chain C Region Expression Levels in the Serum of Obsessive-Compulsive Disorder Patients: A proteomic Approach. *Iran J Pharm Res* **16**, 1264-1271 (2017).

196 Zamanian-Azodi, M. *et al.* Serum Proteomic Study of Women With Obsessive-Compulsive Disorder, Washing Subtype. *Basic Clin Neurosci* **9**, 337-346 (2018). https://doi.org:10.32598/bcn.9.5.337

197 Clarke, R. A., Lee, S. & Eapen, V. Pathogenetic model for Tourette syndrome delineates overlap with related neurodevelopmental disorders including Autism. *Translational Psychiatry* **2**, e158-e158 (2012). https://doi.org:10.1038/tp.2012.75

198 Canitano, R. & Vivanti, G. Tics and Tourette syndrome in autism spectrum disorders. *Autism : the international journal of research and practice* **11**, 19-28 (2007). https://doi.org:10.1177/1362361307070992

199 Darrow, S. M. *et al.* Autism Spectrum Symptoms in a Tourette's Disorder Sample. *J. Am. Acad. Child Adolesc. Psychiatry* **56**, 610-617 e611 (2017). https://doi.org:10.1016/j.jaac.2017.05.002

200 Pringsheim, T. & Hammer, T. Social behavior and comorbidity in children with tics. *Pediatr. Neurol.* **49**, 406-410 (2013). https://doi.org:10.1016/j.pediatrneurol.2013.08.005

201 Wile, D. J. & Pringsheim, T. M. Behavior Therapy for Tourette Syndrome: A Systematic Review and Meta-analysis. *Curr. Treat. Options Neurol.* **15**, 385-395 (2013). https://doi.org:10.1007/s11940-013-0238-5

202 Geschwind, D. H. Autism: many genes, common pathways? *Cell* **135**, 391-395 (2008). https://doi.org:10.1016/j.cell.2008.10.016

203 Jones, K. L. *et al.* Autism with intellectual disability is associated with increased levels of maternal cytokines and chemokines during gestation. *Mol. Psychiatry* **22**, 273-279 (2017). https://doi.org:10.1038/mp.2016.77

204 Patel, S. *et al.* Maternal immune conditions are increased in males with autism spectrum disorders and are associated with behavioural and emotional but not cognitive co-morbidity. *Translational Psychiatry* **10** (2020). https://doi.org:10.1038/s41398-020-00976-2

205 Dalsgaard, S., Waltoft, B. L., Leckman, J. F. & Mortensen, P. B. Maternal history of autoimmune disease and later development of tourette syndrome in offspring. *J. Am. Acad. Child Adolesc. Psychiatry* **54**, 495-501 e491 (2015). https://doi.org:10.1016/j.jaac.2015.03.008

206 Jones, H. F. *et al.* Maternal autoimmunity and inflammation are associated with childhood tics and obsessive-compulsive disorder: Transcriptomic data show common enriched innate immune pathways. *Brain, Behavior, and Immunity* **94**, 308-317 (2021). https://doi.org:https://doi.org/10.1016/j.bbi.2020.12.035

207 Lennington, J. B. *et al.* Transcriptome Analysis of the Human Striatum in Tourette Syndrome. *Biol Psychiatry* **79**, 372-382 (2016). https://doi.org:10.1016/j.biopsych.2014.07.018

208 Wright, C. *et al.* Altered expression of histamine signaling genes in autism spectrum disorder. *Transl Psychiatry* **7**, e1126 (2017). https://doi.org:10.1038/tp.2017.87

209 Li, M. *et al.* Integrative functional genomic analysis of human brain development and neuropsychiatric risks. *Science* **362**, eaat7615 (2018). https://doi.org:10.1126/science.aat7615

210 Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139-140 (2010). https://doi.org:10.1093/bioinformatics/btp616

211 ggplot2: Elegant Grapics for Data Analysis (Springer-Verlag New York, 2016).

212 Ashburner, M. *et al.* Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* **25**, 25-29 (2000). https://doi.org:10.1038/75556

213 The Gene Ontology, C. The Gene Ontology Resource: 20 years and still GOing strong. *Nucleic Acids Res.* **47**, D330-D338 (2019). https://doi.org:10.1093/nar/gky1055

214 Yu, G., Wang, L. G., Han, Y. & He, Q. Y. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* **16**, 284-287 (2012). https://doi.org:10.1089/omi.2011.0118

215 Kanehisa, M. Toward understanding the origin and evolution of cellular organisms. *Protein Sci.* **28**, 1947-1951 (2019). https://doi.org:10.1002/pro.3715

216 Kanehisa, M., Furumichi, M., Sato, Y., Ishiguro-Watanabe, M. & Tanabe, M. KEGG: integrating viruses and cellular organisms. *Nucleic Acids Res.* (2020). https://doi.org:10.1093/nar/gkaa970

217 Kanehisa, M. & Goto, S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**, 27-30 (2000). https://doi.org:10.1093/nar/28.1.27

218 Jassal, B. *et al.* The reactome pathway knowledgebase. *Nucleic Acids Res.* **48**, D498-D503 (2020). https://doi.org:10.1093/nar/gkz1031

219 Shannon, P. *et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **13**, 2498-2504 (2003). https://doi.org:10.1101/gr.1239303

220 Chin, C. H. *et al.* cytoHubba: identifying hub objects and sub-networks from complex interactome. *BMC Syst. Biol.* **8 Suppl 4**, S11 (2014). https://doi.org:10.1186/1752-0509-8-S4-S11

221 Joly-Amado, A. *et al.* CCL2 Overexpression in the Brain Promotes Glial Activation and Accelerates Tau Pathology in a Mouse Model of Tauopathy. *Front. Immunol.* **11**, 997 (2020). https://doi.org:10.3389/fimmu.2020.00997

222 Müller, N. The Role of Intercellular Adhesion Molecule-1 in the Pathogenesis of Psychiatric Disorders. *Front. Pharmacol.* **10**, 1251 (2019). https://doi.org:10.3389/fphar.2019.01251

223 Araujo, J., Zhang, M. & Yin, F. Heme Oxygenase-1, Oxidation, Inflammation, and Atherosclerosis. *Front. Pharmacol.* **3** (2012). https://doi.org:10.3389/fphar.2012.00119

224 Casey, S. C., Baylot, V. & Felsher, D. W. The MYC oncogene is a global regulator of the immune response. *Blood* **131**, 2007-2015 (2018). https://doi.org:10.1182/blood-2017-11-742577

225 Gnanaprakasam, J. N. & Wang, R. MYC in Regulating Immunity: Metabolism and Beyond. *Genes (Basel)* **8** (2017). https://doi.org:10.3390/genes8030088

226 Carow, B. & Rottenberg, M. E. SOCS3, a Major Regulator of Infection and Inflammation. *Front Immunol* **5**, 58 (2014). https://doi.org:10.3389/fimmu.2014.00058

227 Golovina, E. *et al.* Understanding the impact of SNPs associated with autism spectrum disorder on biological pathways in the human fetal and adult cortex. *Sci. Rep.* **11**, 15867 (2021). https://doi.org:10.1038/s41598-021-95447-z

228 Li, X. *et al.* Elevated immune response in the brain of autistic patients. *J. Neuroimmunol.* **207**, 111-116 (2009). https://doi.org:10.1016/j.jneuroim.2008.12.002

229 Dunkelberger, J. R. & Song, W.-C. Complement and its role in innate and adaptive immune responses. *Cell Res.* **20**, 34-50 (2010). https://doi.org:10.1038/cr.2009.139

230 Markiewski, M. M. & Lambris, J. D. The role of complement in inflammatory diseases from behind the scenes into the spotlight. *Am. J. Pathol.* **171**, 715-727 (2007). https://doi.org:10.2353/ajpath.2007.070166

231 Schafer, D. P. *et al.* Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron* **74**, 691-705 (2012). https://doi.org:10.1016/j.neuron.2012.03.026

232 Weber-Stadlbauer, U. Epigenetic and transgenerational mechanisms in infection-mediated neurodevelopmental disorders. *Translational Psychiatry* **7**, e1113-e1113 (2017). https://doi.org:10.1038/tp.2017.78

233 Cortese, S. *et al.* Candidate diagnostic biomarkers for neurodevelopmental disorders in children and adolescents: a systematic review. *World Psychiatry* **22**, 129-149 (2023). https://doi.org:https://doi.org/10.1002/wps.21037

234 Chen, Y.-C., Chang, Y.-W. & Huang, Y.-S. Dysregulated Translation in Neurodevelopmental Disorders: An Overview of Autism-Risk Genes Involved in Translation. *Dev. Neurobiol.* **79**, 60-74 (2019). https://doi.org:https://doi.org/10.1002/dneu.22653

235 Chen, Z. & Van Eyk, J. E. in *Genomic and Precision Medicine (Third Edition)* (eds Geoffrey S. Ginsburg & Huntington F. Willard) 79-87 (Academic Press, 2017).

236 Wilkins, M. R. *et al.* Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol. Genet. Eng. Rev.* **13**, 19-50 (1996). https://doi.org:10.1080/02648725.1996.10647923

237 Hetman, M. & Slomnicki, L. P. Ribosomal biogenesis as an emerging target of neurodevelopmental pathologies. *J. Neurochem.* **148**, 325-347 (2019). https://doi.org:10.1111/jnc.14576

238 Jiao, L. *et al.* Ribosome biogenesis in disease: new players and therapeutic targets. *Signal Transduction and Targeted Therapy* **8**, 15 (2023). https://doi.org:10.1038/s41392-022-01285-4

239 Sharifi, S. & Bierhoff, H. Regulation of RNA Polymerase I Transcription in Development, Disease, and Aging. *Annu. Rev. Biochem.* **87**, 51-73 (2018). https://doi.org:10.1146/annurev-biochem-062917-012612

240 Jia, X. *et al.* Protein translation: biological processes and therapeutic strategies for human diseases. *Signal Transduct Target Ther* **9**, 44 (2024). https://doi.org:10.1038/s41392-024-01749-9

241 Poulin F, S. N. in *Madame Curie Bioscience Database* (Landes Bioscience, Austin (TX), 2000-2013).

242 Rosina, E. *et al.* Disruption of mTOR and MAPK pathways correlates with severity in idiopathic autism. *Transl Psychiatry* **9**, 50 (2019). https://doi.org:10.1038/s41398-018-0335-z

243 Paul, M. S. *et al.* Rare EIF4A2 variants are associated with a neurodevelopmental disorder characterized by intellectual disability, hypotonia, and epilepsy. *Am. J. Hum. Genet.* **110**, 120-145 (2023). https://doi.org:10.1016/j.ajhg.2022.11.011

244 Carbonell, A. U. *et al.* Comparing synaptic proteomes across five mouse models for autism reveals converging molecular similarities including deficits in oxidative phosphorylation and Rho GTPase signaling. *Front. Aging Neurosci.* **15**, 1152562 (2023). https://doi.org:10.3389/fnagi.2023.1152562

245 Abraham, J. R. *et al.* Proteomic Investigations of Autism Brain Identify Known and Novel Pathogenetic Processes. *Sci. Rep.* **9**, 13118 (2019). https://doi.org:10.1038/s41598-019-49533-y

246 Yang, J. *et al.* Association between plasma proteome and childhood neurodevelopmental disorders: A two-sample Mendelian randomization analysis. *EBioMedicine* **78**, 103948 (2022). https://doi.org:10.1016/j.ebiom.2022.103948

247 Castagnola, M. *et al.* Hypo-phosphorylation of salivary peptidome as a clue to the molecular pathogenesis of autism spectrum disorders. *J. Proteome Res.* **7**, 5327-5332 (2008). https://doi.org:10.1021/pr8004088

248 Taurines, R. *et al.* Serum protein profiling and proteomics in autistic spectrum disorder using magnetic bead-assisted mass spectrometry. *Eur. Arch. Psychiatry Clin. Neurosci.* **260**, 249-255 (2010). https://doi.org:10.1007/s00406-009-0066-5

249 Momeni, N. *et al.* A novel blood-based biomarker for detection of autism spectrum disorders. *Transl Psychiatry* **2**, e91 (2012). https://doi.org:10.1038/tp.2012.19

250 Suganya, V., Geetha, A. & Sujatha, S. Urine proteome analysis to evaluate protein biomarkers in children with autism. *Clin. Chim. Acta* **450**, 210-219 (2015). https://doi.org:10.1016/j.cca.2015.08.015

251 Shen, L. *et al.* Proteomics Study of Peripheral Blood Mononuclear Cells (PBMCs) in Autistic Children. *Front. Cell. Neurosci.* **13**, 105 (2019). https://doi.org:10.3389/fncel.2019.00105

252 Buccitelli, C. & Selbach, M. mRNAs, proteins and the emerging principles of gene expression control. *Nat. Rev. Genet.* **21**, 630-644 (2020). https://doi.org:10.1038/s41576-020-0258-4

253 Liu, Y., Beyer, A. & Aebersold, R. On the Dependency of Cellular Protein Levels on mRNA Abundance. *Cell* **165**, 535-550 (2016). https://doi.org:https://doi.org/10.1016/j.cell.2016.03.014

254 Thomas, S. N., French, D., Jannetto, P. J., Rappold, B. A. & Clarke, W. A. Liquid chromatography–tandem mass spectrometry for clinical diagnostics. *Nature Reviews Methods Primers* **2**, 96 (2022). https://doi.org:10.1038/s43586-022-00175-x

255 Gagnon-Bartsch, J. A. & Speed, T. P. Using control genes to correct for unwanted variation in microarray data. *Biostatistics* **13**, 539-552 (2012). https://doi.org:10.1093/biostatistics/kxr034

256 Molania, R., Gagnon-Bartsch, J. A., Dobrovic, A. & Speed, T. P. A new normalization for Nanostring nCounter gene expression data. *Nucleic Acids Res* **47**, 6073-6083 (2019). https://doi.org:10.1093/nar/gkz433

257 Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **43**, e47-e47 (2015). https://doi.org:10.1093/nar/gkv007

258 Storey, J. D. A direct approach to false discovery rates. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)* **64**, 479-498 (2002). https://doi.org:https://doi.org/10.1111/1467-9868.00346

259 Wickham, H. *ggplot2: elegant graphics for data analysis*. (Springer-Verlag New York, 2016).

260 R: A Lanugage and Environment for Statistical Computing, (R Foundation for Statistical Computing, Vienna, Austria, 2020).

261 RStudio: Integrated Development for R (Boston, MA, 2020).

262 ggpubr: 'ggplot2' Based Publication Ready Plots (2023).

263 Guo, L. & Rondina, M. T. The Era of Thromboinflammation: Platelets Are Dynamic Sensors and Effector Cells During Infectious Diseases. *Front. Immunol.* **10** (2019).

264 De Luca, C., Colangelo, A. M., Alberghina, L. & Papa, M. Neuro-Immune Hemostasis: Homeostasis and Diseases in the Central Nervous System. *Front. Cell. Neurosci.* **12** (2018). https://doi.org:10.3389/fncel.2018.00459

265 Szaflarski, W. *et al.* Early rRNA processing is a stress-dependent regulatory event whose inhibition maintains nucleolar integrity. *Nucleic Acids Res* **50**, 1033-1051 (2022). https://doi.org:10.1093/nar/gkab1231

266 Schier, A. C. & Taatjes, D. J. Structure and mechanism of the RNA polymerase II transcription machinery. *Genes Dev.* **34**, 465-488 (2020). https://doi.org:10.1101/gad.335679.119

267 Zheng, B., Han, M., Bernier, M. & Wen, J. K. Nuclear actin and actin-binding proteins in the regulation of transcription and gene expression. *Febs j* **276**, 2669-2685 (2009). https://doi.org:10.1111/j.1742-4658.2009.06986.x

268 Klages-Mundt, N. L., Kumar, A., Zhang, Y., Kapoor, P. & Shen, X. The Nature of Actin-Family Proteins in Chromatin-Modifying Complexes. *Front Genet* **9**, 398 (2018). https://doi.org:10.3389/fgene.2018.00398

269 Kapoor, P. & Shen, X. Mechanisms of nuclear actin in chromatin-remodeling complexes. *Trends Cell Biol.* **24**, 238-246 (2014). https://doi.org:10.1016/j.tcb.2013.10.007

270 Broek, J. A. C., Guest, P. C., 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. *Mol. Autism* **5**, 41 (2014). https://doi.org:10.1186/2040-2392-5-41

271 Murgia, F. *et al.* Metabolomic Characterization of Pediatric Acute-Onset Neuropsychiatric Syndrome (PANS). *Front. Neurosci.* **15**, 645267 (2021). https://doi.org:10.3389/fnins.2021.645267

272 Rohart, F., Gautier, B., Singh, A. & KA, L. C. mixOmics: An R package for 'omics feature selection and multiple data integration. *PLoS Comput. Biol.* **13**, e1005752 (2017). https://doi.org:10.1371/journal.pcbi.1005752

273 Frick, L. R., Williams, K. & Pittenger, C. Microglial dysregulation in psychiatric disease. *Clin. Dev. Immunol.* **2013**, 608654 (2013). https://doi.org:10.1155/2013/608654

274 Jones, H. F. *et al.* Maternal Autoimmunity and Inflammation are Associated with Childhood Tics and Obsessive-Compulsive Disorder: Transcriptomic Data show Common Enriched Innate Immune Pathways. *Brain. Behav. Immun.* (2021). https://doi.org:10.1016/j.bbi.2020.12.035

275 Brown, A. S. Epidemiologic studies of exposure to prenatal infection and risk of schizophrenia and autism. *Dev. Neurobiol.* **72**, 1272-1276 (2012). https://doi.org:10.1002/dneu.22024

276 Paolicelli, R. C. *et al.* Synaptic pruning by microglia is necessary for normal brain development. *Science* **333**, 1456-1458 (2011). https://doi.org:10.1126/science.1202529

277 Atladóttir, H. O. *et al.* Association of hospitalization for infection in childhood with diagnosis of autism spectrum disorders: a Danish cohort study. *Arch. Pediatr. Adolesc. Med.* **164**, 470-477 (2010). https://doi.org:10.1001/archpediatrics.2010.9

278 Zhang, T. *et al.* Prenatal and Early Childhood Infections and Subsequent Risk of Obsessive-Compulsive Disorder and Tic Disorders: A Nationwide, Sibling-Controlled Study. *Biol. Psychiatry* **93**, 1023-1030 (2023). https://doi.org:https://doi.org/10.1016/j.biopsych.2022.07.004

279 Kurlan, R., Johnson, D. & Kaplan, E. L. Streptococcal infection and exacerbations of childhood tics and obsessive-compulsive symptoms: a prospective blinded cohort study. *Pediatrics* **121**, 1188-1197 (2008). https://doi.org:10.1542/peds.2007-2657

280 Nielsen, T. C. *et al.* Association of maternal autoimmune disease and early childhood infections with offspring autism spectrum disorder: A population-based cohort

study. *Autism Res.* **15**, 2371-2380 (2022). https://doi.org:https://doi.org/10.1002/aur.2824

281 Atladottir, H. O. *et al.* Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. *J. Autism Dev. Disord.* **40**, 1423-1430 (2010). https://doi.org:10.1007/s10803-010-1006-y

282 Abdallah, M. W. *et al.* Amniotic fluid inflammatory cytokines: potential markers of immunologic dysfunction in autism spectrum disorders. *World J. Biol. Psychiatry* **14**, 528-538 (2013). https://doi.org:10.3109/15622975.2011.639803

283 Brown, A. S. *et al.* Elevated maternal C-reactive protein and autism in a national birth cohort. *Mol. Psychiatry* **19**, 259-264 (2014). https://doi.org:10.1038/mp.2012.197

284 Jones, H. F. *et al.* Maternal thyroid autoimmunity associated with acute-onset neuropsychiatric disorders and global regression in offspring. *Dev. Med. Child Neurol.* **61**, 984-988 (2019). https://doi.org:10.1111/dmcn.14167

285 Patel, S. *et al.* Maternal immune-related conditions during pregnancy may be a risk factor for neuropsychiatric problems in offspring throughout childhood and adolescence. *Psychol. Med.*, 1-11 (2020). https://doi.org:10.1017/s0033291720001580

286 Masi, A., Glozier, N., Dale, R. & Guastella, A. J. The immune system, cytokines, and biomarkers in autism spectrum disorder. *Neurosci. Bull.* **33**, 194-204 (2017). https://doi.org:10.1007/s12264-017-0103-8

287 Kawasaki, T. & Kawai, T. Toll-like receptor signaling pathways. *Front. Immunol.* **5**, 461 (2014). https://doi.org:10.3389/fimmu.2014.00461

288 Han, V. X. *et al.* Emerging evidence of Toll-like receptors as a putative pathway linking maternal inflammation and neurodevelopmental disorders in human offspring: A systematic review. *Brain. Behav. Immun.* **99**, 91-105 (2022). https://doi.org:https://doi.org/10.1016/j.bbi.2021.09.009

289 Sameer, A. S. & Nissar, S. Toll-Like Receptors (TLRs): Structure, Functions, Signaling, and Role of Their Polymorphisms in Colorectal Cancer Susceptibility. *Biomed Res Int* **2021**, 1157023 (2021). https://doi.org:10.1155/2021/1157023

290 Chen, C.-Y., Shih, Y.-C., Hung, Y.-F. & Hsueh, Y.-P. Beyond defense: regulation of neuronal morphogenesis and brain functions via Toll-like receptors. *J. Biomed. Sci.* **26**, 90 (2019). https://doi.org:10.1186/s12929-019-0584-z

291 Lee, C. C., Avalos, A. M. & Ploegh, H. L. Accessory molecules for Toll-like receptors and their function. *Nat. Rev. Immunol.* **12**, 168-179 (2012). https://doi.org:10.1038/nri3151

292 Kleiveland, C. R. in *The Impact of Food Bioactives on Health: in vitro and ex vivo models* (eds Kitty Verhoeckx *et al.*) 161-167 (Springer International Publishing, 2015).

293 Hornung, V. *et al.* Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG

oligodeoxynucleotides. *J Immunol* **168**, 4531-4537 (2002). https://doi.org:10.4049/jimmunol.168.9.4531

294 Ginhoux, F. *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* **330**, 841-845 (2010). https://doi.org:10.1126/science.1194637

295 Ashwood, P. *et al.* In search of cellular immunophenotypes in the blood of children with autism. *PLoS One* **6**, e19299 (2011). https://doi.org:10.1371/journal.pone.0019299

296 Rodriguez, N. *et al.* Inflammatory dysregulation of monocytes in pediatric patients with obsessive-compulsive disorder. *J. Neuroinflammation* **14**, 261 (2017). https://doi.org:10.1186/s12974-017-1042-z

297 Pu, D. & Wang, W. Toll-like receptor 4 agonist, lipopolysaccharide, increases the expression levels of cytokines and chemokines in human peripheral blood mononuclear cells. *Exp. Ther. Med.* **8**, 1914-1918 (2014). https://doi.org:10.3892/etm.2014.2025

298 Guha, M. & Mackman, N. LPS induction of gene expression in human monocytes. *Cell. Signal.* **13**, 85-94 (2001). https://doi.org:10.1016/s0898-6568(00)00149-2

299 Fitzgerald, K. A. & Kagan, J. C. Toll-like Receptors and the Control of Immunity. *Cell (Cambridge)* **180**, 1044-1066 (2020). https://doi.org:10.1016/j.cell.2020.02.041

300 Ullah, M. O., Sweet, M. J., Mansell, A., Kellie, S. & Kobe, B. TRIF-dependent TLR signaling, its functions in host defense and inflammation, and its potential as a therapeutic target. *J. Leukoc. Biol.* **100**, 27-45 (2016). https://doi.org:10.1189/jlb.2RI1115-531R

301 Chaiwut, R. & Kasinrerk, W. Very low concentration of lipopolysaccharide can induce the production of various cytokines and chemokines in human primary monocytes. *BMC Res. Notes* **15**, 42 (2022). https://doi.org:10.1186/s13104-022-05941-4

302 Jyonouchi, H., Sun, S. & Le, H. Proinflammatory and regulatory cytokine production associated with innate and adaptive immune responses in children with autism spectrum disorders and developmental regression. *J. Neuroimmunol.* **120**, 170-179 (2001). https://doi.org:10.1016/s0165-5728(01)00421-0

303 Enstrom, A. M., Onore, C. E., Van de Water, J. A. & Ashwood, P. Differential monocyte responses to TLR ligands in children with autism spectrum disorders. *Brain. Behav. Immun.* **24**, 64-71 (2010). https://doi.org:10.1016/j.bbi.2009.08.001

304 Jyonouchi, H., Geng, L. & Davidow, A. L. Cytokine profiles by peripheral blood monocytes are associated with changes in behavioral symptoms following immune insults in a subset of ASD subjects: an inflammatory subtype? *J. Neuroinflammation* **11**, 187 (2014). https://doi.org:10.1186/s12974-014-0187-2

305 Jyonouchi, H., Geng, L., Cushing-Ruby, A. & Quraishi, H. Impact of innate immunity in a subset of children with autism spectrum disorders: a case control study. *J. Neuroinflammation* **5**, 52 (2008). https://doi.org:10.1186/1742-2094-5-52

306 Hughes, H. K. *et al.* Dysregulated gene expression associated with inflammatory and translation pathways in activated monocytes from children with autism spectrum disorder. *Translational Psychiatry* **12**, 39 (2022). https://doi.org:10.1038/s41398-021-01766-0

307 Fleige, S. & Pfaffl, M. W. RNA integrity and the effect on the real-time qRT-PCR performance. *Mol. Aspects Med.* **27**, 126-139 (2006). https://doi.org:https://doi.org/10.1016/j.mam.2005.12.003

308 Bantulà, M. *et al.* Reference Gene Validation for RT-qPCR in PBMCs from Asthmatic Patients with or without Obesity. *Methods Protoc* **5** (2022). https://doi.org:10.3390/mps5030035

309 Nielsen, M. C., Andersen, M. N. & Møller, H. J. Monocyte isolation techniques significantly impact the phenotype of both isolated monocytes and derived macrophages in vitro. *Immunology* **159**, 63-74 (2020). https://doi.org:10.1111/imm.13125

310 Livak, K. J. & Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **25**, 402-408 (2001). https://doi.org:https://doi.org/10.1006/meth.2001.1262

311 Schroeder, A. *et al.* The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol. Biol.* **7**, 3 (2006). https://doi.org:10.1186/1471-2199-7-3

312 Masi, A. *et al.* Cytokine aberrations in autism spectrum disorder: a systematic review and meta-analysis. *Mol. Psychiatry* **20**, 440-446 (2015). https://doi.org:10.1038/mp.2014.59

313 Banerjee, A. *et al.* Validation of Induced Microglia-Like Cells (iMG Cells) for Future Studies of Brain Diseases. *Front. Cell. Neurosci.* **15** (2021). https://doi.org:10.3389/fncel.2021.629279

314 Borcherding, N., Jethava, Y. & Vikas, P. Repurposing Anti-Cancer Drugs for COVID-19 Treatment. *Drug Des. Devel. Ther.* **14**, 5045-5058 (2020). https://doi.org:10.2147/dddt.S282252

315 Vempati, U. D. *et al.* Metadata Standard and Data Exchange Specifications to Describe, Model, and Integrate Complex and Diverse High-Throughput Screening Data from the Library of Integrated Network-based Cellular Signatures (LINCS). *SLAS Discovery* **19**, 803-816 (2014). https://doi.org:10.1177/1087057114522514

316 Chakraborty, R. *et al.* In-silico screening and in-vitro assay show the antiviral effect of Indomethacin against SARS-CoV-2. *Comput. Biol. Med.* **147**, 105788 (2022). https://doi.org:10.1016/j.compbiomed.2022.105788

Chapter 7

Appendix

7.1 Chapter 2

7.1.1 Statement of Contribution

Declaration by candidate: Chapter 2 of this thesis has been published in Frontiers Neuroscience, and a reference of this article is as follows:

Alshammery, S, Patel, S, Jones, H. J., Han, V. X., Gloss, B. S., Gold, W. A., Dale, R. C. (2022). Common targetable inflammatory pathways in brain transcriptome of autism spectrum disorders and Tourette syndrome. *Frontiers in Neuroscience* doi: 10.3389/fnins.2022.999346

7.1.2 Published Manuscript

frontiers | Frontiers in Neuroscience

TYPE Original Research
PUBLISHED 15 December 2022
DOI 10.3389/fnins.2022.999346

OPEN ACCESS

EDITED BY
Ming Jin Lim,
Guy's and St Thomas' NHS Foundation Trust, United Kingdom

REVIEWED BY
Michael Absoud,
King's College London, United Kingdom
Yanfa Sun,
Longyan University, China
Michael John Griffiths,
University of Liverpool, United Kingdom

*CORRESPONDENCE
Russell C. Dale
russell.dale@health.nsw.gov.au

SPECIALTY SECTION
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

RECEIVED 20 July 2022
ACCEPTED 24 November 2022
PUBLISHED 15 December 2022

CITATION
Alshammery S, Patel S, Jones HF, Han VX, Gloss BS, Gold WA and Dale RC (2022) Common targetable inflammatory pathways in brain transcriptome of autism spectrum disorders and Tourette syndrome. *Front. Neurosci.* 16:999346. doi: 10.3389/fnins.2022.999346

Common targetable inflammatory pathways in brain transcriptome of autism spectrum disorders and Tourette syndrome

Sarah Alshammery[1,2], Shrujna Patel[1,2], Hannah F. Jones[1,2,3], Velda X. Han[1,4], Brian S. Gloss[5], Wendy A. Gold[1,2] and Russell C. Dale[1,2,6]*

[1]Kids Neuroscience Centre, The Children's Hospital at Westmead, Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia, [2]The Children's Hospital at Westmead Clinical School, Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia, [3]Department of Neuroservices, Starship Children's Hospital, Auckland, New Zealand, [4]Khoo Teck Puat-National University Children's Medical Institute, National University Health System, Singapore, Singapore, [5]Westmead Research Hub, Westmead Institute for Medical Research, Westmead, NSW, Australia, [6]The Brain and Mind Centre, The University of Sydney, Sydney, NSW, Australia

Neurodevelopmental disorders (NDDs), including autism-spectrum disorders (ASD) and Tourette syndrome (TS) are common brain conditions which often co-exist, and have no approved treatments targeting disease mechanisms. Accumulating literature implicates the immune system in NDDs, and transcriptomics of post-mortem brain tissue has revealed an inflammatory signal. We interrogated two RNA-sequencing datasets of ASD and TS and identified differentially expressed genes, to explore commonly enriched pathways through GO, KEGG, and Reactome. The DEGs [False Discovery Rate (*FDR*) <0.05] in the ASD dataset (n = 248) and the TS dataset (n = 156) enriched pathways involving inflammation, cytokines, signal transduction and cell signalling. Of the DEGs from the ASD and TS analyses, 23 were shared, all of which were up-regulated: interaction networks of the common protein-coding genes using STRING revealed 5 central up-regulated hub genes: *CCL2*, *ICAM1*, *HMOX1*, *MYC*, and *SOCS3*. Applying KEGG and Reactome analysis to the 23 common genes identified pathways involving the innate immune response such as interleukin and interferon signalling pathways. These findings bring new evidence of shared immune signalling in ASD and TS brain transcriptome, to support the overlapping symptoms that individuals with these complex disorders experience.

KEYWORDS

inflammation, brain, bioinformatics, neurodevelopmental disorders, immune dysregulation

Introduction

Neurodevelopmental disorders (NDDs), such as autism-spectrum disorders (ASD) and tic disorders including Tourette syndrome (TS), are neurological conditions which commonly co-exist and have shared genetic contributions (Clarke et al., 2012). ASD is characterised by social communication and language deficits, and repetitive stereotypical behaviour. Tics are repetitive stereotyped movements (motor tics) or vocalisations (vocal tics), and when present for more than 12 months, fulfil a diagnosis of TS. Tics are present in 11–22% of children with ASD, while ASD is present in 12% of children diagnosed with TS (Canitano and Vivanti, 2007; Pringsheim and Hammer, 2013; Darrow et al., 2017). Limited disease specific treatments are currently available for NDDs, and management focuses on symptom mitigation and developmental support (Wile and Pringsheim, 2013; Mittal, 2020).

The genetic aetiology of neurodevelopmental disorders is thought to be due to variants in multiple genes that converge on common pathways (Geschwind, 2008; Cross-Disorder Group of the Psychiatric Genomics Consortium, 2019). However, genetic aetiologies in these disorders are unable to explain the wide phenotypic heterogeneity, instead, the interaction between environmental and genetic factors are proposed to play an important role in pathogenesis of NDDs. In addition, immune dysregulation and inflammation have long been suggested to contribute to the pathophysiology, where early insults during gestation, such as maternal immune activation (MIA), can impact the development of the foetal brain (Scharf et al., 2013; Paschou et al., 2014; Sandin et al., 2014; Mataix-Cols et al., 2015; Frick et al., 2016; Tick et al., 2016; Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium, 2017; Han et al., 2021). MIA, encompassing maternal conditions such as infection, asthma, obesity, autoimmune disease, and psychosocial stress, are associated with increased incidence of NDDs in offspring, such as ASD and TS (Dalsgaard et al., 2015; Jones et al., 2017, 2021; Patel et al., 2020). MIA is thought to act as a disease primer, which in addition to genetic predisposition, results in increased expression of neurodevelopmental disorders (Estes and McAllister, 2016). Studies have also shown dysregulation in proinflammatory cytokines such as IL-12, TNF, monocyte chemoattractant protein 2 (MCP-2), and IL-2 in the brains and peripheral blood of individuals with ASD and TS (Leckman et al., 2005; Vargas et al., 2005; Morer et al., 2010; Ashwood et al., 2011).

Transcriptomic analyses (RNA sequencing) of post-mortem brains from individuals with ASD have shown upregulated genes involved in inflammation and microglial dysregulation (Gandal et al., 2018a,b). Similarly, analysis of post-mortem brain striatum from individuals with TS identified up-regulated genes in immune and inflammatory pathways, and implicated microglial activation as a primary source of inflammation (Lennington et al., 2016). In both the ASD and TS brain transcriptome studies, the downregulated genes were enriched in pathways involved in synaptic function and GABA neurotransmission, aligning with the genetic variation found in these disorders (Lennington et al., 2016; Gandal et al., 2018a,b). By contrast, the upregulated inflammatory findings were considered more likely to be due to environmental factors or secondary (Lennington et al., 2016; Gandal et al., 2018a,b).

Given the shared genetic heterogeneity and comorbidity of NDDs, there is an increasing need to examine common disease pathways. As inflammation has been reported in brain transcriptomics in both ASD and TS, we examined for shared gene expression between ASD and TS in order to improve our understanding of the pathophysiology of NDDs and provide future potential therapeutic targets (Lennington et al., 2016; Gandal et al., 2018a,b).

Materials and methods

Data availability and open-source bioinformatic analysis

Human brain transcriptome data (RNA-seq) from two independent published studies were obtained with authors permission from synapse.org and analysed for differential gene expression and pathway enrichment analysis (Lennington et al., 2016; Gandal et al., 2018b). Unlike TS, where only one study interrogating the brain transcriptome exists, there are a number of studies investigating ASD brain transcriptome (Wright et al., 2017; Gandal et al., 2018a,b; Li et al., 2018; He et al., 2019). The current ASD dataset was chosen as it presented the largest cohort of samples (Gandal et al., 2018a,b). The ASD data were downloaded from synapse.org (ID: syn8234507) as count files, and RNA-seq metadata of 42 ASD cases were matched with 43 neurotypical controls (NC) (Gandal et al., 2018b). The pre-frontal cortex (PFC) region was chosen for the ASD analysis given the large sample size with matched controls. The TS data was downloaded as BAM files from synapse.org (ID: syn3158906), which included putamen and the caudate nucleus regions from 9 TS cases to 9 normal controls (Lennington et al., 2016). The bioinformatic workflow, including all utilised code and quality control figures can be found at https://github.com/sarahalshammery/ASDTS.

Demographic and clinical variables of cases and controls

Autism spectrum disorder

A total of 42 ASD cases and 43 normal control PFC samples were utilised in this analysis (**Supplementary Table 1**;

Gandal et al., 2018b). The ASD cohort selected (n = 42) consisted of nine female cases (21.43%) and 33 male cases (78.57%), with mean age of 26.38, median of 22.5, and range of 2–67 years. The normal control cohort selected (n = 43) comprised of nine females (20.93%) and 34 males (79.07%), with mean age of 28.63, median of 24, and range of 4–60 years. A Mann–Whitney test indicated no significant difference (U = 831, P-value = 0.5295) between the ages of the ASD and normal control cohorts. The full demographic data can be accessed from https://doi.org/10.7303/syn12080241.

Tourette syndrome

A total of 9 TS cases and 9 normal control caudate nucleus and putamen samples were included (**Supplementary Table 1**; Lennington et al., 2016). The TS cohort (n = 9) entailed four female cases (44.44%), and five male cases (55.56%) with mean age of 62.77, median of 52, and range of 29–84 years. The normal control (NC) cohort (n = 9) consisted of four (44.44%) females and five males (55.6%) with mean age of 58, median of 52, and range of 4–60 years. The full demographic data is in the **Supplementary material** of the original study [See their **Supplementary Table 2** (Lennington et al., 2016)]. There was no statistical differences in the age of the TS cases in comparison to normal controls (Lennington et al., 2016).

Data quality control

The ASD dataset was prepared and sequenced as described,[1] reads were mapped against the Genome Reference Consortium Human Build 37 (GRCh37, otherwise known as hg19). The TS dataset were mapped against GRCh37 (hg19), and gene level counts for reference sequence (RefSeq) genes were assessed using HTSeq-count (Lennington et al., 2016). The raw counts for each dataset were converted to the counts per million (cpm) scale and filtered by expression using the *filterByExpr* function (Robinson et al., 2010). The data was normalised as per the EdgeR guide using Trimmed Mean of M-values (TMM) normalisation (Lennington et al., 2016).

Differential gene expression analysis

Genes with an False Discovery Rate (*FDR*) of <0.05 following differential gene expression analysis of each dataset were considered differentially expressed genes (DEGs) in this investigation. The DEGs were identified by a quasi-likelihood (QL) negative binomial (NB) generalised log-linear model (glmQLF). Genes with a logFC > = 0 were considered to be up-regulated, and those below 0 were down-regulated. DEGs were visualised through a volcano plot using the ggplot 2 package (Wickham, 2016).

Pathway and network enrichment analysis

Enrichments of the DEGs were identified through an over-representation analysis using Gene Ontology (GO) Biological Process, Reactome and the Kyoto Encyclopedia of Genes Genomes (KEGG), through the ClusterProfiler package [False Discovery Rate (*FDR*) <0.05] (Ashburner et al., 2000; Kanehisa and Goto, 2000; Yu et al., 2012; Kanehisa, 2019; The Gene Ontology Consortium, 2019; Jassal et al., 2020; Kanehisa et al., 2020). These are databases which allow genes to be grouped based on their relationships (GO), or the participation in pathways (Reactome and KEGG). For the main individual analyses, pathways enriched by less than 10 genes were excluded. Given the perceived more significant mechanistic insights of the Reactome results, they are presented in the main text, whereas GO and KEGG are presented in the **supplementary material**.

The protein-coding DEGs which were common to both the ASD and the TS DGE analyses, were visualised using a protein-protein interaction (PPI) network through the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING)[2], with an interaction score >0.4, and default active interaction sources (Jassal et al., 2020). The PPI network from the DEGs common to both ASD and TS datasets were further imported into Cytoscape (Shannon et al., 2003). CytoHubba, an app for Cytoscape was used to identify hub genes by ranking nodes by network features through the multiple correlation clustering (MCC) method (Chin et al., 2014). The expression of the hub genes in the disease cohorts compared to controls were visualised using the ggplot 2 package (Wickham, 2016). A Shapiro–Wilk test was utilised to test normality of the hub genes' counts.

Results

Transcriptional signatures

To identify relationships within the cases and their respective controls, we set out to explore differences based on transcriptome signatures. The ASD and TS cases were not observed to be transcriptionally distinct from their respective controls using hierarchal clustering analyses (**Supplementary Figures 1, 2**).

1 http://www.doi.org/10.7303/syn4587615

2 https://string-db.org/

Differential gene expression analysis

Autism spectrum disorder

The DEGs within the PFC of ASD cases compared to neurotypical controls consisted of 239 upregulated genes and 9 downregulated genes, represented through a volcano plot (Figure 1A). Results of the DGE analysis can be accessed in Supplementary material (Supplementary Tables 2A,B).

Tourette syndrome

The DEGs within the striatum of individuals with TS compared to neurotypical controls consisted of 143 upregulated genes and 13 downregulated genes, as shown in the volcano plot (Figure 1B). Results of the DGE analysis can be accessed in Supplementary material (Supplementary Tables 3A,B).

Immune pathways are enriched in autism spectrum disorders and Tourette syndrome brain transcriptome

Autism spectrum disorder

To explore enriched terms and pathways in the ASD DEGs, over-representation pathway analyses were conducted through three databases (*FDR* < 0.05). The GO analysis revealed 337 terms, consisting mainly of upregulated DEGs, and involved many immune response and inflammatory signalling, along with epigenetic terms (Supplementary Table 2C and Supplementary Figure 3). The top 3 GO terms were "humoral immune response," "leukocyte mediated immunity," and "lymphocyte mediated immunity." Over-representation analysis using KEGG revealed 9 pathways, majority of which were enriched by up-regulated genes (Supplementary Figure 4). The top 3 KEGG pathways (based on *FDR*) were "Systemic lupus erythematosus," "Neutrophil extracellular trap formation," and "Staphylococcus aureus infection" (Supplementary Table 2D). Enrichment of the DEGs using Reactome revealed 9 pathways, mostly enriched by up-regulated DEGs (Figures 2A,C). Of the 9 pathways, the top 3 Reactome pathways (based on *FDR* and count) were "Interleukin-4 and Interleukin-13 signalling," "Signalling by interleukins," and "Interferon signalling." Overall, 4/9 Reactome pathways were involved in the immune response consisting of cytokine signalling, innate and adaptive immune response pathways, 2/9 pathways were involved in signal transduction, 2/9 pathways were disease related, and 1/9 pathway belonged to gene expression and transcription. A full list of pathways from the three databases can be found in Supplementary material (Supplementary Tables 2C–E).

Tourette syndrome

The DEGs within the TS analysis enriched several terms and pathways from the three databases (*FDR* < 0.05). GO

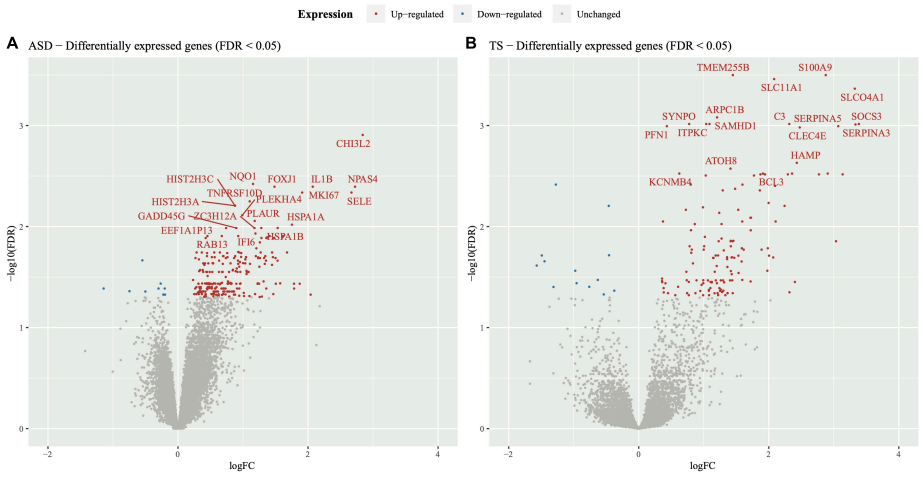


FIGURE 1
Volcano plot of differentially expressed genes in autism spectrum disorders (ASD) and Tourette syndrome (TS). Differential gene expression analysis was performed on the transcripts of **(A)** ASD cases and neurotypical controls and **(B)** TS cases and neurotypical control samples. The y-axis represents statistical significance (−log10FDR) and the x-axis represents gene expression log fold change (logFC). The top 20 differentially expressed genes were labelled for ease of viewing, all of which had an upregulated expression in both the ASD and TS datasets.

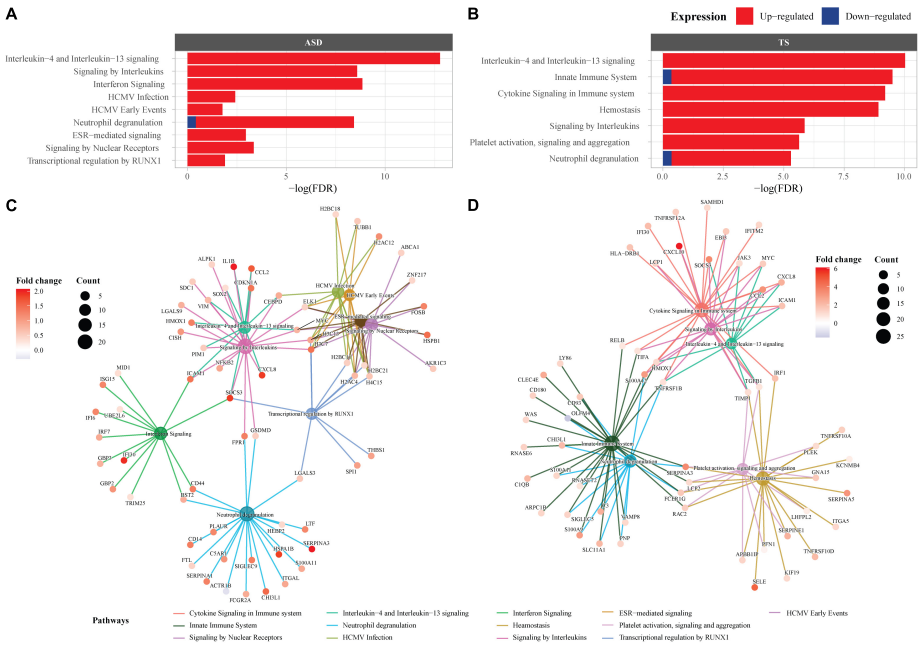


FIGURE 2
Pathway enrichment analysis in autism spectrum disorders (ASD) and Tourette syndrome (TS). Reactome enrichment analysis (*FDR* < 0.05) of the differentially expressed genes (DEGs) in the brain transcriptome of individuals with ASD compared with controls **(A,C)** and the DEGs of individuals with TS compared with controls **(B,D)**. Panels **(A,B)**: ASD enrichment results **(A)** and TS enrichment results **(B)** presented as bar plots of the top 10 pathways (y-axis), with the statistical significance of the pathways presented by the x-axis. Panels **(C,D)**: Connectivity network (CNET) plots of the top 5 enriched pathways and the interactions of genes that make up the pathways, represented by each gene's fold change. The enriched pathways in ASD **(C)** and TS **(D)** are represented by a colour.

over-representation analysis revealed 135 terms, majority of which were enriched by up-regulated genes (Supplementary Table 3C and Supplementary Figure 5). The top 3 enriched GO terms were "immune response," "cell activation," and "leukocyte activation." Over-representation analysis using KEGG did not enrich any pathways. Enrichment of the DEGs using Reactome revealed 7 pathways, most of which were enriched by up-regulated DEGs (Figures 2B,D). Of the 7 pathways, the top 3 Reactome pathways (sorted by *FDR* and count) were "Interleukin-4 and Interleukin-13 signalling," "Innate Immune System," "Cytokine Signalling in Immune system." Overall, 5/7 Reactome pathways were involved in the immune response consisting of cytokine signalling, innate and adaptive immune response pathways, and 2/7 pathways were involved in the homeostasis pathway. The full list of pathways can be found in Supplementary material (Supplementary Tables 3C,D).

Differentially expressed genes common to autism spectrum disorders and Tourette syndrome

Of the DEGs from the ASD analysis, and the DEGs from the TS analysis, 23 DEGs were found to be shared. In both the ASD and TS datasets, 23/23 of the common genes had an up-regulated expression. The common protein-coding DEGs were mapped into a PPI network, and their expression in the ASD and TS cohorts was visualised (Figure 3A). From this network, we identified the top five hub genes using Cytoscape and CytoHubba, which consisted of C-C Motif Chemokine Ligand 2 (*CCL2*), Intercellular Adhesion Molecule 1 (*ICAM1*), Heme Oxygenase 1 (*HMOX1*), MYC Proto-Oncogene (*MYC*), and Suppressor Of Cytokine Signalling 3 (*SOCS3*; Table 1; Shannon et al., 2003; Chin et al., 2014). The raw data are presented in log scale for the five hub genes in cases compared to controls, shown

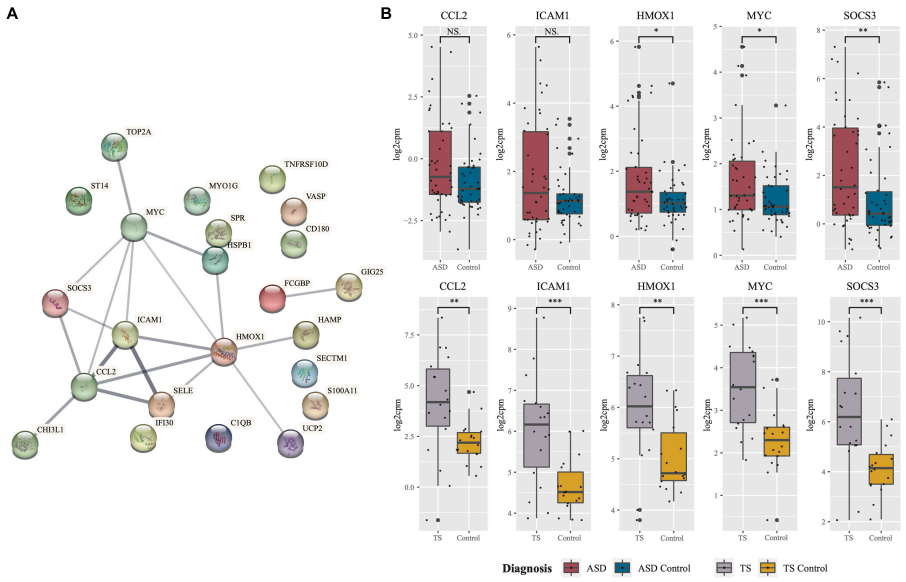


FIGURE 3
Protein network of common differentially expressed genes and expression of hub genes in autism spectrum disorders (ASD and Tourette syndrome (TS). **(A)** Protein-protein-interaction (PPI) network of genes found to be commonly differentially expressed in ASD and TS. The network consists of nodes (circles) and edges (lines) representative of predicted functional associations of the common protein-coding genes. Edge thickness is indicative of the strength of predicted evidence. **(B)** Hub genes central to the network were identified using Cytoscape, and the expression (log2cpm; y-axis) of the five hub genes in cases and neurotypical control (NC) cohorts. Expression of the five hub genes in ASD cases (n = 42; red) against neurotypical controls (n = 43; blue) and TS cases (n = 9; purple) and neurotypical controls (n = 9; yellow). The median is shown as the black line in each group's box, with the small black dots representing each sample. The whiskers on either side of the boxes represent the minimum (Q1-1.5*IQR) and maximum (Q3 + 1.5*IQR) log2cpm excluding outliers. The big black dots represent potential outliers (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, NS, non-significant, Mann–Whitney–Wilcoxon Test). Network generated using STRING.

for ASD and TS (Figure 3B). A full list of the common DEGs can be found in Supplementary material (Supplementary Table 4).

Common differentially expressed genes in autism spectrum disorders and Tourette syndrome enrich immune pathways

As many of the enriched dysregulated pathways in ASD and TS overlapped, we set out to explore enriched pathways from the 23 DEGs common to both disorders, using overrepresentation analyses through Reactome. The Reactome analysis revealed up-regulated genes enriched in 6 pathways in ASD and 6 pathways in TS, with the top three common pathways involved in "Interleukin-4 and Interleukin-13 signalling," "Interferon gamma signalling," and "Signalling by Interleukins" (Figure 4). The full list of pathways can be found in the Supplementary material (Supplementary Table 4).

Discussion

In this study we investigated enriched immune and inflammatory pathways in post-mortem brain tissue of individuals with ASD and TS, as well as pathways common to both disorders. As the focus of our hypothesis was to explore the immune response present in the seminal datasets, the paper's focal point will be the inflammatory findings. Differential gene expression of the PFC region in ASD revealed that the majority (239 genes) of the 248 DEGs were upregulated compared to normal controls. Analogous to this, in the striatum of TS, the majority (143 genes) of the identified 156 DEGs were also upregulated compared to controls. This analysis validates the previous studies of upregulated genes in post-mortem brains of individuals with ASD and TS (Voineagu et al., 2011; Lennington et al., 2016).

The identified dominant signal of immune response and inflammation from the ASD GO enrichment analysis aligns with studies investigating brain transcriptome and pathology

TABLE 1 Up-regulated hub genes in autism spectrum disorders (ASD) and Tourette syndrome (TS).

Gene	Gene name	Type of protein	Protein function	Reference
CCL2/MCP-1	C-C motif chemokine ligand 2/monocyte chemotactic and activating factor 1	Chemotactic cytokine.	Produced by microglia, neurons, astrocytes and mononuclear phagocytes, CCL2 recruits monocytes to the site of infection during inflammatory events.	Morer et al., 2010; Joly-Amado et al., 2020
ICAM1	Intercellular adhesion molecule 1	Immunoglobulin-like transmembrane glycoprotein expressed in the endothelial lumen.	Injury to the blood brain barrier results in microglia and astrocytes surrounding the capillary endothelial cells, where release of ICAM1 is responsible for eliminating antigens.	Müller, 2019
HMOX1	Heme oxygenase 1	Rate limiting enzymes that catalyses degradation of heme into biliverdin, ferrous ion, and carbon monoxide.	As a by-product of catabolising heme, HMOX1 has protective effects in vascular inflammation.	Araujo et al., 2012
MYC	Myelocytomatosis proto-oncogene	Transcription factor, binds DNA in a non-specific manner.	Involved in the regulation of immune checkpoints such as CD47 and PD-L1, and regulates expression of cells within the innate and adaptive immune responses.	Gnanaprakasam and Wang, 2017; Casey et al., 2018
SOCS3	Suppressor of cytokine Signalling 3	Suppressor of cytokine signalling family, part of a negative feedback system	Regulates cytokine signal transduction through STAT3 activation, using the gp130 receptor.	Carow and Rottenberg, 2014

Hub genes shared in ASD and TS following differential expression analyses. The common differentially expressed genes (23) from each disorder's analysis were imported into STRING and Cytoscape to identify hub genes. The top 5 hub genes using the MCC method were selected.

of individuals with ASD, and supports the involvement of astrocytes and activated microglia (Voineagu et al., 2011; Gandal et al., 2018b; Golovina et al., 2021). Of interest, the top 3 GO terms (by *FDR*) involved the humoral immune response and leukocyte mediated immunity. These terms were enriched by genes including *IL1b*, *TLR8*, complement genes (*C1QB*, *C1R*, *C2*), and chemokines (*CXCL5*, *CXCL8*)–all of which are involved in inflammation.

The enriched pathways established by the KEGG and Reactome analyses in the ASD cases identified major cellular pathways with therapeutic potential. The differential expression of central immune genes comprising cytokines, and CD cell markers (such as *IL1B*, *CD14*, *CD44*), support the reports of dysregulated cytokine levels in brains of individuals with ASD (Vargas et al., 2005; Li et al., 2009). Next, involvement of complement genes vital in phagocytosis (*C1QA*, *C1QB*, *C1QC*, *C1R*), which play a central role in immunity, response to infection, as well as synaptic pruning, further implicate the involvement of the immune system in ASD (Markiewski and Lambris, 2007; Dunkelberger and Song, 2010; Schafer et al., 2012). In addition, the enrichment of histone subunits fundamental to gene expression and epigenetic regulation (*H3C13*, *H3C7*, *H2BC11*, *H2BC3*), supports the concept of potential association between epigenetic regulation and inflammation (Weber-Stadlbauer, 2017).

Analysis of the TS differentially expressed genes using GO identified numerous enriched immune response and inflammatory signalling terms. The enriched pathways highlighted by the Reactome analysis in TS identified upregulated DEGs involved in the immune response such as cytokine signalling (*CXCL8*, *CXCL10*, *CCL2*) (Morer et al., 2010). In addition, pathways involving genes within major histocompatibility complexes II (i.e., *ICAM1*, *HLA-DRB1*) and the S100 family (*S100A9*, *S100A11*, *S100A12*) were enriched. These findings were similarly observed in the original analysis of these TS cases (Lennington et al., 2016).

Given the substantial comorbidity and overlap between NDDs, we identified genes and pathways common to both ASD and TS. We identified 23 common DEGs, all of which were upregulated in both disorders. From the 23 common genes, five were determined hub genes: *CCL2*, *ICAM1*, *HMOX1*, *MYC*, and *SOCS3*, all of which are involved in the immune response.

Our investigation has confirmed immune and inflammatory pathways are commonly enriched by up-regulated genes in ASD and TS. To further explore these intersecting findings, the 23 genes common to ASD and TS were analysed separately, which repeatedly identified enriched inflammatory pathways involving interleukin and interferon signalling. These pathways were enriched by the hub genes, which have a role in the immune response. We utilised this approach as it allowed for comparison of the same genes within both disorders, while employing the distinct *FDRs* from each analysis, offering insight into the strength of each disorder's signal.

Our current study identified commonly enriched inflammatory pathways, however, several questions regarding the involvement of the immune response in ASD and TS remain unanswered. The cause of the identified inflammatory signals is still ambiguous, in addition to its nature. Research

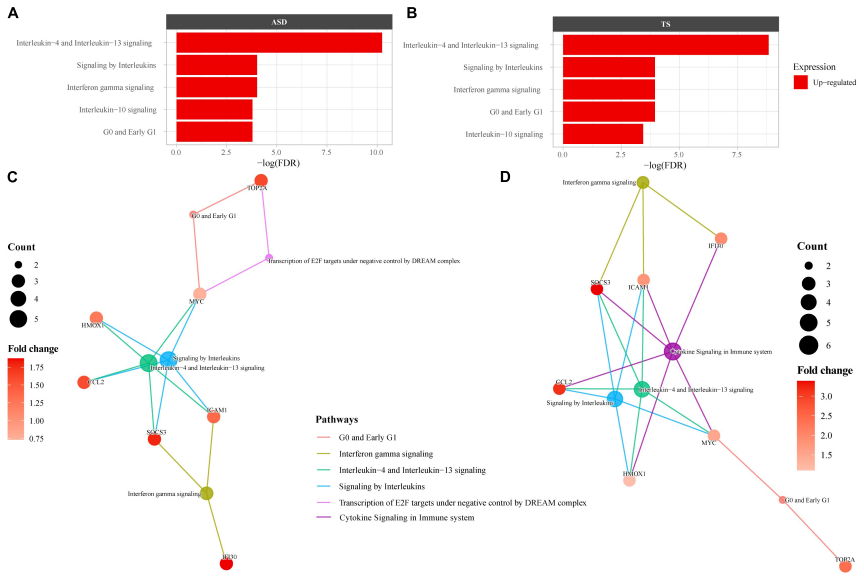


FIGURE 4
Overrepresented Reactome pathways common to autism spectrum disorders (ASD) and Tourette syndrome (TS). Reactome enrichment analysis (*FDR* < 0.05) of the common differentially expressed genes (DEGs) in the brain transcriptome of individuals with ASD compared with controls **(A,C)** and individuals with TS) compared with controls **(B,D)**. Panels **(A,B)**: ASD enrichment results **(A)** and TS enrichment results **(B)** presented as bar plots of the top 10 pathways (y-axis), with the statistical significance of the pathways presented by the x-axis. Panels **(C,D)**: Connectivity network (CNET) plots of the top 5 enriched pathways and the interactions of genes that make up the pathways, represented by each gene's fold change. The enriched pathways in ASD **(C)** and TS **(D)** are represented by a colour.

investigating the source of inflammation in NDDs has suggested it is an environmental or secondary component, rather than genetic (Voineagu et al., 2011; Lennington et al., 2016). In particular, the influence of MIA, which could create a neuroinflammatory environment in offspring, may alter immune signalling pathways and epigenetic control of cell function during the critical periods of development (Han et al., 2021). In addition, the identified inflammatory signal might be casual and pathogenic, or alternatively reactive or protective in origin, which cannot be deduced from the current investigation. Further functional and mechanistic explorations of tissue from individuals with NDDs might elucidate the nature of this inflammation.

Despite our findings, this study has a number of caveats. Firstly, our analysis involved different brain regions from the two disorders, prefrontal cortex for ASD, and caudate and putamen for TS, as corresponding brain region data was not available for the two disorders at the time of analysis.

Secondly, the majority of the samples within the two datasets were not children, as cohorts of paediatric post-mortem brain samples are scarce. Therefore, our analysis represents late-stage disease, and it is unclear if the findings will be reflected in younger cohorts. It is not known whether the inflammatory signal seen in ASD and TS accumulates over the course of life or is present in childhood.

Inflammation and the involvement of a dysregulated immune response is present in brain transcriptome data of both ASD and TS. Although classified as clinically distinct disorders, ASD and TS have common genetic aetiologies, along with overlaps in symptoms and comorbidities. We provide biological evidence that there is shared dysregulation of immune response and inflammatory signalling pathways in NDDs. Further studies to understand the cause and potential gene-environmental contribution to this inflammatory signal in these complex disorders is warranted.

Data availability statement

Publicly available datasets were analysed in this study. This data can be found here: https://www.synapse.org/#!Synapse:syn12080241.

Ethics statement

The studies involving human participants were reviewed and approved by National Institute of Mental Health. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SA analysed, interpreted, and wrote the results of this investigation. SP, HJ, VH, WG, and RD assisted in the interpretation and writing of the results. BG assisted in the analysis and interpretation of the results. All authors read and approved the final manuscript.

Funding

Financial support for this study was granted by the Petre Foundation, Brain Foundation, and NHMRC Investigator Grant (APP1193648).

Acknowledgments

We gratefully acknowledged the use of the PsychENCODE Consortium data in the Capstone Collection "UCLA-ASD" study, and data published as, "Transcriptome Analysis of the Human Striatum in Tourette Syndrome."

Conflict of interest

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

The handling editor declared a past collaboration with one of the author RD.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.999346/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Transcriptional clustering of ASD patients and normal controls. Cluster analyses identifying transcriptional differences within the autism-spectrum disorder (ASD; pink) patients and normal controls (NC; blue) using Euclidean distances. **(A)** Hierarchal cluster dendrogram using the agglomerative method. **(B)** Variance histogram identifies the amount of variance (y-axis) explained by each principal component (x-axis; dimension). **(C)** Principal component analysis (PCA) scatter plot of PC2 (y-axis) and PC1 (x-axis) which account for most of the variance in the data set. ASD, autism-spectrum disorder (n = 42), NC, normal control (n = 43).

SUPPLEMENTARY FIGURE 2
Transcriptional clustering of TS and normal controls. Cluster analyses identifying transcriptional differences within individuals with Tourette syndrome (TS; purple) and normal controls (NC; yellow) using Euclidean distances. **(A)** Hierarchal cluster dendrogram using the agglomerative method. **(B)** Variance histogram identifies the amount of variance (y-axis) explained by each principal component (x-axis; dimension). **(C)** Principal component analysis (PCA) scatter plot of PC2 (y-axis) and PC1 (x-axis) which account for most of the variance in the data set. TS, Tourette syndrome (n = 9), NC, normal control (n = 9).

SUPPLEMENTARY FIGURE 3
Autism spectrum disorders (ASD) Gene Ontology (GO) enrichment analysis. GO enrichment analysis (FDR/p.adjust $<$ 0.05) of the top differentially expressed genes (P value $<$ 0.05) in ASD. Statistical significance of the pathway (FDR) enriched is shown on the y-axis, while the enriched term is shown on the x-axis.

SUPPLEMENTARY FIGURE 4
Autism spectrum disorders (ASD) Kyoto Encyclopedia of Genes Genomes (KEGG) enrichment analysis. KEGG enrichment analysis (FDR/p.adjust $<$ 0.05) of the top differentially expressed genes (P value $<$ 0.05) in ASD. Statistical significance of the pathway (FDR) enriched is shown on the y-axis, while the enriched pathway is shown on the x-axis.

SUPPLEMENTARY FIGURE 5
Tourette syndrome Gene Ontology (GO) enrichment analysis. GO enrichment analysis (FDR/p.adjust $<$ 0.05) of the top differentially expressed genes (P value $<$ 0.05) in Tourette syndrome. Statistical significance of the pathway (FDR) enriched is shown on the y-axis, while the enriched term is shown on the x-axis.

References

Araujo, J., Zhang, M., and Yin, F. (2012). Heme oxygenase-1, oxidation, inflammation, and atherosclerosis. *Front. Pharmacol.* 3:119. doi: 10.3389/fphar.2012.00119

Ashburner, M., Ball, C., Blake, J., Botstein, D., Butler, H., Cherry, J., et al. (2000). Gene ontology: Tool for the unification of biology. The gene ontology consortium. *Nat. Genet.* 25, 25–29. doi: 10.1038/75556

Ashwood, P., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Pessah, I., and Van de Water, J. (2011). Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. *Brain Behav. Immun.* 25, 40–45. doi: 10.1016/j.bbi.2010.08.003

Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium (2017). Meta-analysis of GWAS of over 16,000 individuals with autism spectrum disorder highlights a novel locus at 10q24.32 and a significant overlap with schizophrenia. *Mol. Autism* 8:21. doi: 10.1186/s13229-017-0137-9

Canitano, R., and Vivanti, G. (2007). Tics and Tourette syndrome in autism spectrum disorders. *Autism* 11, 19–28. doi: 10.1177/1362361307070992

Carow, B., and Rottenberg, M. (2014). SOCS3, a major regulator of infection and inflammation. *Front. Immunol.* 5:58. doi: 10.3389/fimmu.2014.00058

Casey, S., Baylot, V., and Felsher, D. (2018). The MYC oncogene is a global regulator of the immune response. *Blood* 131, 2007–2015. doi: 10.1182/blood-2017-11-742577

Chin, C., Chen, S., Wu, H., Ho, C., Ko, M., and Lin, C. (2014). cytoHubba: Identifying hub objects and sub-networks from complex interactome. *BMC Syst. Biol.* 8(Suppl. 4):S11. doi: 10.1186/1752-0509-8-S4-S11

Clarke, R., Lee, S., and Eapen, V. (2012). Pathogenetic model for Tourette syndrome delineates overlap with related neurodevelopmental disorders including autism. *Transl. Psychiatry* 2:e158. doi: 10.1038/tp.2012.75

Cross-Disorder Group of the Psychiatric Genomics Consortium (2019). Genomic relationships, novel loci, and pleiotropic mechanisms across eight psychiatric disorders. *Cell* 179, 1469–1482.e11.

Dalsgaard, S., Waltoft, B., Leckman, J., and Mortensen, P. (2015). Maternal history of autoimmune disease and later development of Tourette syndrome in offspring. *J. Am. Acad. Child Adolesc. Psychiatry* 54, 495–501.e1. doi: 10.1016/j.jaac.2015.03.008

Darrow, S., Grados, M., Sandor, P., Hirschtritt, M., Illmann, C., Osiecki, L., et al. (2017). Autism spectrum symptoms in a Tourette's disorder sample. *J. Am. Acad. Child Adolesc. Psychiatry* 56, 610–617.e1. doi: 10.1016/j.jaac.2017.05.002

Dunkelberger, J., and Song, W. (2010). Complement and its role in innate and adaptive immune responses. *Cell Res.* 20, 34–50. doi: 10.1038/cr.2009.139

Estes, M., and McAllister, A. (2016). Maternal immune activation: Implications for neuropsychiatric disorders. *Science* 353, 772–777. doi: 10.1126/science.aag3194

Frick, L., Rapanelli, M., Abbasi, E., Ohtsu, H., and Pittenger, C. (2016). Histamine regulation of microglia: Gene-environment interaction in the regulation of central nervous system inflammation. *Brain Behav. Immun.* 57, 326–337. doi: 10.1016/j.bbi.2016.07.002

Gandal, M., Haney, J., Parikshak, N., Leppa, V., Ramaswami, G., Hartl, C., et al. (2018a). Shared molecular neuropathology across major psychiatric disorders parallels polygenic overlap. *Science* 359, 693–697.

Gandal, M., Zhang, P., Hadjimichael, E., Walker, R., Chen, C., Liu, S., et al. (2018b). Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. *Science* 362:eaat8127.

Geschwind, D. (2008). Autism: Many genes, common pathways? *Cell* 135, 391–395. doi: 10.1016/j.cell.2008.10.016

Gnanaprakasam, J., and Wang, R. (2017). MYC in regulating immunity: Metabolism and beyond. *Genes (Basel)* 8:88. doi: 10.3390/genes8030088

Golovina, E., Fadason, T., Lints, T., Walker, C., Vickers, M., and O'Sullivan, J. (2021). Understanding the impact of SNPs associated with autism spectrum disorder on biological pathways in the human fetal and adult cortex. *Sci. Rep.* 11:15867. doi: 10.1038/s41598-021-95447-z

Han, V., Patel, S., Jones, H., and Dale, R. (2021). Maternal immune activation and neuroinflammation in human neurodevelopmental disorders. *Nat. Rev. Neurol.* 17, 564–579. doi: 10.1038/s41582-021-00530-8

He, Y., Zhou, Y., Ma, W., and Wang, J. (2019). An integrated transcriptomic analysis of autism spectrum disorder. *Sci. Rep.* 9:11818. doi: 10.1038/s41598-019-48160-x

Jassal, B., Matthews, L., Viteri, G., Gong, C., Lorente, P., Fabregat, A., et al. (2020). The reactome pathway knowledgebase. *Nucleic Acids Res.* 48, D498–D503. doi: 10.1093/nar/gkz1031

Joly-Amado, A., Hunter, J., Quadri, Z., Zamudio, F., Rocha-Rangel, P., Chan, D., et al. (2020). CCL2 overexpression in the brain promotes glial activation and accelerates tau pathology in a mouse model of tauopathy. *Front. Immunol.* 11:997. doi: 10.3389/fimmu.2020.00997

Jones, H., Han, V., Patel, S., Gloss, B., Soler, N., Ho, A., et al. (2021). Maternal autoimmunity and inflammation are associated with childhood tics and obsessive-compulsive disorder: Transcriptomic data show common enriched innate immune pathways. *Brain Behav. Immun.* 94, 308–317. doi: 10.1016/j.bbi.2020.12.035

Jones, K., Croen, L., Yoshida, C., Heuer, L., Hansen, R., Zerbo, O., et al. (2017). Autism with intellectual disability is associated with increased levels of maternal cytokines and chemokines during gestation. *Mol. Psychiatry* 22, 273–279. doi: 10.1038/mp.2016.77

Kanehisa, M. (2019). Toward understanding the origin and evolution of cellular organisms. *Protein Sci.* 28, 1947–1951. doi: 10.1002/pro.3715

Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 28, 27–30. doi: 10.1093/nar/28.1.27

Kanehisa, M., Furumichi, M., Sato, Y., Ishiguro-Watanabe, M., and Tanabe, M. (2020). KEGG: Integrating viruses and cellular organisms. *Nucleic Acids Res.* 49, D545–D551. doi: 10.1093/nar/gkaa970

Leckman, J., Katsovich, L., Kawikova, I., Lin, H., Zhang, H., Kronig, H., et al. (2005). Increased serum levels of interleukin-12 and tumor necrosis factor-alpha in Tourette's syndrome. *Biol. Psychiatry* 57, 667–673. doi: 10.1016/j.biopsych.2004.12.004

Lennington, J., Coppola, G., Kataoka-Sasaki, Y., Fernandez, T., Palejev, D., Li, Y., et al. (2016). Transcriptome analysis of the human striatum in Tourette syndrome. *Biol. Psychiatry* 79, 372–382. doi: 10.1016/j.biopsych.2014.07.018

Li, M., Santpere, G., Imamura Kawasawa, Y., Evgrafov, O., Gulden, F., Pochareddy, S., et al. (2018). Integrative functional genomic analysis of human brain development and neuropsychiatric risks. *Science* 362:eaat7615.

Li, X., Chauhan, A., Sheikh, A., Patil, S., Chauhan, V., Li, X., et al. (2009). Elevated immune response in the brain of autistic patients. *J. Neuroimmunol.* 207, 111–116. doi: 10.1016/j.jneuroim.2008.12.002

Markiewski, M., and Lambris, J. (2007). The role of complement in inflammatory diseases from behind the scenes into the spotlight. *Am. J. Pathol.* 171, 715–727. doi: 10.2353/ajpath.2007.070166

Mataix-Cols, D., Isomura, K., Perez-Vigil, A., Chang, Z., Ruck, C., Larsson, K., et al. (2015). Familial risks of Tourette syndrome and chronic tic disorders. A population-based cohort study. *JAMA Psychiatry* 72, 787–793. doi: 10.1001/jamapsychiatry.2015.0627

Mittal, S. (2020). Tics and Tourette's syndrome. *Drugs Context* 9, 2019–2012. doi: 10.7573/dic.2019-12-2

Morer, A., Chae, W., Henegariu, O., Bothwell, A., Leckman, J., and Kawikova, I. (2010). Elevated expression of MCP-1. IL-2 and PTPR-N in basal ganglia of Tourette syndrome cases. *Brain Behav. Immun.* 24, 1069–1073. doi: 10.1016/j.bbi.2010.02.007

Müller, N. (2019). The role of intercellular adhesion molecule-1 in the pathogenesis of psychiatric disorders. *Front. Pharmacol.* 10:1251. doi: 10.3389/fphar.2019.01251

Paschou, P., Yu, D., Gerber, G., Evans, P., Tsetsos, F., Davis, L., et al. (2014). Genetic association signal near NTN4 in Tourette syndrome. *Ann. Neurol.* 76, 310–315. doi: 10.1002/ana.24215

Patel, S., Dale, R., Rose, D., Heath, B., Nordahl, C., Rogers, S., et al. (2020). Maternal immune conditions are increased in males with autism spectrum disorders and are associated with behavioural and emotional but not cognitive co-morbidity. *Transl. Psychiatry* 10:286. doi: 10.1038/s41398-020-00976-2

Pringsheim, T., and Hammer, T. (2013). Social behavior and comorbidity in children with tics. *Pediatr. Neurol.* 49, 406–410. doi: 10.1016/j.pediatrneurol.2013.08.005

Robinson, M., McCarthy, D., and Smyth, G. (2010). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140. doi: 10.1093/bioinformatics/btp616

Sandin, S., Lichtenstein, P., Kuja-Halkola, R., Larsson, H., Hultman, C., and Reichenberg, A. (2014). The familial risk of autism. *JAMA* 311, 1770–1777. doi: 10.1001/jama.2014.4144

Schafer, D., Lehrman, E., Kautzman, A., Koyama, R., Mardinly, A., Yamasaki, R., et al. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron* 74, 691–705. doi: 10.1016/j.neuron.2012.03.026

Scharf, J., Yu, D., Mathews, C., Neale, B., Stewart, S., Fagerness, J., et al. (2013). Genome-wide association study of Tourette's syndrome. *Mol. Psychiatry* 18, 721–728. doi: 10.1038/mp.2012.69

Shannon, P., Markiel, A., Ozier, O., Baliga, N., Wang, J., Ramage, D., et al. (2003). Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

The Gene Ontology Consortium. (2019). The gene ontology resource: 20 years and still GOing strong. *Nucleic Acids Res.* 47, D330–D338. doi: 10.1093/nar/gky1055

Tick, B., Bolton, P., Happe, F., Rutter, M., and Rijsdijk, F. (2016). Heritability of autism spectrum disorders: A meta-analysis of twin studies. *J. Child Psychol. Psychiatry* 57, 585–595. doi: 10.1111/jcpp.12499

Vargas, D., Nascimbene, C., Krishnan, C., Zimmerman, A., and Pardo, C. (2005). Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann. Neurol.* 57, 67–81. doi: 10.1002/ana.20315

Voineagu, I., Wang, X., Johnston, P., Lowe, J., Tian, Y., Horvath, S., et al. (2011). Transcriptomic analysis of autistic brain reveals convergent molecular pathology. *Nature* 474, 380–384. doi: 10.1038/nature10110

Weber-Stadlbauer, U. (2017). Epigenetic and transgenerational mechanisms in infection-mediated neurodevelopmental disorders. *Transl. Psychiatry* 7:e1113. doi: 10.1038/tp.2017.78

Wickham, H. (2016). *ggplot2: Elegant grapics for data analysis*. New York, NY: Springer-Verlag. doi: 10.1007/978-3-319-24277-4

Wile, D., and Pringsheim, T. (2013). Behavior therapy for Tourette syndrome: A systematic review and meta-analysis. *Curr. Treat Options Neurol.* 15, 385–395. doi: 10.1007/s11940-013-0238-5

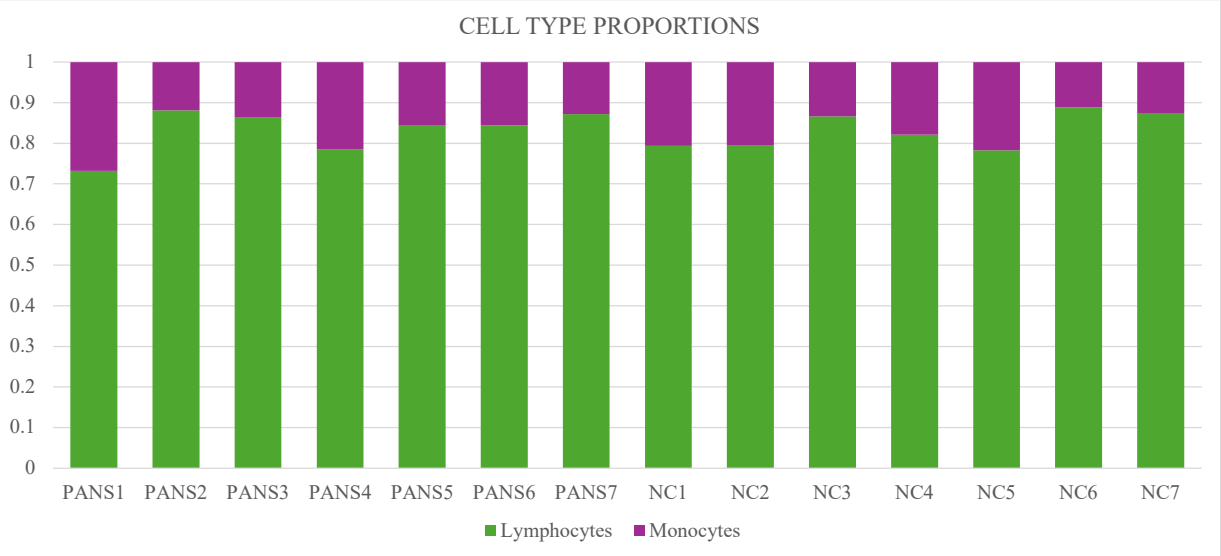
Wright, C., Shin, J., Rajpurohit, A., Deep-Soboslay, A., Collado-Torres, L., Brandon, N., et al. (2017). Altered expression of histamine signaling genes in autism spectrum disorder. *Transl. Psychiatry* 7:e1126. doi: 10.1038/tp.2017.87

Yu, G., Wang, L., Han, Y., and He, Q. (2012). clusterProfiler: An R package for comparing biological themes among gene clusters. *OMICS* 16, 284–287. doi: 10.1089/omi.2011.0118

7.2 Chapter 4

7.2.1 Routine testing: proportion of immune cells

As mentioned in section 3.7.1, routine blood testing for the patients included in this assay was performed to assess the proportion of immune cells in the blood. In addition, this was validated with testing blood from the controls utilised in this assay. The proportions of immune cells (monocytes and lymphocytes) were comparable in patients compared to controls as can be observed in the figure below:



7.2.2 Statement of Contribution

Declaration by candidate: Chapter 4 of this thesis has been included in a manuscript under review in Molecular Psychiatry, and I am joint first-author. A reference of this article is as follows:

Velda X Han^, **Sarah Alshammery**^, Brooke A Keating, Brian S Gloss, Markus J Hofer, Mark E Graham, Nader Aryamanesh, Lee L Marshall, Songyi Yuan, Emma Maple-Brown, Jingya Yan, Sushil Bandodkar, Kavitha Kothur, Hiroya Nishida, Hannah Jones, Erica Tsang, Xianzhong Lau, Ruwani Dissanayake, Iain Perkes, Shekeeb Mohammad, Fabienne Brilot, Wendy Gold, Shrujna Patel, Russell C Dale

My contribution to this manuscript includes:

- Design of research study
- Conduction of experiments
- Acquisition and data analysis
- Performing computational analyses
- Drafting of manuscript

7.2.3 Manuscript

Epigenetic, ribosomal, and immune dysregulation in Paediatric Acute-Onset Neuropsychiatric Syndrome

Velda X Han[1,2,3]^, MMED; Sarah Alshammery[1,4]^, BSc Hons; Brooke A Keating[1,4], PhD; Brian S Gloss[5], PhD; Markus J Hofer[6,7], PhD; Mark E Graham[8], PhD; Nader Aryamanesh[9,10], PhD; Lee L Marshall[9], PhD; Songyi Yuan[10], MD, Emma Maple-Brown[1,11], Bsc Hons, Jingya Yan[1,4,12], PhD; Sushil Bandodkar[4,12], PhD; Kavitha Kothur[1], PhD; Hiroya Nishida[1], PhD; Hannah Jones[13], PhD; Erica Tsang[1], MNutrDiet; Xianzhong Lau[14], PhD; Ruwani Dissanayake[15], PhD; Iain Perkes[16,17], MD; Shekeeb Mohammad[1,4], PhD; Fabienne Brilot[1,4,6,18],PhD; Wendy Gold[1,19,20], PhD; Shrujna Patel[1,4*], PhD; Russell C Dale[1,4,19*]PhD

^ Joint first author *Joint last author

[1]Kids Neuroscience Centre, The Children's Hospital at Westmead, Faculty of Medicine and Health, University of Sydney, NSW, Australia

[2]Khoo Teck Puat-National University Children's Medical Institute, National University Health System, Singapore, Singapore

[3]Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

[4]The Children's Hospital at Westmead Clinical School, Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia

[5]Westmead Research Hub, Westmead Institute for Medical Research, Westmead, NSW, Australia

[6]The Brain and Mind Centre, The University of Sydney, Sydney, NSW, Australia

[7]School of Life and Environmental Sciences and Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia

[8]Synapse Proteomics Group, Children's Medical Research Institute, The University of Sydney

[9]Bioinformatics Group, Children's Medical Research Institute, University of Sydney, Westmead, NSW, Australia

[10]Faculty of Medicine and Health, Sydney Medical School, University of Sydney, Camperdown, NSW, Australia

[11]Graduate School of Medicine, Faculty of Science, Medicine and Health, University of Wollongong

[12]Department of Biochemistry, The Children's Hospital at Westmead, NSW, Australia

[13]Starship Children's Hospital, Auckland, New Zealand

[14]Australian Genome Research Facility, Melbourne, VIC, Australia

[15]Australian Genome Research Facility, Westmead, NSW, Australia

[16]Department of Psychological Medicine, Sydney Children's Hospitals Network, Westmead, NSW, Australia

[17]Discipline of Psychiatry and Mental Health, School of Clinical Medicine, Faculty of Medicine and Health, University of New South Wales, Sydney, NSW, Australia

[18]School of Medical Sciences, Discipline of Applied Medical Science, Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, Australia.

[19]The University of Sydney, School of Medical Sciences and Discipline of Child and Adolescent Health, Faculty of Medicine and Health, Sydney, NSW, Australia.

[20]Molecular Neurobiology Research Laboratory, Kids Research, Children's Hospital at Westmead, and The Children's Medical Research Institute, Westmead, NSW, Australia.

Corresponding author:

Prof Russell Dale
Kids Neuroscience Centre, The Children's Hospital at Westmead, Faculty of Medicine and Health, University of Sydney, Sydney, New South Wales, Australia

The Children's Hospital at Westmead Clinical School, Faculty of Medicine and Health, University of Sydney, Sydney, New South Wales, Australia

The Brain and Mind Centre, The University of Sydney, Sydney, New South Wales, Australia

Fax: +61 2 9845 3389
Email: russell.dale@sydney.edu.au

Conflict of interest

There are no competing interests to declare.

Abstract

Paediatric Acute-Onset Neuropsychiatric Syndrome (PANS) is characterised by abrupt onset obsessive compulsive disorder and regression in neurodevelopmental skills, triggered by infection or stress. Whether PANS is a distinct entity or part of a neurodevelopmental spectrum is uncertain, and its pathophysiology remains unclear. We show that children with PANS and other non-PANS neurodevelopmental disorders have higher reported early childhood infections and a loss of previously acquired developmental skills compared to neurotypical controls. Children with PANS have normal routine immune testing, however bulk RNA-sequencing revealed upregulated pathways in ribosomal biogenesis and RNA methyltransferases, and downregulated pathways in diverse cellular functions such as mitochondrial activity, cell signalling, endocytosis, and immune responses. Single-cell RNA-sequencing confirmed these findings but showed heterogeneity across immune cell types. Toll-like receptor stimulation assay using peripheral blood mononuclear cells revealed reduced TNF and interleukin-6 responses in PANS patients compared to controls. RNA sequencing before and after intravenous immunoglobulin treatment in PANS patients revealed reversal of the dysregulated ribosomal, epigenetic, and cell signaling pathways. Given the central role of the immune system in synaptic pruning and neurodevelopment, these insights provide rationale for novel epigenetic and immune modulating therapies to optimize neurodevelopmental trajectories and minimize neuropsychiatric impairment.

Introduction

Neurodevelopmental disorders (NDDs) including autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), Tourette syndrome/tics disorder, and obsessive-compulsive disorder (OCD) are increasing in prevalence, and now affect 1 in 6 children[1]. Overlaps in clinical symptoms and comorbidities of NDDs suggest shared genetic etiologies[2]. While genetic factors exert a significant influence on NDDs, most cases are attributed to common susceptibility loci, rather than rare gene variants[2, 3]. The interplay of genetic and environmental factors in the pathogenesis of childhood NDDs is increasingly recognized, starting from preconception and extending into early adulthood[4]. During this period, the central nervous system undergoes dynamic changes including neurogenesis, cell migration, cell differentiation, and synaptogenesis. Additionally, brain immune cells, primarily microglia, actively refine neural networks, through synaptic pruning[5, 6]. Early life programming, influenced by environmental exposures during critical developmental periods, can affect a child's developmental trajectory[4]. This lasting impact is proposed to occur through cellular stress on the epigenetic and gene regulatory program of brain and immune cells [7, 8].

Environmental exposures in the postnatal period, such as infections and stress which represent second 'hits', have been observed to trigger the onset or worsening of neurodevelopmental (inattention, tics, autistic features) or neuropsychiatric (obsessive-compulsive, anxiety) symptoms in children [9-12]. These observations have led to the proposal of clinical syndromes such as Paediatric acute-onset neuropsychiatric syndrome (PANS) or Paediatric autoimmune neuropsychiatric disorder associated with Streptococcal infection (PANDAS), which is a subset of PANS [13, 14]. Although these syndromes have been described in

literature, their aetiology remains enigmatic, thus not fully recognized as 'discrete biological entities'. Early clinical observations of PANS/PANDAS proposed these conditions as distinct autoimmune entities, akin to autoimmune encephalitis [14]. However, an alternative hypothesis is that PANS is part of the broader NDD continuum, representing a phenotype that demonstrates ongoing interactions between the immune system and brain in a subset of people with NDDs[15]. Studies that investigate the underlying pathophysiology of PANS/PANDAS are lacking. Furthermore, antibiotics and immune modulation, such as intravenous immunoglobulin (IVIg), have been proposed to benefit individuals with PANS/PANDAS, but these treatments remain contentious [16, 17].

We developed a program to explore the influence of environmental factors, in particular recurrent infections, on neuropsychiatric symptoms, as well as associated loss of developmental skills. We focussed on children who fulfil criteria for PANS, who have abrupt infection or stress triggered neuropsychiatric symptoms, as a model to examine gene-environment interactions in NDDs (Figure 1A). We hypothesize that epigenetic and immune processes operate at the gene-environment interface and are central to the pathogenesis of PANS and other NDDs. We performed bulk blood transcriptomic analyses in PANS, and then further explored these findings in PANS via single cell transcriptomics and functional immune assays. We also conducted blood transcriptomic analysis on children with PANS both before and after intravenous immunoglobulin administration, aiming to define the biological effect of IVIg in these individuals.

Methods

Participant selection

NDD cohort: We performed a prospective study of 100 sequential children (<18 years) referred with complex NDDs to a specialist clinic at the Children's Hospital at Westmead, Sydney from January 2020 to January 2023. In this clinic, there is a referral enrichment of PANS, Tourette syndrome, and OCD, often with accompanying disorders, such as ASD and ADHD. Children seen at this clinic are screened using a structured clinical assessment on REDCap, followed by a clinical directed interview.

PANS cohort: Within the NDD cohort, 32 children met the criteria for PANS [13]. Additionally, 4 more children who met the criteria for PANS were recruited prospectively. We used the 2013 PANS diagnostic criterion which included an abrupt, dramatic onset of OCD or severely restricted food intake[13]. There was concurrent presence of at least two of the seven categories including (i) anxiety, (ii) emotional lability and/or depression, (iii) irritability, aggression, and/or oppositional behaviours, (iv) behavioural regression, (v) deterioration in school performance, (vi) sensory or motor abnormalities, (vii) somatic signs and symptoms, including sleep disturbances, enuresis or urinary frequency[14]. Abrupt and dramatic onset of neuropsychiatric symptoms was defined as within 48 hours. Children with PANS included in the biological investigations all had new or ongoing neuropsychiatric symptoms (Supplementary Table 1) [13].

PANS-IVIg cohort: Within the PANS cohort, a subgroup of 9 children were about to commence or were receiving ongoing open-label intravenous immunoglobulin (IVIg) treatment (1.5-2g/kg per month) [14]. In Australia, PANS is currently a Medicare-approved indication for IVIg.

These patients who received IVIg had debilitating neuropsychiatric symptoms, despite optimization of psychology and conventional psychiatric treatments.

Controls: We recruited age- and sex- matched healthy children of hospital workers or patients being investigated for non-neurological and non-inflammatory disorders (eg. short stature) (Supplementary Table 2). We screened 58 healthy controls using the same structured clinical assessment on REDCap as the NDD patients. Within this group, 29 controls were involved in biological investigations. The inclusion criterion for controls involved in biological investigations was the absence of NDDs, autoimmune diseases and severe allergic conditions. Four controls had previous neurological symptoms (epilepsy in 3, benign intracranial hypertension in 1) that were resolved prior to blood taking.

All children with PANS and controls used in these biological blood studies were screened to ensure absence of any infections, allergic reactions/anaphylaxis, other major medical conditions, or admissions in the four weeks before blood draw.

Structured clinical assessment on REDCap and clinical directed interview

A structured assessment using REDCap was completed by parents of participants. Childhood infection history was captured using a purpose-built screening tool for infection (Supplementary Figure 1). There is currently no standardised tool to quantify infection rates in children, and most questionnaires are designed to screen for immunodeficiencies. Thus, we developed a screening tool that records frequencies of common viral and bacterial infections in childhood, involving respiratory tract, urinary tract, recurrent mouth ulcers, skin infections, and more serious bacterial infections (e.g. pneumonia, meningitis, bone

infections). Additional questions on frequency of visits to general practitioners, emergency department, or hospitalisations due to infection, and frequency of antibiotic courses, were also included. A 5-point Likert scale was used to assess frequency of these infections, with options including never, occasional (less than once per year), sometimes (1-3 times per year), often (4-6 times per year) and almost always (so frequent, hard to count), in the first 5 years of life and the last 12 months of life (scored 0-4). Objective scores of functioning, including the Strengths and Difficulties Questionnaire (SDQ), was performed for each child [18]. The presence or absence of loss of skills at any time were also recorded (eg. loss of learning ability attention/concentration, language, social skills, fine and gross motor skills).

For the NDD cohort, the clinicians (RCD, SSM, VXH) performed a clinical directed interview and recorded the child's diagnosis using DSM-5 criteria, including PANS criteria[13, 19]. In addition, markers of disease severity, including emergency department attendance due to neurodevelopmental symptoms, and absence from school for more than three months, were recorded. Triggers (e.g., stress, infection, excitement) that worsen neurodevelopmental symptoms were also recorded.

For the PANS-IVIg cohort, we used the Clinical Global Impression Scale-Severity score (CGI-S) as a quantitative measure for the therapeutic response to IVIg in PANS, and charted their daily CGI-S scores over four-week cycles[20]. The CGI-S question is: "Considering your total clinical experience with this particular population, how mentally ill is the patient at this time?"[20]. This rating is based upon observed and reported symptoms, behaviour, and function. A seven-point rating scale is used which ranged from 1=normal to 7= extremely ill.

223 *Sample collection*

224 After written consent, venous blood samples were collected for testing in PAXgene™ blood
225 RNA tubes (Qiagen, Hilden, Germany) and Acid Citric Dextrose tubes (BD).

226

227 *Bulk blood RNA-sequencing*

228 We performed bulk blood RNA sequencing in the PANS cohort comprising 20 children with
229 PANS versus 15 gender and age-matched controls. There were no significant differences in
230 age, gender or clinical characteristics between the 20 children with PANS who underwent bulk
231 blood RNA sequencing, versus the other 12 in the cohort. Subsequently, we performed bulk
232 blood RNA sequencing in the PANS-IVIg cohort comprising another 9 children with PANS
233 before and after IVIg treatment versus 10 gender and age-matched controls. Blood samples
234 were collected immediately prior to IVIg administration, and 10-14 days post IVIg. In the
235 PANS-IVIg cohort, bulk RNA sequencing was performed via two batches, 4 patients versus 4
236 controls in the first batch, and 5 patients versus 6 controls in the second batch. There was no
237 addition of new medications during the period when omics analyses were performed. Bulk
238 blood RNA sequencing was conducted by the Australian Genome Research Facility (AGRF,
239 Melbourne, Australia), as described in Supplementary material.

240

241 *Single-cell blood RNA sequencing*

242 We performed single-cell RNA sequencing using the 10X Genomics platform on an additional
243 two children with PANS who had not previously undergone bulk RNA sequencing. compared
244 with two matched control subjects, as described in Supplementary material.

245

246 *Toll-like receptor immune assay*

We developed an in-house Toll-like receptor (TLR) immune assays on PBMCs from 7 children with PANS versus 7 gender and age-matched controls, as described in Supplementary material.

Bioinformatic analysis

Bulk/single-cell RNA sequencing bioinformatic analysis and enrichment analysis

RNA seq data were analyzed (by VXH, SA, EM, BG, NA, ML) in the R statistical environment [21] with *tidyverse*[22], described in Supplementary material. For 10X Genomics single cell RNA sequencing, the *Seurat* package was used for analysis[23], described in Supplementary material. In this study, we focused on a pathway driven analysis, demonstrating cumulative effects of the differential expression of multiple genes, instead of focusing on individual genes. Pathway enrichment analysis was performed via Gene Set Enrichment Analysis (GSEA) to obtain enriched Gene Ontology (GO) and reactome pathways using the *fgsea* package, described in Supplementary material.

Statistical analysis

We performed statistical analyses using GraphPad Prism v8.2.0. We compared characteristics of NDDs versus controls using independent t-tests, chi-squared tests, and Mann-Whitney U tests.

Ethics approval

Ethical approval was granted by the Sydney Children's Hospitals Network Human Research Ethics Committee (HREC/18/SCHN/227, 2021/ETH00356). Informed consent was obtained from all subjects.

Results

Neurodevelopmental disorders (NDD) cohort: clinical characteristics

A total of 100 sequentially reviewed children with NDDs (mean age 10.1 years 4-17), 68% males) and 58 neurotypical controls (mean age 9.5 (5-14 years), 60% males) were interviewed using the standardized assessment tool. A low socioeconomic background was disproportionately higher in the NDD group compared to controls (median decile 8 versus 10, $p<0.00001$, Supplementary Figure 2). The NDD group also had significantly higher rates of gastroesophageal reflux (27% vs 1.7%, $p = 0.000061$), constipation (33% vs 10.3%, $p=0.001$), asthma (24% vs 5.2%, $p=0.002$), eczema (16% vs 5.2%, $p=0.044$), hay fever (13% vs 3.4%, $p=0.048$), and allergies (38% vs 5.2%, $p<0.00001$), compared to controls.

Parents reported significantly more frequent childhood infections in the NDD group compared to controls in first 5 years of life (in red) and last 12 months of life (in blue) (heat map presented in Figure 1B). In the first 5 years of life, there were higher rates of throat infection ($p=0.0009$), ear infection ($p=0.0014$), mouth ulcers ($p=0.0033$), general practitioner visits ($p<0.0001$), and antibiotic courses ($p<0.0001$) in NDDs versus controls (Figure 1C). In the 12 months prior to interview, there were higher rates of mouth ulcers ($p=0.0006$) and antibiotic courses ($p<0.0001$) in NDDs versus controls (Supplementary Figure 3).

The main diagnoses in the NDD group included tic disorder/Tourette syndrome (68%), OCD (48%), ADHD (46%), ASD (35%), plus anxiety (63%), depression (9%) and learning disability (6%). As expected, using the strengths and difficulties questionnaire (SDQ), children with NDDs had more total difficulties compared to healthy controls, and higher emotional

symptoms, conduct problems, hyperactivity/inattention, and problems with peer relationships (all p<0.0001, Figure 1D and Supplementary Figure 4).

Reported environmental triggers at onset of tics/OCD were common, and occurred in 45% of the children with NDDs, including infection (26%), stress (11%) and medications (3%). Loss of skills associated with neurodevelopmental symptoms were reported in 82% of the NDD group, compared to 3.4% of controls (p<0.00001). Loss of skills in the NDD group were in the domains of learning ability (69%), fine motor skills (46%), social skills (45%), gross motor skills (37%) and language (27%) (Figure 1E). The NDD cohort were significantly impaired: 67% of children were taking one or more conventional psychiatric medication, 31% presented to emergency department due to their neuropsychiatric symptoms, and 24% had prolonged school absence (>3 months). Treatment included alpha agonist (35%), selective serotonin reuptake inhibitors (31%), neuroleptics (27%), and stimulants (27%). Immune modulators initiated or used during the time of the study included intravenous immunoglobulin (17%), antibiotics (21%), and oral corticosteroids (14%).

PANS cohort: Clinical characteristics

Out of the 100 patients with NDDs, 32 fulfilled the PANS criteria (mean age 10.4 years (4-12), 62.5% males)[13]. Children with PANS had predominant OCD (81.2%), restrictive eating (46.7%), and separation anxiety (84%). Additional symptoms included emotional lability/depression (68.8%), irritability/aggression/oppositional behaviours (53.1%), deterioration in school performance (87.5%), sensory or motor abnormalities (53.1%), tics (46.9%), somatic symptoms including sleep disturbance and enuresis (59.4%). A significant proportion of children with PANS had pre-existing ASD (38%), ADHD (28%), and learning disabilities (28%),

prior to onset of first PANS episode (patient details in Supplementary Table 3). Although all children in the PANS cohort had a convincing history of acute onset of neuropsychiatric symptoms, infection was associated with onset in 66%, and in the other 34%, the onset was associated with manifest stress or unclear reasons. The PANS cohort was significantly impaired: 63% had prolonged school absence (>3 months), 81% report a relapsing remitting clinical course, and the mean number of conventional psychiatric drugs trialed was 2.4 (0-9). A large proportion of them previously tried infection/immune therapies including antibiotics (70%), steroids (47%) and IVIg (57%). The mean duration of time from first PANS episode to time of research blood sampling was 3.7 years (0.5-10 years). Although some of the children were in the chronic phase of PANS, all of them had ongoing developmental and neuropsychiatric symptoms at time of blood sampling.

PANS versus non-PANS NDD cohort: Clinical characteristics

Children with PANS (n=32) compared to non-PANS NDDs (n=68, mean age: 10.6 years (4-18), 70.6% males) were more likely to have OCD (81% vs 37%, p=0.0001), anxiety (84% vs 57%, p=0.001), depression (69% vs 7%, p=0.00001), and more severe NDD symptoms resulting in prolonged school absence (63% vs 18%, p=0.0001) (Supplementary Table 3). There were no significant differences between childhood infection rates or loss of skills between the two groups (Supplementary Figure 3 and 4)

Cerebrospinal fluid and blood inflammatory markers in PANS

Due to the infection provoked nature and abrupt onset of neuropsychiatric symptoms in PANS, the children often presented to hospital acutely, and some required cerebrospinal fluid (CSF) examination to exclude encephalitis. Within the PANS cohort, six children (mean age: 7

343 years (4-11), 67% males) had CSF evaluation during the first or subsequent PANS relapse.
344 These children had negative antibody testing (NMAR, LGI1, CASPR2, GABA-BR, AMAP-R) in
345 serum and cerebrospinal fluid. We compared CSF cytokines and inflammatory metabolites in
346 these six children with PANS with two other control groups (1) children with non-
347 inflammatory neurogenetic disorders (n= 11, mean age: 6.3 years (0.3-16), 36.4%), and (2)
348 children with proven autoimmune encephalitis (n= 8, mean age: 9 years (3-14), 62.5% males)
349 (Supplementary Table 4). We measured CSF tumor necrosis factor (TNF), interferon (IFN)-
350 alpha, interleukin (IL)-10 and interleukin-6 as these have been shown to be the most
351 differentiating cytokines of encephalitis compared to controls[24]. In addition, we measured
352 CSF neopterin and kynurenine-tryptophan ratio which are sensitive markers of
353 neuroinflammation (described previously)[25, 26]. These CSF biomarkers were elevated in
354 encephalitis but not elevated in PANS, and the findings in PANS did not differ from the
355 neurogenetic 'negative' controls (Figure 2A). In addition, there were no quantitative cell count
356 differences in the peripheral blood white cell, neutrophil, lymphocyte, C-reactive protein, and
357 erythrocyte sedimentation rate in children with PANS compared to the controls (taken at time
358 of blood omics samples, Supplementary Figure 5).
359
360 *PANS cohort: Bulk whole blood RNA sequencing*
361 We first performed whole blood bulk RNA sequencing in 20 children with PANS (mean age:
362 10.5 years (4-16), 62.5% males) versus 15 age and gender matched controls (mean age: 10.3
363 years (4-16), 60% males) (Supplementary Table 2 for patient details). Post RUV normalization,
364 the principal component analysis of the bulk gene expression showed discrimination between
365 the children with PANS versus controls (Figure 2B). There were 6914 differentially expressed

366 genes (FDR <0.05), 3544 were upregulated (Figure 2C: in red) and 3369 were downregulated
367 (Figure 2C: in blue).
368
369 Based on GSEA analysis using a ranked gene list, the top 5 upregulated Gene Ontology (GO)
370 and Reactome pathways predominantly involved ribosomal biogenesis, translational
371 processes, and RNA methyltransferase pathways (Figure 2D: in red, Supplementary Figure 7).
372 In particular, the top three upregulated GO molecular function pathways were identified as
373 structural constituents of ribosome, rRNA binding, and RNA methyltransferase activity
374 (presented as CNET plot in Figure 3A: in red). Key genes that enriched the upregulated
375 pathways included ribosomal genes associated with small subunit (40S) or large subunit (60S)
376 of the eukaryotic and mitochondrial ribosome: *RPS, RPL, MRPS, MRPL*, as well as RNA and
377 tRNA methyltransferase genes: *METTL, TRMT* (Figure 3B).
378
379 The top 5 downregulated GSEA GO and Reactome pathways involved diverse cellular
380 functions, including mitochondrial activity, protein kinase signaling, and immune function
381 involving autophagosome organization, exocytosis, receptor mediated endocytosis,
382 phagocytic vesicle, secretory granule, and neutrophil degranulation (Figure 2D: in blue). The
383 top downregulated Reactome pathway involved neutrophil degranulation (presented as CNET
384 plot in Figure 3A: in blue). The corresponding downregulated genes included integrin genes:
385 *ITGAM, ITGAL, ITGAX, ITGB2*, toll-like receptor (TLR) genes: *TLR2, MAPK1, MAPK14, NFKB1*,
386 cytokine/chemokine genes: *CXCR1, CXCR2, CXCL1, TNFRSF1B*, complement genes: *C5AR1*, and
387 Fc-gamma receptor genes: *FCGR2A, FCGR3B* (Figure 3C).
388
389 *PANS cohort: Single-cell blood RNA sequencing*

Based on the findings from bulk RNA sequencing, we conducted a deeper investigation into cell-specific gene expression patterns through single-cell RNA sequencing. We performed single-cell RNA sequencing in two children with PANS (mean age: 8 years, 50% male, case summaries in Supplementary text), and compared them to 2 neurotypical controls (mean age: 11.5 years, 50% male).

Based on cell markers, we identified nine distinct cell types (Figure 4A). Adaptive immune cells, T cells (CD8 and CD4), and B cells constituted the largest proportion of cells across all samples (Figure 4B). Additionally, UMAP analysis of integrated biological samples revealed distinct cell clusters (Figure 4C). There were no significant differences observed in the distribution of cell clusters between individual PANS patients and controls (Supplementary Figure 8).

The most significant enriched pathways were observed in CD8 T cells and B cells, likely attributed to their large population sizes in the sequencing data, indicating their substantial contribution to the identified pathway enrichments (Figure 4D, Figure 4E, Supplementary Figure 9, 10). GSEA cellular component (Figure 4D) and reactome analysis (Figure 4E) revealed predominantly upregulated pathways related to ribosome biogenesis and translation. Upon further investigation, ribosome biogenesis and translational pathways showed upregulation in adaptive immune cells, but the direction of these pathways was more varied in innate immune cells. Specifically, ribosomal and translational pathways were upregulated in classical monocytes and dendritic cells, but showed downregulation in non-classical monocytes and natural killer cells. Conversely, predominantly downregulated pathways included cellular pathways including mitochondrial, respiratory electron transport (Figure 4D and Figure 4E).

Immune pathways including IFN-gamma signaling and adaptive immune system were predominantly downregulated in adaptive immune cells but upregulated in innate immune cells. Additionally, immune pathways including endocytic vesicle, phagocytic vesicle and secretory granule which were downregulated in bulk RNA sequencing, were upregulated in CD4 T cells and innate immune cells including classical monocytes, non-classical monocytes, and natural killer cells (Supplementary Figure 9, 10).

PANS cohort: Toll like-receptor immune assay

Given the dysregulation of immune pathways uncovered by both bulk and single-cell RNA sequencing analyses, we aimed to determine the functional immune status of PBMCs in PANS. We assessed cytokine responsiveness upon stimulation of a critical innate immune pathway, the TLR pathway. TLR proteins expressed on immune cells recognize pathogen-associated molecular patterns like lipopolysaccharide (LPS), triggering a signaling cascade that produces pro-inflammatory cytokines and chemokines (Figure 5A). We measured the gene expression and cytokine production of LPS-stimulated PBMCs of 7 children with PANS (mean age 10.6 years (range 5-15), 57.1% male) versus 7 neurotypical controls (mean age 11.9 years (range 8-15), 57.1% male) (Fig. 5B).

In PBMCs stimulated with LPS, *IL6* expression was reduced at both 30 minutes ($p < 0.05$) and 3 hours ($p < 0.05$) in PANS compared to controls, and IL6 and TNF (protein) was reduced at 3 hours ($p < 0.01$) in PANS compared to controls (Figure 5C). This indicated a reduced responsiveness to LPS stimulation in PBMCs of PANS compared to controls.

PANS IVIg cohort: Clinical response to IVIg treatment

Within the PANS cohort, we observed a clinical pattern of response to IVIg in 9 children who received open-label 4 weekly IVIg. Although the therapeutic response to IVIg varied, the effect commenced early (often a few days after infusion), but only lasted for around two to three weeks, and the beneficial effect often waned before the next infusion (Figure 6A). Most of our patients required chronic monthly use of IVIg due to the ongoing nature of their symptoms. For example, in one patient, we observed gradual improvement of OCD symptoms with monthly IVIg treatment over 6 months, however OCD symptoms appeared to worsen after stopping IVIg, requiring re-commencement (Figure 6B).

PANS IVIg cohort: Bulk whole blood RNA sequencing

Given the abnormalities found in gene translation and cellular function at baseline in PANS, we next explored the mechanistic action of IVIg in PANS. We performed bulk RNA sequencing in 9 children with PANS (mean age: 10.1 years (7-15), 55.6% males) before and after receiving IVIg treatment compared to 10 healthy controls (mean age: 10.8 years (8-17), 50% males). Following RUV normalization, principal component analysis (PCA) of the bulk RNA sequencing data revealed robust discrimination between children with PANS at baseline and controls. Additionally, PANS patients post-IVIg treatment appeared to exhibit a closer proximity to the control group, suggesting a potential therapeutic effect of IVIg treatment on RNA sequencing profiles in PANS patients (Supplementary Figure 11). There were 541 differentially expressed genes (FDR <0.05) in PANS at baseline versus controls, and 4200 differentially expressed genes (FDR <0.05) in PANS post IVIg treatment compared to pre IVIg treatment.

We compared the normalized enrichment scores (NES) for the top 50 GSEA GO pathways present in: (1) PANS pre IVIg versus control (left column), and (2) PANS post IVIg versus PANS

pre IVIg (right column) (Figure 6C). We were able to reproduce our baseline RNA sequencing findings in PANS versus controls in this second (replication) PANS cohort. In PANS pre IVIG versus control, the most upregulated pathways involved ribosomal function and translation, whereas the most downregulated genes involved immune function, endocytosis and cell signalling (Figure 6C). The pathways that were upregulated in PANS pre IVIg treatment were downregulated post IVIg treatment (ribosomal biogenesis, translational, post translational protein modifications). Pathways that were downregulated in the PANS pre IVIg treatment were upregulated post IVIg treatment (immune, epigenetic, and cellular function) (Figure 6C, Figure 6D, Supplementary Figure 12).

Discussion

The prevailing paradigm regarding the pathogenesis of NDDs recognizes a complex interplay between genetic, epigenetic, and environmental factors. Environmental influences at critical windows of vulnerability have been shown to affect fetal brain development in animal models[27-29]. Proposed mechanisms of early-life programming in neurodevelopment involve epigenetic 'priming' of microglial and peripheral immune cells, resulting in systemic immune dysregulation[30-32]. A 'two-hit' model explains how the prenatal disruptions in brain development increases offspring susceptibility to recurrent infections and abnormal neurodevelopmental trajectories[27, 29]. While prenatal first 'hits', such as maternal inflammation during pregnancy, have been firmly linked to NDD risk, the connection between postnatal 'hits' and NDD symptoms in humans remains less defined[27].

Brain development is a highly dynamic process, involving a fine orchestration of neurological functions. Within this context, episodic "regressions" in developmental skills have been

485 previously described and diagnosed as autistic regression or PANS[11, 13, 14]. In our cohort,
486 children with NDDs unsurprisingly exhibited a high degree of emotional and behavioural
487 issues. Notably, we also observed a high frequency of loss of developmental skills, often
488 triggered by infection or stress, in children with NDD compared to controls. This observation
489 suggests that loss of developmental skills may be a common occurrence in NDDs, and not
490 exclusive to autistic regression or PANS. We observed increased reported childhood
491 infections and allergic conditions in children with NDDs compared to controls, consistent with
492 previous studies[9, 33-35]. The underlying mechanisms behind the association between recurrent
493 infections and neurodevelopmental or psychiatric manifestations is poorly understood. To
494 better delineate disease pathways underlying this association, we focussed our investigation
495 on children fulfilling PANS criteria. Our hypothesis was that PANS is not an autoimmune
496 encephalitis, but instead a gene regulatory disorder associated with peripheral and brain
497 immune dysregulation.

498 We showed that CSF examination of children with PANS is typically normal, lacking evidence
499 of encephalitis. In addition, routine immune testing in PANS revealed normal quantitative
500 immune cells in peripheral blood, and no evidence of systemic inflammation. In a clinical
501 cohort, while brain cell RNA sequencing is not possible, peripheral blood immune analysis is
502 feasible. In this study, children experienced recurrent infections and exhibited abnormal
503 immune responses, making peripheral blood immune cell transcriptomics highly relevant in
504 understanding their condition. Key findings from our blood RNA-sequencing analyses of PANS
505 patients included upregulation of translational pathways enriched with ribosomal proteins,
506 and downregulated pathways of broad cellular function and immune pathways. The
507 translation of information encoded by mRNA into functional proteins occurs in ribosomes,

508 which plays critical roles in brain development[36]. There are established associations between
509 disruption of translational machinery and NDDs. Firstly, monogenic mutations affecting
510 components of the translational process, such as ribosomal proteins or translational factors
511 can cause NDDs[37-39]. Secondly, environmental exposures like infections and stressors can
512 disrupt translational processes by impacting ribosomal biogenesis[40]. Ribosomal biogenesis is
513 critical for maintaining homeostasis during stress such as proteostasis imbalance, nutrient
514 deprivation, and oxidative stress[40]. Perturbations to ribosomal biogenesis can lead to
515 reduction of protein synthesis crucial for dendritic and synaptic function, contributing to
516 changes in neuronal connectivity associated with NDDs[39]. In our study, we observed a large
517 number of upregulated *RPL* and *RPS* genes coding for 40S and 60S ribosomal subunits in PANS
518 versus controls, also seen in in blood leukocytes and post-mortem cortical tissue of individuals
519 with ASD[41]. Consistent findings across these studies supports the notion that ribosomal
520 protein dysregulation in both brain and peripheral blood is a central feature in the
521 pathophysiology of NDDs in general, and not specific to PANS [41].

522 We hypothesize that early life environmental factors alter the epigenetic regulation of
523 immune cells in NDDs, resulting in disturbance to the translational processes via ribosomal
524 biogenesis. We found epigenetic pathways including RNA methyltransferase and chromatin
525 organization to be dysregulated in children with PANS. Recent studies highlight the
526 environmental impact on epigenetics and epitranscriptomics, which play pivotal roles in
527 regulating translational processes[42, 43]. Histone methyltransferases (*KMT)*, histone
528 demethylase (*KDM)*, and RNA methyltransferases (*METTL*), play crucial roles in regulating
529 ribosomal gene transcription[44-47]. Modifications to histone lysine methylation have been
530 shown to modulate ribosomal expression via post translational modifications, aiding cell

adaptation to stressful environmental conditions[48]. In addition, ribosomal proteins also have non-ribosomal functions, such as immune functions[49]. Ribosomal stress results in the release and accumulation of ribosome-free ribosomal proteins that are involved in immune signaling pathways, which may also be contributing to downstream immune dysregulation in PANS[49]. However, other interactions between immune, translational, and epigenetic processes are also plausible. Immune cells are highly adaptable to environmental stimuli, such as infections, and their responses involve complex interactions with epigenetic mechanisms and ribosomal biogenesis. For example, T cell activation, marked by the release of effector molecules, can initiate reprogramming of cellular metabolism, typically accompanied by increased ribosomal biogenesis and enhanced activity of RNA cap methyltransferases[50, 51]. Thus, it is possible that our RNA sequencing data reflects activation of certain immune cell populations, in early life or in response to recent environmental stimuli. Nevertheless, the precise interactions between epigenetic, translational, and immune mechanisms warrants further investigations in the context of PANS.

This study observed a diverse downregulation of cellular functions using RNA sequencing in PANS, encompassing mitochondrial activity, receptor-mediated endocytosis, exocytosis, and immune pathways. These findings are consistent with RNA sequencing studies of blood and brain samples of other NDDs[52, 53]. Specifically, immune factors are increasingly recognised to operate at the gene-environment interface during NDD pathogenesis. Integrated brain transcriptome and epigenetic analyses of individuals with NDD demonstrate convergent dysregulated immune pathways[54, 55]. Abnormal peripheral immune responses, including abnormal cytokine production, immunoglobulin levels, and altered cellular response to stimuli, have also been reported in individuals with NDDs [56]. We identified a downregulated

554 immune signal in blood RNA sequencing, implicating various innate immune pathways,
555 enriched in genes related to TLR, integrin, complement, cytokine, and chemokine signaling.
556 The cytokine hypo-responsiveness to stimulation via LPS in the children with PANS in our
557 study provides additional evidence of innate immune dysfunction in PANS. While this study
558 primarily highlights innate immune dysregulation, our single-cell RNA sequencing also reveals
559 immune dysregulation in adaptive immune cells, such as CD8+ T cells and B cells. Although
560 TLR is primarily associated with innate immune responses, TLR also has direct and indirect
561 effects of T cells function[57]. Given the fact that we used a PBMC fraction, further studies
562 should explore differential TLR response in individual cell types, such as monocytes and
563 lymphocytes. Further immunological testing on both innate and adaptive immune cells is
564 needed to determine whether this dysregulation is global or restricted to specific cell types.
565 We propose that children with PANS have global suppression of immune function, with
566 downstream deficits in cell debris clearance, and potential secondary inflammation. This
567 finding correlates with the recurrent infections in children with NDDs that we and others have
568 observed[10, 58].

569 Early-life exposures to environmental factors, can lead to 'epigenetic' programming,
570 impacting immune cells both peripherally and in the brain[59, 60]. There is a dynamic cross-talk
571 between microglia and other myeloid-derived cells[61]. While our study has revealed peripheral
572 blood immune dysregulation in PANS, it is plausible that microglia also have impaired function
573 in individuals with PANS. Microglia are crucial for immune surveillance, regulating neuronal
574 functions, and influencing synaptic connections through synaptic pruning[6, 62]. Disruption to
575 microglia-mediated synaptic pruning is a key process in NDDs and psychiatric disorders [62].
576 Previous positron emission tomography imaging studies of individuals with PANS revealed

reactive microglial states[63]. Animal models of maternal immune activation show perturbations in offspring microglia function, including impaired phagocytosis, resulting in aberrant proinflammatory states, contributing to neurobehavioral abnormalities[64]. We propose that early life environmentally-driven epigenetic changes to the neuroimmune axis can result in neuroimmune dysfunction, contributing to neurodevelopmental and psychiatric disorders [61, 65].

The use of immunotherapy in PANS, particularly IVIg is reported to be clinically beneficial, but its effectiveness is controversial and its immunomodulatory effects remain unknown [16, 17, 66]. IVIg has multiple mechanisms of action in inflammatory diseases, including modulation of expression of Fc receptors, interference with complement activation and cytokines, and regulation of immune cell activation[67]. Our study is the first to provide insights on the biological effects of IVIg in PANS. Firstly, we found that IVIg modified dysregulated pathways involving both innate and adaptive immunity present at baseline in PANS, demonstrating the broad immunomodulatory effects of IVIg in PANS. Secondly, IVIg appeared to modify other key dysregulated pathways in PANS related to ribosomal biogenesis, translational, and post-translational protein modifications, as well as epigenetic pathways. Although studies on the effects of IVIg on the translational program are limited, other mechanisms affecting gene regulation, via methylation and microRNA alterations, have been reported in Kawasaki disease and immunodeficiency[68, 69]. Thirdly, we found upregulated pathways in inositol phosphate phosphatase activity post IVIg treatment in PANS. The effects of IVIg on the Fc gamma receptor via inositol phosphate phosphatase pathways have also been demonstrated in immune thrombocytopenia purpura [70]. It should be emphasized that although we describe the benefit of IVIg in some individuals with PANS and demonstrate potential biological effects

of IVIg on gene regulation, this does not provide proof of effect. Robust randomised-controlled clinical trials with significant clinical benefits are needed to provide evidence for IVIg in PANS. Further investigations into the impacts of IVIg on peripheral-brain immune crosstalk, along with identifying gene signatures to predict individual treatment responsiveness to IVIg are crucial for personalizing treatment options for individuals with PANS. Additionally, correlating clinical responsiveness to IVIg with biological changes in gene expression, will help to delineate key pathways critical for alleviating PANS symptoms. In vitro cell-based studies are needed to delineate specific mechanisms of IVIg in PANS, as opposed to general actions of IVIg on blood cells. Additionally, exploring oral treatments with sustained effects is important considering the short-lived clinical response to IVIg.

In this study, we demonstrate a common gene regulatory signature in PANS, replicated across two cohorts using bulk RNA sequencing and further confirmed in two patients through single-cell RNA sequencing. We propose that this signature arises from the interaction between environmental factors and genetic vulnerability, mediated by the epigenetic machinery. Nonetheless, we acknowledge the possible influence of DNA variants on this signature. Limitations of our study include the retrospective nature of the childhood infection and loss of skills history, which may be susceptible to recall bias and overreporting among patients enrolled from a specialized clinic. Additionally, controlling for complex human variables and potential confounders was challenging. We were unable to control for socioeconomic status, which is an important confounder as lower socioeconomic status seen in our NDD cohort could be associated with higher rates of childhood infections and other allergic diseases. Second, our omics analysis involved a small sample size with considerable heterogeneity in symptomology and phase of disease. Although some of the children with PANS were in the

623 chronic phase of disease, all of them had ongoing debilitating neuropsychiatric symptoms,
624 requiring psychotherapy and psychiatric medications. Longitudinal RNA sequencing at
625 different time points in PANS (acute and chronic phase) will help us better understand the
626 stability of the gene expression signature. Additionally, while there may be persistent gene
627 expression changes in people with chronic infections, we ensured that the children with PANS
628 did not have significant acute or uncontrolled chronic infections 4 weeks before blood taking.
629 Gaining appropriate controls was challenging, half of our controls were being investigated for
630 growth or puberty delay. Thirdly, patients were on conventional psychiatric medications
631 which could potentially affect gene expression; however subgroup analysis of PANS patients
632 did not reveal significant differences in gene expression between patients receiving
633 psychiatric medications versus those who were not (data not shown). Fourth, it is important
634 to note that bulk RNA sequencing of whole blood includes all leukocyte populations, whereas
635 10X Genomics single-cell RNA sequencing is based on PBMCs (excluding neutrophils). Fifth,
636 our current study is based on peripheral blood and not brain, as brain is not a feasible tissue
637 to investigate in humans. However, we hypothesize that peripheral blood cells are
638 representative of brain cells, given the fact that putative environmental drivers of immune
639 signature have effects across organs with different cellular origins. We propose that RNA
640 sequencing signatures in peripheral blood will transpire to be a valuable diagnostic tool in
641 NDDs[71]. Further studies to examine blood and brain RNA signatures in animal models are
642 needed to further explore the blood-brain correlation. Larger prospective cohort studies are
643 needed, combining environmental exposures, omics analyses (DNA, RNA and epigenetic
644 modifications) with detailed immune function testing, to unpick gene-environment
645 interactions in PANS compared to the broader NDD spectrum. In our PANS cohort, a
646 significant proportion of children had pre-existing NDDs. Although PANS commonly occurs in

previously healthy children, emerging clinical evidence indicates that the constellation of PANS symptoms can also manifest in children with pre-existing NDDs, with an overlapping genetic background observed between PANS and NDDs[3]. Future studies including subgroups of patients with PANS and NDDs will be critical to determine if PANS is a subgroup with a unique biological mechanism, or if children with PANS represent the more severe end of NDD spectrum with similar genetic, environmental and epigenetic underpinnings. These efforts aim to advance precision medicine in diagnostics, prognostication, and therapeutics for NDDs.

In conclusion, we propose that the pathogenesis of PANS involves epigenetic dysregulation which results in downstream immune dysregulation [52]. We propose that PANS is not a discrete 'autoimmune' entity but represents an important clinical phenotype of NDDs centred around epigenetic neuroimmunology. Epigenetic and immune modulating therapies require further investigation in PANS and other NDDs [72, 73].

Data availability

All relevant data are included in the manuscript or supplemental material. Bulk RNA sequencing and single-cell RNA sequencing data has been uploaded to Gene Expression Omnibus (GEO). Raw data files and R code are available to be shared upon request to the corresponding author russell.dale@sydney.edu.au

Acknowledgements

We are grateful for the study participants who contributed to this research. We would like to thank the doctors and nurses in the Department of Paediatric Endocrinology at The Children's Hospital at Westmead, Sydney for their help and support to recruit controls. We would like

to thank Children's Medical Research Institute and Australian Genome Research Facility for providing expertise in sequencing and bioinformatic support. We would like to thank PANS Australia, Stepping Stones Therapy for Children, and Dr Leila Masson for their kind donations for this study.

Author contributions

VH, SA, SP and RD designed the research studies. VH, BK, SY, HN, HJ, IP, SM, RD recruited the patients and performed clinical assessments. VH, SA, BK, MG, EM, JY, SB, KK, ET, PL, RD conducted experiments and acquired and analyzed the data. MH, MG, FB, WG, SP and RD oversaw the experiments and data analysis. VH, SA, BG, NA, ML, EM performed computational analyses. VH and SA drafted the manuscript and shared responsibilities as co-first authors. The order of the co-first author's names was determined by workload. SP and RD critically revised the manuscript and shared responsibilities as senior authors.

Funding

Financial support for the study was granted by the Dale NHMRC Investigator Grant AP1193648, Petre Foundation, Cerebral Palsy Alliance, PANS Australia, PANDAS Network and International OCD Foundation. VXH was supported by ExxonMobil NUS Research Scholarship and National University Hospital Singapore (NUHS) Clinician-Scientist Program during the course of this study.

Figures

Figure 1 Clinical data of 100 children with neurodevelopmental disorders (NDD) versus 58 healthy controls

(A) The study workflow involved clinical interview of children with NDDs and controls. Bulk blood RNA sequencing, single-cell blood RNA sequencing, and Toll-like receptor stimulation assay were performed for children with Paediatric acute-onset neuropsychiatric syndrome (PANS). Children with PANS receiving intravenous immunoglobulin (IVIg) treatment were categorized as the PANS-IVIg cohort. Bulk blood RNA sequencing was performed before and after IVIg treatment. Image generated with *Biorender.*

(B) Heatmap of reported childhood infections of 100 children with NDDs (top half) versus 58 healthy controls (bottom half). Responses to infection screening tool in the first 5 years of life (in red) and 12 months prior to interview (in blue). On the x axis, numbers 0 to 14 represent the 14 questions in the infection screening tool (Supplementary Figure 1). The intensity of the colour represents the frequency based on the 5-point Likert scale. There is increased intensity in the heatmap in the children with NDDs versus controls reflecting higher frequency of reported infections in those with NDDs, particularly in the first 5 years of life.

(C) Bar charts of frequency of reported infection in the first 5 years of life in children with NDDs versus healthy controls. The frequency is based on a 5-point Likert scale including - never, occasional (less than once per year), sometimes (1-3 times per year), often (4-6 times per year) and almost always (almost always). In the first 5 years of life, there were significantly higher rates of throat infection, ear infection, mouth ulcers, general practitioner visits for infection, antibiotic courses and emergency visits for infection in the NDD group compared to controls.

(D) Dot plots showing the total difficulties and impact score of the Strengths Difficulties Questionnaire (SDQ). Children with NDDs compared to healthy controls have a higher average total difficulties and impact score ($p<0.0001$).

(E) Bar plot showing the percentage of children with NDDs (in yellow) and controls (in blue) with reported loss of skills. A significantly higher proportion of children with NDDs compared to healthy controls report loss of skills at any time in their childhood, including learning ability, social skills, fine and gross motor skills.

Figure 2 Bulk blood RNA sequencing of Paediatric acute-onset neuropsychiatric syndrome patients (PANS) versus controls

(A) Cerebrospinal fluid cytokine levels (TNF, IL10, IL6, IFN alpha), neopterin, and kynurenine/tryptophan (KYN/TRP) levels in children with PANS (n=6) did not significantly differ from controls with non-inflammatory neurogenetic conditions (n=11). On the other hand, children with anti-NMDA receptor encephalitis (n=8) had markedly elevated cytokine levels, neopterin and KYN/TRP compared to controls with neurogenetic conditions and PANS. The CSF findings in PANS were not in keeping with autoimmune encephalitis.

(B) Principal component analysis (PCA) performed on bulk RNA sequencing performed in children with PANS and controls. The x-axis represents Principal Component 1 (PC1), while the y-axis represents Principal Component 2 (PC2). Unbiased hierarchical clustering of gene expression between children with PANS and controls showed separation of data indicating strong group discrimination post RUV.

(C) Volcano plot with annotation of 6914 differentially expressed genes (3544 upregulated genes in red and 3369 downregulated genes in blue) (FDR<0.05).

(D) Bar plot of Gene Set Enrichment Analysis (GSEA) Gene Ontology (GO) Biological Process (BP), Molecular Function (MF), Cellular Component (CC) and Reactome pathways.

The top 5 upregulated GSEA GO and Reactome pathways were predominantly ribosomal biogenesis, translational processes, as well as RNA methyltransferase pathways.

The top 5 downregulated GSEA GO and Reactome pathways involved broad diverse cellular functions, namely in mitochondrial activity, protein kinase signaling, and immune function including autophagosome organization, exocytosis, receptor mediated endocytosis, phagocytic vesicle, secretory granule, and neutrophil degranulation.

Figure 3 Connectivity network plots of top upregulated and downregulated bulk RNA sequencing in Paediatric acute neuropsychiatric syndrome patients, compared to controls
(A) Top panel:
Connectivity network plot of the top 3 up-regulated enriched Gene Set Enrichment Analysis (GSEA) Gene Ontology molecular function (GO MF) pathways: structural constituent of ribosome, rRNA binding and RNA methyltransferase (in red). The enriched pathways are represented by their respective colors, and corresponding genes' adjusted p value. Upregulated genes that enrich the pathways included ribosomal genes associated with small subunit (40S) or large subunit (60S) of the eukaryotic and mitochondrial ribosome: *RPS, RPL, MRPS, MRPL*, as well as RNA and tRNA methyltransferase genes: *METTL, TRMT*.
Lower panel:
Connectivity network (CNET) plot of the top down-regulated enriched GSEA Reactome pathway: neutrophil degranulation (in blue). The enriched pathway is represented by the respective colors, and corresponding genes' adjusted p value. Downregulated genes that enrich the pathway included integrin genes: *ITGAM, ITGAL, ITGAX, ITGB2*, TLR genes: *TLR2, MAPK1, MAPK14, NFKB1*, cytokine/chemokine genes: *CXCR1, CXCR2, CXCL1, TNFRSF1B*, complement genes: *C5AR1*, and Fc-gamma receptor genes: *FCGR2A, FCGR3B*.
(B) The y-axis depicts the log counts per million after the removal of unwanted variation (RUV) normalization, illustrating the upregulation of gene expression for representative genes *RPS19* and *METTL3* in PANS compared to controls.
(C) The y-axis depicts the log counts per million after the removal of unwanted variation (RUV) normalization, illustrating the downregulation of gene expression for representative genes *ITGAM*, *MAPK1* and *NFKB1* in PANS compared to controls.

Figure 4 Single- cell blood RNA sequencing of Paediatric acute neuropsychiatric syndrome patients versus controls

(A) Heatmap of top differentially expressed genes based on cell type. Genes are the top markers of differential analysis between cell types (FDR <0.05) ranked by difference in proportion of cells expressing the respective gene.

(**B)** Bar chart of cell population composition of the two controls and two PANS patient samples

(C) Uniform manifold approximation and projection (UMAP) projection of all 4 samples, including 2 children with PANS and 2 control samples in single cell transcriptomics identified 9 unique clusters: NK cells, CD8 T cell, B cell, CD4 T cell, classical monocytes (cMono), non-classical monocytes (ncMono), plasmacytoid dendritic cell (pDC), classical dendritic cell (cDC).

(D) Dot plot visualizing the top 30 GSEA GO cellular component pathways for CD8T cells. The dot's colour represents the normalized enrichment score (NES), with red indicating upregulation, blue indicating downregulation, and zero represented by white. The size of each dot corresponds to the -log10(padj value) of the pathway. The NES scores of these pathways were mapped across all other cell types, demonstrating heterogeneity in cell type pathway enrichment. In terms of ribosome biogenesis pathways, it was found that adaptive immune cells exhibited upregulation, whereas the direction of these pathways was more varied in innate immune cells. In addition, cellular function, including mitochondrial activity, were downregulated across all cell types.

(E) Dot plot visualizing the top 30 Reactome pathways for CD8T cells. The NES scores of these pathways were mapped across all other cell types, demonstrating heterogeneity in cell type pathway enrichment. In terms of translational pathways, it was found that adaptive immune cells exhibited upregulation, whereas the direction of these pathways was more varied in innate immune cells. Immune pathways were predominantly downregulated in adaptive immune cells and upregulated in innate immune cells.

Figure 5 Toll-like receptor stimulation analysis in Paediatric acute neuropsychiatric syndrome patients compared to controls

(A) The Toll-like receptor (TLR) pathway is a crucial component of the innate immune system responsible for detecting and responding to various pathogens. TLR proteins expressed on immune cells recognize pathogen-associated molecular patterns like lipopolysaccharide (LPS), triggering a signaling cascade that produces pro-inflammatory cytokines, such as interleukin (IL)6 and tumour necrosis factor (TNF). TLR4= Toll-like receptor 4, TIRAP= Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein, TRIF= TIR-domain-containing adaptor-inducing interferon-beta, TRAM= TRIF-related adaptor molecule, MyD88= myeloid differentiation primary response protein 88, IRAK= interleukin-1 receptor associated kinase, NF-κB (nuclear factor kappa light chain enhancer of activated B cells)

(B) Gene expression and cytokine production of LPS stimulated peripheral blood mononuclear cells (PBMCs) of PANS patients compared to controls. PBMCs of PANS and controls were stimulated with 500ng/mL LPS for 30 minutes, 3 hours, and 24 hours. Gene expression of IL6, and TNF were measured using quantitative reverse transcription polymerase chain reaction. Target gene expression was normalized to household gene, Beta-2-Microglobulin. In addition, release of IL6 and TNF into the cell culture media was measured using ELISA.

(C) In PBMCs stimulated with LPS, IL6 gene (RNA) expression was lower at both 30 minutes ($p < 0.05$) and 3 hours ($p < 0.05$) in PANS compared to controls, and IL6 and TNF protein production (ELISA) was significantly reduced at 3 hours ($p < 0.01$) in PANS compared to controls (Figure 5C). Bars and whiskers represent median with interquartile range; n = 7. Mann-Whitney test, *P < 0·05, **P < 0·01.

Figure 6 Bulk blood RNA sequencing of children with Paediatric acute-onset neuropsychiatric syndrome (PANS) before and after intravenous immunoglobulin (IVIg) treatment, compared to controls

(A) Clinical response of three children with PANS who received IVIg was prospectively assessed using the Clinical Global Impression Scale- Severity (CGI-S) to document the therapeutic response to IVIg . We charted their daily CGI-S scores over 4-8 four-week cycles (different colour represents a separate 4-week cycle). CGI-S followed a seven-point scale where 1 is normal and 7 is extremely ill. The clinical benefit of IVIg is typically seen 1-7 days after infusion and lasts for 2-3 weeks, and then the clinical benefit wanes.

(B) Clinical response (OCD symptoms) of a child with PANS who received monthly IVIg treatment over 6 months using the Children's Yale-Brown Obsessive Compulsive Scale (CY-BOCS) score. CY-BOCS score has a total severity score of 0-50 to chart the progress of OCD symptoms. IVIg infusions are marked with X. We observed gradual improvement in OCD symptoms this child over 6 months. However, OCD symptoms were observed to relapse after stopping IVIg, requiring re-commencement of IVIg.

(C) We compared the normalized enrichment scores (NES) for the top 50 Gene Set Enrichment Analysis (GSEA) Gene Ontology (GO) pathways present in: (1) PANS pre IVIg versus control (left column), and (2) PANS post IVIg versus PANS pre IVIg (right column). Pathways upregulated (red) in PANS pre-IVIg versus controls including ribosomal biogenesis, translational, post-translational protein modifications, and immune pathways, were downregulated (blue) post-IVIg treatment. Pathways that were downregulated in the PANS pre-IVIG versus controls including immune pathways, epigenetics, and broad cellular function, showed upregulation after IVIg treatment.

*Regulation of transcription of nucleolar large rRNA by RNA polymerase I

**Nuclear transcribed mRNA catabolic process deadenylation-dependent decay

(D) The expression of representative genes *RPS19, RPL41*, and *METTL3* was upregulated in PANS Pre IVIg compared to controls. After IVIg treatment, the gene expression of these genes was decreased, comparable to levels in controls. The log counts per million post-removal of unwanted variation (RUV) normalization are plotted on the y-axis.

Supplementary material

Supplementary Text

Supplementary Table

Supplementary Figure

References

1. Zablotsky B, Black LI, Maenner MJ, Schieve LA, Danielson ML, Bitsko RH *et al.* Prevalence and Trends of Developmental Disabilities among Children in the United States: 2009-2017. *Pediatrics* 2019; **144**(4).

2. 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* 2018; **360**(6395).

3. Trifiletti R, Lachman HM, Manusama O, Zheng D, Spalice A, Chiurazzi P *et al.* Identification of ultra-rare genetic variants in pediatric acute onset neuropsychiatric syndrome (PANS) by exome and whole genome sequencing. *Scientific Reports* 2022; **12**(1)**:** 11106.

4. Bale TL, Baram TZ, Brown AS, Goldstein JM, Insel TR, McCarthy MM *et al.* Early life programming and neurodevelopmental disorders. *Biol Psychiatry* 2010; **68**(4)**:** 314-319.

5. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P *et al.* Synaptic pruning by microglia is necessary for normal brain development. *Science* 2011; **333**(6048)**:** 1456-1458.

6. Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R *et al.* Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron* 2012; **74**(4)**:** 691-705.

7. Weber-Stadlbauer U, Richetto J, Zwamborn RAJ, Slieker RC, Meyer U. Transgenerational modification of dopaminergic dysfunctions induced by maternal immune activation. *Neuropsychopharmacology* 2021; **46**(2)**:** 404-412.

8. Zahir FR, Brown CJ. Epigenetic Impacts on Neurodevelopment: Pathophysiological Mechanisms and Genetic Modes of Action. *Pediatric Research* 2011; **69**(8)**:** 92-100.

9. Lin J-N, Lin C-L, Yen H-R, Yang C-H, Lai C-H, Lin H-H *et al.* Increased Risk of Tics in Children Infected with Enterovirus: A Nationwide Population-Based Study. *Journal of Developmental & Behavioral Pediatrics* 2017; **38**(4)**:** 276-282.

10. Karlsson H, Sjöqvist H, Brynge M, Gardner R, Dalman C. Childhood infections and autism spectrum disorders and/or intellectual disability: a register-based cohort study. *Journal of Neurodevelopmental Disorders* 2022; **14**(1)**:** 12.

11. Jyonouchi H, Geng L, Davidow AL. Cytokine profiles by peripheral blood monocytes are associated with changes in behavioral symptoms following immune insults in a subset of ASD subjects: an inflammatory subtype? *J Neuroinflammation* 2014; **11:** 187.

12. Merzon E, Israel A, Ashkenazi S, Rotem A, Schneider T, Faraone SV *et al.* Attention-Deficit/Hyperactivity Disorder Is Associated With Increased Rates of Childhood Infectious Diseases: A Population-Based Case-Control Study. *J Am Acad Child Adolesc Psychiatry* 2023; **62**(2)**:** 253-260.e251.

13. Chang K, Frankovich J, Cooperstock M, Cunningham MW, Latimer ME, Murphy TK *et al.* Clinical evaluation of youth with pediatric acute-onset neuropsychiatric syndrome (PANS): recommendations from the 2013 PANS Consensus Conference. *J Child Adolesc Psychopharmacol* 2015; **25**(1)**:** 3-13.

14. Swedo SE, Leckman JF, Rose NR. From research subgroup to clinical syndrome: modifying the PANDAS criteria to describe PANS (pediatric acute-onset neuropsychiatric syndrome). *Pediatr Therapeut* 2012; **2**(2)**:** 113.

15. Jones HF, Han VX, Patel S, Gloss BS, Soler N, Ho A *et al.* Maternal autoimmunity and inflammation are associated with childhood tics and obsessive-compulsive disorder: Transcriptomic data show common enriched innate immune pathways. *Brain Behav Immun* 2021; **94:** 308-317.

16. Williams KA, Swedo SE, Farmer CA, Grantz H, Grant PJ, D'Souza P *et al.* Randomized, Controlled Trial of Intravenous Immunoglobulin for Pediatric Autoimmune Neuropsychiatric Disorders Associated With Streptococcal Infections. *J Am Acad Child Adolesc Psychiatry* 2016; **55**(10)**:** 860-867.e862.

17. Perlmutter SJ, Leitman SF, Garvey MA, Hamburger S, Feldman E, Leonard HL *et al.* Therapeutic plasma exchange and intravenous immunoglobulin for obsessive-compulsive disorder and tic disorders in childhood. *Lancet* 1999; **354**(9185)**:** 1153-1158.

18. Goodman R. The Strengths and Difficulties Questionnaire: a research note. *J Child Psychol Psychiatry* 1997; **38**(5)**:** 581-586.

19. Regier DA, Kuhl EA, Kupfer DJ. The DSM-5: Classification and criteria changes. *World Psychiatry* 2013; **12**(2)**:** 92-98.

20. Busner J, Targum SD. The clinical global impressions scale: applying a research tool in clinical practice. *Psychiatry (Edgmont)* 2007; **4**(7)**:** 28-37.

21. R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/. 2013.

22. Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R *et al.* Welcome to the Tidyverse. *Journal of Open Source Software* 2019; **4:** 1686.

23. Hao Y, Hao S, Andersen-Nissen E, Mauck WM, 3rd, Zheng S, Butler A *et al.* Integrated analysis of multimodal single-cell data. *Cell* 2021; **184**(13)**:** 3573-3587.e3529.

24. Kothur K, Wienholt L, Mohammad SS, Tantsis EM, Pillai S, Britton PN *et al.* Utility of CSF Cytokine/Chemokines as Markers of Active Intrathecal Inflammation: Comparison of Demyelinating, Anti-NMDAR and Enteroviral Encephalitis. *PLoS One* 2016; **11**(8)**:** e0161656.

25. Yan J, Han V, Heng B, Guillemin G, Bandodkar S, Dale R. Development of a translational inflammation panel for the quantification of cerebrospinal fluid Pterin, Tryptophan-Kynurenine and Nitric oxide pathway metabolites. *eBioMedicine* 2022; **77:** 103917.

26. Yan J, Kothur K, Mohammad S, Chung J, Patel S, Jones HF *et al.* CSF neopterin, quinolinic acid and kynurenine/tryptophan ratio are biomarkers of active neuroinflammation. *eBioMedicine* 2023; **91**.

27. Han VX, Patel S, Jones HF, Dale RC. Maternal immune activation and neuroinflammation in human neurodevelopmental disorders. *Nature Reviews Neurology* 2021; **17**(9)**:** 564-579.

28. Bucknor MC, Gururajan A, Dale RC, Hofer MJ. A comprehensive approach to modeling maternal immune activation in rodents. *Frontiers in Neuroscience* 2022; **16**.

29. 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.

30. Bordeleau M, Lacabanne C, Fernández de Cossío L, Vernoux N, Savage JC, González-Ibáñez F *et al.* Microglial and peripheral immune priming is partially sexually dimorphic in adolescent mouse offspring exposed to maternal high-fat diet. *Journal of Neuroinflammation* 2020; **17**(1)**:** 264.

31. Lin CW, Septyaningtrias DE, Chao HW, Konda M, Atarashi K, Takeshita K *et al.* A common epigenetic mechanism across different cellular origins underlies systemic immune dysregulation in an idiopathic autism mouse model. *Mol Psychiatry* 2022; **27**(8)**:** 3343-3354.

32. Hayes LN, An K, Carloni E, Li F, Vincent E, Trippaers C *et al.* Prenatal immune stress blunts microglia reactivity, impairing neurocircuitry. *Nature* 2022; **610**(7931)**:** 327-334.

33. Zhang T, Brander G, Isung J, Isomura K, Sidorchuk A, Larsson H *et al.* Prenatal and Early Childhood Infections and Subsequent Risk of Obsessive-Compulsive Disorder and Tic Disorders: A Nationwide, Sibling-Controlled Study. *Biol Psychiatry* 2023; **93**(11)**:** 1023-1030.

34. Kurlan R, Johnson D, Kaplan EL. Streptococcal infection and exacerbations of childhood tics and obsessive-compulsive symptoms: a prospective blinded cohort study. *Pediatrics* 2008; **121**(6)**:** 1188-1197.

35. Atladóttir HO, Thorsen P, Schendel DE, Østergaard L, Lemcke S, Parner ET. Association of hospitalization for infection in childhood with diagnosis of autism spectrum disorders: a Danish cohort study. *Arch Pediatr Adolesc Med* 2010; **164**(5)**:** 470-477.

36. Jishi A, Qi X, Miranda HC. Implications of mRNA translation dysregulation for neurological disorders. *Semin Cell Dev Biol* 2021; **114:** 11-19.

37. Paul MS, Duncan AR, Genetti CA, Pan H, Jackson A, Grant PE *et al.* Rare EIF4A2 variants are associated with a neurodevelopmental disorder characterized by intellectual disability, hypotonia, and epilepsy. *Am J Hum Genet* 2023; **110**(1)**:** 120-145.

38. Hüffmeier U, Kraus C, Reuter MS, Uebe S, Abbott M-A, Ahmed SA *et al.* EIF3F-related neurodevelopmental disorder: refining the phenotypic and expanding the molecular spectrum. *Orphanet Journal of Rare Diseases* 2021; **16**(1)**:** 136.

39. Hetman M, Slomnicki LP. Ribosomal biogenesis as an emerging target of neurodevelopmental pathologies. *J Neurochem* 2019; **148**(3)**:** 325-347.

40. Szaflarski W, Leśniczak-Staszak M, Sowiński M, Ojha S, Aulas A, Dave D *et al.* Early rRNA processing is a stress-dependent regulatory event whose inhibition maintains nucleolar integrity. *Nucleic Acids Res* 2022; **50**(2)**:** 1033-1051.

41. Lombardo MV. Ribosomal protein genes in post-mortem cortical tissue and iPSC-derived neural progenitor cells are commonly upregulated in expression in autism. *Molecular Psychiatry* 2021; **26**(5)**:** 1432-1435.

42. Qu W, Li Q, Wang M, Zhao X, Wu J, Liu D *et al.* m(6)A Modification Involves in Enriched Environment-Induced Neurogenesis and Cognition Enhancement. *Front Cell Dev Biol* 2022; **10:** 903179.

43. Jakobsson ME, Malecki J, Nilges BS, Moen A, Leidel SA, Falnes P. Methylation of human eukaryotic elongation factor alpha (eEF1A) by a member of a novel protein lysine methyltransferase family modulates mRNA translation. *Nucleic Acids Res* 2017; **45**(14)**:** 8239-8254.

44. Alejandro-Osorio AL, Huebert DJ, Porcaro DT, Sonntag ME, Nillasithanukroh S, Will JL *et al.* The histone deacetylase Rpd3p is required for transient changes in genomic expression in response to stress. *Genome Biology* 2009; **10**(5)**:** R57.

45. Salifou K, Ray S, Verrier L, Aguirrebengoa M, Trouche D, Panov KI *et al.* The histone demethylase JMJD2A/KDM4A links ribosomal RNA transcription to nutrients and growth factors availability. *Nat Commun* 2016; **7:** 10174.

46. Del Valle-Morales D, Romano G, Le P, Saviana M, Brown R, Micalo L *et al.* METTL3 alters capping enzyme expression and its activity on ribosomal proteins. *bioRxiv* 2023.

47. Lin S, Choe J, Du P, Triboulet R, Gregory RI. The m(6)A Methyltransferase METTL3 Promotes Translation in Human Cancer Cells. *Mol Cell* 2016; **62**(3)**:** 335-345.

48. Van Rechem C, Black JC, Boukhali M, Aryee MJ, Gräslund S, Haas W *et al.* Lysine demethylase KDM4A associates with translation machinery and regulates protein synthesis. *Cancer Discov* 2015; **5**(3)**:** 255-263.

49. Zhou X, Liao WJ, Liao JM, Liao P, Lu H. Ribosomal proteins: functions beyond the ribosome. *J Mol Cell Biol* 2015; **7**(2)**:** 92-104.

50. Asmal M, Colgan J, Naef F, Yu B, Lee Y, Magnasco M *et al.* Production of ribosome components in effector CD4+ T cells is accelerated by TCR stimulation and coordinated by ERK-MAPK. *Immunity* 2003; **19**(4)**:** 535-548.

51. Galloway A, Kaskar A, Ditsova D, Atrih A, Yoshikawa H, Gomez-Moreira C *et al.* Upregulation of RNA cap methyltransferase RNMT drives ribosome biogenesis during T cell activation. *Nucleic Acids Research* 2021; **49**(12)**:** 6722-6738.

52. Pramparo T, Pierce K, Lombardo MV, Carter Barnes C, Marinero S, Ahrens-Barbeau C *et al.* Prediction of Autism by Translation and Immune/Inflammation Coexpressed Genes in Toddlers From Pediatric Community Practices. *JAMA Psychiatry* 2015; **72**(4)**:** 386-394.

53. He Y, Zhou Y, Ma W, Wang J. An integrated transcriptomic analysis of autism spectrum disorder. *Scientific Reports* 2019; **9**(1)**:** 11818.

54. Nardone S, Sharan Sams D, Reuveni E, Getselter D, Oron O, Karpuj M *et al.* DNA methylation analysis of the autistic brain reveals multiple dysregulated biological pathways. *Translational Psychiatry* 2014; **4**(9)**:** e433-e433.

55. Ramaswami G, Won H, Gandal MJ, Haney J, Wang JC, Wong CCY *et al.* Integrative genomics identifies a convergent molecular subtype that links epigenomic with transcriptomic differences in autism. *Nature Communications* 2020; **11**(1)**:** 4873.

56. Estes ML, McAllister AK. Immune mediators in the brain and peripheral tissues in autism spectrum disorder. *Nat Rev Neurosci* 2015; **16**(8)**:** 469-486.

57. Duan T, Du Y, Xing C, Wang HY, Wang RF. Toll-Like Receptor Signaling and Its Role in Cell-Mediated Immunity. *Front Immunol* 2022; **13:** 812774.

58. Sabourin KR, Reynolds A, Schendel D, Rosenberg S, Croen LA, Pinto-Martin JA *et al.* Infections in children with autism spectrum disorder: Study to Explore Early Development (SEED). *Autism Res* 2019; **12**(1)**:** 136-146.

59. Bale TL. Epigenetic and transgenerational reprogramming of brain development. *Nat Rev Neurosci* 2015; **16**(6)**:** 332-344.

60. Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E *et al.* Defining trained immunity and its role in health and disease. *Nature Reviews Immunology* 2020; **20**(6)**:** 375-388.

61. Wohleb ES, Delpech JC. Dynamic cross-talk between microglia and peripheral monocytes underlies stress-induced neuroinflammation and behavioral consequences. *Prog Neuropsychopharmacol Biol Psychiatry* 2017; **79**(Pt A)**:** 40-48.

62. Ginhoux F, Prinz M. Origin of microglia: current concepts and past controversies. *Cold Spring Harb Perspect Biol* 2015; **7**(8)**:** a020537.

63. Frick L, Pittenger C. Microglial Dysregulation in OCD, Tourette Syndrome, and PANDAS. *J Immunol Res* 2016; **2016:** 8606057.

64. Antonson AM, Lawson MA, Caputo MP, Matt SM, Leyshon BJ, Johnson RW. Maternal viral infection causes global alterations in porcine fetal microglia. *Proc Natl Acad Sci U S A* 2019; **116**(40)**:** 20190-20200.

65. Weber-Stadlbauer U. Epigenetic and transgenerational mechanisms in infection-mediated neurodevelopmental disorders. *Translational Psychiatry* 2017; **7**(5)**:** e1113-e1113.

66. Melamed I, Kobayashi RH, O'Connor M, Kobayashi AL, Schechterman A, Heffron M *et al.* Evaluation of intravenous immunoglobulin in pediatric acute-onset neuropsychiatric syndrome. *Journal of child and adolescent psychopharmacology* 2021; **31**(2)**:** 118-128.

67. Negi V, Elluru SR, Siberil S, Graff-Dubois S, Mouthon L, Kazatchkine M *et al.* Intravenous Immunoglobulin: An Update on the Clinical Use and Mechanisms of Action. *Journal of clinical immunology* 2007; **27:** 233-245.

68. Li SC, Chan WC, Huang YH, Guo MM, Yu HR, Huang FC *et al.* Major methylation alterations on the CpG markers of inflammatory immune associated genes after IVIG treatment in Kawasaki disease. *BMC Med Genomics* 2016; **9 Suppl 1**(Suppl 1)**:** 37.

69. De Felice B, Nigro E, Polito R, Rossi FW, Pecoraro A, Spadaro G *et al.* Differently expressed microRNA in response to the first Ig replacement therapy in common variable immunodeficiency patients. *Scientific Reports* 2020; **10**(1)**:** 21482.

70. Lin SY, Kinet JP. Immunology. Giving inhibitory receptors a boost. *Science* 2001; **291**(5503)**:** 445-446.

71. Awamleh Z, Goodman S, Choufani S, Weksberg R. DNA methylation signatures for chromatinopathies: current challenges and future applications. *Hum Genet* 2023.

72. Ghasemi S. Cancer's epigenetic drugs: where are they in the cancer medicines? *The Pharmacogenomics Journal* 2020; **20**(3)**:** 367-379.

73. Dougan M, Dranoff G. Immune Therapy for Cancer. *Annual Review of Immunology* 2009; **27**(1)**:** 83-117.

Figure 1

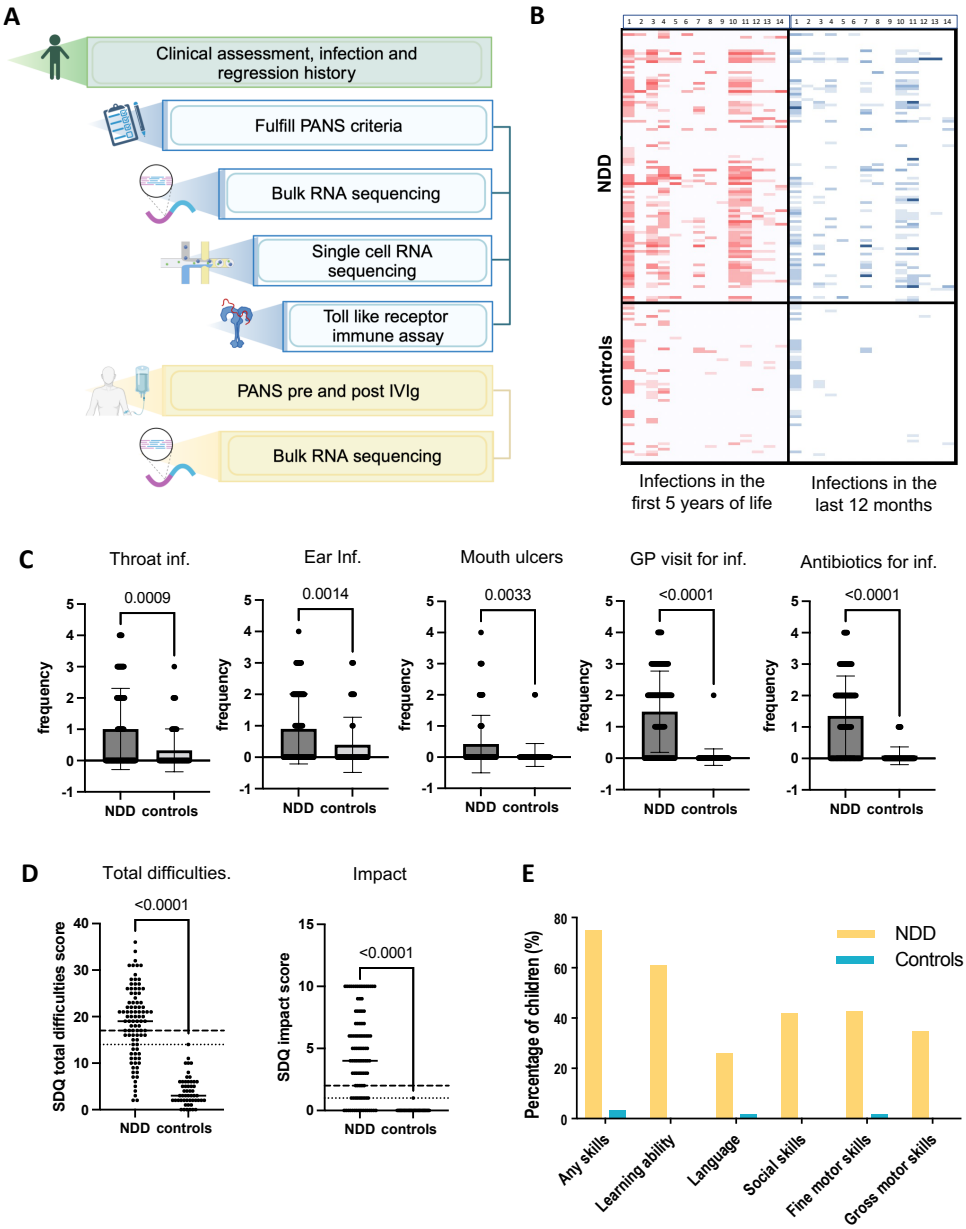

A
Clinical assessment, infection and regression history
Fulfill PANS criteria
Bulk RNA sequencing
Single cell RNA sequencing
Toll like receptor immune assay
PANS pre and post IVIg
Bulk RNA sequencing
B
NDD
controls
Infections in the first 5 years of life
Infections in the last 12 months
C
Throat inf.
Ear Inf.
Mouth ulcers
GP visit for inf.
Antibiotics for inf.
0.0009
0.0014
0.0033
<0.0001
<0.0001
frequency
NDD controls
D
Total difficulties.
Impact
<0.0001
<0.0001
SDQ total difficulties score
SDQ impact score
NDD controls
E
Percentage of children (%)
NDD
Controls
Any skills
Learning ability
Language
Social skills
Fine motor skills
Gross motor skills

Figure 2

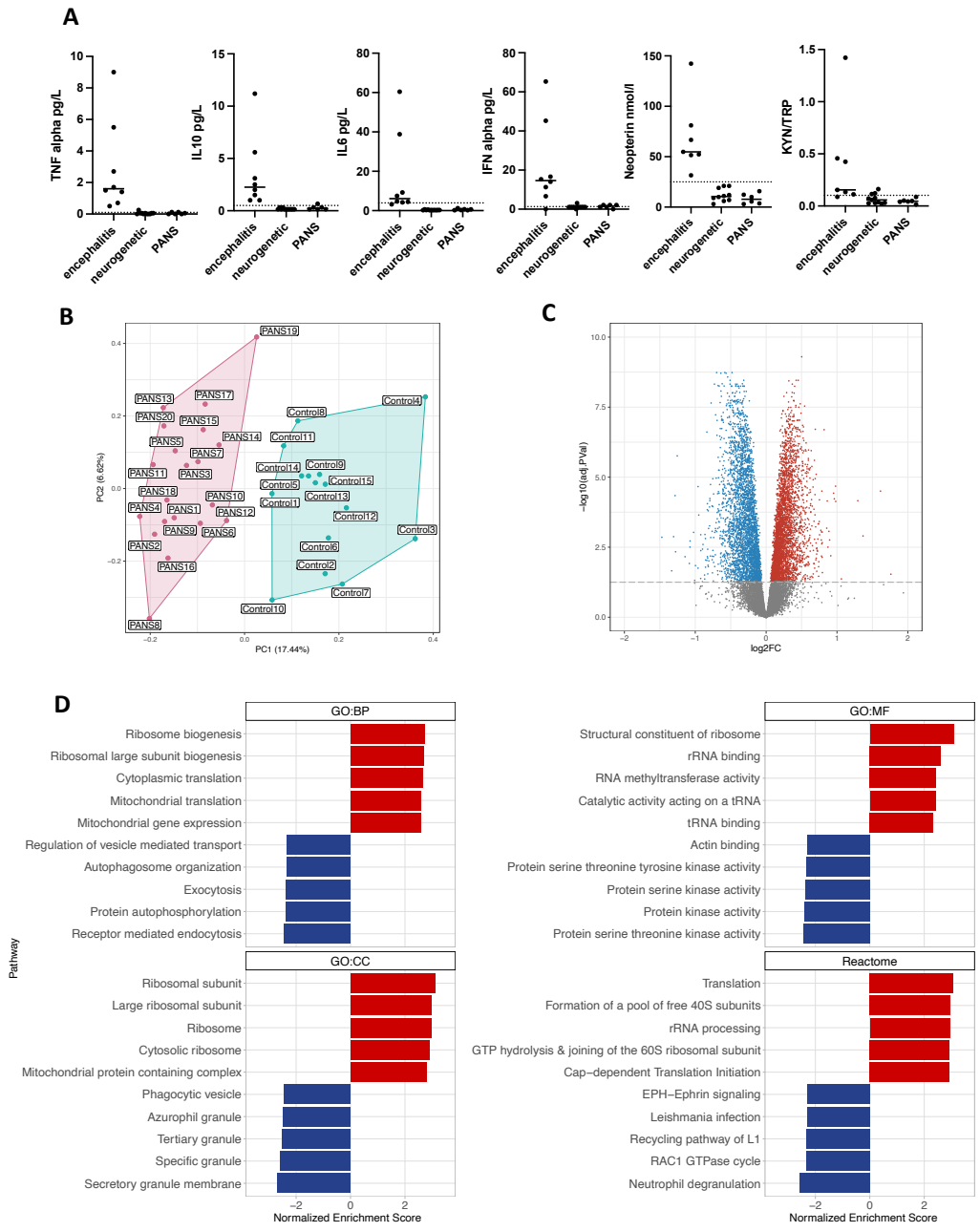

A
TNF alpha pg/L
IL10 pg/L
IL6 pg/L
IFN alpha pg/L
Neopterin nmol/l
KYN/TRP
encephalitis
neurogenetic
PANS
B
PC1 (17.44%)
PC2 (6.62%)
C
−log10(adj.PVal)
log2FC
D
GO:BP
Ribosome biogenesis
Ribosomal large subunit biogenesis
Cytoplasmic translation
Mitochondrial translation
Mitochondrial gene expression
Regulation of vesicle mediated transport
Autophagosome organization
Exocytosis
Protein autophosphorylation
Receptor mediated endocytosis
GO:MF
Structural constituent of ribosome
rRNA binding
RNA methyltransferase activity
Catalytic activity acting on a tRNA
tRNA binding
Actin binding
Protein serine threonine tyrosine kinase activity
Protein serine kinase activity
Protein kinase activity
Protein serine threonine kinase activity
GO:CC
Ribosomal subunit
Large ribosomal subunit
Ribosome
Cytosolic ribosome
Mitochondrial protein containing complex
Phagocytic vesicle
Azurophil granule
Tertiary granule
Specific granule
Secretory granule membrane
Reactome
Translation
Formation of a pool of free 40S subunits
rRNA processing
GTP hydrolysis & joining of the 60S ribosomal subunit
Cap−dependent Translation Initiation
EPH−Ephrin signaling
Leishmania infection
Recycling pathway of L1
RAC1 GTPase cycle
Neutrophil degranulation
Pathway
Normalized Enrichment Score

Figure 3

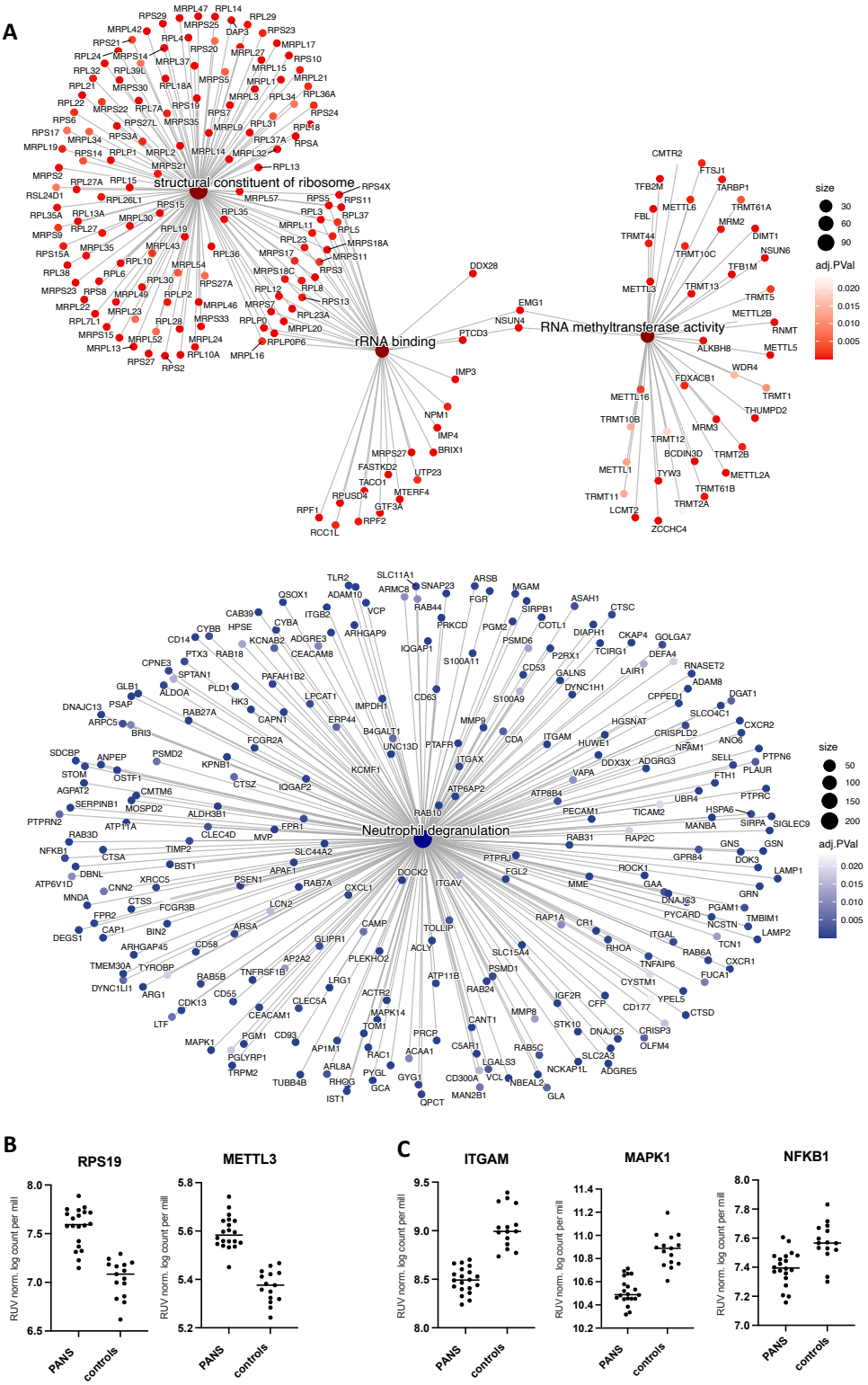

A
structural constituent of ribosome
rRNA binding
RNA methyltransferase activity
Neutrophil degranulation
size
adj.PVal
B
RPS19
METTL3
C
ITGAM
MAPK1
NFKB1
RUV norm. log count per mill
PANS
controls

Figure 4

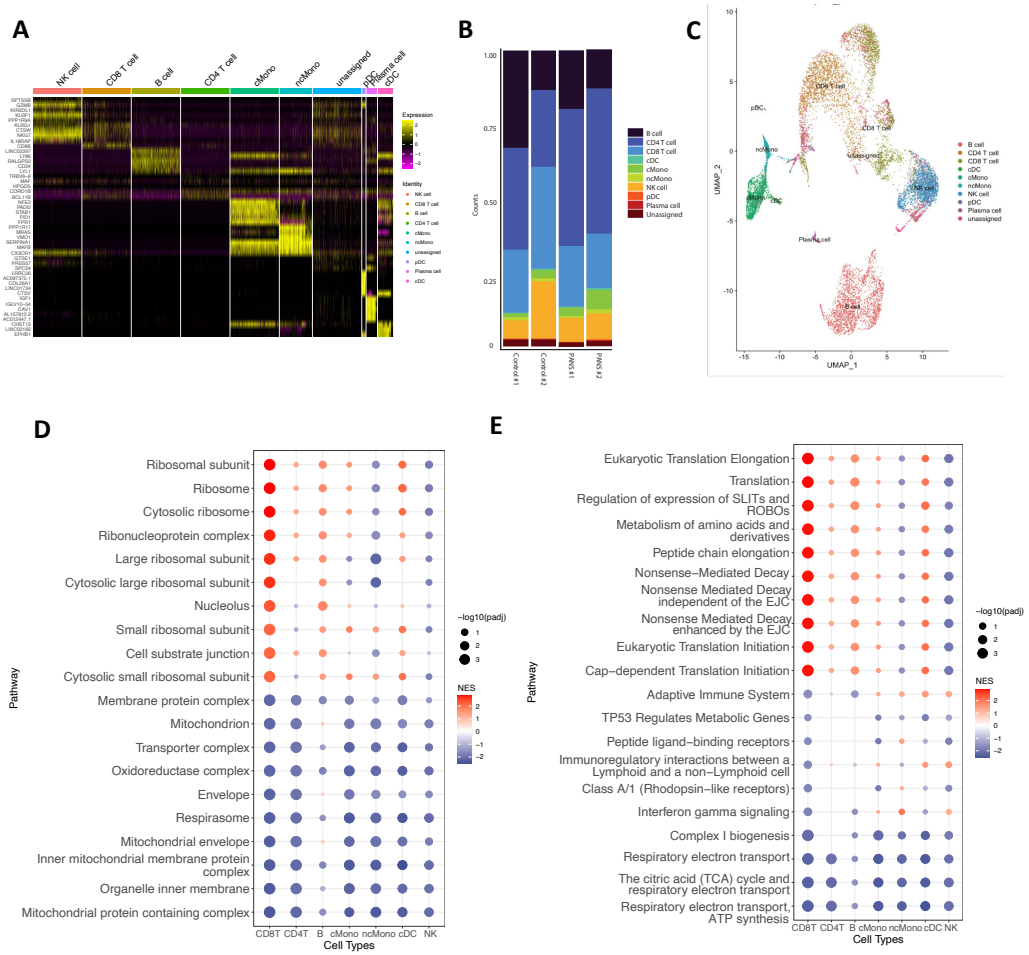

A
NK cell
CD8 T cell
B cell
CD4 T cell
cMono
ncMono
unassigned
pDC
Plasma cell
cDC
Expression
Identity
B
Counts
Control #1
Control #2
PANS #1
PANS #2
B cell
CD4T cell
CD8T cell
cDC
cMono
ncMono
NK cell
pDC
Plasma cell
Unassigned
C
UMAP_1
UMAP_2
D
Ribosomal subunit
Ribosome
Cytosolic ribosome
Ribonucleoprotein complex
Large ribosomal subunit
Cytosolic large ribosomal subunit
Nucleolus
Small ribosomal subunit
Cell substrate junction
Cytosolic small ribosomal subunit
Membrane protein complex
Mitochondrion
Transporter complex
Oxidoreductase complex
Envelope
Respirasome
Mitochondrial envelope
Inner mitochondrial membrane protein complex
Organelle inner membrane
Mitochondrial protein containing complex
Pathway
CD8T CD4T B cMono ncMono cDC NK
Cell Types
−log10(padj)
NES
E
Eukaryotic Translation Elongation
Translation
Regulation of expression of SLITs and ROBOs
Metabolism of amino acids and derivatives
Peptide chain elongation
Nonsense−Mediated Decay
Nonsense Mediated Decay independent of the EJC
Nonsense Mediated Decay enhanced by the EJC
Eukaryotic Translation Initiation
Cap−dependent Translation Initiation
Adaptive Immune System
TP53 Regulates Metabolic Genes
Peptide ligand−binding receptors
Immunoregulatory interactions between a Lymphoid and a non−Lymphoid cell
Class A/1 (Rhodopsin−like receptors)
Interferon gamma signaling
Complex I biogenesis
Respiratory electron transport
The citric acid (TCA) cycle and respiratory electron transport
Respiratory electron transport, ATP synthesis
Pathway
CD8T CD4T B cMono ncMono cDC NK
Cell Types
−log10(padj)
NES

Figure 5

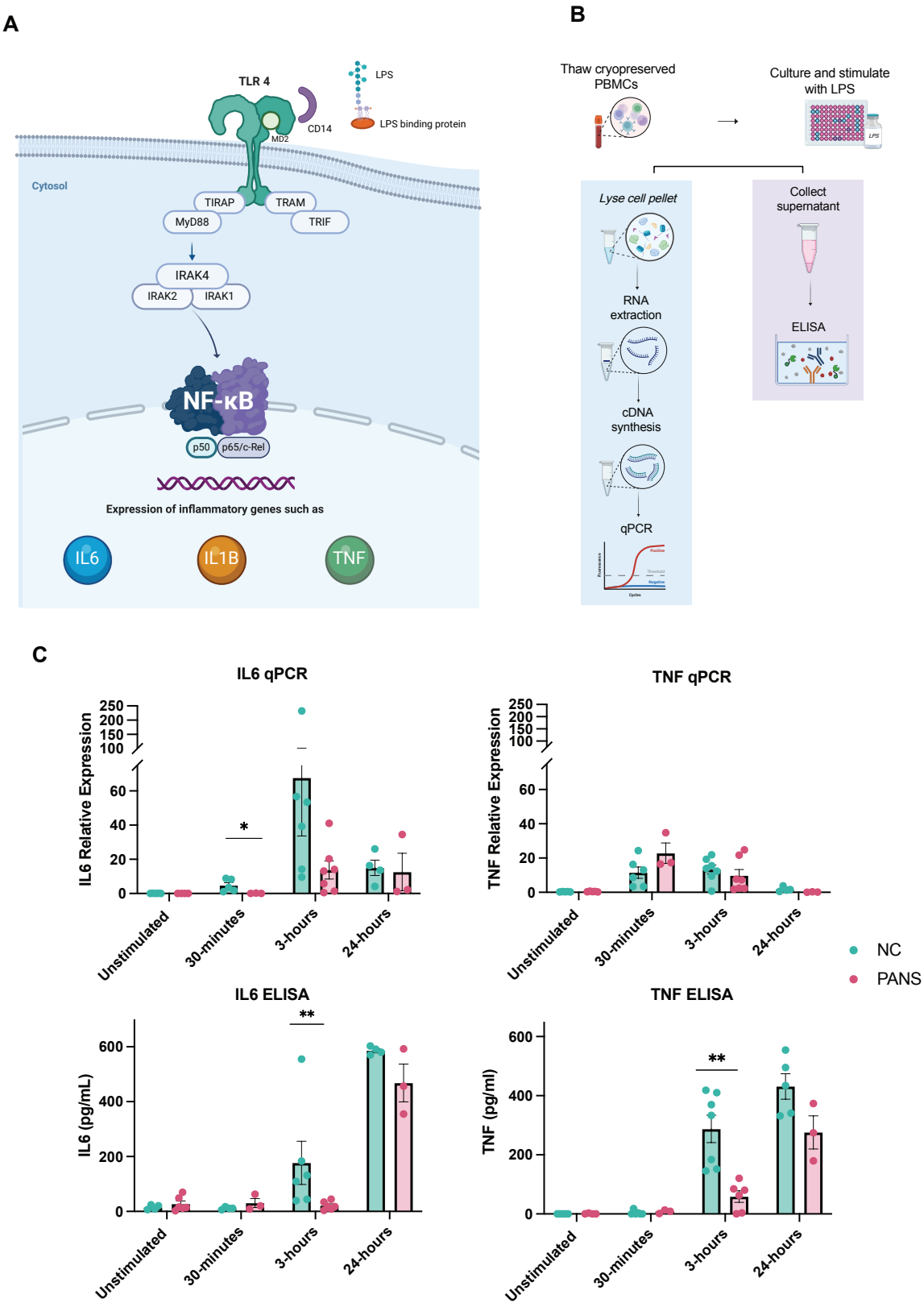
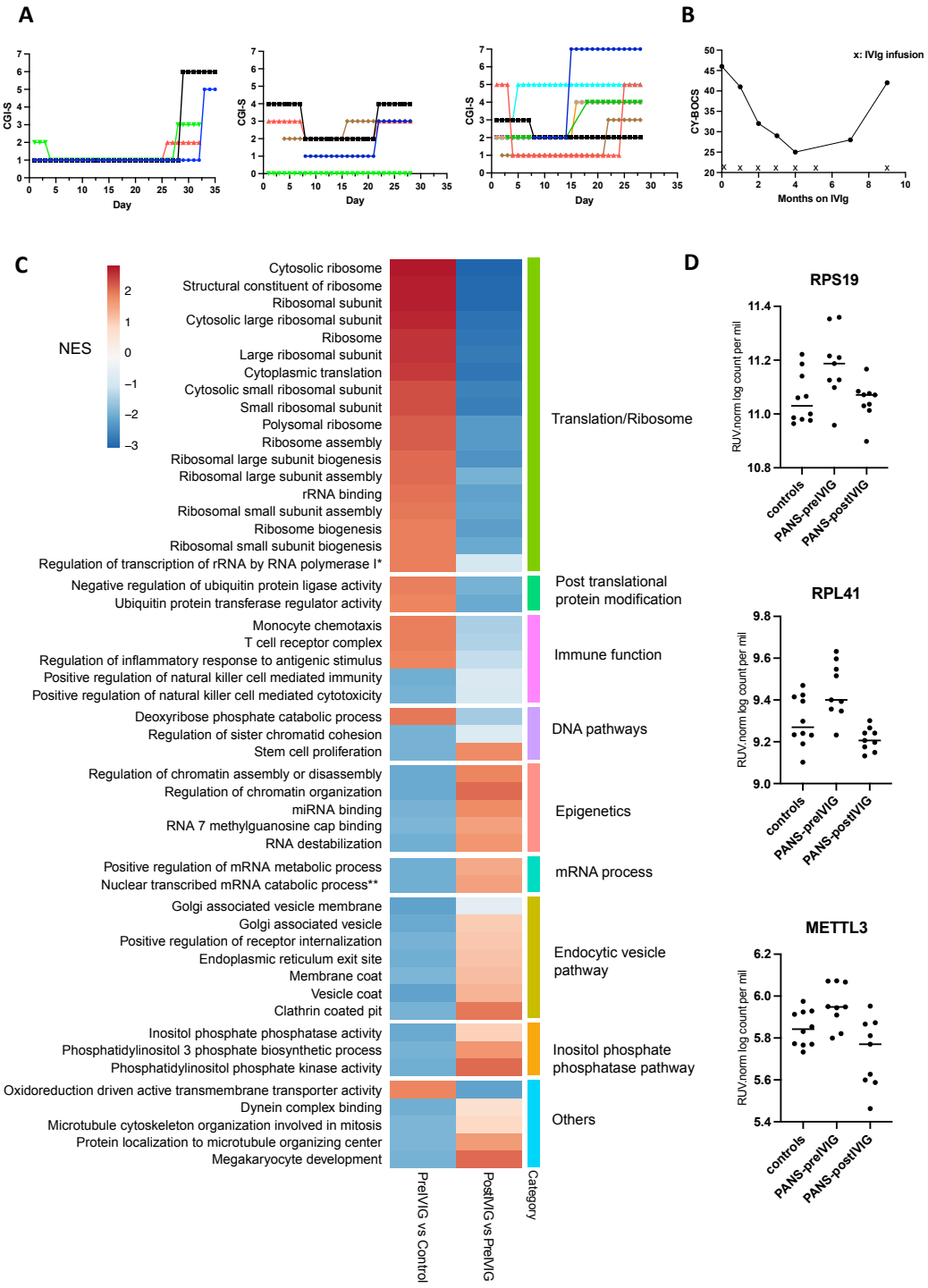

A
TLR 4
LPS
CD14
MD2
LPS binding protein
Cytosol
TIRAP
TRAM
MyD88
TRIF
IRAK4
IRAK2
IRAK1
NF-κB
p50
p65/c-Rel
Expression of inflammatory genes such as
IL6
IL1B
TNF
B
Thaw cryopreserved PBMCs
Culture and stimulate with LPS
Lyse cell pellet
Collect supernatant
RNA extraction
cDNA synthesis
qPCR
ELISA
C
IL6 qPCR
IL6 Relative Expression
TNF qPCR
TNF Relative Expression
Unstimulated
30-minutes
3-hours
24-hours
NC
PANS
IL6 ELISA
IL6 (pg/mL)
TNF ELISA
TNF (pg/ml)

Figure 6


A
CGI-S
Day
B
CY-BOCS
x: IVIg infusion
Months on IVIg
C
NES
Cytosolic ribosome
Structural constituent of ribosome
Ribosomal subunit
Cytosolic large ribosomal subunit
Ribosome
Large ribosomal subunit
Cytoplasmic translation
Cytosolic small ribosomal subunit
Small ribosomal subunit
Polysomal ribosome
Ribosome assembly
Ribosomal large subunit biogenesis
Ribosomal large subunit assembly
rRNA binding
Ribosomal small subunit assembly
Ribosome biogenesis
Ribosomal small subunit biogenesis
Regulation of transcription of rRNA by RNA polymerase I*
Translation/Ribosome
Negative regulation of ubiquitin protein ligase activity
Ubiquitin protein transferase regulator activity
Post translational protein modification
Monocyte chemotaxis
T cell receptor complex
Regulation of inflammatory response to antigenic stimulus
Positive regulation of natural killer cell mediated immunity
Positive regulation of natural killer cell mediated cytotoxicity
Immune function
Deoxyribose phosphate catabolic process
Regulation of sister chromatid cohesion
Stem cell proliferation
DNA pathways
Regulation of chromatin assembly or disassembly
Regulation of chromatin organization
miRNA binding
RNA 7 methylguanosine cap binding
RNA destabilization
Epigenetics
Positive regulation of mRNA metabolic process
Nuclear transcribed mRNA catabolic process**
mRNA process
Golgi associated vesicle membrane
Golgi associated vesicle
Positive regulation of receptor internalization
Endoplasmic reticulum exit site
Membrane coat
Vesicle coat
Clathrin coated pit
Endocytic vesicle pathway
Inositol phosphate phosphatase activity
Phosphatidylinositol 3 phosphate biosynthetic process
Phosphatidylinositol phosphate kinase activity
Inositol phosphate phosphatase pathway
Oxidoreduction driven active transmembrane transporter activity
Dynein complex binding
Microtubule cytoskeleton organization involved in mitosis
Protein localization to microtubule organizing center
Megakaryocyte development
Others
PreIVIG vs Control
PostIVIG vs PreIVIG
Category
D
RPS19
RPL41
METTL3
RUV.norm log count per mil
controls
PANS-preIVIG
PANS-postIVIG