Investigating the use of steroids in children with Infantile Epileptic Spasms Syndrome: A multi-omics evaluation of gene and epigenetic regulation

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Statement of originality

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

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

No content produced by generative AI tools has been used in the preparation of this thesis.

Dr Emily Innes

28th September 2025

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Author attribution statement

Chapter two of the thesis is presented as a scoping review, previously published by first author Dr Emily Innes.

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

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2. Oral presentation on “Infantile Epileptic Spasms Syndrome and Steroids: preliminary clinical, proteomic and phosphoproteomic results”, Neuroimmune Research Group, at Kids Research Centre, 9th September 2024

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Abbreviations

ACTH	Adrenocorticotrophin hormone
ADHD	Attention Deficit Hyperactivity Disorder
ASD	Autism Spectrum Disorder
ASM	Anti-seizure medication
ASQ-3	Ages and Stages Questionnaire – Third edition
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BP	Biological processes
CC	Cellular components
CI	Confidence Interval
CMRI	Children's Medical Research Institute
CNS	Central nervous system
CSF	Cerebrospinal fluid
CP	Cerebral Palsy
CT	Computed Tomography
DEE	Developmental and epileptic encephalopathy
DEG	Differential expressed genes
DNA	Deoxyribonucleic acid
EE	Epileptic Encephalopathy
EEG	Electroencephalogram
ELISA	Enzyme-linked immunosorbent assay
EMG	Electromyography
FDR	False discovery rate
FBC	Full blood count
GESA	Gene Set Enrichment Analyses
GO	Gene-Ontology
GDD	Global Developmental Delay
HIE	Hypoxic ischemic encephalopathy
HPA-axis	Hypothalamic Pituitary adrenal-axis
Hz	Hertz
ICISS	International Collaborative Infantile Spasms Study
IESS	Infantile Epileptic Spasm Syndrome
ILAE	International League Against Epilepsy
L	Left
LC-MS	Liquid Chromatography-Mass Spectroscopy
mRNA	Messenger ribonucleic acid
MCA	Middle cerebral artery
MF	Molecular function
MRI	Magnetic resonance imaging
NES	Normalised Enrichment Score
NDD	Neurodevelopmental disorder

ORA	Overrepresentation analysis
PCA	Principal Component Analysis
PBMC	Peripheral blood mononuclear cell
PSS	Perceived Stress Scale
PTM	Post-translational modifications
R	Right
RUV	Removal of unwanted variation
RNA	Ribonucleic acid
SD	Standard deviation
SCHN	Sydney Children's Hospital Network
TSC	Tuberous Sclerosis Complex
UKISS	United Kingdom Infantile Spasms Study

THESIS ABSTRACT

Introduction

Infantile Epileptic Spasms Syndrome (IESS) is a severe developmental and epileptic encephalopathy of infancy. Prednisolone, adrenocorticotrophin hormone and vigabatrin achieve epileptic spasms cessation yet do not improve long-term outcomes including epilepsy and developmental/intellectual impairment. The evidence exploring aetiopathogenic and therapeutic mechanisms in IESS remains limited.

Aims

The primary aims of this thesis were to investigate possible mechanisms of disease underlying IESS and how prednisolone treatment exerts an anti-seizure effect in IESS. My hypothesis was that infants with IESS have altered gene and epigenetic regulation with distinct immune profiles, and that prednisolone controls epileptic spasms by altering gene expression and/or modifying immune responses. The secondary aims were determining if response, or non-response to prednisolone treatment altered long-term epilepsy and neurodevelopmental outcomes. My hypothesis was that treatment responders and those with unknown aetiology would have more favourable outcomes.

Methods

To address these aims my work had three parts, a systematic review followed by a prospective clinical cohort study following infants with IESS for 12 months combined with a biological “multi-omics” analysis examining disease and treatment mechanisms. Blood samples were collected from infants with IESS in the clinical study pre and post prednisolone treatment and compared to an age and sex matched control group. The biological analysis included neuroinflammation panel testing, whole blood bulk RNA sequencing, proteomic and phosphoproteomic sequencing. A pathway directed analysis was performed to determine common pathways enriched by differentially expressed genes and abundant proteins.

Results

From my systematic review, I identified five key aetiopathogenic mechanisms of IESS that corticosteroids may modify. These included altering gene regulation, stress, neuroinflammation, neuronal excitability and metabolic pathways. I highlighted that altered gene and epigenetic regulation may influence the expression of vulnerability genes in IESS and may be modified by corticosteroids. However, the evidence was constrained by limited reproducible findings in large cohorts of children with IESS.

The clinical cohort study enrolled 11 infants with IESS (4M, 7F), six had unknown aetiology (55%) and seven (45%) had structural or structural-genetic aetiologies. Median age at epileptic spasms onset was 6.9 months and all achieved epileptic spasms cessation by day 14 of treatment. At 12 months follow-up, infants with unknown-IESS aetiology had a more favourable prognosis with lower rates of epilepsy and developmental delay.

The multi-omics comparison between IESS pre-treatment and controls identified lower serum levels of brain-derived neurotrophic factor and dysregulation of ribosomal, immune and chromatin pathways in addition to other cellular pathways. Dysregulated immune pathways included downregulated secretory granule membrane and upregulated complement activation pathways. Prednisolone treatment reversed and upregulated immune pathways, specifically the secretory granule membrane pathway at an RNA and protein level. Genes enriching this pathway were involved in vesicle mediated transport, regulation of cytokine production and regulation of phagocytosis. Prednisolone treatment also led to downregulation of ribosomal and T-cell pathways and dysregulated chromatin and actin pathways.

Conclusion

These findings have contributed to our understanding of IESS as a neurodevelopmental disorder and that altered gene, and epigenetic regulation may be an aetiological mechanism underlying IESS. Prednisolone treatment appears to control epileptic spasms by altering gene expression through immune-mediated and ribosomal/mRNA and chromatin pathways. It is not clear if these effects are sustained as

the long-term prognosis in IESS remains poor. Future studies examining gene regulation and targeted disease-modifying treatments are required to improve long-term disability in IESS.

CHAPTER 1 - Introduction

1.1 Researcher background

I completed three years of subspeciality paediatric neurology training in Sydney, Australia from 2020-2022. During my first year of training, as the George Gregan Epilepsy Fellow at the Children's Hospital at Westmead, I found the management of complex epilepsies in children quite challenging and was intrigued by the successful use of immune-modifying treatments including steroids and intravenous immunoglobulin for developmental and epileptic encephalopathy (DEE). During my final year of training, my supervisors Professor Russell Dale and Associate Professor Shekeeb Mohammad offered me an opportunity to join researchers at the Kids Research Centre, investigating mechanisms of disease and therapeutics in neurology, which I gratefully accepted to further refine my clinical and research skills. Fortunately I was able to explore mechanisms by which steroids exert an anti-seizure effect in infantile epileptic spasm syndrome (IESS)- one of the most common and severe DEEs in childhood.

1.2 Content and scope of thesis

This thesis will examine the possible mechanisms of disease underlying infantile epileptic spasm syndrome (IESS) and how prednisolone treatment exerts an anti-seizure effect in IESS. Prednisolone, rather than adrenocorticotrophic hormone (ACTH), was chosen as it is the accepted standard of care for IESS in Australia. This introductory chapter provides an overview of IESS, the current accepted definitions and clinical features and key trials examining treatment efficacy and long-term outcomes. Chapter two, includes a literature review published during my Masters candidature (1) examining the possible aetiopathogenic mechanisms leading to IESS and anti-seizure mechanisms of action of ACTH/prednisolone reported in children with IESS. Chapter three details the methodology and results of a prospective cohort study of infants with IESS, including 12-month epilepsy and developmental outcome measures. Chapter four presents the multi-omics investigation exploring gene regulation and immune profiles of infants with IESS from the clinical cohort study compared to healthy controls as well as a comparative analysis of the pre- and post-

treatment effects of prednisolone. The “multi-omics” analysis includes bulk-RNA transcriptomics, proteomics and phosphoproteomics of peripheral immune cells. Chapter five discusses the findings and how they may inform future studies investigating treatment effects or disease-modifying therapies in IESS.

1.3 Historical Overview

IESS is the most common epilepsy syndrome in the first year of life. (2) It is a severe neurodevelopmental disorder (NDD) characterised by three key features; epileptic spasms, a highly disorganised electrical pattern on electroencephalogram (EEG) known as hypsarrhythmia, and a developmental arrest or loss of previously attained skills. (2) Dr William James West published the first clinical description of epileptic spasms in his own son, James, in a letter to the editor of The Lancet in 1841. (3) At the age of 4 months, James, a previously well child developed “slight bobbings of the head forward” which developed into powerful truncal contractions, which clustered and occurred multiple times per day resulting in a decline in his “intellectual vivacity” and motor skills. (3) Experts in London, Dr Clarke and Dr Locock recognised this condition, having diagnosed it in six children and referred to it as “salaam convulsions”, referencing the Arabic “salaam” greeting of bowing forward. (4) Dr Newnham, published a case series of four children, including James West, in 1849, describing the clusters of spasms as “nodding seizures” noting that they may also involve the truncal extensor muscles, often occurred around sleep and progressed “into epilepsy, or some other form of infantile convulsions”. (5) Despite over 60 case reports adding to the clinical phenotype, and approximately 76 world-wide terms describing epileptic spasms, (4) very little progress was made understanding the pathophysiology and treatment options until the characteristic EEG changes were identified in the 1950s.

Three parties, Lennox and Davies (1950), Vasquez and Turner (1951) and Gastaut and Remond (1952) independently described EEG abnormalities associated with epileptic spasms. (6-8) Lennox and Davies described “massive myoclonic jerks” associated with a slow 2 Hertz (Hz) EEG correlate, yet it is possible they were referencing an epilepsy now known as Lennox-Gastaut syndrome. Vasquez et al., (1951) and Gastaut et al., (1952) provided a clear description of IESS, which they referred to as “Epilepsia en flexion generalizida” and

"myoclonies type B". They termed the abnormal EEG background "cerebral dysrhythmia", consisting of a diffuse, high voltage, slow and disorganised rhythm, and multifocal discharges and described the seizure/ictal correlate of spike and slow wave discharges. Gibbs and Gibbs formally defined this pattern as "hypsarrhythmia ", from the Greek words "hypsi" meaning high/mountainous and "arrhythmos" meaning arhythmical, in their 1952 Atlas of Electroencephalography. (9) This was a significant breakthrough that further defined the triad of findings in this disorder, that herein, was consistently referred to as "West syndrome" and prompted further treatment trials, as medications commonly used at the time (phenobarbitone, phenurone etc) were ineffective. (4)

In 1958, two publications reported that hormonal therapy, adrenocorticotrophin hormone (ACTH) (10, 11) and oral cortisone (11) had a "spectacular" effect resulting in epileptic spasm cessation and improvement in the EEG. Trials of hormonal therapies were popular at the time, following McQuarrie et al., (1942, 1946) observations that seizures induced by increased water intake and anti-diuretic hormone (ADH) in adults with epilepsy were reversed by the mineralocorticoid, deoxycortisone, thought to counteract the effects of ADH and possess anti-seizure properties. (12, 13) Despite the development of over 40 new anti-seizure medications (ASM), over the subsequent few decades, only vigabatrin, a GABA-ergic agent, developed in 1977, has proven to be an effective treatment for IESS when used in combination with ACTH/steroids (14) or alone for epileptic spasms due to Tuberous Sclerosis Complex (TSC). (15, 16)

Currently, ACTH/prednisolone remain first line treatment for IESS based on treatment trials that have shown definitive benefit (described in detail below). (17, 18)

1.4 Epidemiology and aetiology of IESS

Although IESS is a rare type of epilepsy, it is the most common epilepsy syndrome affecting infants aged one-24 months. (19) A meta-analysis examining the global incidence and prevalence of IESS from 54 studies including 1792 infants between 1946-2016 identified a pooled incidence of 2.5 per 10,000 live births and prevalence of 1.5 per 100,000 children. (20) Higher incidences were seen in continents with higher geographical latitudes such as

Europe and North America, although Scandinavia had a particularly elevated incidence, not explained by the latitudinal effect. (20)

Onset of IESS peaks between three to seven months of age. (21) Ninety-four percent of cases present before the age of 12 months (22) and onset after 18 months is rare. (4) Both sexes are affected, several studies have reported males are more frequently affected than females with a ratio of 60:40. (4, 18, 23-25). However, the meta-analysis examining global IESS rates concluded that compared to the reference population, males were not significantly more affected than females (p=0.056). (20)

The aetiology of IESS is heterogenous, comprising both known and unknown causes. The known causes include both structural brain changes, either congenital or acquired, as well as monogenic aetiologies. Currently, the International League Against Epilepsy (ILAE) recommends classifying aetiology according to seven subgroups, including structural, genetic, metabolic, acquired, infectious, immune or unknown aetiologies. The proportion of IESS due to a known aetiology varies between 61-69% in large multi-centre cohort studies, (19, 26, 27) higher than earlier reports of 51% suggesting that diagnostic accuracy may have increased with the advancement of brain magnetic resonance imaging (MRI) and genetic testing. (26, 28) While these aetiological associations define underlying causes in some patients, it is unclear how these varied causes lead to a similar appearing phenomenology occurring in the same time window of early childhood. The aetiology and prevalence of aetiological subgroups, as well as proposed pathophysiological mechanisms, will be explored in detail in Chapter two.

1.5 Differential diagnoses of epileptic spasms

Imitators of epileptic spasms are widely recognised and include a range of normal and abnormal infant movements. (4, 29) Normal phenomena that may be mistaken for IESS, include the Moro or “startle” reflex, other arousal behaviours and sleep myoclonus. (2, 4, 30) Typically the history of a precipitating stimulus (auditory, tactile, visual) and infrequent singular events suggests startle responses. Abnormal infant movements that are non-epileptic in nature can be seen as part of medical conditions including abdominal pain (or

colic), gastroesophageal reflux or Sandifer syndrome, characterised by distress or opisthotonic posturing and movement disorders including sleep-related rhythmic movement disorder, paroxysmal dystonia, spasmus nutans, and benign disorders including benign paroxysmal tonic upgaze and shuddering. (4, 29)

There is an entity termed “benign spasms” or “benign myoclonus of infancy” that manifests in the first year of life, usually between four to nine months of age. (31-34) The “spasms” are extremely similar appearance to epileptic spasms, characterised by brief one to second tonic or myoclonic contractions affecting the head, neck, torso and upper limbs and may cluster. (29, 31) In contrast to IESS, there is no developmental regression. (33) The key differentiating feature from IESS is a normal EEG recording throughout the event, highlighting the crucial role of video EEG differentiating epileptic to non-epileptic events. (31-34) The disorder does not require treatment and is self-limiting often resolving in the second year of life. (31-34)

Epilepsy syndromes occurring in the first year of life that predominantly involve myoclonus should be differentiated from IESS. Myoclonic epilepsy of infancy is a self-limiting epilepsy characterised by brief (less than one second) myoclonus that occurs during sleep and wakefulness. (2) Early infantile DEEs with prominent myoclonus include Otahara syndrome which presents in the first week to three months of life and Dravet Syndrome, characterised by recurrent febrile and afebrile tonic or focal clonic seizures with onset between three and nine months of life. (2, 35, 36) In older infants and children, alternate epilepsy diagnoses to consider include myoclonic-astatic epilepsy and early-onset Lennox-Gastaut syndrome, although the predominant seizure types are atonic or tonic seizures. (2, 37).

Electromyography (EMG) of the ictal event may help differentiate epileptic spasms from myoclonic and tonic seizures. There is no consensus definition for the duration of an epileptic spasm, however, there are two recognised components, a fast phasic contraction lasting 0.5-2 seconds followed by a tonic “hold” or “hung-up” phase often of two seconds duration. (38, 39) Surface EMG burst morphology shows a characteristic rhomboid shaped discharge with low amplitude spikes that build in amplitude to the peak of the rhomboid

reflecting recruitment of motor units during the phasic contraction then a decrescendo/tapering off pattern as part of the tonic then relaxation phase. (38, 39) The EMG pattern helps differentiate from myoclonic seizures, the briefest seizure type with a much shorter “spike-like” EMG pattern typically lasting less than 100 milliseconds. Similarly, EMG of tonic seizures demonstrates a prolonged high frequency “hold” lasting from 2-10 seconds. (38, 39) Distinguishing epileptic spasms from short tonic or myoclonic seizures can be clinically challenging due to the overlap in ictal duration and morphology, often the presence of clustering is an alert for physicians. (40)

A small number of studies have examined the prevalence of epileptic spasms imitators reporting 51-60% of infants referred for suspected IESS receive an alternate diagnosis. (29, 40) The most frequent imitators were myoclonus both non-epileptic and epileptic, tonic seizures, GORD related movements or other movement disorders. (4, 29, 41) One study reported clustering of events and pre-existing neurodevelopmental diagnoses were strongly associated with IESS (p<0.0001). (40)

1.6 Current definitions of IESS and key clinical features

There have been many changes to the nomenclature and definitions of IESS over the years. In 2017, the ILAE underwent a major revision to their classification guidelines and adopted a standardised “three-tiered” approach to epilepsy classification by defining the seizure type, epilepsy type and epilepsy syndrome with consideration to aetiology at all stages. (42, 43) In this classification, the seizure type was “infantile spasms”, and the syndromic term was “West Syndrome”. The epilepsy type was considered “generalised”, implying bilateral/symmetric or unknown seizure onset however, focal onset epileptic spasms were known to occur in structural pathologies.

In 2022, the ILAE released a position statement on classification and definition of epilepsy syndromes in neonates and infants. (2) This introduced the term infantile epileptic spasms syndrome, and necessary features for diagnosis, which is referred to in this thesis. The paper acknowledged the presence of focal seizures preceding spasms onset or co-occurring with IESS in structural aetiologies however, these revised terms and concepts were only incorporated in the 2025 update to the “three-tiered” epilepsy classification system. (44) The update stated that the seizure type should be referred to as “epileptic spasms” which in IESS could be focal, generalised or unknown onset. (44) The ILAE emphasised using a multimodal approach with EEG, imaging and biochemical or genetic investigations to help classify the onset of epileptic spasms. Two main syndromes were proposed, “IESS” incorporating focal, unknown and generalised onset epileptic spasms or “structural epilepsy” with focal seizures and epileptic spasms only. (44) The emphasis was to consider early surgical treatment for structural epilepsy if spasms-specific treatment failed. As the majority of my thesis was completed prior to the 2025 update, I have referred to the classification and definition of IESS from the 2022 paper.

A standardised classification for the aetiology of IESS was necessary due to the complexity of earlier terms such as “symptomatic”, “idiopathic” and “cryptogenic” (45) which described both aetiology and developmental status, yet were used inconsistently by researchers who defined these terms differently. (46) The "symptomatic" group included infants with a either a recognised aetiology or pre-existing epilepsy or developmental delay

that strongly implied the presence of an underlying disorder. (45) The “idiopathic” group had no clearly defined aetiology and required normal development before and/or after epileptic spasm diagnosis. (45, 47) “Cryptogenic” implied that an underlying disorder was suspected but not proven and there was general agreement that development was affected. (45) Ambiguity arose from the overlapping definitions and the synonymous use of the terms “cryptogenic” and “idiopathic” at the time of IESS diagnosis, prior to confirming final aetiology and/or outcomes. (45, 46) The ILAE improved the standardisation and classification of IESS, accounting for these various sub-groups in their 2022 position statement. (2)

1.6.1 ILAE classification of IESS (2022 iteration)

The ILAE proposed the term IESS to include two groups of infants, those that presented with the classical triad of “West syndrome” meeting the criteria listed in Table 1.1 and infants that only partially fulfill criteria. (2) For a diagnosis of IESS, infants must have a confirmed clinical diagnosis of typical epileptic spasms with onset between one-24 months of age. (2) The ILAE recommendation acknowledges that developmental changes and/or hypsarrhythmia are not always present, especially early in the disease course. (2) The EEG may have “atypical” or “modified” features of hypsarrhythmia such as focal abnormalities or an organised rather than chaotic background. The ILAE recommended describing the atypical features, rather than using the terms “atypical” or “modified”. (2, 47)

Table 1.1. Diagnostic criteria for infantile epileptic spasm syndrome (Excerpt from Zuberi et al., 2022). (2)

	Mandatory	**Alerts**	**Exclusionary**
Seizures	Flexor, extensor or mixed epileptic spasms which often occur in clusters		
EEG	Interictal: Hypsarrhythmia, multifocal or focal epileptiform discharges (that might be seen quickly after the spasms onset)	Interictal: Normal EEG Suppression-burst pattern	Ictal: Normal EEG during recorded clinical events of suspected spasms
Age at onset	1-24 months (while epileptic spasms may begin later, this would not be IESS)	Age at onset 1-2 months	
Comorbidities	Developmental slowing after spasms onset but may be absent early in the disease course (difficult to determine in a child with existing significant developmental disorders)		
Is MRI or ictal EEG required for diagnosis? An MRI is not required for diagnosis but is highly recommended to evaluate for underlying cause. An ictal EEG is not required for diagnosis provided the interictal study shows hypsarrhythmia or epileptiform abnormalities or developmental delay. In the absence of hypsarrhythmia or epileptiform anomalies, an ictal recording is required			
Possible evolving syndrome: Infants with preceding brain injury, developmental brain malformations, or specific genetic conditions, including early infantile DEE, who show significant interictal EEG abnormalities (high amplitude, background slowing, and/or multifocal discharges) should be watched carefully for the development of clinical epileptic spasms. However, the syndrome of IESS cannot be diagnosed prior to onset of the mandatory seizure type			
Syndrome without laboratory confirmation: In resource-limited regions, an interictal EEG is highly recommended. However, if EEG is unavailable, if clear clusters of typical epileptic spasms are witnessed by an experienced clinician (in person or on video recording), with the other clinical mandatory and exclusionary criteria, IESS can be diagnosed			

Please note the following sections detail the clinical features seen in IESS. The ILAE has provided brief descriptions in their position paper which are referenced accordingly and further expanded upon as needed.

1.6.2 Epileptic Spasms

1.6.2.1 Semiology

Epileptic spasms are mandatory for the diagnosis of IESS. They are sudden brief tonic contractions of the axial (neck and trunk) and proximal limb muscles. (2, 48) They are termed “flexor” or “extensor” based on the muscles involved, or “mixed” meaning both types of epileptic spasms are present. (48) Epileptic spasms may be symmetric or asymmetric, indicating a possible focal pathology. Infants with asymmetric epileptic spasms

may adopt a “fencers posture” with one arm flexed into the body whilst the other extends or have subtle focal signs such as versive eye deviation or fisting of one hand. Asymmetric events may also be asynchronous in that one side contracts prior to the other. (48)

Flexor epileptic spasms include the semiology described by Dr West of “head nodding” and “jack-knifing”. (4) “Head nodding” refers to the sudden forward flexion of the neck and shoulders with minimal torso and arm involvement. Upward eye deviation may occur. (4) The more powerful events were named “jack-knifing” as the sudden flexion involved the torso as if “jack-knifing” at the waist, such that the head and hips are held in close proximity. The arms are typically stretched out in-front, either adducted across the body as if hugging someone or abducted in a wide embrace. (4) Extensor epileptic spasms resemble a stretching movement, with extension of the torso, hips, knees and arms, which may be held adducted, close to the body or abducted/outstretched. (4) One study examined 5,042 epileptic spasms that occurred in 24 infants and found mixed events (42%) occurred most frequently followed by flexor (34%) and then extensor (23%) epileptic spams. (38) Asymmetry was rare (1%). (38)

There are many features associated with epileptic spasms, commonly infants have a fearful expression or ocular abnormalities (nystagmus, versive movements, eyelid fluttering) during the event. (7, 49) Following their cessation, infants may grunt or cry, they are typically distressed or appear exhausted. (4, 38) Rarely a behavioural arrest may occur. (38) Autonomic features including flushing, sweating, cyanosis, pupillary dilatation and altered respiration have also been described. (4, 38) Although not typical, epileptic spasms may also be accompanied by other seizure types, particularly focal motor or focal impaired awareness seizures. (4) Co-occurrence of seizures is reported in up to 27-40% of IESS cohorts, (23, 50, 51) typically associated with a structural aetiology, such as TSC, yet was also seen in those with unknown (cryptogenic) aetiology. (52)

1.6.2.2 Duration and timing of epileptic spasms

The ILAE notes that epileptic spasms typically last "< 3 seconds" and may occur repetitively or "cluster" over several minutes. As this varies significantly between infants, there is no stipulated time criteria. (2) The duration has been examined using electromyogram (EMG) recordings of the deltoid, (39) truncal and appendicular muscles. (38) Epileptic spasms have an initial phasic contraction lasting one to two seconds, followed by a less intense tonic contraction lasting two-10 seconds, (38, 39) however, the tonic phase is not always present.

Clustering of epileptic spasms occurs in up to 70-88% of infants (38, 52, 53) however, the number of epileptic spasms per cluster varies widely. One study reported an average of 24 epileptic spasms per cluster which lasted between two-10 minutes, (54) whereas others have described up to 125 events in one cluster, (38) and clusters more than 30 minutes duration. Typically, the intensity and frequency of epileptic spasms increases and then decreases within the cluster following a crescendo-decrescendo pattern. (38) Clusters occur sporadically during both wakefulness and the transition states between sleep and wakefulness. (38) Studies have confirmed observations that epileptic spasms occur infrequently during sleep. (38, 50, 52)

1.6.3 EEG features

1.6.3.1 Interictal EEG features – hypsarrhythmia and modified hypsarrhythmia

There are no universally accepted definitions of hypsarrhythmia, the highly disorganised EEG pattern found in IESS. The 2022 ILAE position paper included interictal hypsarrhythmia as a mandatory feature of IESS, although acknowledged a "typical hypsarrhythmia may not be present" and that "infants with IESS often lack one of these three criteria". (2) Definitions of "typical" hypsarrhythmia including voltage criteria compared to "atypical" or "modified" features were not described.

Historically, Gibbs and Gibbs described hypsarrhythmia in their 1952 Atlas of Electroencephalography (9):

> *"random high voltage slow waves and spikes. These spikes vary from moment to moment, both in duration and location. At times they appear to be focal, and a few seconds later they seem to originate from multiple foci. Occasionally, the spike discharge becomes generalized, but it never appears as a rhythmically repetitive and highly organized pattern that could be confused with a discharge of the petit mal variant type. The abnormality is almost continuous and in most cases it shows as clearly in the waking as in the sleeping record."*

Since this time key features that have become synonymous with "typical" or "classical" hypsarrhythmia are summarised in Table 1.2. The terms "atypical" or "modified" were introduced later by several authors, who recognised clear variations of these key EEG features. (49, 55, 56) Variants or "modified" hypsarrhythmia patterns adapted from these authors are described in Table 1.3. (4, 22, 57-65)

A large prospective study of 447 infants with IESS reported that 82% had hypsarrhythmia or modified hypsarrhythmia on EEG at presentation. (66) The ILAE acknowledges this as part of the syndrome of IESS stating that hypsarrhythmia may be absent early in the disease course or in older children. (2) The temporal and electrophysiological mechanisms underlying hypsarrhythmia are not clearly understood. However, it is recognised that modified patterns may have different aetiological/structural and prognostic significance compared to typical hypsarrhythmia. (62, 66)

Table 1.2 Key interictal EEG abnormalities and distinguishing features between hypsarrhythmia and modified hypsarrhythmia, adapted from several sources (57-65)

EEG feature	Hypsarrhythmia	Modified Hypsarrhythmia
High voltage	Hypsarrhythmia is characterized by high-voltage EEG activity, often exceeding 200 or 300 microvolts based on age at IESS onset. Voltages may reduce in amplitude particularly during non-REM sleep.	In modified forms, there may be periods of generalised, regional or localised voltage attenuation, lasting from two to 10 seconds or similar burst-suppressed patterns. Hemispheric asymmetry or "hemihypsarrhythmia" may dominate, where voltage and background waveforms consistently differ over one hemisphere, often linked to structural abnormalities.
Irregular Slow Waves	Generalised irregular, high-amplitude slow waves interspersed with spikes.	Slow waves may become more periodic or focal, suggesting abnormal anatomical focus.
Continuous slow wave activity	Slow wave activity in hypsarrhythmia is nearly continuous, present during both wakefulness and sleep. Activity is often seen in NREM sleep and may be absent or minimal in REM sleep.	Periods of more organised background rhythms or other waveforms are seen including voltage attenuation. Temporal variations are also seen during sleep-wake states.
Disorganisation - Arrhythmia	EEG appears chaotic or highly disorganised with no consistent rhythm or pattern, random high-voltage slow waves and multifocal spikes are seen that vary in duration and location.	Periods of arrhythmia may alternate with more organised or recognisable awake or sleep rhythms "relative normalisation". Hemispheric asymmetry or "hemihypsarrhythmia" may be present.
Disorganisation - Asynchrony	Inter and intra-hemispheric asynchrony is present characterised by independent waveforms occurring at asynchronous times within the same and contralateral hemisphere.	Bursts of synchronous waveforms and/or synchronisation between hemispheres suggesting periodicity.
Spike and sharp waves	Classically waveforms are multifocal, random or independent, they may be generalised.	There is a focality to waveforms, they may be rhythmic or present in a highly organised pattern suggesting consistent anatomical focus. Or there may be very few spike/sharp waves seen.

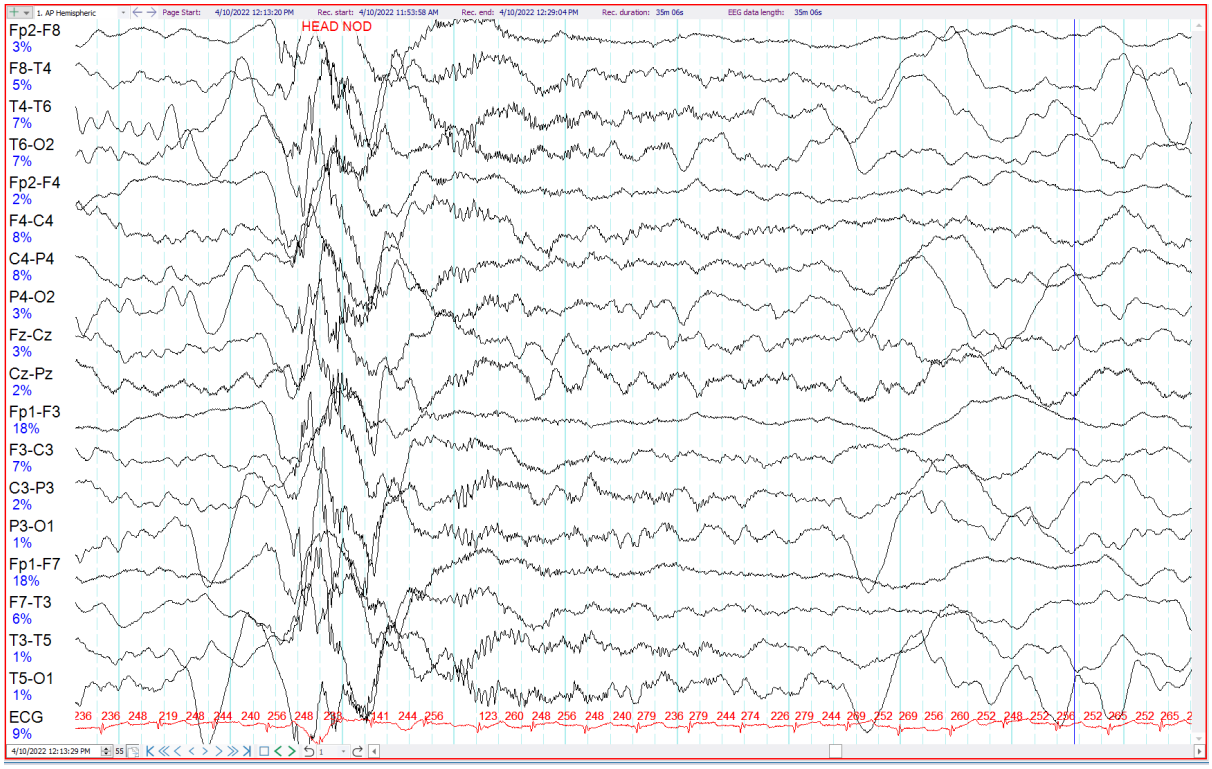
1.6.3.2 Ictal EEG features

The ictal features of epileptic spasms have been well described following the advent of video EEG time-locked with electromyogram (EMG), electrocardiogram (ECG) and electro-oculogram (EOG) channels. In 1979, Kellaway et al reviewed 5, 042 epileptic spasms from 24 infants and described 11 ictal patterns. (38) The three most common EEG patterns seen were, a high-voltage generalised slow wave followed by voltage attenuation in 37.9% of events (See Figure 1.1), a generalised sharp-slow-wave complex in 17.4%, and a generalised sharp-slow-wave complex followed by attenuation in 13.2%. (38) Several studies replicated these findings and noted that voltage attenuation or electro-decrement may not be seen and may or may not represent a post-ictal rather than ictal phenomenon. (39, 67-70) Most authors would agree that the electro-decrement is typical of a tonic contraction. Similarly, there are reported variations of the paroxysms of fast activity, either >15Hz (70) or lower frequency spindle-like activity overriding the slow/sharp wave complex. (39) Another study identified bursts of 15–23 Hz activity preceded the onset of epileptic spasms by 500 milliseconds. (71) The ictal EEG pattern is often generalised but can be focal, particularly in IESS with structural abnormalities. (39, 67-70) When generalised, the location of the waveform with maximal amplitude may differ, although is typically seen in the frontal or central regions. (4, 38, 39)

Overall, there are variations to the ictal EEG pattern, and studies have confirmed there is not always a clear correlation between this pattern and type of epileptic spasm (flexor/extensor/mixed).(68) In summary, the most recognisable ictal EEG pattern of an epileptic spasm is a high amplitude generalised slow-sharp wave complex with overriding fast activity followed by electro-decrement.

Figure 1.1 Ictal EEG pattern of an epileptic spasm

Exemplar EEG demonstrating a generalised high voltage slow wave with overriding fast activity followed by a generalised voltage attenuation known as electro-decrement.



1.6.4 IESS as a neurodevelopmental syndrome

The ILAE classes IESS as a developmental and epileptic encephalopathy (DEE). (2, 43) IESS is the most common DEE and considered the prototypic example. DEE recognises there is a spectrum encompassing a "developmental encephalopathy" as well as an "epileptic encephalopathy, which patients may move between. (43) The "developmental encephalopathy" emphasises that developmental impairment or delay is present throughout the syndrome, and part of the underlying NDD. (37, 43) Whereas "epileptic encephalopathy" refers to periods of increased or frequent epileptiform activity and seizures that interferes with development causing a cognitive slowing or regression which may be associated with behavioural or psychiatric disturbances. (37, 43)

Several older studies have reported 52-86% of infants have developmental impairment that precedes the onset of IESS. (49, 72-75) This was more common in infants with known aetiology including structural or genetic disorders. (49, 72-75) One recent cohort study examined the developmental profile of 95 infants with newly diagnosed IESS

using the Bayley Scales of Infant and Toddler Development and a clinical assessment of visual, auditory and social behaviours. (76) More than 90% of infants had developmental delay in all domains and 99% had cognitive impairment. (76) After cognition, motor and communication domains were most frequently affected. Within each domain, the majority of infants (66-85%) had severe delays. (76) The relationship to IESS aetiology was not explicitly explored, although risk factors associated with developmental impairment included delay in presentation of more than 28 days, pre-term birth, HIE and neonatal infections. (76) Delay to presentation and treatment has also been associated with poor long-term neurodevelopmental outcomes, (41, 77-81) suggesting a prolonged period of "epileptic encephalopathy" may have significant long-term impacts, regardless of treatment response.

1.7 Treatment of IESS

It is widely recognised that IESS is a drug-resistant epilepsy, that is refractory to standard anti-seizure medications. Since ACTH and prednisolone were first used in 1958 (10) and 1962 respectively (82), over 200 studies have assessed the efficacy of various medications including vigabatrin, valproate, pyridoxine, nitrazepam, zonisamide, topiramate, sulthiame, levetiracetam. (4, 83) ACTH, steroids and vigabatrin are considered the most effective, however treatment regimens including dose, frequency and duration of therapy varied widely based on medication accessibility, cost, side-effect profile and geographical preferences. (84) For example, natural porcine-derived ACTH preparations (Acthar Gel) were commonly used in the United States of America, whilst synthetic ACTH tetracosactide and Synacthen were preferred in the United Kingdom.

Between 2004 and 2018, two landmark multi-centre randomised controlled trials (RCTs), the United Kingdom Infantile Spasms Study (UKISS, 2004) (18) and the International Collaborative Infantile Spasms Study (ICISS, 2017) (14) established the evidence base for standardised treatment protocols in IESS. UKISS compared hormonal therapy, ACTH (tetracosactide) to vigabatrin, while ICISS evaluated hormonal therapy combined with vigabatrin versus hormonal therapy alone. (14, 18) Both trials included longitudinal follow-

up studies assessing epilepsy and neurodevelopmental outcomes at age 14-18 months (17, 85) and UKISS also included outcomes at age four years. (86). Both examined the impact of aetiology, age at IESS onset and lead-time from IESS onset to treatment response on epilepsy and neurodevelopment. (14, 77, 85) The study design, primary and secondary outcomes are presented in Table 1.3 and summarised below. Importantly both trials excluded IESS secondary to TSC.

Table 1.3 UKISS (2004, 2005, 2010, 2011), ICISS (2017, 2018) and combined (2023) studies comparing short term efficacy of hormonal treatment (ACTH, or prednisolone), vigabatrin or combination treatment and long-term epilepsy and neurodevelopmental outcomes

Study, Author (year)	Study design, Eligibility criteria	Participants and Follow-up	Intervention	Epilepsy Outcomes	Developmental Outcomes
UKISS Lux et al. (2004)	Multi-centre RCT Inclusion criteria: IESS, clinically diagnosed with hypsarrhythmia (or variant) on EEG Exclusion criteria: Age onset <2m or > 12m, TSC, contraindication to Rx	Participants: *n*=107 (64M/43F) Median age 5m *Hormonal Rx* *n*=55 *Vigabatrin Rx* *n*=52 Duration FU: 14d	*Hormonal Rx* Prednisolone 40mg/d (*up to 60mg/d) *n*=30 ACTH 40IU alt days (*up to 60IU) *n*=25 for 14d then 2w wean *Vigabatrin Rx* 100mg/kg/d [#up to 150mg/kg/d] for 14d	**EPILEPSY – Primary outcomes** Epileptic spasm cessation at 13-14d of Rx: *Hormonal Rx*: *n*=40/55 (73%) vs - Prednisolone: *n*=21/30 (70%) - ACTH: *n*=19/25 (76%) *Vigabatrin Rx*: *n*=28/52 (54%) *Hormonal vs Vigabatrin*, Difference 19%, *p*=0.043 *Prednisolone vs ACTH*, Difference 6%, *p*=0.61	N/A
UKISS Lux et al. (2005)	UKISS (2004) RCT	Participants: *n*=101/107 (5 died, 1 withdrew) median age 14m Duration FU: Aged 12-14m	As above	**EPILEPSY (aged 12-14m) – Primary outcomes** Absence of spasms: *Hormonal Rx*: *n*=41/55 (75%) vs *Vigabatrin Rx*: *n*=39/51 (76%) Difference 1%, *p*=0.82 Absence of spasms and seizures *Hormonal Rx*: *n*=28/55 (51%) vs *Vigabatrin Rx*: *n*=32/51 (63%) Difference 12%, *p*=0.17	**DEVELOPMENT (aged 12-14m)-Primary outcomes** VABS score -all IESS aetiology *Hormonal Rx*: 78.6 (*n*=55) vs *Vigabatrin Rx:* 77.5 (*n*=52) Difference 1.1, *p*=0.73 **Secondary outcomes** *VABS score* - unknown aetiology *Hormonal Rx:* 88.2 (*n*=24) vs *Vigabatrin Rx:* 78.9 (*n*=21) *D*ifference 9.3, *p*=0.025 VABS score - if epileptic spasms present or not *Absence spasms: 80.9 (SD 15) vs* *Presence spasms: 72.7 (SD 13.4)* Difference: 8.2, *p=0.008*

UKISS Darke et al (2010)	UKISS (2004) RCT	Participants: *n*=77/107 (9 died, 14 declined, 7 lost to FU) mean age 4.2y Duration FU: Aged 4y	As above	**EPILEPSY (aged 4y) – Primary outcomes** Presence of epilepsy *Hormonal Rx*: *n*=17/39 (44%) vs *Vigabatrin Rx*: *n*=21/38 (55%) Difference 10%, *p*-value not reported	**DEVELOPMENT (aged 4y) – Primary outcomes** VABS score- all IESS aetiology *Hormonal Rx*: 60 (*n*=39) vs *Vigabatrin Rx*: 50 (*n*=38) Difference 10, *p*=0.091 **Secondary outcomes** VABS score-Unknown aetiology *Hormonal Rx:* 96 (*n*=21) vs *Vigabatrin Rx:* 63 (*n*=16) Difference 33, *p*=0.033
UKISS O'Callaghan et al (2011)	UKISS (2004) RCT	Participants: *n*=77/107 (9 died, 14 declined, 7 lost to FU) mean age 4.2y Duration FU: Aged 4y	As above	**EPILEPSY (aged 4y) -Secondary outcomes** Longer lead-time to Rx associated with higher prevalence of epilepsy <7d: 30% 8-14d: 35% 15-28d: 30% 1-2m: 37% >2m: 57%, *p*=0.023	**DEVELOPMENT (aged 4y) -Secondary outcomes** Longer lead-time to Rx associated with lower VABS score (mean) <7d: 76.2, *p*=0.014 8-14d: 62.8, *p*=0.003 15-28d: 65.4, *p*=0.03 1-2m: 65.3, *p*=0.003 >2m: 55.5, *p*=0.014
ICISS O'Callaghan et al (2017)	Multi-centre RCT Inclusion criteria: IESS, clinically diagnosed with hypsarrhythmia (or variant) on EEG Exclusion criteria: Age onset <2m or > 14m, >7d delay from dx to Rx, TSC, past Rx for IESS, unavailable for FU,	Participants: *n*=377 (210M/167F) Median age 6.8m *Hormonal Rx* *n*=191 *Combination Rx (Hormonal + Vigabatrin)* *n*=186 Duration FU: 14d	*Hormonal Rx* Prednisolone 40mg/d (*up to 60mg/d) *n*=131 ACTH 40IU alt days (*up to 60IU) *n*=60 for 14d then 2w wean *Combination Rx:* Vigabatrin100mg/kg/d [$^{\alpha}$up to 150mg/kg/d] for 3m then 4w wean Hormonal as above *n*=135 prednisolone, *n*=51 ACTH	**EPILEPSY – Primary outcomes** Epileptic spasm cessation between d 14-42 of Rx: *Hormonal Rx*: *n*=108/191 (57%) vs *Combination Rx*: *n*=133/186 (72%) Difference 15%, *p*=0.002	**DEVELOPMENT** N/A

	contraindication to Rx				
O'Callaghan et al (2018)	ICISS (2017) RCT	Participants: *n*=362/377 (7 died, 6 lost to FU, 2 withdrew) median age not provided Duration FU: Aged 18m	As above	**EPILEPSY (aged 18m) – Primary outcomes** Presence of epilepsy *Hormonal Rx*: *n*=52/178 (29%) vs *Combination Rx: n*=54/180 (29%) Difference 0.8%, *p*=0.90 Presence of epileptic spasms *Hormonal Rx*: *n*=27/180 (15%) vs *Combination Rx*: *n*=28/178 (15%) Difference 0.7%, *p*=0.85	**DEVELOPMENT (aged 18m) – Primary outcomes** VABS score - all IESS aetiology *Hormonal Rx*: 72.7 (*n*=181) vs *Combination Rx:* 73.9 (*n*=181) Difference 1.2%, *p*=0.55 **Secondary outcomes** VABS score- unknown aetiology *Hormonal Rx*: 81.5 vs *Combination Rx:* 83.5 Difference 2, *p*=0.52 VABS score (if epileptic seizures present or not) *Absence seizures:* 79 vs *Presence seizures:* 60.5 Difference: 18.5, *p*<0.001
Osbourne et al (2023)	UKISS (2004) & ICISS (2017) RCTs	Participants: *n*=126 Median age 6 & 7m *ACTH Rx* *n*=191 *Prednisolone Rx* *n*=186 Duration FU: 14d, 14-42d	As per UKISS	**EPILEPSY – Primary outcomes** Epileptic spasm cessation at 13-14d of Rx *ACTH Rx*: *n*=47/62 (76%) vs *Prednisolone Rx*: *n*=43/64 (67%) Difference 9%, *p*=0.28	**DEVELOPMENT** N/A
		Participants: *n*=121-124/126 Duration FU: Aged 14 & 18m	As above	**EPILEPSY (aged 14, 18m) – Primary outcomes** Presence of epilepsy *ACTH Rx*: *n*=20/61 (33%) vs *Prednisolone Rx*: *n*=26/643 (41%)	**DEVELOPMENT (aged 14, 18m)) – Primary outcome** VABS score - all IESS aetiology *ACTH Rx*: 78 (*n*=61) vs *Prednisolone Rx*: 74.8 (*n*=60)

				Difference 8%, *p*=0.33 <u>Presence of epileptic spasms</u> *ACTH Rx*: 19% vs *Prednisolone Rx*: 20% Difference 1%, *p*-value not provided	Difference 3.2, *p*=0.36

Key: ACTH=adrenocorticotrophin hormone, d=day, EEG=electroencephalogram, F=female, FU=follow up, ICISS= International Collaborative Infantile Spasms Study, IESS=infantile epileptic spasm syndrome, IU=international units, M=male, m=month, N/A=not applicable, Rx=treatment, TSC=tuberous sclerosis complex, VABS=vineland adaptive behaviour scales, vs=versus, w=week, UKISS=United Kingdom Infantile Spasms Study, y=year, *doses escalated on day eight if epileptic spasms were ongoing, #dose escalated on day five if epileptic spasms ongoing, α=dose escalated on day three if epileptic spasms ongoing. Please note this table includes selected clinical measures from the seven trials included.

1.7.1 Epilepsy outcomes

ACTH or prednisolone remain first-line treatment for IESS (apart from TSC), based on evidence from the UKISS RCT (18) and three follow-up studies. (17, 77, 86) The UKISS trial compared the efficacy of hormonal therapy (ACTH or prednisolone) versus vigabatrin in achieving cessation of epileptic spasms by day 13–14 of treatment. The trial enrolled 107 infants with median age of five months at IESS onset. Of 107 infants randomised, 55 received hormonal treatment (30 prednisolone, 25 ACTH) and 52 received vigabatrin. After 14 days, ACTH/prednisolone achieved a significantly higher rate of epileptic spasm cessation (73%, *n*=40/55) compared to vigabatrin (54%, *n*=28/52), with a 19% difference (95% CI 1%–36%, *p*=0.043). (18) No significant differences were observed between ACTH and prednisolone treatments (76% vs 70%, *p*=0.61), or based on aetiology. (18) Electroclinical response including resolution of hypsarrhythmia was higher following ACTH/prednisolone treatment compared to vigabatrin (81% vs 56%, *p*=0.024). (18) Adverse event frequencies were similar between groups, with ACTH/prednisolone causing irritability, hunger, and hypertension, and vigabatrin causing drowsiness. (18)

At age 12-14 months, 101 infants (94%) were available for follow-up. (17) Seventy-five percent of infants were free of epileptic spasms, with similar rates between ACTH/prednisolone (75%) and vigabatrin (76%) treatment groups. (17) The proportion of infants that remained free of epileptic spasms without relapse was also comparable (ACTH/prednisolone 40% vs vigabatrin 37%), as was the freedom rate from epileptic spasms and seizures (ACTH/prednisolone 51% vs vigabatrin 63%). (17) Although ACTH/prednisolone led to earlier cessation of epileptic spasms, long-term spasm and seizure freedom rates were similar between all treatment groups.

Similar findings were seen at age four years, 77 infants (72%) from the original cohort were assessed, however, analysis was by intention-to-treat. (86) The proportion of infants with ongoing epileptic spasms (ACTH/prednisolone 13% vs vigabatrin 13%) and/or epilepsy (ACTH/prednisolone 44% vs vigabatrin 55%) was comparable between treatment groups. Irrespective of treatment, infants with known aetiology had significantly higher rates of epilepsy (62%) compared to those with unknown aetiology (35%), *p*=0.021. (86)

Longer lead times between epileptic spasms onset and initiation of treatment also correlated with higher epilepsy rates. (77) Among infants treated within seven days from epileptic spasm onset, 30% had epilepsy at four years, compared to 57% in those with lead times exceeding two months (p=0.023). (77)

The ICISS trial published in 2017 expanded on UKISS findings by evaluating whether combining vigabatrin with ACTH/prednisolone was superior in achieving epileptic spasms cessation between days 14-42 of treatment compared to ACTH/prednisolone alone. (14) Three hundred and seventy-seven infants, with median age 6.8 months at IESS onset were randomized, 186 received combination treatment, vigabatrin with hormonal treatment (135 prednisolone, 51 ACTH) and 191 received hormonal treatment (131 prednisolone, 60 ACTH) alone. Combination therapy resulted in a higher rate of epileptic spasm cessation (72% vs 57%, difference 15.0%, 95% CI 5.1–24.9; p=0.002) and a shorter median time to cessation (two days vs four days, p<0.001). (14) Electro-clinical improvement was also more frequent with combination therapy (66% vs 55%, p=0.023). (14) Rates of epileptic spasms cessation were higher for infants with unknown aetiology that received combination treatment compared to hormonal therapy alone (85% vs 60%, p<0.001). (14) Adverse events did not significantly differ between treatment groups. (14)

In the ICISS trial, 362 infants (96%) were re-assessed at 18 months. (85) Similar findings to UKISS were identified, namely that the proportion of infants with ongoing epileptic spasms (combination 15% vs hormonal 15%) and/or epilepsy (combination 29% vs hormonal 29%) were comparable between treatment groups. (85) Similarly, infants with known aetiology had significantly higher rates of epilepsy compared to those with unknown aetiology (35 % vs 22.4%, p=0.021) irrespective of the treatment received. (85) Longer lead times were again associated with higher rates of epilepsy (p=0.023) and a higher likelihood of ongoing IESS at 18 months (p=0.0007). (85)

In 2023, a subsequent analysis examined epilepsy outcomes comparing ACTH and prednisolone treatment, drawing on data from both the UKISS and ICISS trials. (87) Of 126 infants randomised, 62 received ACTH while 64 received prednisolone. The rates of epileptic spasms cessation at days 13-14 and between days 14-42 were comparable between ACTH

(76% and 67% respectively) and prednisolone (67% and 56% respectively) treatment groups. (87) The presence of a known aetiology and longer lead time to treatment were not significantly associated with epileptic spasms cessation. (87) At 14-18 months of age, the prevalence of epilepsy was similar between ACTH and prednisolone treatment groups (33% vs 41%, *p*=0.33). (87) The rates of ongoing epileptic spasms were also comparable between ACTH (20%) and prednisolone (19%) treatment groups. (87)

These studies indicated that although combination and hormonal treatment achieve earlier cessation of epileptic spasms than vigabatrin alone, the long-term rates of epilepsy and presence of ongoing epileptic spasms are comparable. (14, 17, 18, 77, 85-87) Only infants with unknown aetiology and shorter lead time to treatment appear to have more favorable epilepsy outcomes. (77, 85-87) ACTH and prednisolone demonstrated similar efficacy. (87) In Australia, prednisolone is preferred as first-line therapy for IESS with the sequential addition of vigabatrin for non-responders. (88) Prednisolone is preferred given its lower cost, (89) oral administration, and comparatively favourable side-effect profile relative to ACTH (90-93) and vigabatrin. (94, 95) ACTH requires special handling under medical supervision whilst some infants may be precluded from using vigabatrin due to pre-existing visual field defects and long-term concerns regarding risk of retinal toxicity, (95-98) movement disorder and/or MRI brain abnormalities. (95, 98-102)

1.7.2 Developmental outcomes

Two UKISS follow-up studies assessed whether early cessation of epileptic spasms improved developmental outcomes at 12-14 months (17) and four years (86) using the Vineland Adaptive Behaviour Scales (VABS). (103) At 12-14 months, mean VABS scores were within the average range (70-120) and did not significantly differ between ACTH/prednisolone (78.6, SD 16.8) and vigabatrin (77.5, SD 12.7) groups (*p*=0.73). (17) However, a sub-analysis showed that infants with unknown aetiology treated with ACTH/prednisolone had significantly higher mean VABS scores (88.2) than those treated with vigabatrin (78.9), with a 9.3% difference (95% CI 1.2–17.3, *p*=0.025). (17) Infants without ongoing epileptic spasms also had higher VABS scores than those with ongoing

epileptic spasms (80.9 vs 72.7, *p*=0.008), (17) suggesting that epileptic spasm and seizure freedom are associated with improved development.

At age four years, median VABS scores were lower than at 14 months, falling into the "mildly impaired functioning" category, although they did not significantly differ between ACTH/prednisolone and vigabatrin treatment (60 vs 50, *p*=0.09). (86) Infants with unknown aetiology assigned to ACTH/prednisolone again had higher VABS scores compared to vigabatrin (96 vs 63, *p*=0.033). (86) The association between epilepsy and VABS scores was not explored. (86) A further analysis demonstrated that earlier age at IESS onset and longer lead time from onset to treatment were associated with lower VABS scores at four years. (77) Lead time from diagnosis to treatment was categorised into five timepoints, less than seven days, eight to 14 days, 15 days to one month, one to two months and more than two months. For each month earlier in the age that epileptic spasms began, VABS scores reduced by 3.1 points (95% CI 0.64-5.5, *p*=0.03), and for each increase in lead time category, scores dropped by 3.9 points (95% CI 7.3-0.4, *p*=0.014). (77) There was no defined age or lead time category associated with a poor prognosis. Of note, ACTH/steroids appeared to offer a protective effect on development for infants with unknown aetiology (*p*=0.04). (77)

The ICISS follow-up study at 18 months of age, found mean VABS scores did not significantly differ between the combination (vigabatrin and hormonal) treatment group compared to hormonal treatment alone (73.9 vs 72.7, *p*=0.55). (85) VABS scores were significantly higher in infants with unknown compared to known aetiologies (82.5 vs 66.8, *p*<0.001), independent of treatment. (85) Mean VABS scores were higher in infants with earlier spasms cessation between days 14-42 compared to those without (79.1 vs 63.2, *p*<0.001). (85) Similarly, at 18 months, infants without seizures had higher VABS scores compared to those with seizures (79 vs 60.5, SD 18.2, *p*<0.001). (85) Longer lead times were again associated with lower VABS scores (*p*<0.001). (85) These findings indicated that a known IESS aetiology and ongoing epilepsy strongly impacted development.

Unpublished results from the ICISS follow-up of 351 infants (93%) demonstrated at age 3.5 years median VABS scores were lower than at 14 months (64 vs 73), although they did not differ between combination treatment or hormonal treatment alone (63 vs 65, *p*=0.99).

(104) Early clinical responders had higher median VABS score compared to non-responders (72.5 vs 52, $p<0.001$). (104) The influence of lead-time and aetiology were not presented.

The 2023 analysis comparing ACTH to prednisolone treatment found no significant difference in mean VABS score aged 14-18 months between the 61 infants who received ACTH (78·0, SD 20.2) versus the 60 infants who received prednisolone (74·8, SD 18·3, $p=0·36$). (87) Similar trends were seen that longer lead times to treatment, known IESS aetiology and ongoing epilepsy strongly affected development. (87)

Key findings from these studies indicate that initial treatment type does not influence long-term developmental outcomes and that VABS score decline and deviate from age-appropriate norms between the second and fourth year of life. ACTH and steroids may confer a protective effect on development for those with unknown aetiology however, this requires further confirmation. Factors impacting development include underlying aetiology, lead time and initial response to treatment and presence of ongoing epilepsy.

1.7.3 Summary of key findings from UKISS and ICISS

Overall, while ACTH, prednisolone and vigabatrin may initially control epileptic spasms, it remains unclear whether long-term epilepsy or neurodevelopmental outcomes are altered due to initial epilepsy control or treatment changing the disease course. Infants with unknown aetiology and shorter lead time to treatment appear to have more favorable outcomes. Better development is associated with epileptic spasm and seizure freedom, and some benefit is seen for infants with unknown aetiology treated with ACTH/steroids. These studies highlight that IESS is a DEE where the “epileptic encephalopathy” may initially respond to treatment however, the underlying “developmental encephalopathy” persists in a large proportion of infants across different aetiological groups. In Australia, prednisolone is a preferred first line treatment option, given its low cost, ease of administration and comparably better side-effect profile.

1.8 Prognosis and Long-term Outcomes

Prior to the availability of effective treatments for IESS, large cohort studies described the natural history and spontaneous resolution of epileptic spasms and hypsarrhythmia. Two large cohort studies examining 837 infants found that in the majority of infants both the clinical and EEG features resolved between three (75) and five years of age. (72) Additional cohort studies described spontaneous cessation of epileptic spasms in 25-28% of infants by one year of age, (23, 105) 49% by age two years, 65% by age three years and 74% by four years. (23) A proportion of these infants were treated with phenytoin, phenobarbitone or other derivates, generally considered ineffective for IESS, (105) whilst a minority had hormonal treatment. (23)

The UKISS and ICISS trials demonstrated that although first-line treatments effectively stop epileptic spasms, at four years of age the rates of ongoing epileptic spasms (13%) and epilepsy (50%) are unchanged. (86) IESS progresses to other seizure types and epilepsy syndromes in 50-70% of infants, and the majority are considered drug-resistant epilepsies (40-60%). (4, 22, 46, 78, 106). Lennox-Gastaut syndrome, a severe DEE, develops in 15-25% of these infants, (22, 78, 106, 107) although others have reported rates of up to 50%. (24)

Cognitive impairment including moderate to severe learning difficulties occurs in 70-90% of infants with IESS. (24, 46, 78, 107) A minority with normal cognitive intelligence have specific learning difficulties in areas such as fine motor function, reading or mathematics. (46) Autism Spectrum Disorder (ASD) develops in 35-40% of infants (78, 108) and there is a long-term risk of other psychiatric disorders. (46)

Mortality can be significant ranging between 10-35%. (24, 46) Long-term studies following over 200 infants with IESS for 20-50 years have reported 25% died at mean age of 17.2 years and 50% by 48.6 years. (109) Death at a younger age tended to relate to treatment complications (sepsis, cardiomyopathy), (46) whereas cause of death in older adults was related to medical conditions including pneumonia (47%) or epilepsy (17%). (109) Known IESS aetiology was associated with a fivefold risk of death compared to unknown aetiologies. (109)

Favourable prognostic factors in IESS include an unknown aetiology, (46, 70, 77, 78, 107, 110) normal development before IESS onset, (22, 46, 111, 112) prompt treatment (41, 78-81) within 28 days from epileptic spasms onset (77, 112-114) and an early response to treatment. (77, 78, 85, 86) Other factors include a short duration of hypsarrhythmia , (66, 112, 115) treatment of multifocal epileptic discharges (70, 116, 117) and early management of epileptic spasms relapses and adverse effects of treatment. (46, 78) As demonstrated by the UKISS and ICISS trials, the type of treatment does not significantly alter long-term outcomes. (14, 17, 85-87) However, time to treatment is a major modifiable risk factor. (118) One study surveying 100 parents identified a median of 24.5 days from IESS onset to the first visit to an effective care provider skilled at diagnosing and managing IESS. (118)

1.9 Summary

IESS is the most common epilepsy syndrome in infants and has been the focus of research for the past 180 years. However the mechanisms underlying this disorder remain unclear, particularly regarding the biological mechanisms associated with poor prognosis. Our current first-line treatments may stop epileptic spasms however long-term outcomes are unchanged and include significant morbidity and disability. To improve outcomes we need to improve our understanding of underlying disease and treatment mechanisms. This represents a significant gap in our knowledge and is explored in Chapter two.

CHAPTER 2 – Literature review: Mechanisms of action of ACTH/corticosteroids in IESS

2.1 Introduction

IESS is the most common epilepsy syndrome in the first year of life, however despite its early description by Dr West in 1841, there have been very few advancements to our knowledge regarding the mechanisms underlying this disorder over the last 180 years. Animal studies have reproduced models of IESS with variable success, however insights remain limited as these animal models cannot capture the complexity and heterogeneity of aetiological mechanisms underlying IESS in children. Similarly, treatment for IESS remains limited to a small number of therapies with established efficacy including ACTH/steroids, vigabatrin and the ketogenic diet. Despite significant advancements to precision medicine in neurology, ACTH/steroids remain first-line treatment for IESS. Currently there are no targeted or disease-modifying treatments available for IESS. This gap in treatment reflects our limited understanding of both IESS pathophysiology and the mechanisms by which ACTH/steroids exert an anti-seizure effect in IESS. These knowledge gaps formed the basis of this thesis. As part of my literature review, numerous hypotheses have been put forward, yet there were limited studies synthesising the evidence in children with IESS that explored disease and therapeutic mechanisms of ACTH/steroids. This formed the basis of my systematic scoping review, presented in this chapter in manuscript form, addressing the current state of understanding, knowledge gaps and areas for future research.

2.2 Aetiopathogenesis of infantile epileptic spasms syndrome and mechanisms of action of adrenocorticotrophin hormone/corticosteroids in children: A scoping review

This manuscript forming Chapter two has been published in Developmental Medicine and Child Neurology, 2025. (1) All pertinent references are contained within the publication and/or listed in supplementary materials in section 2.3. The naming and numbering of the supplementary texts and figures is retained as in the published manuscript for easy referencing.

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SCOPING REVIEW

Aetiopathogenesis of infantile epileptic spasms syndrome and mechanisms of action of adrenocorticotrophin hormone/corticosteroids in children: A scoping review

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Abstract

Aim: To review the aetiopathogenesis of infantile epileptic spasms syndrome (IESS) and mechanisms of action of adrenocorticotrophin hormone (ACTH)/corticosteroids established in humans.

Method: MEDLINE, PubMed, and Embase were systematically searched from inception to December 2023 to identify studies related to IESS aetiology and treatment response. Mechanistic themes were identified and through consensus meetings refined and grouped into five overarching hypotheses.

Results: Five hypotheses were generated from 17 mechanistic themes: (1) gene and epigenetic regulation altering expression of 'vulnerability' genes; (2) stress and hypothalamic–pituitary–adrenal axis activation; (3) neuroinflammation and altered immune function; (4) altered neuronal transmission and pathways; and (5) dysfunction of metabolic pathways.

Interpretation: The evidence that ACTH/corticosteroids alter these processes remains limited. It is plausible that these processes interact with one another, rather than existing independently, and affect maturational and regulatory processes in the central nervous system, consistent with proposals that IESS is a neurodevelopmental disorder. Understanding how ACTH/corticosteroids work in IESS may facilitate disease-modifying treatments and improve neurodevelopmental outcomes.

Abbreviations: ACTH, adrenocorticotrophin hormone; BBB, blood–brain barrier; CRH, corticotrophin-releasing hormone; CRHR1, corticotrophin-releasing hormone receptor type 1; CSF, cerebrospinal fluid; GABA, γ-aminobutyric acid; GO, gene ontology; HPA, hypothalamic–pituitary–adrenal; IESS, infantile epileptic spasms syndrome; Ig, immunoglobulin; IGF-1, insulin-like growth factor 1; IL, interleukin; KYN, kynurenine; KYNA, kynurenic acid; MMP-9, metallopeptidase-9; mRNA, messenger RNA; β-NGF, β-nerve growth factor; TSC, tuberous sclerosis complex; TNF, tumour-necrosis factor.

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Infantile spasms is the most common epilepsy syndrome in the first year of life, with an incidence of 3 per 10 000 live births.[1] Peak onset is between 3 months and 7 months with a slight male predominance.[1] The syndrome is defined by epileptic spasms, which are clusters of brief tonic contractions of the axial musculature, in addition to developmental arrest or regression of skills with a disorganized, high-amplitude background on electroencephalogram (EEG) known as hypsarrhythmia.[1] This triad was previously referred to as 'West syndrome'; however, some infants may have epileptic spasms with a 'modified' hypsarrhythmia or focal EEG abnormalities without developmental changes. These two groups are incorporated by the International League Against Epilepsy as 'infantile epileptic spasms syndrome' (IESS).[1] The aetiology of IESS is highly heterogenous, including more than 200 known causes, classed according to the International League Against Epilepsy as structural, genetic, metabolic, acquired, infectious, and immune aetiologies.[2] Descriptors such as symptomatic (pre-, peri-, post-natal), cryptogenic, and idiopathic spasms are no longer recommended. It is unclear how multiple aetiologies lead to IESS; however, it is proposed that there is a disruption during a critical developmental period, or multiple insults at various time points causing a 'desynchronization' of developmental processes.[3] It is possible that multiple biological pathways converge or intersect to result in the IESS phenotype.[3]

IESS is often refractory to standard antiseizure medications, and first-line treatment is hormonal therapy such as intravenous or intramuscular adrenocorticotrophin hormone (ACTH) or high-dose oral corticosteroids. Vigabatrin is recommended for IESS due to tuberous sclerosis complex (TSC) or may be used in combination with ACTH/steroids for IESS more generally.[4] Recent meta-analyses confirm ACTH and corticosteroids have comparable short-term efficacy, achieving epileptic spasm cessation and resolution of hypsarrthymia in 60% to 70% of children.[5,6] Corticosteroids offer a cost-effective and possibly safer alternative to ACTH.[5,7]

Despite worldwide adoption of these aggressive treatment strategies, IESS has a poor long-term prognosis, 50% to 70% have ongoing epilepsy, 75% are affected by developmental delay/intellectual disability, and 35% to 40% have autism spectrum disorder.[7] Favourable prognostic factors include an unknown aetiology, typical development before IESS onset, and early initiation and response to treatment.[7–10]

ACTH and corticosteroids have been used since the 1950s; however, it is not clear how they exert their mechanism of action in the context of IESS. Reviews exploring IESS aetiology and treatment have largely focused on animal models,[11–13] which are inherently limited by their inability to represent a wide range of aetiological mechanisms, generate a typical epileptic spasm with hypsarrthymia on EEG, and use ACTH/corticosteroid formulations generally not prescribed in children. A recent narrative review and cohort study summarized human and animal studies on neurotrophic factors, neurotransmitters, and markers of inflammation to generate therapeutic proposals.[14] In this review we adopt a systematic approach to explore all published evidence in children with IESS, analysing aetiopathogenesis and the possible mechanism of actions of ACTH/corticosteroids used in clinical practice.

What this paper adds

- Summarizes five aetiopathogenic mechanisms of infantile epileptic spasms syndrome that adrenocorticotrophin hormone (ACTH)/corticosteroids may modify.
- These include altering gene regulation, stress, neuroinflammation, neuronal excitability, and metabolic pathways.
- Limited reproducible findings in large cohorts constrain the evidence in children.
- Altered gene regulation seems plausible for exploration in future studies of large cohorts.
- Future treatments could target mechanisms underlying this neurodevelopmental disorder rather than one-off ACTH/corticosteroids.

METHOD

This scoping review was guided by previously described methodological frameworks that emphasize a flexible and iterative search strategy[15] and written according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR).[16]

Search strategy

To perform this review, MEDLINE, PubMed, and Embase online databases were searched from inception until 20th December 2023. Search terms included a comprehensive list of keywords related to IESS, aetiology, pathophysiology, biomarkers, treatment, and response (further methodology and Medical Subject Headings [MeSH] terms in Appendix S1). To outline genetic causes of IESS, we searched these databases from January 2010 to December 2023 for studies evaluating the yield of genetic testing in IESS cohorts and the associated monogenic and chromosomal disorders (full method in Appendix S2). We only included studies that provided sufficient data to confirm the genetic diagnosis and classify aetiological groups as per International League Against Epilepsy guidelines. Single-gene studies were excluded. References and citing articles were hand-searched.

Eligibility criteria

Eligibility criteria included: (1) a confirmed IESS diagnosis following International League Against Epilepsy definitions; (2) children aged between 0 years and 2 years; (3) aetiology and/or treatment mechanisms explored; (4) ACTH or steroid formulation used in clinical practice for treatment studies; and (5) full-text journal articles. We excluded studies that: (1) evaluated animal and/or experimental data or; (2) investigated alternative hypotheses, for example insulin tolerance, head circumference, and EEG changes without a clear exploration of underlying biological or treatment effects. Studies were not excluded on the basis of their date of publication and every effort was made to translate publications in languages other than English.

Study selection and data extraction

Figure S1 outlines the study selection process. The initial search and screening were performed by EAI; duplicates and articles that did not meet eligibility criteria were excluded. EAI, SSM, and RCD reviewed these studies and undertook three consensus meetings to categorize articles into themes describing both aetiological and therapeutic mechanisms (Figure S1).

RESULTS

The search retained 124 studies, which were categorized into 17 themes (Figure S1). These themes could be grouped into five hypotheses (Figure 1): altered gene regulation, stress, neuroinflammation, altered neuronal networks, and altered metabolic pathways. These hypotheses are explored sequentially, focusing on the possible aetiopathogenic mechanisms of IESS and potential antiseizure mechanism of action of ACTH/steroids aligned to each hypothesis. Where possible results were summarized as heat maps to highlight any statistically significant differences in the hormonal, immune, and metabolic profiles of children with IESS compared with controls at baseline and the effect of treatment. Studies without a useful control population or clear pretreatment baseline data were excluded from the heat maps.

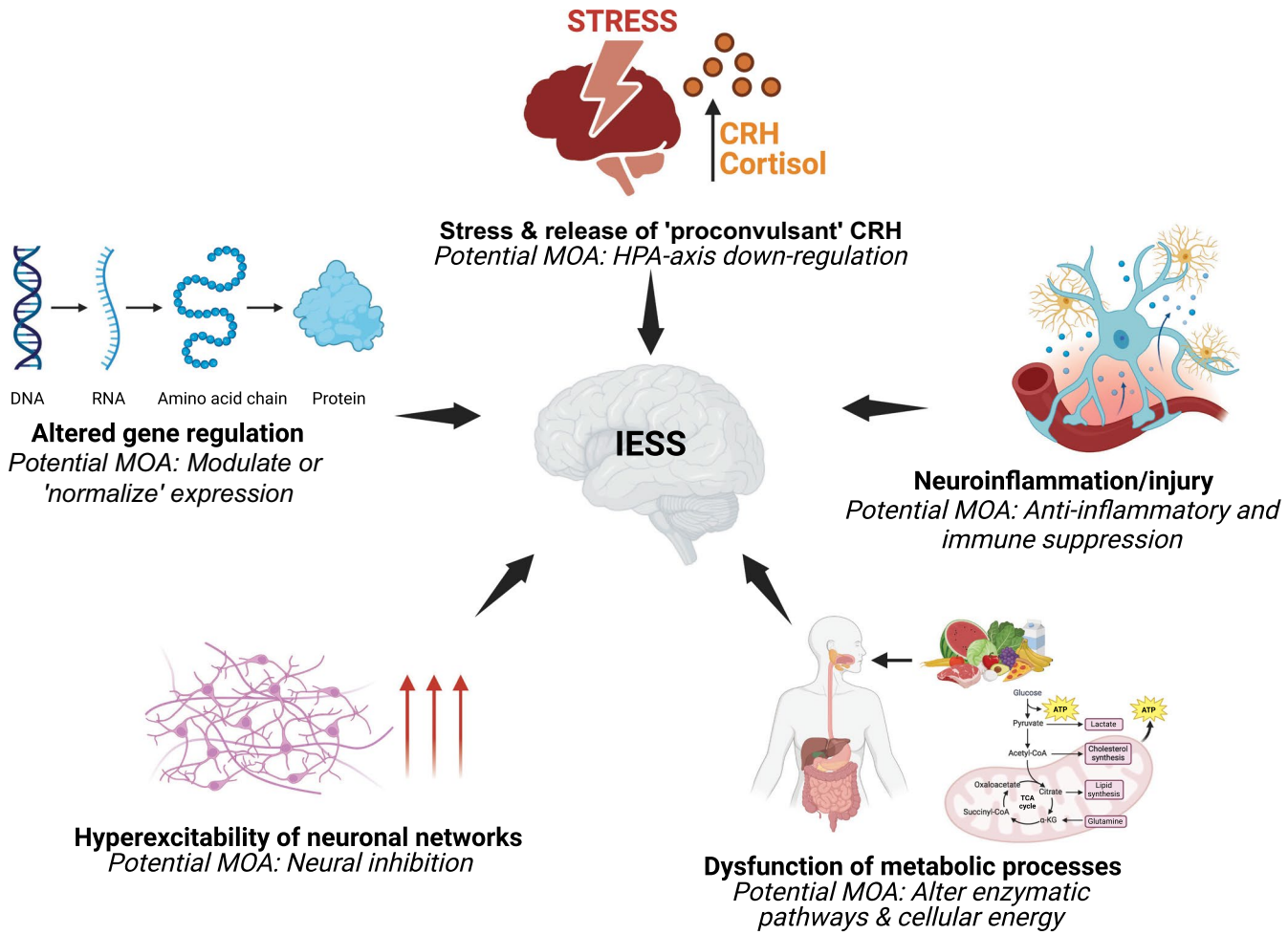

FIGURE 1 The five aetiopathogenic mechanisms that have been proposed to cause IESS, and the potential mechanism of action of ACTH/corticosteroids. Created with BioRender.com. Abbreviations: CRH, corticotrophin-releasing hormone; HPA, hypothalamic–pituitary–adrenal; IESS, infantile epileptic spasms syndrome; MOA, mechanism of action.

Putative mechanisms underlying IESS and the action of ACTH/steroids

Gene variation and gene regulation

This hypothesis was composed of three themes (Figure S1): (1) highly penetrant DNA variations including 24 studies that described pathogenic or likely pathogenic genetic aetiologies for IESS and two studies that analysed biological pathways associated with IESS; (2) DNA polymorphisms explored by nine studies; and (3) one study examining gene regulation and epigenetics. In total, 35 studies explored how altered gene variation/expression may influence IESS onset and whether ACTH/steroids modify gene expression and one study explored epigenetic aspects and gene regulation in the context of IESS.

Highly penetrant DNA variation

An aetiological cause for IESS is identified in 56% to 64% of children,[8,17] leaving a significant proportion 'unknown', which are often presumed to be genetic. Twenty-four studies that describe the genetic aetiology of IESS are outlined in Tables 1–3. These studies are presented in three groups: (1) IESS cohorts including 'all aetiologies' to identify the prevalence of aetiological subcategories and yield of genetic testing (Table 1);[17–24] (2) IESS with 'presumed/confirmed' genetic aetiology and yield of testing (Table 2);[25–39] and (3) IESS with confirmed genetic diagnoses (Table 3).[40]

From IESS studies examining 'all aetiologies', 51% of 2116 children had 'unknown aetiology', as presented in Figure 2a. This is probably a reflection of the variability of investigations performed (details in Table 1). The second largest aetiological group was structural (acquired/congenital) disorders (21%) followed by genetic (14%), genetic–structural (11%), metabolic (2%), and infectious (1%) aetiologies. The diagnostic yield from all genetic tests combined was 22% (467 out of 2116) in IESS including 'all aetiologies' (Table 1), and 20% (189 out of 936) in IESS 'presumed/confirmed genetic aetiology' (Table 2). The yield of individual tests was similar in 'all aetiology' and 'presumed/confirmed genetic aetiology' cohorts: chromosomal microarray (8% vs 9%), epileptic encephalopathy panel (23% vs 25%), and whole-exome/genome sequencing (29% vs 21%). Karyotyping was diagnostic in 6% of the IESS 'all aetiology' cohort, with trisomy 21 the most prevalent chromosomal disorder.

Figure 2b demonstrates the significant genetic heterogeneity of monogenic disorders associated with IESS. On the basis of our review, we identified 51 genes that were reported as pathogenic/likely pathogenic in two or more individuals from the IESS cohorts in Tables 1–3. The 10 most prevalent monogenic disorders were *TSC2* (22% of all monogenic cases), *TSC1* (5%), *STXBP1* (13%), *CDKL5* (12%), *SCN2A* (5%), *KCNQ2* (5%), *ALG13* (3%), *ALDH-7A1* (3%), *KCNB1* (2%), and *SCN1A* (2%). We further performed a gene ontology (GO) pathway analysis[41] using slim terms to identify the key biological processes (Figure 2c) and molecular functions (Figure 2d) shared by these 51 genes (method summarized in Figure 2). The top GO biological processes pathways enriched by these genes included anatomical structural development, signalling, and transmembrane transport whereas the top GO molecular functions pathways involved catalytic and transporter activity and RNA binding.

A previous study used GO pathway analysis to compare pathogenic variants in IESS to other infantile epilepsies:[42] the GO pathways enriched by genes in IESS included broad immunological, developmental, and regulatory pathways of the central nervous system (CNS) including cell cycle regulation and tumorigenic processes (microRNA regulation).[42] Another study performed GO pathway analysis and protein–protein interaction networks to investigate pathways enriched by genes in novel copy number variants in IESS.[43] Pathways over-represented involved ventral forebrain development and synaptic function (transport and signalling pathways).[43] Haploinsufficiency was examined; however, the pathogenicity of certain novel microduplications was not able to be assigned.[43]

DNA polymorphisms

DNA polymorphisms, or single-nucleotide polymorphisms, are the most common type of genetic variation, which may confer susceptibility or resistance to disease. Single-nucleotide polymorphisms are defined by their presence in more than 1% of the population.[44] Investigators have pursued hypotheses that polymorphisms in genes encoding the glucocorticoid receptor (*NRC3C1*), melanocortin receptor (*MC2R*, *MC3R*, *MC4R*), and corticotrophin-releasing hormone receptor (*CRH1*) could affect the stress response by increasing the risk of IESS or modifying response to ACTH.[45–49] Polymorphisms of genes involved in neurotransmission were also studied including *SCN1A* (encoding sodium channels), *GRIN1* (*N*-methyl-D-aspartate, NMDA receptor), *5-HTT* (serotonin transporter), and *ABCB1*, a gene encoding P-glycoprotein, an efflux pump of the blood–brain barrier (BBB).[50–53] Although single-nucleotide polymorphisms in *MC2R*, *MC4R*, *NRC3C1*, *SCN1A*, *GRIN1*, and *5-HTT* were associated with ACTH response,[50–52] the significance of these findings is unclear and limited by their targeted hypothesis-driven approach (as opposed to genome-wide association studies) and lack of replication by other studies.

Gene regulation and epigenetics

Gene regulation can be analysed through RNA sequencing, proteomics, and epigenetic studies. No studies were identified that have used untargeted RNA or proteomic sequencing in blood or brain tissue to identify aetiological or therapeutic mechanisms in IESS.

Epigenetic regulation involves chemical modifications to the genome that switches genes 'on' or 'off' without altering the DNA sequence. This includes DNA methylation, histone modification, or altering non-coding RNAs. Epigenetic modifications can be induced by stress and environmental and lifestyle factors. One study demonstrated lower global DNA

TABLE 1 Studies of IESS which included all aetiologies and subgroups are presented (eight studies with total 2116 cases), including inclusion/exclusion criteria, the breakdown of aetiological subgroups, genetic testing used and yield, and the monogenic causes found.

Study	IESS cohort (*n*)	Selection criteria	Aetiological subgroups (*n*, %)	Method and yield of genetic testing (*n*, %)	Genes identified by aetiological subgroup (*n*, % of total cohort)
Peng et al.[19]	541	Included: 0–2 years Excluded: nil	Unknown (253, 47%) Structural–acquired (137, 25%) Genetic (70, 13%) Genetic–structural (39, 7%) Structural–congenital (27, 5%) Metabolic (13, 2%) Infection (2, 0.4%)	Total: 105 out of 541 (19%) CMA: 12 out of 207 (6%) Karyotype: 2 out of 183 (1%) Epileptic encephalopathy panel: 27 out of 105 (26%) WES: 63 out of 234 (27%) Mitochondrial genome analysis: 1 out of 24 (3%)	**Genetic (70, 13%):** Single-gene disorders (59, 11%): 25 genes *Pathogenic/likely pathogenic: STXBP1* (12), *CDKL5* (12), *KCNQ2* (5), *IRF2BPL* (4), *CLCN4* (2), *GNAO1* (2), *SCN8A* (2), *KCNB1* (2), *SCN2A* (2), *CYFIP2 + KMT2D* (1), *DNM1* (1), *ARX* (1), *GRIN2B* (1), *AARS* (1), *NTRK2* (1), *STPAN1* (1), *GNB1* (1), *KMT2D* (1), *SMARCA2* (1) *CLCN4 (1)*, *SCN10A (1)*, *CACNA1A (1)*, *GABRE (1)*, *UFC1 (1)* Chromosomal disorders/CNV (11, 2%): 8 genes *Pathogenic/likely pathogenic:* 1p36.33del (2) 1p36.33-32del (1), Xp22.11–21.3dup (1), Xp22.13del (1), 20q13.33del (1), 15q11.2dup (1), 9q33.3–34.11 del (1), 9p24.3–22.del (1) 5p12-11dup (1), 3p25.3del (1) Genes involved: *CDKL5*, *EEF1A2*, *KCNQ2*, *STXBP1*, *HCN1*, *SETD5*, *GNB1*, *ARX* **Genetic–structural (39, 7%): 6 genes** Single-gene disorders *Pathogenic/likely pathogenic: TSC2* (10), *TSC1* (4), TSC, no gene (17), *NF1* (2), NF, no gene (2), *NEDD4L* (1), *DCX* (1), *NPRL3* (1), Chromosomal: 17p13.3del (1) **Metabolic (13, 2%): 10 genes** *Pathogenic/likely pathogenic: WDR45* (3), *SLC35A2* (1), *ALG1* (1), *ALG13* (1), *ATP7A* (1), *MMACHC* (1), *ACADS* (1), *SLC35A2 (1)*, *HEXA* (1), *ALDH7A1* (1) Mitochondrial: *MT-ND1* (1)
Liu et al.[18]	728	Included: 0–2 years Excluded: nil[a]	Unknown (442, 61%)[b] Genetic–structural (129, 18%) Structural (79, 11%) Genetic (76, 10%) Metabolic (2, <1%)	Total: 185 out of 728 (25%) CMA/epileptic encephalopathy / WES: 75 out of 442 (17%) Karyotype: 1 out of 1 (100%) Targeted gene PCR: 108 out of 129 (84%) Epileptic encephalopathy panel: 0 out of 13 (0%) Metabolic panel: 2 out of 2 (100%)	**Genetic (76, 10%):** Single-gene disorders (62, 9%): 27 genes *Pathogenic/likely pathogenic: STXBP1* (21), *SCN2A* (6), *CDKL5* (6), *ARX* (2), *KCNQ2* (2), *KCNB1* (2), *RARS* (2), *RYR3* (2), *DIAPH3 + STXBP1* (1), *ALG13* (1), *CACNA1A* (1), *GRIN2B* (1), *IQSEC2* (1), *KCNMA1* (1), *MEF2C* (1), *NID2* (1), *SCN8A* (1), *TCF4* (1), *SHANK3* (1), *ALDH7A1* (1), *ASAH1* (1), *CUBN* (1), *DOCK7* (1), *NRXN1* (1), *TBC1D24* (1), *TNK2* (1), *VRK* (1) Chromosomal (14, 2%): 7 genes 1p36 del (2), 17pdel (2), 7q11del (2), 15q Dup (1), trisomy 21 (1), other (6) Genes involved: *UBE3A*, *GABRB3*, *CHRNA7*, *NDE1*, *MYH11*, *GABRD*, *MAGI2* **Genetic–structural (129, 18%): 3 genes:** *TSC2* (91), *TSC1* (15), TSC-no gene (21), *NF1* (2)
Jiang et al.[20]	441	Included: 2 months-2 years Excluded: no MRI, ACTH or vigabatrin treatment	Unknown (223, 51%) Structural–acquired (104, 24%) Genetic (68, 15%) Congenital–structural (31, 7%) Infection (10, 2%) Metabolic (5, 1%)	Total: 68 out of 441 (15%) CMA: N/A Karyotype: N/ A Epileptic encephalopathy panel: N/ A WES: N/ A	**Genetic (68, 15%):** Single-gene disorders (68, 15%): 24 genes *Pathogenic: TSC1/2* (30), *CDKL5* (4), *KCNQ2* (4), *SCN2A* (4), *DEPDC5* (3) *COL4A1* (2), *FOXG1* (2), Other (19)- N/A Chromosomal disorders (0%)

(Continues)

TABLE 1 (Continued)

Study	IESS cohort (*n*)	Selection criteria	Aetiological subgroups (*n*, %)	Method and yield of genetic testing (*n*, %)	Genes identified by aetiological subgroup (*n*, % of total cohort)
Yan et al.[21]	41	Included: >1 year //- 3.5 years Excluded: <1 year	Structural (21, 51%) Unknown (11, 27%) Genetic (9, 22%)	Total: 9 out of 41 (22%) WES: 9 out of 21 (31%)	**Genetic (9, 22%):** Single-gene disorders (9, 22%): 7 genes *Pathogenic/likely pathogenic: POLR2A* (1), *SCN2A* (1), *WDR45* (1), *SCN1A* (1), *GRIA2* (1), *GTPBP3* (1), *CDKL5* (1), *ADA2* (1) Mitochondrial: chrM-144485 T > C, ChrM-3736 M > A Chromosomal disorders (0%)
Demarest et al.[23]	21	Included: 0–1 year, had ACTH treatment, referred for WES Excluded: nil	Genetic and genetic–structural (12, 57%) Unknown (7, 33%) Structural–acquired (2, 10%)	Total: 5 out of 21 (24%) CMA: 0 out of 13 Epileptic encephalopathy panel: 0 out of 11 WES (trio): 5 out of 21 (24%)	**Genetic and genetic–structural (12, 57%):** Single-gene disorders (10, 48%): 10 genes *Pathogenic*: *NR2F1* (1), *GNB1* (1), *NEUROD2* (1), *NDUFAF5* (1) *GABRA2* (1) *VOUS*: *PEMT* (1), *ASXL2* (1), *RALGAPB* (1), STRADA (1), *DYNC1I1* (1) Chromosomal disorders (2, 10%) Trisomy 21 (2)
Symonds et al.[22]	52	Included: 0–3 years[c] Excluded: nil	Unknown (16, 31%) Structural (14, 27%) Genetic–structural (12, 24%) Genetic (9, 17%) Genetic–metabolic (1, 1%)	Total: 22 out of 52 (42%) CMA: N/A Epileptic encephalopathy panel: N/ A WGS (trio): N/ A	**Genetic, genetic–structural and metabolic (22, 42%):** Single-gene disorders (13, 25%): 9 genes *Pathogenic/likely pathogenic: TSC2* (3), *TSC1* (2), *CDKL5* (2) *IDIC15* (1), *ASAH1* (1), *CACNA1G* (1), *DEPDC5* (1), *PAFAH1B1* (1), *POLR1A* (1) Chromosomal disorders/CNV (9, 17%) Trisomy 21 (6), trisomy 13 (1) 16p13.11 del (1), 17p13.3 del (Miller–Dieker) (1)
Helbig et al.[24]	41	Included: 0–18 years[c] referred for WES Excluded: nil	Genetic (16, 39%) Other diagnoses N/A	Total: 16 out of 41 (39%) WES (singleton or trio): 16 out of 41 (39%)	**Genetic (16, 39%):** Single-gene disorders (16, 39%): 18 genes *Pathogenic/likely pathogenic: FOXG1* (2), *CACNA1C* (1), *CCND2* (1), *COL4A1* (1), *DYNC1H1* (1), *GNAO1* + *ATP2B3* (1), *KMT2A* (1), *RARS2* (1), *ANO3* + *NALCN* (1), *DNM1* (1) *VOUS (possibly pathogenic): CACNA1E* (1), *AIMP2* + *LRFN2* (1), *COQ4* (1), *HNRNPR* (1), *HTR2C* (1)
Wirrell et al.[17]	251	Included: 0–2 years Excluded: nil	Unknown (90, 36%) Structural–acquired (55, 22%) Genetic (36, 14%) Structural–congenital (27, 11%) Genetic–structural (25, 10%) Metabolic (12, 5%) Infection (5, 2%)	Total: 57 out of 251 (23%) CMA: 12 out of 87 (14%) Karyotype: 17 out of 32 (53%) Chromosomal SNP: 2 out of 4 (50%) Targeted gene SNP: 11 out of 24 (46%) Epileptic encephalopathy panel: 11 out of 34 (32%) WES//WGS: 0 out of 4 (0%) Mitochondrial SNP: 1 out of 4 (25%) Mitochondrial gene panel: 3 out of 9 (33%)	**Genetic (36, 14%):** Single-gene disorders (11, 4%): 7 genes *Pathogenic/likely pathogenic: CDKL5* (3), *KCNQ3* (2) *LYK5* (2), *STXBP1* (1), *SCN1A* (1), *KRAS* (1), *KANSL* (1) Chromosomal disorders/CNV (25, 10%) *Pathogenic/likely pathogenic:* risomy 21 (15), 15q11 mut (3) chr2 mut (2), Williams syndrome (1), trisomy 13 (1) 15q21 mut (1), chr6 mut (1), other (1) **Genetic–structural (25, 10%): 7 genes** *Pathogenic/likely pathogenic: TS1/2* (12), *NF1* (2), *DCX* (1), *LIS1* (1) Miller–Dieker (1), *AGS* (1), *SETBP1* (1) 15q11.2 dup (1), cortical malformations with genetic change (5) **Metabolic (12, 5%): 3 genes** *Pathogenic/likely pathogenic: POMPT1* (1), *POLG1* (1), Leigh disease, *ATP6* (1)

TABLE 1 (Continued)

Study	IESS cohort (*n*)	Selection criteria	Aetiological subgroups (*n*, %)	Method and yield of genetic testing (*n*, %)	Genes identified by aetiological subgroup (*n*, % of total cohort)
	Total cohort 2116			Positive genetic result Total: 467 out of 2116 (22%) Mean: 26% Median: 23% Range: 15–42%	

[a]No genetic testing for acquired cases.
[b]Seventy-two had no genetic testing.
[c]Part of DEE cohort.
Abbreviations: ACTH, adrenocorticotrophin hormone; CMA, chromosomal microarray; CNV, copy number variant; DEE, developmental and epileptic encephalopathy; FCD, focal cortical dysplasia; IESS, infantile epileptic spasms syndrome; MRI, magnetic resonance imaging; N/A, not available; NF, neurofibromatosis; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism; TSC, tuberous sclerosis complex; VOUS, variant of uncertain significance; WES, whole-exome sequencing; WGS, whole-genome sequencing (singleton, duo, or trio, stated if known).

methylation in lymphocytes of children with IESS of unknown aetiology, compared with typically developing controls.[54] Pretreatment methylation levels did not differ between ACTH responders and non-responders; however, samples were not re-collected to determine whether ACTH altered methylation.

Hypothalamic–pituitary–adrenal axis

This hypothesis consisted of three themes and 24 studies that described hypothalamic–pituitary–adrenal (HPA)-axis hormones to determine whether the stress system is activated at IESS onset and whether ACTH/steroids modify the stress response. Five studies examined hypothalamic hormones, 13 studied pituitary and thyroid hormones, and 15 examined adrenal hormones, with several studies overlapping (Figure S1).

Hypothalamic hormones (corticotrophin-releasing hormone, somatostatin)

Three studies examined the hypothesis that there is excessive release of 'stress' hormones in IESS, particularly the 'pro-convulsant' corticotrophin-releasing hormone (CRH), known to trigger seizures in animal studies.[55]

One study examined messenger RNA (mRNA) and protein expression of CRH and CRH receptor type 1 (CRHR1) in brain tissue of children with IESS.[56] Tissue was resected during epilepsy surgery and compared with autopsy controls. In IESS, CRH and CRHR1 were significantly upregulated.[56] No studies measured CRH levels or CRH mRNA expression in blood. Two studies quantified CRH levels in cerebrospinal fluid (CSF) compared with controls undergoing CSF testing for fever, seizures, or neurological conditions (Figure S2). CSF CRH level was no different in IESS compared with controls.[57,58] One study found CSF CRH inversely correlated with low CSF cortisol, indicating possible suppression of downstream 'stress' hormones.[57] No studies have analysed the effect of treatment on CRH.

Two studies examined CSF somatostatin, which is an excitatory neurotransmitter. CSF somatostatin was higher in IESS than controls in one study[59] and similar to controls in another.[60] ACTH reduced CSF somatostatin levels, but not significantly.[59,60] Blood somatostatin levels have not been measured in IESS.

Pituitary and thyroid hormones

Several studies have quantified ACTH in blood and CSF, hypothesizing that IESS results from an ACTH deficiency which treatment corrects.[61–64] Authors have proposed a primary and/or secondary ACTH deficiency due to stress.[64]

At IESS onset, blood ACTH levels were no different to controls,[62] or within a normal range (Figure S3).[65,66] In contrast, five studies reported CSF ACTH levels were significantly lower in IESS than controls[57,58,61,62,67] (Figure S2) and lowest in IESS secondary to known aetiologies (e.g. brain injury, trisomy 21).[63,68,69] ACTH treatment did not 'correct' the CSF ACTH deficiency[61–63,69] or lead to a significant increase in blood ACTH (Figures S2 and S3).[65,66]

TABLE 2 Studies of IESS which included presumed/confirmed genetic causes of IESS (15 studies with total 936 cases), including inclusion/exclusion criteria, genetic testing used and yield, and the monogenic causes found.

Study	IESS cohort (*n*)	Selection criteria	Method and yield of genetic testing	Genes identified by aetiological subgroup (*n*, % of total cohort)
D'Gama et al.[25]	32	Included: 0–1 year[b] Excluded: known genetic, acquired causes	Total: 6 out of 32 (19%) WGS (singleton, duo, or trio): 6 out of 32 (19%)	**Genetic (6, 19%):** Single-gene disorders (5, 16%): 5 genes *Pathogenic/likely pathogenic: STXBP1* (1), *PTEN* (1), *TED5* (1), *KCNJ6* (1), *DYNC1H1* (1) Chromosomal disorders/CNV (1, 3%) 9pterq22.23 15q22.2qter 9
Koh et al.[26]	46	Included: 0–18 years[b], IESS onset < 1 year Excluded: known genetic, acquired causes	Total: 9 out of 46 (20%) WGS (singleton, duo, or trio): 9 out of 46 (20%)	**Genetic and genetic–structural (9, 20%):** Single-gene disorders (8, 17%): 8 genes *Pathogenic/likely pathogenic: STXBP1* (1), *CYFIP2* (1), *DYNC1H1* (1), *GRIN2B* (1), *NPRL2* (1), *OTUD6B* (1), *PGAP2* (1) *VOUS: MTR* (1) Chromosomal disorders/CNV (1, 2%): Chr16:138446–140150
Scheffer et al.[27]	38	Included: 0–18 years[b], IESS onset presumed < 2 years, negative CMA Excluded: known genetic, structural, causes	Total: 4 out of 38 (11%) WES (singleton, duo, or trio): 4 out of 38 (11%)	**Genetic (4, 11%):** Single-gene disorders (3, 8%): 3 genes *Pathogenic/likely pathogenic: STXBP1* (1) *ALG13* (1), *HECW2* (1) Chromosomal disorders/CNV (1, 3%): 1 gene 9q33.3q34.11 del (*STXBP1*)
Lee et al.[28]	16	Included: 0–1 year Excluded: known structural, acquired, metabolic causes	Total: 4 out of 16 (25%) WGS (singleton or trio): 4 out of 16 (25%)	**Genetic (4, 25%):** Single-gene disorders (4, 25%): 4 genes *Pathogenic/likely pathogenic: HDAC4 (1), GRM7 (1), CACNA1E (1), KMT2E (1) new line before VOUS*: SOX5 (1), SHROOM4 (1)
Choi et al.[29]	58	Included: 0–1 year Excluded: known genetic cause (from CMA/karyotype), structural, metabolic causes	Total: 17 out of 58 (29%) Epileptic encephalopathy panel: 17 out of 58 (29%)	**Genetic (17, 29%):** Single-gene disorders (14, 24%): 7 genes *Pathogenic: CDKL5* (4), *STXBP1* (3), *KCNB1* (2), *SCN2A* (2), *EEF1A2* (1), *KANSL1* (1), *MECP2* (1) CNV (3, 5%): not specified
Krey et al.[30]	45	Included: 0–40 years, IESS onset < 1 year Excluded: TSC, trisomy 21, structural–acquired causes	Total: 13 out of 45 (29%) Epileptic encephalopathy panel: 13 out of 45 (29%)	**Genetic (13, 29%):** Single-gene disorders (11, 24%): 7 genes *Pathogenic/likely pathogenic: CDKL5* (5), *ARX* (1), *SCN1A* (1), *KCNB1* (1), *DEPDC5* (1), *AARS* (1), *WDR45* (1) Chromosomal disorders/CNV (2, 4%): 20 genes 2q24.1q24.3 dup (1), 15q11.1q13.1 trip (1) Genes involved: *GABRB3*, *TUBGCP5*, *NIPA1*, *MKRN3*, *MAGEL2*, *NDN*, *SNRPN*, *UBE3A*, *ATP10A*; *SCN2A*, *SCN3A*, *CD302*, *FAP*, *IFIHI*, *GCA*, *KCNH7*, *FIGN*, *GRB14*, *COBLL1*, *TANK*
Muir et al.[31]	92	Included: 0–2 years Excluded: known structural, metabolic causes	Total: 7 out of 92 (8%) Epileptic encephalopathy panel: 7 out of 92 (8%)	**Genetic (7, 8%):** Single-gene disorders (7, 8%): 6 genes *Pathogenic: KCNB1* (2), *GNAO1* (1), *KIF1A* (1), *SLC35A2* (1), *STXBP1* (1), and *TBL1XR1* (1) *VOUS*: *FASN* (1), *HDAC4* (1), *PNMAL1* (1), *PPP3CA* (1)

TABLE 2 (Continued)

Study	IESS cohort (*n*)	Selection criteria	Method and yield of genetic testing	Genes identified by aetiological subgroup (*n*, % of total cohort)
EPGP/ Yuskaitis et al.[32]	133	Included: 0–1 year, 6 months follow-up Excluded: known genetic, structural, metabolic causes, severe developmental delay before IESS	Total: 15 out of 100 (15%) WES (trio): 15 out of 100 (15%)	**Genetic (15, 15%):** Single-gene disorders (15, 15%): 12 genes *Pathogenic: STXBP1* (3), *KCNQ2* (2), *ALG13* (1), *DNM1* (1), *GABRA1* (1), *GNAO1* (1), *GRIN1* (1), *KCNT1* (1), *PTEN* (1), *SCN2A* (1), *SCN8A* (1), *TUBB2A* (1)
Ko et al.[33]	128	Included: 0–3 years[a] normal CMA, cause confirmed by epileptic encephalopathy NGS panel Excluded: structural, metabolic causes	Total: 34 out of 128 (27%) Epileptic encephalopathy panel: 34 out of 128 (27%)	**Genetic (34, 27%):** Single-gene disorders (34, 27%): 14 genes *Pathogenic/likely pathogenic: CDKL5 (7), STXBP1 (6), SCN8A (5), KCNQ2 (3), KCNB1 (2), DNM1 (2), SCN2A (2), ARX (1), WWOX (1), BRAT (1), EEF1A2 (1), HCN1 (1), MECP2 (1), PRODH (1)*
Dimassi et al.[34]	10	Included: 0–1 year, negative CMA, negative *CDKL5*, *STXBP1* and *ARX* Excluded: known genetic, metabolic structural, acquired causes, family history of seizure, consanguinity	Total: 4 out of 10 (40%) WES (trio): 4 out of 10 (40%)	**Genetic (4, 40%):** Single-gene disorders (4, 40%): 4 genes *Possibly pathogenic: CDKL5* (1), *STXBP1* (1), *ALG13* (1), *NR2F1* (1) Chromosomal disorders/CNV (0)
Boutry-Kryza et al.[35]	73	Included: 0–11 years, IESS onset <2 years, negative CMA, karyotype, *ARX* testing Excluded: known genetic, structural, metabolic, acquired causes	Total: 11 out of 73 (15%) CMA and SNP analysis for 5 genes (*CDKL5*, *STXBP1*, *KCNQ2*, *GRIN2A*, *MAGI2)*: 11 out of 73 (15%)	**Genetic (11, 15%):** Single-gene disorders (6, 8%): 2 genes *Pathogenic/likely pathogenic: CDKL5* (3), *STXBP1* (3) Chromosomal disorders/CNV (5, 7%): 30 genes *Pathogenic*: Xq27.1 dup (1), 2q22.3 q24.2 del (1), 5q14.3 del (1), 9q3.3 del (1), 2q24.2 dup (1) Genes involved: *MIR3660*, *CETN3*, *LOC731157*, *MBLAC2*, *POLR3G*, *LSMD3*, *GPR98*, *LUCAT1*, *ARRDC3*, *EHMT1*, *CACNA1B*, *SCN2A*, *CSRNP3*, *GLANT3*, *TTC21B*, *NEDD4*, *CALN1*, *OTOA*, *RRN3P1*, *UQCRC2*, *PDZD9*, C16orf52, *VWA3A*, *EEF2K*, *POLR3E*, *CDR2*, *RRN3P3*, *SMG1P1*, *LOC653786*, *NPIPB5* *Potential risk factor:* (3)-see original
Hino-Fukuyo et al.[36]	18	Included: 0–2 years Excluded: known genetic, structural, metabolic, acquired causes; neonatal onset IESS	Total: 9 out of 18 (50%) CMA: 4 out of 18 (22%) Epileptic encephalopathy panel: 0 out of 14 (0%) WES (trio): 5 out of 14 (36%)	**Genetic (9, 50%):** Single-gene disorders (5, 27%): 5 genes *Pathogenic/likely pathogenic: SLC35A2* (1), *NR2F1* (1), *CACNA2D1* (1), *ALG13* (1), *BRWD3* (1) Chromosomal disorders/ CNV (4, 22%): > 100 genes *Likely pathogenic*: Xq28 dup (1), 19p13.2 del (1), *Possibly pathogenic*: 16p13.1 del (1), 19p13.2 del (1) *Benign* (5)

(Continues)

TABLE 2 (Continued)

Study	IESS cohort (*n*)	Selection criteria	Method and yield of genetic testing	Genes identified by aetiological subgroup (*n*, % of total cohort)
Michaud et al.[37]	44	Included: 0–2 years, non-specific MRI brain findings Excluded: structural, metabolic, causes	Total: 11 out of 44 (25%) CMA: 4 out of 44 (9%) Epileptic encephalopathy panel: 0 out of 8 (0%) Targeted sequencing (*ARX/ CDKL/ STXBP1*): 2 out of 38 (5%) WES (trio): 5 out of 18 (28%)	**Genetic (11, 25%):** Single-gene disorders (7, 16%): 7 genes *Pathogenic: STXBP1* (2), *ARX* (1), *PNPO* (1), *ADSL* (1), *CASK* (1) *ALG13* (1) Eleven new candidate genes (8, 18%) predicted pathogenic *SQSTM1*, *MYO9B*, *NR2F1*, *NPC1L1*, *TNFA1P6*, *EIF2C4*, *TENM2*, *HEG1*, *LAMA2*, *SEMA5B*, *HSPG2* Chromosomal disorders/CNV (4, 9%): 5 genes *Likely pathogenic:* 2q21.3-q22.1 del (1), 15q11.1q13.1 tetrasomy (1), 16p11.2 dup (1) Genes involved: *CXCR4*, *NXPH2*, *GABRA5*, *GABRA3*, *UBE3A*
EuroEPIGENOMICS, Epi4K, EPGP et al.[38]	159	Included: 0–2 years[a] Excluded: structural, metabolic, acquired causes, family history of epilepsy	Total: 42 out of 159 (26%) WES (trio): 42 out of 159 (26%)	**Genetic:** **Single-gene disorders (42, 26%): 46 genes** *De novo probably damaging: STXBP1* (3), *CDKL5* (1), *CNTN5* (1), *GABRA1* (1), *GABRB1* (1), *GABRB3* (1), *GRIN1* (1), *KCNB1* (1), *SCN8A* (1), *KCNQ2* (1), *SCN2A* (1), *TIFA* (1), *FAM86C1* (1), *SLC16A3* (1), *PNMAL1* (1), *PIK3AP1* (1), *NLRP8* (1), *TRRAP* (1), *SMURF1* (1), *C1QTNF6* (1), *ATP2B4* (1), *SLAMF1* (1), *GNAO1* (1), *OR10S1* (1), *NGLN2* (1), *PLA1A* (1), *FAM50A* (1), *ASXL1* (1) *DHTKD1* (1), *TAF1* (1), *ETS1* (1), *TRIM29* (1), *AKR1C4* (1), *CSNK1E* (1), *KIAA2018* (1), *ITGAM* (1), *PRDM12* (1), *THOC2* (1), *SMG9* (1), *DNM1* (1), *NR1H2* (1), *HIST2H2BE* (1), *SLC1A2* (1), *RRP1B* (1), *ANKRD12* (1) *De novo possibly damaging: AGL13* (1), NCOR2 (1), *KCNT1* (1), *NFASC* (1), *SGK223* (1), *C1QTNF6* (1), *NLRP5* (1), *TNNI3K* (1) *PALLD* (1), *MSANTD1* (1), *RFX3* (1), *GAS2* (1), *DIAPH3* (1), *PACS2* (1), *NEDD4L* (1), *PDIK1L* (1)
Mefford et al.[39]	44	Included: 0–2 years[a] Excluded: known causes (various physician-directed investigations)	Total: 3 out of 44 (7%) CMA: 3 out of 44 (7%)	**Genetic (3, 7%):** Chromosomal disorders/CNV (3, 7%): 4 genes *Pathogenic/likely pathogenic:* Xp22 del, 16p11.2dup Genes involved: *LRRK2*, *SCLT1*, *EPHA6*, *GABRR3* VOUS: 12q12del and 4q28del, 3q11 dup
	Total cohort 936		Positive genetic result Total: 189 out of 936 (20%) Mean: 23% Median: 23% Range: 7–50%	

[a]Part of DEE cohort.

Abbreviations: ACTH, adrenocorticotrophin hormone; ASD, autism spectrum disorder; CMA, chromosomal microarray; CNV, copy number variant; DEE, developmental and epileptic encephalopathy; FCD, focal cortical dysplasia; IESS, infantile epileptic spasms syndrome; MRI, magnetic resonance imaging; N/A, not available; NF, neurofibromatosis; NGS, next-generation sequencing; SNP, single-nucleotide polymorphism; TSC, tuberous sclerosis complex; VOUS, variant of uncertain significance; WES, whole-exome sequencing; WGS, whole-genome sequencing (singleton, duo, or trio, stated if known).

TABLE 3 Studies of IESS reporting confirmed genetic causes (one study with 124 cases), including inclusion/exclusion criteria and monogenic causes found.

Study	IESS cohort (*n*)	Selection criteria	Genes identified by aetiological subgroup (*n*, % of total cohort)
Nagarajan et al.[40]	124	Included: 2 months to 2 years, genetic cause confirmed by CMA, karyotype, triplet repeat PCR or methylation MLPA, Sanger sequencing, or NGS Excluded: structural–genetic, metabolic causes	**Genetic (124, 100%):** Single-gene disorders (105, 85%): 51 genes *Pathogenic/likely pathogenic: ALDH7A1* (10), *SCN2A* (7), *CDKL5* (6), *ALG13* (5), *KCNQ2* (4), *STXBP1* (4), *WWOX* (4), *SCN1A* (4), *NTRK2* (4), *KCNT1* (3), *SYNGPA1* (3), *SCN3A* (3), *SLC2A1* (3), *MECP2* (2), *CPLX1* (2), *UGP2* (2), *PP3CA* (2), *PLPBP* (2), *GRM7* (1), *TBCD* (1), *CHD2* (1), *CDK19* (1), *FOXG1* (1), *NRROS* (1), *PURA* (1), *KANSL1* (1), *GABBR2* (1), *GRIN1* (1), *CSNK2A1* (1), *PNPO* (1), *CACNA1A* (1), *NPRL3* (1), *IQSEC2*, (1), *CYFIP2* (1), *MBOAT7* (1), *MBD5* (1), *PPP2R1A* (1), *DNM1* (1), *NONO* (1), *EHMT1* (1), *GNAO1* (1), *PRRT2* (1), *AMT* (1), KMT2C (1), *ADSL* (1), *SATB1* (1), *PACS2* (1), *HUWE1* (1), *ASNS* (1), *MIPEP* (1), *PLEKHG2* (1), *SCN8A* (1) Mitochondrial: *likely pathogenic MT-ND5* (1) Chromosomal disorders/CNV (19, 15%) *Pathogenic*: trisomy 21 (14), Xq28 dup (2), cri-du-chat (1), 15q dup (1), 1p36 del 18 g dup (1)

Abbreviations: CMA, chromosomal microarray; CNV, copy number variant; DEE, developmental and epileptic encephalopathy; FCD, focal cortical dysplasia; IESS, infantile epileptic spasms syndrome; MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; PCR, polymerase chain reaction.

β-Endorphin was investigated as a surrogate marker of ACTH, as they share the same precursor hormone, pro-opiomelanocortin. Most studies found no differences in blood[62] or CSF β-endorphin levels[61,62] between IESS and controls. Only one study found CSF β-endorphin was significantly lower in IESS.[58] In a separate study, ACTH treatment led to a non-significant reduction of CSF β-endorphin (Figure S2).[69]

At IESS onset, other pituitary (luteinizing hormone, follicle-stimulating hormone, growth hormone) and thyroid hormones (thyroid stimulating hormone, T3, T4) were within normal ranges (Figure S3).[60,70,71] ACTH transiently suppressed these hormones after 2 to 3 weeks;[60,70,71] however, levels returned to baseline at treatment completion.[60,70,71]

Adrenal hormones (cortisol, intermediary steroids)

At IESS onset, only one study found blood cortisol was significantly higher in IESS compared with hospitalized controls.[72] In studies without controls, blood cortisol was either in the normal range[66,73–76] or elevated (Figure S3).[71,72,77] CSF cortisol was lower in IESS than controls which reached significance in one study.[57,67,72] Urinary cortisol was normal in two studies, examined without controls.[73,74]

Eleven studies analysed the treatment effect of ACTH,[60,65,66,70–72,74,76,77] hydrocortisone,[72,77,78] and prednisone[75] on cortisol. Most studies found ACTH/steroid treatment did not significantly alter blood cortisol levels from baseline (Figure S3).[60,65,66,70–72,74–78] During the first 2 weeks of treatment, most studies found ACTH and hydrocortisone caused a transient increase in blood cortisol levels[60,66,70,72,74,76,77] whereas prednisone suppressed levels.[65] ACTH and hydrocortisone increased CSF cortisol in one study, yet no statistical analysis was provided.[72] After 24 hours of ACTH, urinary cortisol increased from baseline[73,74] yet there was no control comparison or correlation with treatment response.[74]

Another hypothesis is that ACTH/steroids exert their antiseizure effect through 'intermediary steroids', such as the precursors of cortisol, aldosterone, and sex hormones. This was based on observations that progesterone and deoxycorticosterone prevented and reduced seizures in adults.[79] No studies have compared intermediary steroid levels at IESS onset with controls. Testosterone precursors, dehydroepiandrosterone sulfate, and androstenedione are reportedly elevated or within normal range at baseline in IESS.[65,73,74] ACTH significantly lowered dehydroepiandrosterone sulfate but not androstenendione,[65] and higher baseline ratios of dehydroepiandrosterone sulfate:androstenedione correlated with ACTH response in some studies.[73,74] No correlations were found for cortisol or aldosterone precursors.[73,74] Other studies reported ACTH and hydrocortisone increased plasma/CSF 11-hydroxycorticosteroid[78] and general excretion of steroids;[80] however, the findings lacked statistical comparison and have not been replicated.

Inflammation

We found 18 studies that examined possible neuroinflammation and immune dysregulation underlying IESS and the anti-inflammatory effect of ACTH/steroids. Six studies quantified lymphocyte populations, five examined immunoglobulins, nine quantified cytokines/chemokines, one examined microglial activation, and three assessed integrity of the BBB.

Lymphocyte, T-cell, and B-cell population

We have summarized the results from included studies examining immune cell types and cytokines/chemokines in the blood (Figure 3) and CSF (Figure S4) of children with IESS, compared with matched controls, and the treatment effect of ACTH and prednisone.

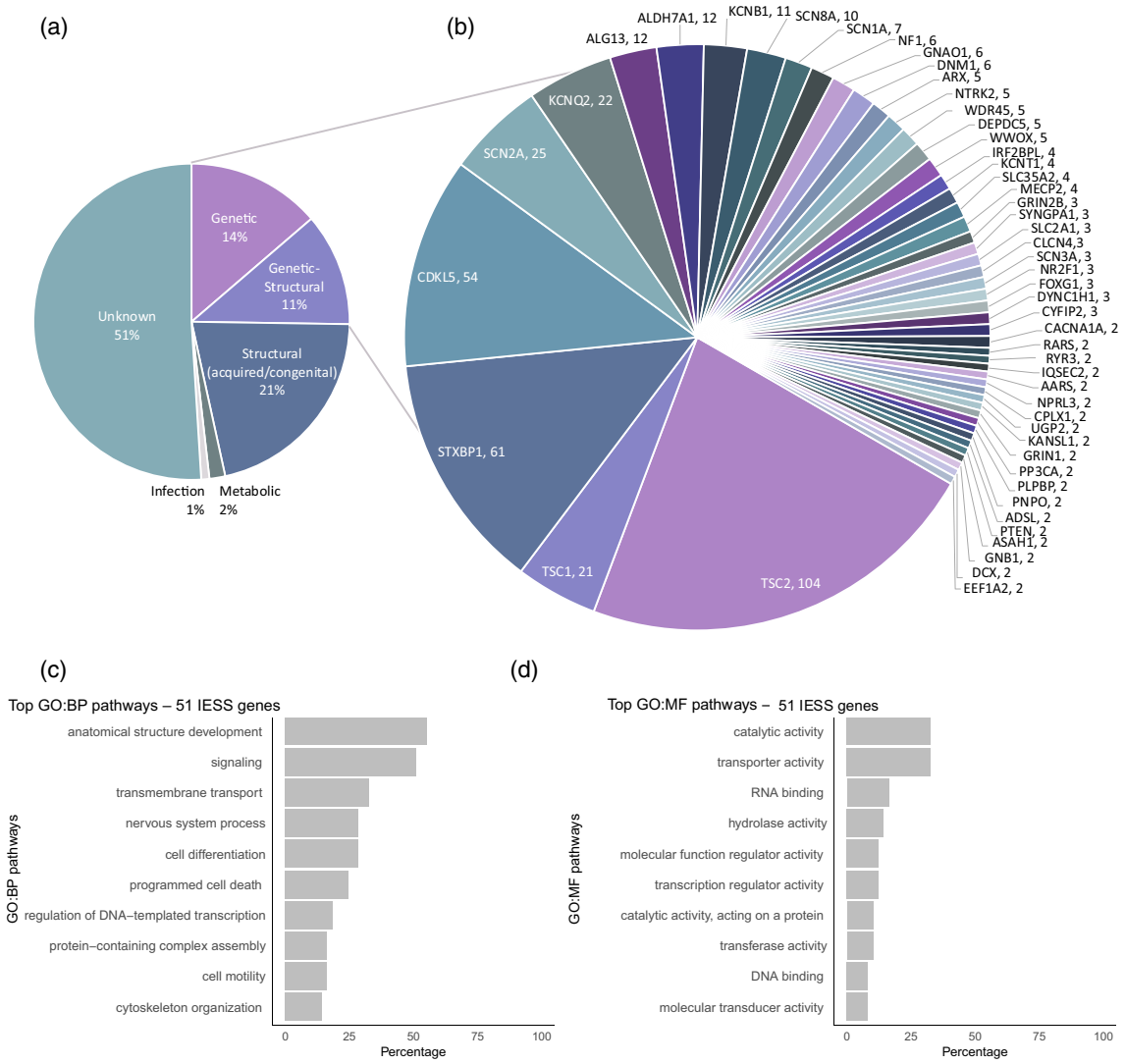

FIGURE 2 The aetiologies of IESS in studies of IESS (Tables 1–3). (a) The combined prevalence of aetiological subgroups in IESS combined from Table 1: eight studies with 2116 children (including all known aetiologies). Aetiology was classified as per International League Against Epilepsy guidelines. (b) Prevalence of 51 pathogenic/likely pathogenic genes associated with IESS identified in two or more individuals from 25 studies with 3176 children (combined from Tables 1–3). Note: a small proportion of cases with TSC and NF were excluded, as no gene was identified or *TSC1* was not differentiated from *TSC2*. (c, d) These 51 genes were categorized using GO slim BP and MF terms. The GO Term Mapper from the Lewis-Sigler Institute for integrative genomics was used and graphs were generated using the ggplot2 package on the R platform (R Foundation for Statistical Computing, Vienna, Austria). The top 10 GO BP pathways enriched by the genes included anatomical structure development (55% of genes), signalling (51% of genes), and transmembrane transport (33% of genes). The top 10 GO MF pathways enriched by the genes included catalytic activity (33% of genes), transporter activity (33% of genes), and RNA binding (16% of genes). Abbreviations: BP, biological processes; GO, gene ontology; IESS, infantile epileptic spasms syndrome; MF, molecular function.

In a single study, lymphocyte count at IESS onset was no different to controls (Figure 3).[81] ACTH significantly reduced lymphocytes in one study,[82] whereas another found no change.[81] Three of five studies found T-cell subsets were significantly lower at IESS onset compared with controls.[81,83–85] After ACTH and prednisone, three studies reported reduced CD3, CD4, CD8 cell counts and CD4:8 ratio, consistent with suppression of cell-mediated immunity.[81,82,85] Helper T cells were more suppressed than cytotoxic T cells (Figure 3).[81,82,85] Recovery of these T-cell subsets was seen at 6 months after treatment.[82] At IESS onset, B-cell counts were either lower[81] or no different to controls,[86] and were unchanged by ACTH in a single study.[81]

Functional T-cell studies were limited. At IESS onset, two studies reported reduced proliferation and migration of T cells after phytohaemagglutinin stimulation[83,84] although responses following ACTH/steroids were not performed. Dinitrochlorobenzene skin testing, which measures delayed hypersensitivity reactions, found that after ACTH the erythematous skin reaction was reduced or absent, indicating a significantly suppressed T-cell response.[83,84]

	BASELINE: IESS vs controls							TREATMENT effect: IESS during/post Rx				
	Chen et al.[85]	Ture et al.[88]	Ozaydin et al.[87]	Shiihara et al.[81]	Montelli et al.[84]	Liu et al.[89]	Montelli et al.[83]	Chen et al.[85]	Ture et al.[88]	Yamanaka et al.[90]	Shiihara et al.[81]	Ohya et al.[82]
								Pred	ACTH			
A: Quantification of cell types, proteins (Immunoglobulins) and growth factors												
WBC count				0							0	0
Lymphocyte count				0							0	-2
T cells							-2					
CD1					-1							
CD3	0		-1	-2	-1			0			-2	
CD4	0		-1	0	-1			-3			-2	-2
CD8	0		0	0	2			-3			0	0
CD4:CD8	0			0	-2			-3			-3	-2
CD 25				-2							0	
CD 95				-3							0	
B cells							0					
CD 19			0	-3							0	
IgA	0		-1		-1		0	0				1
IgG	0		-1		1		0	0				1
IgM	0		-2		1		0	-1				1
Exotaxin				3							0	
bFGF				3							0	
GCSF				3							0	
G-MCSF				0							0	
PDGF				0							0	
VEGF				0							0	
B: Cytokines and Chemokines												
IL-1RA				0						3	0	
IL-1β	1	-1		2				1	-1	-1	-3	
IL-2	-1	1		0		3			0		0	
IL-2R	3							-3				
IL-4				0							0	
IL-5				2							0	
IL-6	1	3		3				1	-1		0	
IL-7				0							0	
IL-7A												
IL-8	3			0				-3			0	
IL-9				0							0	
IL-10				0							0	
IL-12				0							-1	
IL-13				0							0	
IL-15				2							0	
IL-17				0							0	
IL-17A		3							-1			
IL-23		-1							1			
IFN-γ				0							0	
IFN-IP-10				0							0	
IFN-α						3						
TNF-α	3			0		3		-3	-1		0	
TNF-β												
CCL-2 (MCP-1)				0							0	
CCL-3 (MIP-1α)				0							0	
CCL-4 (MIP-1β)				0							-2	
CCL-5 (RANTES)				0							0	

-3	↓ p<0.01
-2	↓ p<0.05
-1	↓ p>0.05
0	no change
1	↑ p>0.05
2	↑ p<0.05
3	↑ p<0.01
	Not tested

FIGURE 3 Inflammatory and immune profile in the blood of children with IESS at baseline and following treatment. Preliminary evidence that children with IESS have elevated pro-inflammatory cytokines in blood compared with controls and may have altered T-cell responses. ACTH and prednisone appear to suppress T-cell-mediated immunity yet do not consistently alter cytokine/chemokine levels. Variances were graded as follows: 0, no change; 1, elevated with $p > 0.05$; 2, elevated with $p < 0.05$; 3, elevated with $p < 0.01$; –1, reduced with $p > 0.05$; –2, reduced with $p < 0.05$; –3, reduced with $p < 0.01$; grading of CSF chemokine/cytokines in encephalitis based on Kothur et al.[153] Abbreviations: ACTH, adrenocorticotrophin hormone; bFGF, basic fibroflast growth factor; CCL, chemokine ligand; CD, cluster of differentiation; GCSF, granulocyte colony-stimulating factor; G-MCSF, granulocyte-macrophage colony-stimulating factor; IESS, infantile epileptic spasms syndrome; IFN, interferon; Ig, immunoglobulin; IL, interleukin; PDGF, platelet-derived growth factor; Pred, prednisone; Rx, treatment; TNF, tumour-necrosis factor; VEGF, vascular endothelial growth factor; WBC, white blood cell count.

Immunoglobulins

Studies quantifying immunoglobulin-A (IgA), IgG, IgM found no differences in children with IESS at baseline[83–85,87] and no changes after prednisone[85] or ACTH treatment (Figure 3).[82] At baseline, one study reported antibody formation against normal brain tissue, suggesting an abnormal immune response.[86]

Cytokines/chemokines

Four studies analysed the cytokine/chemokine profile in serum (Figure 3).[81,85,88,89] At IESS onset, children had significantly higher serum levels of the following pro-inflammatory cytokines: interleukin-1β (IL-1β), IL-2R, IL-6, IL-8, and IL-15, IL-17A, tumour-necrosis factor-α (TNF-α), and interferon-α[81,85,88,89] whereas the anti-inflammatory cytokines IL-1RA, IL-4, and IL-10 were not significantly different (Figure 3).[81] Most studies found IL-1β was unaltered by prednisone/ACTH;[85,88,90] however, one reported a significant reduction.[81] Prednisone,[85] but not ACTH, reduced IL-2R, IL-8, and TNF-α; however, IL-2 and IL-6 were unchanged.[81,85,88] A single study found chemokines did not differ from controls, and were not changed by ACTH.[81]

Two studies[57,91] examined the cytokine/chemokine profile in CSF (Figure S4). CSF IL-1RA was significantly lower in IESS than controls,[57,91] which was not observed in blood.[81] One study found detectable or elevated IL-1β levels in IESS with a known aetiology (compared with unknown).[92] Two infants successfully treated with ACTH had repeat CSF testing; IL-1β normalized in one and remained detectable in the other.[92] One study investigated CSF neopterin, finding one of four infants had elevated levels; however, treatment effect was not analysed.[93]

Microglial activation

One study[94] examined for microglial activation in children with unknown IESS aetiology using positron emission tomography scans and a tracer that binds to a translocator protein which is expressed by activated microglia. Five of eight children had focal areas of increased binding, suggesting neuroinflammation, which reduced or normalized following ACTH.[94] The significance of these findings was limited by the lack of control comparison and lack of replication.

BBB function

Three studies measured CSF and serum protein and albumin levels as indirect evidence of BBB damage, with conflicting results.[63,95,96] Two studies found no evidence of BBB damage, as CSF albumin and CSF:serum albumin ratios were normal and were unaltered following ACTH.[63,95] One study reported elevated CSF protein/albumin which normalized after dexamethasone and ACTH.[96] Findings were limited by the lack of comparison with typically developing controls and by the presumption that CSF protein/albumin levels reliably reflect BBB damage.

One study analysed levels of serum matrix metallopeptidase-9 (MMP-9) as a marker of BBB damage. MMP-9 is an enzyme in the cerebral endothelium, which degrades collagen and proteins of the BBB.[95] MMP-9 levels did not differ between aetiological subgroups of IESS. ACTH responders had significantly higher MMP-9 levels, and a higher ratio of MMP-9 to its inhibitor (TIMP-1). The authors inferred ACTH may restore BBB integrity, yet their findings were limited by the lack of control comparison and serial measurements. Another study measured CSF:serum IgG index as evidence of a CNS immune response; however, it was within normal ranges before and during ACTH treatment.[63]

Neural transmission and connectivity

Twenty-two studies explored the hypothesis that IESS results from altered neural transmission and connectivity and that ACTH/steroids may modify signalling pathways or neuronal function (Figure S1). Twelve studies examined neurotransmitters and excitatory or inhibitory amino acids, seven studies analysed neural pathways, and four examined markers of neuronal health as a measure of neuronal excitability.

Neurotransmitters and amino acids

Several studies investigated whether IESS is associated with disturbances of primary monoamines in the CSF: noradrenaline,[97,98] dopamine,[60,93,97–101] and serotonin.[60,93,97–100,102] Most studies found metabolites in IESS were either lower[97,100,102] or no different[60,93,99] to controls (Figure 4). Only one study found dopamine metabolites were elevated.[98] ACTH treatment did not significantly alter primary monoamines.[60,97–99]

Studies examining whether an imbalance of CSF inhibitory (γ-aminobutyric acid [GABA], glycine) or excitatory (aspartate, glutamate) amino acids may lead to IESS have produced variable results (Figure 4). In two of three studies, CSF GABA was lower in IESS than controls[101,103] and significantly lower in IESS with a known aetiology.[99] ACTH reduced CSF GABA, reaching significance in one of two studies.[99,101] ACTH also reduced homocarnosine, an inhibitory hormone produced from GABA and histidine[104] yet these findings were limited by the lack statistical analyses and comparison with controls. One study found ACTH significantly increased aspartate;[105] however, other amino acids were unchanged.[105] Magnetic resonance spectroscopy found no difference in glutamine/glutamate metabolites in IESS compared with controls at baseline.[106] ACTH treatment lowered the glutamine/glutamate signal; however, this normalized on repeat imaging 6 to 11 months later.[106]

Neural pathways and connectivity

Evidence supporting altered neuronal pathways in IESS was based on early autopsy findings that children with IESS and intellectual impairment had reduced dendritic branching and growth of cortical neurons compared with controls.[107,108] One group proposed that IESS originates from brainstem dysfunction and altered serotonergic pathways after identifying common brainstem abnormalities and altered histological expression of neurotransmitters.[109] Subsequent larger autopsy case series have disputed these brainstem abnormalities.[110] Three studies used functional MRI (fMRI) analyses in IESS to assess

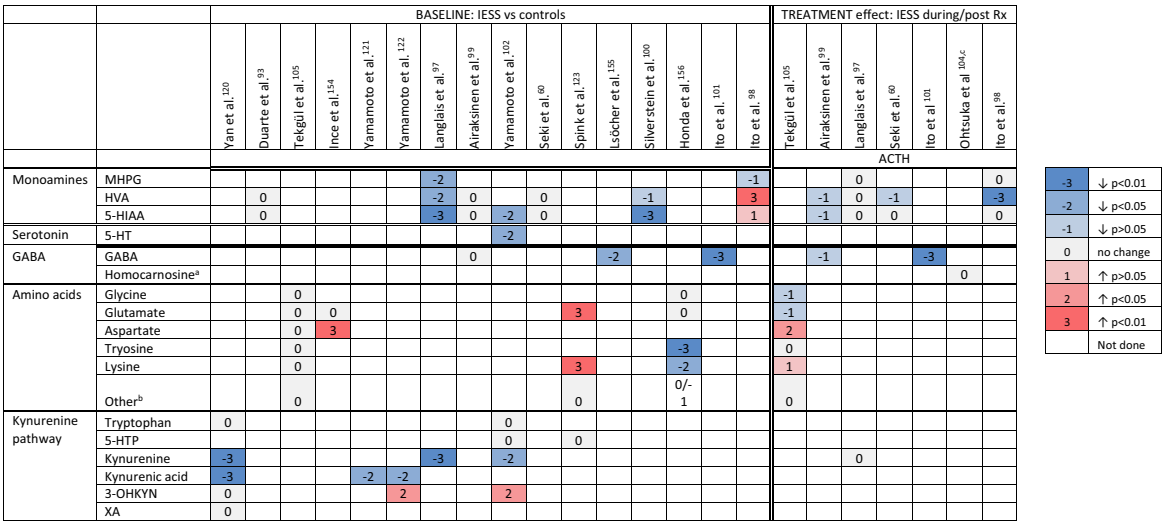

FIGURE 4 Metabolite profile of monoamines, amino acids, and the kynurenine pathway in the cerebrospinal fluid of children with IESS at baseline and following treatment. Evidence indicating CSF GABA and kynurenine pathway metabolites are reduced in children with IESS at epileptic spasm onset without a clear or consistent response to ACTH treatment. [a]An inhibitory neurotransmitter produced from GABA and histidine. [b]Phenylalanine, methionine, threonine, proline, arginine, isoleucine, leucine, alanine, tyrosine, serine, hydroxyproline, cysteine, valine, ornithine, glutamine, histidine, taurine. [c]Baseline higher than reference range (no control comparison). Abbreviations: 3-OHKYN, 3-hydroxykynurenine; 5-HIAA, 5 hydroxyindoleacetic acid; 5-HT, serotonin; 5-HTP, 5-hydroxytryptophan; ACTH, adrenocorticotrophin hormone; GABA, γ-aminobutyric acid; HVA, homovanillic acid; IESS, infantile epileptic spasms syndrome; MHPG, 3-methoxy-4-hydroxyphenylglycol; XA, xanthurenic acid.

default motor networks or connectivity between brain regions. One study investigated neuronal networks that were activated with EEG changes in IESS compared with controls with focal seizures. In IESS, cortical areas were activated in regions with EEG activity and activation was also seen in the deep grey nuclei and occipital regions correlating with EEG slowing.[111] Two studies described the same IESS cohort compared with non-seizure controls and found infants with IESS had disrupted connectivity between regions of the brain that should be well connected in a resting state, the so-called default mode network.[112,113] None of the studies examined the effect of ACTH/steroids on neuronal connectivity. One study found improvement of previously disrupted inter-regional brain connectivity after epilepsy surgery; however, specific data for the two IESS infants were not available.[114]

Markers of neuronal health

Neuronal cell health was analysed by measuring four CSF markers of neuronal injury. Prolactin and nitric oxide metabolites are seen as markers of excitotoxicity. Tau protein is an axonal microtubule-associated protein and neuron-specific enolase is an enzyme within neurons released by damaged cells.[60,115] All four markers, prolactin,[116] tau protein,[115] neuron-specific enolase,[60] and nitric oxide metabolites,[117] were higher in IESS than controls and rose significantly during ACTH treatment. This is indirect evidence of neuronal cell injury/dysfunction in IESS. The authors hypothesized that ACTH may promote death of damaged neurons followed by neuronal repair or regeneration. However, this is speculative; it could also imply that ACTH does not alter the natural history of IESS.

Metabolic pathways

Thirty-three studies explored whether altered metabolic pathways and/or cellular stress contribute to IESS onset and whether ACTH/steroids modify these processes (Figure S1). Nine studies examined the kynurenine pathway, 19 investigated neuronal cell and cerebral metabolism, and five examined growth factors that may regulate neuronal cell survival.

Kynurenine pathway

Early studies identified that ACTH, prednisone, and dexamethasone normalized the urinary excretion of metabolites following a tryptophan load, providing the first evidence that ACTH/steroids alter the kynurenine (KYN) pathway.[118,119] Several studies have since confirmed that children with IESS have significantly lower CSF levels of neuroprotective metabolites KYN[97,102,120] and kynurenic acid (KYNA),[120–122] and a lower KYNA/KYN ratio[120] than controls. CSF 3-hydroxykynurenine (3-OHKY), a neurotoxic subsidiary of KYN, is significantly elevated[102,122] whereas tryptophan did not differ.[102,123] One study analysed ACTH treatment, which did not alter CSF KYN level,[97] while another noted that responders to prednisone had lower baseline CSF KYN and KYNA/KYN than partial or non-responders.[120]

One case series analysed stool microbiota and serum metabolites of the KYN pathway to determine whether microbiome–metabolome interactions affected ACTH treatment response.[124] Before ACTH, non-responders had larger populations of *Clostridioides* and *Peptoclostridium*. This bacterial upregulation correlated with the upregulation of xanthurenic acid. This implied that ACTH treatment failure

may result from increased gut microbiota disturbing KYN metabolism.

Neuronal cell and cerebral metabolism

Neuronal cell metabolism was analysed by serum and CSF markers and various imaging modalities. Serum and CSF markers indicating cellular stress, including lactate, pyruvate, and lactate dehydrogenase, were normal at IESS onset.[125,126] Following ACTH, lactate and pyruvate significantly increased.[125]

Using magnetic resonance spectroscopy, *N*-acetylaspartate, a metabolite produced by neurons/axons, was no different to controls at IESS onset,[106] and ACTH significantly lowered the *N*-acetylaspartate peak, suggesting reduced neuronal activity/energy metabolism.[106,127] Choline peaks, which reflect cell membrane synthesis and degradation, were unaltered.[106,127] Phosphorus metabolites were no different to controls at baseline and were unaltered by ACTH, suggesting oxidative metabolism was not affected.[128] ACTH reduced the ratio of myelin and phospholipid precursors, which is observed as the brain matures, indicating ACTH may alter phospholipid metabolism and myelination, although this is speculative.[128]

Positron emission tomography and single photon-emission computed tomography studies have identified both focal and diffuse areas of cortical and subcortical hypo- and hypermetabolism.[129–135] Only one study compared IESS to controls and did not identify a consistent anatomical focus.[136] Focal imaging changes correlated with focal EEG abnormalities[129,132] and generally resolved on follow-up scans confirming their transient nature (apart from cases of cortical dysplasia).[129,131,132] Single photon-emission computed tomography and carotid ultrasound studies were performed to determine whether ACTH/steroids alter cerebral blood flow and metabolism.[137–142] Only one study compared IESS with controls, reporting increased cerebral blood flow compared with typically developing children, yet no difference to epilepsy controls.[142] ACTH and hydrocortisone reduced cerebral blood flow; however, it remained within normal ranges[142] or no statistical analyses were provided.[137–142]

Growth factors

β-Nerve growth factor (β-NGF) and insulin-like growth factor 1 (IGF-1) regulate neuronal cell growth/survival and early brain development. CSF β-NGF and IGF-1 did not differ in IESS with unknown aetiology from controls yet were significantly lower in IESS with a known cause such as trisomy 21 or stroke.[68,143,144] Histological studies also confirmed loss of IGF-1 cortical neurons in children with IESS due to stroke.[145] ACTH response correlated with higher β-NGF and IGF-1 at baseline;[68,144] however, pre–post-treatment response lacked statistical comparisons.[144]

One study quantified growth factors and found elevated exotoxin, basic fibroblast growth factor, and granulocyte colony-stimulating factor in IESS compared with controls (Figure 3); however, no changes were detected following ACTH.[81] The hypothesis that ACTH enhances the action of growth factors to rescue neurons from apoptosis and improves neuronal or synaptic connections remains speculative.

DISCUSSION

The body of clinical research over the past 65 years summarized here reflects five key hypotheses about the aetiology of IESS and how ACTH/corticosteroids may exert their effect. They include altered gene regulation, stress, neuroinflammation, altered neuronal networks, and altered metabolic pathways. It is likely that these mechanisms interact and coexist, rather than function independently, and supports the concept that IESS is a complex neurodevelopmental disorder. For example, it is described that early life stressors, trauma, and/or inflammation may cause epigenetic modifications which alter gene expression[146] and may increase expression of vulnerability genes in IESS. Given recent advances in bioinformatic analysis, it may be that multi-omics analyses could provide further insights into how these pathways interact or differ in IESS compared with controls or between aetiological subgroups at the DNA, RNA, protein, and metabolite levels. More integrated and standardized approaches may advance or refine some of the earlier hypotheses, for example by investigating the effects of stress through epigenetic analyses rather than the quantification of HPA-axis hormones.

The current evidence supporting altered gene and epigenetic regulation in IESS is limited. Only 23% to 25% of IESS cases[17,19,29,30,32] are explained by monogenic highly penetrant variants which is lower than the 30% to 35% frequency in developmental and epileptic encephalopathies.[22,27] The GO pathways identified in IESS in our review and previous studies encompassed themes of brain development, epigenetic control of cellular processes, and neurotransmission. While cellular transport was a common theme in IESS and other infantile-onset epilepsies, GO pathways in the latter group differed in that they involved motor activity pathways such as cell motility and ion transport and cellular behavioural responses due to stimuli.[42] The heterogeneity in biological pathways indicates that the therapeutic effect of ACTH/steroids is plausible at a more regulatory rather than specific gene/protein level. Given 10% to 20% of the genome is directly regulated by glucocorticoids,[147] it is plausible that ACTH/steroids directly alter gene expression by activating or repressing transcription/translation[148,149] or modify gene expression by indirect mechanisms such as altering transcription factors, RNA (mRNA, non-coding RNA), or epigenetic modifications.[148,149] However, no studies have definitively demonstrated this in IESS.

Evidence supporting HPA-axis activation or 'CRH excess' at IESS onset remains largely speculative. Although one study demonstrated increased expression of CRH and CRHR1 in the brain tissue of infants with IESS, this was

not a clear baseline comparison as infants were undergoing surgery for refractive epilepsy and had been treated with numerous medications.[56] Five studies have reliably identified a CSF ACTH deficiency in IESS compared with controls; however, treatment fails to correct this deficiency. The few studies examining other HPA-axis hormones have failed to identify direct evidence of HPA-axis activation or clear changes following treatment. The methodological limitations of the current evidence make it difficult to definitively exclude the hypothesis that ACTH/steroids alter the HPA-axis hormones, although it seems unlikely that this mechanism alone controls epileptic spasms. Most studies have been limited by small sample sizes, the lack of comparison with a typically developing control group, failure to repeat baseline measures, or inadequate method of assessment and analysis.

The literature supporting neuroinflammation in IESS is limited. Four studies consistently found elevated serum cytokines compared with controls; however, CSF cytokine studies, measures of BBB integrity, and functional imaging studies were either normal or lacked controls or replication studies. Most studies have examined blood or CSF which are less specific than brain tissue and may not reflect processes within the CNS. There is some evidence suggesting ACTH/steroids may suppress T-cell-mediated responses and certain cytokines; however, these studies have not been replicated, and it is not clear whether this is truly altering the disease process in IESS or is an expected effect of ACTH/steroids. The lack of direct evidence supporting neuroinflammation makes it difficult to draw definitive conclusions. We acknowledge the inherent difficulties accessing brain tissue in this population and even future use of CSF biomarkers may prove challenging, as in our experience infants with clear structural aetiologies do not routinely have CSF collected and access to post-treatment CSF samples may be limited by ethical research practices.

The current evidence examining neural transmission in IESS suggests there are no significant measurable and consistent disturbances in monoamine, excitatory, or inhibitory neurotransmission in IESS. CSF GABA was lower in IESS than controls in two studies, yet there were no consistent changes following treatment, even though vigabatrin is known to have efficacy in TSC and non-TSC related IESS. The quality of these studies is limited by similar factors described in the literature on the HPA-axis, including small samples, lack of control comparison, and lack of replication studies. There is minimal evidence supporting abnormalities in neuronal cell function. The evidence is largely derived from early autopsy studies or indirect measures of neuronal cell health. Studies interpreting brain connectivity are limited by the absence of normative fMRI data in the developing brain. Future studies examining neural pathways and connectivity in IESS may be strengthened by analysing a combination of neuroimaging modalities including fMRI or tractography accompanied by non-invasive testing such as EEG or, if feasible, transcranial stimulation.

Lastly, evidence examining the KYN pathway in IESS identified consistent findings that CSF KYN, KYNA, and the KYNA:KYN ratio were lower in IESS than controls and predicted response to steroid treatment in a single study. At a biological level, KYN crosses the BBB and is either converted to KYNA in astrocytes or metabolized in the microglia to quinolinic acid. These metabolites are known to be involved in epilepsy and affect synaptic transmission, inflammation, and immune responses.[150,151] KYNA is a glutamate (NMDA) receptor antagonist and has anti-inflammatory properties, whereas quinolinic acid stimulates the NMDA receptor and may be responsible for excitatory neuronal damage.[150] Further longitudinal studies may confirm the utility of CSF KYN metabolites as a biomarker of treatment response, albeit in a limited population of infants with non-structural pathologies. Evidence of cellular stress and cerebral/neuronal cell dysfunction is inconsistent and assumptions that ACTH may regulate cell survival and neurogenesis remain hypothetical.

The current evidence examining the effect of ACTH/steroids in children with IESS is limited and largely hypothetical. Findings have been inconsistent, which may reflect the heterogeneity in IESS aetiology and treatment response. Reliable and definitive conclusions have been limited by the methodological heterogeneity of studies and the lack of replication of findings. The quality of most studies is limited by small sample sizes, the lack of comparison with a typically developing control group, failure to repeat baseline measures, or inadequate method of assessment and analysis. Most evidence is indirect as there are inherent difficulties accessing brain tissue in this population. As a result, many studies used blood and CSF which are less specific than brain tissue and may not reflect processes within the central nervous system.

It is recommended that future prospective studies use multi-omics sequencing to compare large IESS cohorts with controls to explore gene regulation and the effects of stress/injury/inflammation. Bulk and single-cell approaches including transcriptomics, proteomics, and metabolomics may highlight differences between individuals and aetiological groups at a biological level to explain the heterogeneity of IESS and treatment response. Longitudinal data collection, as evidenced by the EPISTOP multi-omics analysis in TSC,[152] may clearly identify biomarkers of disease progression or treatment response. This may enable individualized treatment protocols in IESS based on the biological mechanisms identified, rather than the current 'one-size-fits-all' approach. Longitudinal studies examining ACTH, steroids, and vigabatrin will better inform us of the long-term neurodevelopmental outcomes or potential harms of treatment. To improve comparability across studies, from the past and future, it is recommended that studies adopt standardized protocols for the measurement of biomarkers and collection of clinical data such as EEG and imaging.

CONCLUSION

The current evidence examining the aetiology and effect of ACTH/steroids in children with IESS is limited and largely hypothetical. Findings have been inconsistent, which may reflect both the heterogeneity in IESS aetiology and treatment response as well as the methodological heterogeneity of studies and lack of reproducibility. Large prospective IESS cohorts with appropriately matched controls will be required to determine differences and commonalities between IESS with a known compared with unknown aetiology and whether ACTH/steroids alter common pathways to control epileptic spasms. An improved understanding of the pathophysiology of IESS and the mechanism of action of ACTH/corticosteroids may lead to new disease-modifying treatments to improve long-term neurodevelopment outcomes.

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CONFLICT OF INTEREST STATEMENT

The authors have stated that they had no interests that might be perceived as posing a conflict or bias.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

The following additional material may be found online:
Appendix S1: Methodology for review.
Appendix S2: Methodology for assessing genetic disorders in IESS.
Figure S1: Flow diagram of studies selected for inclusion in review.
Figure S2: Hormonal profile of the HPA-axis in the CSF of children with IESS at baseline and following treatment.
Figure S3: Hormonal profile of the HPA-axis in the blood of children with IESS at baseline and following treatment.
Figure S4: Inflammatory and immune profile in the CSF children with IESS at baseline and following treatment.

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2.3 Supplementary Material

Supplementary Text 1: Methodology for review

The methodological workflow and process of selecting studies for inclusion is presented in Supplementary Figure 1. Exemplar PubMED search strategy below with limits applied.

Search History	
1	Infantile spasms
2	infantile epileptic spasm syndrome.mp
3	spasms
4	epileptic spasms.mp
5	west syndrome
6	1 or 2 or 3 or 4 or 5 or 6
7	steroids
8	corticosteroids.mp
9	prednisolone
10	hydrocortisone
11	dexamethasone
12	methylprednisolone
13	adrenocorticotrophin hormone
14	ACTH.mp
15	Tetracosactide.mp
16	treatment
17	7 or 8 or 9 or 10 or 11 or 12 or 12 or 14 or 15 or 16
18	biomarkers
19	Effect.mp
20	Action.mp
21	Change.mp
22	mechanism
23	18 or 19 or 20 or 21 or 22 or 23 or 24
24	6 AND 17 AND 23
25	Limit 24 to (humans and “all child (0 to 18 years)” and full-text article)
26	aetiology
27	cause
28	Gene.mp
29	Genetic
30	metabolic
31	inflammation
32	immune
33	excitation
34	neurotransmitters
35	26 or 27 or 28 or 29 or 30 or 31 or 32 or 33 or 34
36	6 AND 23 AND 35
37	Limit 24 to (humans and “all child (0 to 18 years)” and full-text article)

After initial search and three consensus meetings to finalise hypotheses (supplementary figure 1), keywords based on each hypothesis were used to search the same databases and a similar filtering process for inclusion/exclusion was applied to these search results. For example, Kynurenine was used as key word and a title term combined with “AND” (Infantile spasms OR infantile epileptic spasm syndrome.mp OR IESS). The last search was conducted on 20th December 2023.

Supplementary Text 2: Methodology for assessing genetic disorders in IESS

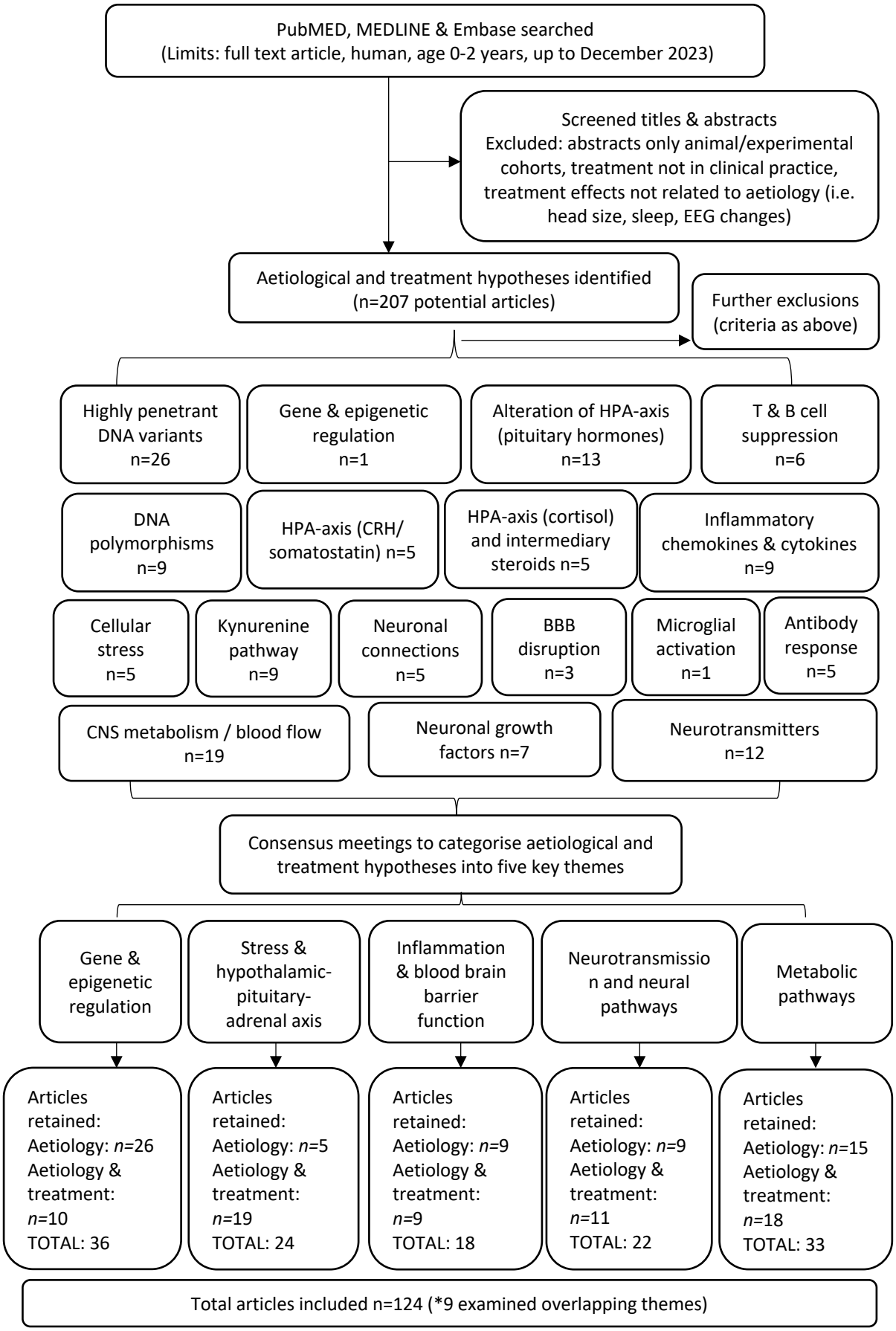
Three online databases, PubMED, MEDLINE and EMBASE databases were searched from 2010-December 2023 for IESS cohort studies examining genetic aetiology and the yield of genetic testing. Studies included met the following criteria: (1) a clearly defined IESS cohort with 2) individual data confirming the genetic diagnosis (obtained from the main publication or supplementary data), (3) IESS cohort of ≥ 5 participants, (4) sufficient data to confirm and categorise aetiological groups according to ILAE guidelines (structural, genetic, infectious, metabolic, immune and unknown). To present the genetic aetiology clearly, we further divided the structural group into structural (acquired/congenital) causes such as brain injury, stroke and genetic-structural disorders such as TSC and disorders of neuronal migration. Studies were excluded that (1) described IESS cohorts associated with a single gene, (2) generalised results across other cohorts such as IESS and Lennox-Gaustaut Syndrome without providing individual IESS data or (3) were written in languages other than English.

Search terms included the following keywords and derivatives: “infantile epileptic spasm syndrome”, “infantile spasms”, “spasms”, “epileptic spasms”, “West syndrome”, “aetiology”, “cause”, “classification”, “diagnosis”, “gene”, “genetic”, “genotype”, “genomic”, “test”, “yield”, “sequencing”, “exome”. References were also hand-searched.

After screening titles and abstracts, 32 studies were identified that met inclusion criteria. Two studies included previously published cohorts (119, 120)[1] as such the most recent publication was included. After reading full-text articles, 6 studies were excluded as they had insufficient data to categorise aetiological groups/confirm genetic diagnosis, included less than five IESS participants or were not written in English. (121-126)

[1] 1. Peng J, Wang Y, He F, Chen C, Wu LW, Yang LF, et al. Novel West syndrome candidate genes in a Chinese cohort. CNS Neurosci Ther. 2018;24(12):1196-206.
2. Allen AS, Berkovic SF, Cossette P, Delanty N, Dlugos D, Eichler EE, et al. De novo mutations in epileptic encephalopathies. Nature. 2013;501(7466):217-21.
3. Chourasia N, Yuskaitis CJ, Libenson MH, Bergin AM, Liu S, Zhang B, et al. Infantile spasms: Assessing the diagnostic yield of an institutional guideline and the impact of etiology on long-term treatment response. Epilepsia. 2022;63(5):1164-76.
4. Hu C, Liu D, Luo T, Wang Y, Liu Z. Phenotypic spectrum and long-term outcome of children with genetic early-infantile-onset developmental and epileptic encephalopathy. Epileptic disorders. 2022;24(2):343-52.
5. Mitta N, Menon RN, McTague A, Radhakrishnan A, Sundaram S, Cherian A, et al. Genotype-phenotype correlates of infantile-onset developmental & epileptic encephalopathy syndromes in South India: A single centre experience. Epilepsy Res. 2020;166:106398.
6. Mercimek-Mahmutoglu S, Patel J, Cordeiro D, Hewson S, Callen D, Donner EJ, et al. Diagnostic yield of genetic testing in epileptic encephalopathy in childhood. Epilepsia. 2015;56(5):707-16.
7. Juanes M, Loos M, Reyes G, Veneruzzo G, García FM, Aschettino G, et al. Clinical and genetic study of developmental and epileptic encephalopathy in Argentinean pediatric patients. Medicina (B Aires). 2022;82(6):856-65.
8. Duc NM, Thu NTM, Bui CB, Hoa G, Le Trung Hieu N. Genotype and phenotype characteristics of West syndrome in 20 Vietnamese children: Two novel variants detected by next-generation sequencing. Epilepsy Res. 2023;190:107094.

Supplementary Figure 1: Flow diagram of studies selected for inclusion in review



Supplementary Figure 2: Hormonal profile of the HPA-axis in the CSF of children with IESS at baseline and following treatment

		BASELINE: IESS vs controls									TREATMENT effect: IESS during/post Rx										
		Aydln et al 2002	Nagamitsu et al 2001	Hirai et al 1998	Baram et al 1995	Baram et al 1992	Seki et al 1990	Nalin et al 1985	Facchinetti et al 1985	Hagishima et al 1972a	Aydln et al 2002	Hirai et al 1998*	Heiskala et al 1997	Seki et al 1990*	Riikonen et al 1988	Nalin et al 1985	Facchinetti et al 1985	Hagishima et al 1972a	Fukazawa et al 1972	Hagishima et al 1972a	Fukazawa et al 1972
											ACTH									Hydrocortisone	
Hypothalamic	CRH		-1			0															
	Somatostatin			2			-1					-1		-1							
Pituitary	ACTH		-3		-3	-2		-2	-2				-1		-1	1	1				
	β-endorphin		-3					0	0				-1			0	0				
	Prolactin	3									2										
Adrenal	Cortisol				-3	-1				1								1		1	
	11-OHCS																		1		1

-3	↓ p<0.01
-2	↓ p<0.05
-1	↓ p>0.05
0	no change
1	↑ p>0.05
2	↑ p<0.05
3	↑ p<0.01
	Not done

Key: CRH=corticotrophin releasing hormone, ACTH=adrenocorticotrophin hormone, 11-OHCS=11-hydroxycorticosteroid, *pyridoxal phosphate given prior to ACTH

Supplementary Figure 3: Hormonal profile of the HPA-axis in the blood of children with IESS at baseline and following treatment

		BASELINE: IESS vs controls		TREATMENT effect: IESS during/post Rx												
		Nalin et al 1985	Hagishima et al 1972a	Yamamoto et al 1998	Kusse et al 1993	Seki et al 1990*	Snead et al 1989	Riikonen et al 1986	Izumi et al 1984/85	Sugie 1983b	Hagishima et al 1972a/b	Fukazawa et al 1972	Yamamoto et al 1998	Hagishima et al 1972a/b	Fukazawa et al 1972	Farwell et al 1984
				ACTH									Dexamethasone	Hydrocortisone		Prednisolone
Hypothalamic	CRH															
	Somatostatin															
Pituitary	ACTH	0			1		1									
	β-endorphin	0														
	LH			0		0							0			
	FSH			0		0							0			
	GH			0		0			0				0			
	TSH			0		0	0						0			
	Prolactin					0	0									
Adrenal	Cortisol		3	0	-1	0	1	1	1	1	1		0	1		-1
	DHEAS				-2			0								
	Androstenedione				0											
	11-OHCS											1			1	
Thyroid	T3, T4					0	0		0				0			

-3	↓ p<0.01
-2	↓ p<0.05
-1	↓ p>0.05
0	no change
1	↑ p>0.05
2	↑ p<0.05
3	↑ p<0.01
	Not done

Key: CRH=corticotrophin releasing hormone, ACTH=adrenocorticotrophin hormone, LH=Luteinising hormone, FSH=Follicle-stimulating hormone, GH=growth hormone, TSH=thyroid stimulating hormone, DHEAS=dehydroepiandrosterone sulfate, 17-OHP=17-hydroxyprogesterone, 11-OHCS=11-hydroxycorticosteroid, T3=triiodothyronine, T4=thyroxine, *pyridoxal phosphate given prior to ACTH

Supplementary Figure 4: Inflammatory and immune profile in the CSF children with IESS at baseline and following treatment

	BASELINE: IESS vs controls		TREATMENT effect: IESS during/post Rx
	Haginoya et al 2009	Baram et al 1992	Sousa et al 2012
			ACTH
IL-1RA	-2		
IL-1β	-1	-1	-1
IL-2			
IL-2R			
IL-4			
IL-5			
IL-6	-1		
IL-7			
IL-7A			
IL-8			
IL-9			
IL-10			
IL-12			
IL-13			
IL-15			
IL-17			
IL-17A			
IL-23			
IFN-γ			
IFN-IP-10			
IFN-α			
TNF-α	-1		
TNF-β			

-3	↓ p<0.01
-2	↓ p<0.05
-1	↓ p>0.05
0	no change
1	↑ p>0.05
2	↑ p<0.05
3	↑ p<0.01
	Not done

2.4 Summary

The evidence from this review suggests that the aetiopathogenic mechanisms underlying IESS and the therapeutic effects of prednisolone may involve five key pathways, including altered gene regulation, stress, neuroinflammation, altered neuronal networks, and altered metabolic pathways. The evidence for each individual hypothesis or pathway is limited, however we propose that these pathways are more likely to interact with one another, rather than exist independently, and affect the maturational and regulatory processes of the CNS more broadly through changes in gene expression.

This aligns to the concept that IESS is a complex NDD and supports earlier proposals that IESS may result from a disruption of developmental processes at any critical timepoint during ontogenesis or pre-, peri- and post-natal neurodevelopment. This may also explain the heterogeneity within IESS, as there are known vulnerability genes directly responsible for neuronal signalling/migration (*SCN2A*, *DCX* etc), whereas other "regulatory" genes that alter gene regulation more broadly (genes encoding transcription factors/other genetic modulators) may be expressed due to stress, infection or inflammation and influence processes across these five pathways. Further, it is plausible that corticosteroids including prednisolone exert an anti-seizure effect in IESS by altering gene regulation directly through transcription/translation dependent processes or indirectly through transcription factors, RNA and epigenetic modifications.

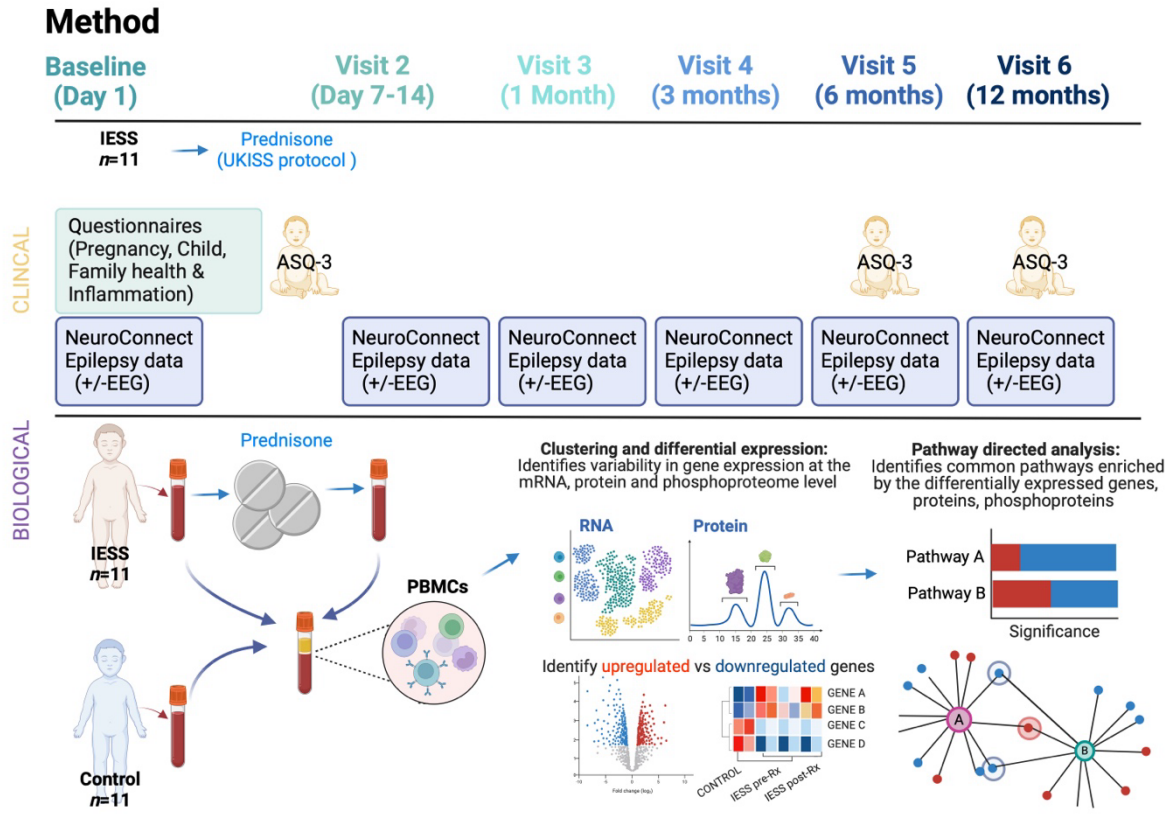
This literature review revealed a gap in the evidence examining the gene regulation hypothesis in IESS. This formed the basis of my multi-omics investigation, detailed in chapters three and four, exploring gene regulation and immune profiles in infants with IESS.

CHAPTER 3 – IESS clinical cohort study

3.1 Introduction and study overview

The primary aims of this study were to investigate possible mechanisms of disease underlying IESS and how prednisolone treatment exerts an anti-seizure effect in IESS. Secondary aims were determining if response to prednisolone treatment altered longer-term epilepsy and neurodevelopmental outcomes. My research involved two key components, a prospective clinical cohort study and a multi-omics biological investigation, further described in Figure 3.1. The methods and results will be presented across two chapters. Chapter three will describe key clinical features, investigations and clinical follow up. Chapter four will describe biological investigations undertaken using a multi-omics approach to analyse gene regulation and immune profiles of infants with IESS pre and post prednisolone treatment, compared to healthy controls. Prednisolone treatment was investigated as it is the accepted standard treatment for IESS in Australia. Patient recruitment and blood sampling for this project was designed to occur in tandem with routine clinical care.

Figure 3.1 Overview of study methodology comprising a clinical cohort study and biological investigation into disease and therapeutic mechanisms of prednisolone in IESS
Eleven infants with IESS were prospectively enrolled into a clinical cohort study to extensivey phenotype epilepsy and neurodevelopmental outcomes at baseline before prednisolone treatment and followed for 12 months post treatment. Prednisolone treatment followed the United Kingdom Infantile Spasms Study (UKISS) protocol. Standardised data collection tools were used including the "NeuroConnect" database at each of the six assessments over the 12 month period and the Ages and Stages Questionnaire-3rd edition (ASQ-3) was performed at baseline, 6- and 12- months post treatment. Venous blood samples were taken prior to commencing prednisolone and repeated at visit 2, after 7-14 days of treatment when routine clinical monitoring bloods were performed. Whole blood samples were used for bulk RNA sequencing and neuro-immune panel testing, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood and used for proteomic and phosphoproteomic sequencing. Bioinformatic analysis identified differentially expressed genes, proteins and phosphoproteins. Pathway-directed analysis was performed to identify common biological pathways enriched by these differentially expressed genes, proteins and phosphoproteins. N.B. Authors own figure created using BioRender.com



Key: ASQ-3=Ages and Stages Questionnaire – 3rd Edition, EEG=electroencephalogram, IESS=Infantile Epileptic Spasms Syndrome, PBMCs=peripheral blood mononuclear cells, Rx=Treatment, UKISS=United Kingdom Infantile Spasms Study.

3.2 Hypotheses and aims

Hypotheses

1) It was hypothesised that the majority of infants with IESS would achieve epileptic spasms cessation within the first 14 days of prednisolone treatment. A smaller proportion, up to 30% as described in the literature, were expected to be non-responders to prednisolone treatment alone.
2) It was hypothesised that infants with IESS of unknown aetiology with early cessation of spasms are more likely to remain spasm and/or seizure free with typical development at 12-months post-treatment compared to infants with structural, immune or infectious IESS aetiologies and/or pre-existing epilepsy and developmental delays.
3) At 12 months follow-up, non-responders were expected to have higher rates of epilepsy requiring two or more medications and are more likely to be affected by moderate-severe global developmental delays rather than isolated or mild global development delay.

Aims

1) Phenotype a cohort of infants with IESS and evaluate the short-term effectiveness of prednisolone in achieving cessation of epileptic spasms by day 14 of treatment
2) Assess neurodevelopmental outcomes at a 12-month follow-up including
   i) Recurrence of epileptic spasms
   ii) Neurological co-morbidities including presence of epilepsy and
   iii) Developmental status using Ages and Stages Questionnaire-Third edition (ASQ-3)
3) Compare clinical outcomes between “responders” and “non-responders” to prednisolone, where responders were defined as those who achieved epileptic spasm cessation and improvement in the EEG by day 14 of treatment, compared to non-responders that continued to have epileptic spasms after 14 days of treatment, or required earlier escalation to ACTH or other anti-seizure medications.

3.3 Methodology for IESS clinical cohort study

3.3.1 IESS cohort selection criteria, recruitment and ethics approval

3.3.1.1 Selection Criteria

Eligible infants were aged between one to 24 months with newly diagnosed IESS due to receive prednisolone treatment. IESS diagnosis had to be made by the treating child neurologist in accordance with the 2022 ILAE recommendations. (2) The presence of epileptic spasms was mandatory for IESS diagnosis, defined as a sudden brief contraction of the axial/truncal muscles and extremities, which could be flexor, extensor or mixed. I confirmed the diagnosis of IESS by reviewing all video evidence of epileptic spasms to visualise and confirm the type of epileptic spasm and the presence of clustering. I also reviewed all routine or prolonged video EEG recordings to ensure the EEG features were supportive of IESS.

For inclusion, we stipulated that treatment doses of prednisolone must follow the Lux et al., (2004, 2005) (17, 18) UKISS protocol. The starting prednisolone dose is 10mg four times a day for 14 days however, if epileptic spasms are ongoing from day seven of treatment, the dose may escalate to 20mg three times a day. Clinicians were not strictly required to reduce prednisolone every five days as in clinical practice it is much easier for families to follow weekly changes. Infants that received both prednisolone and vigabatrin following the ICISS protocol (14) were still eligible to participate, as this is commonly prescribed for conditions such as TSC, however, the additional vigabatrin was captured as a confounder.

Participants were not excluded based on their IESS aetiological diagnosis or if they had a history of seizures or NDDs prior to epileptic spasm onset, as both are common co-morbidities. Participants were excluded if they had received corticosteroids at any time point before IESS diagnosis, if prednisolone dose deviated from the UKISS protocol or if they were unable to attend the second blood collection (visit two in Figure 3.1) within the recruitment sites. Participants with insufficient English language skills to understand the study demands and complete questionnaires were excluded.

3.3.1.2 Recruitment

Infants with IESS were recruited from the Sydney Children's Hospital, Randwick and The Children's Hospital at Westmead, both part of the Sydney Children's Hospital Network (SCHN) and two of three tertiary paediatric hospitals in NSW, Australia. As IESS is an urgent and serious diagnosis it was expected that all cases would be identified as acute inpatient admissions. Treating clinicians confirmed cases of IESS, provided the families with a participant information sheet and referred them to the study team. I explained the study to parents/carers in person or via phone and obtained informed written consent.

3.3.1.3 Ethics and study design

The project was undertaken as part of two existing ethical frameworks within SCHN-Neuro-Tx: Investigating mechanisms of disease and therapeutics in neurology (HREC 2021/ETH00356) and NeuroCONNECT: The SCHN Registry of Neurological Disorders (HREC 2020/ ETH03064). The latter includes the use of a standardised data collection tool. Informed written consent was collected at enrolment and verbal consent for ongoing participation at each visit.

Importantly, my research was designed such that the collection of clinical and biological data would complement routine clinical care. We wanted to ensure that research did not alter or interfere with clinical care yet occurred in parallel. Our goal was to facilitate research participation through ethically responsible approaches that minimise demands on families, caregivers and clinicians. Therefore, as part of the study design which will be discussed below there were no investigations that were considered mandatory for inclusion in the study and clinicians were free to select the type and timing of investigations.

3.3.2 Clinical phenotyping and longitudinal monitoring including standardised tools and questionnaires utilised

3.3.2.1 Longitudinal time points

To clinically phenotype infants with IESS and monitor their epilepsy and developmental outcomes over time, there were six time points of data collection as presented in Figure 3.1. Infants were assessed at baseline (visit one) prior to the first dose/day one of prednisolone treatment and reviewed at day 7-14 (visit two), one month (visit three), three months (visit four), six months (visit five) and 12 months (visit six) from baseline. I chose to follow infants for 12 months to allow sufficient time for an aetiological diagnosis to be established, particularly as genomic results may take six to 12 months. In addition, the 12-month follow-up allowed me to better assess neurodevelopmental outcomes as the UKISS findings demonstrated that rates of epilepsy and developmental impairment were similar at 12 months compared to four years of follow-up. (17, 18) I performed the visits and data collection in person, or if required by telephone.

The following standardised tools were used at each visit and will be discussed in detail in the next section:

- *Visit 1: Baseline (Day one)*
    - Standardised medical history and epilepsy phenotyping using NeuroCONNECT.
    - Baseline questionnaires: Family and Pregnancy Screener and Maternal Current Health Screener, the Child Health and Inflammation Screener
    - Developmental phenotyping using ASQ-3
- *Visit 2: Day 7-14*
    - Standardised medical history and epilepsy phenotyping using NeuroCONNECT.
- *Visit 3: One month*
    - Standardised medical history and epilepsy phenotyping using NeuroCONNECT
- *Visit 4: Three months*
    - Standardised medical history and epilepsy phenotyping using NeuroCONNECT
- *Visit 5: Six months*
    - Standardised medical history and epilepsy phenotyping using NeuroCONNECT
    - Developmental phenotyping using ASQ-3

- *Visit 6: 12 months*
    - Standardised medical history and epilepsy phenotyping using NeuroCONNECT
    - Developmental phenotyping using ASQ-3

3.3.2.2 Standardised medical history using NeuroCONNECT (electronic data capture tool)

At each time point, I used "NeuroCONNECT" (127) an electronic data collection tool created at SCHN that has allowed the standardised, detailed phenotyping of individuals with neurological disorders over time. I completed this during a 20-minute interview with the parents/caregivers. Data was entered using the University of Sydney Redcap database. See Appendix 1 for the full copy of NeuroCONNECT.

NeuroCONNECT incorporates the child's medical history in five sections. The first section captured demographic data, including the infant's date of birth and details of the interviewee and interviewer's name. The second section identified the neurological diagnoses including, but not limited to, epilepsy, cerebral palsy (CP), NDDs as well as disorders of other organs or systems such as visual or hearing impairment and cardiac disease. Each neurological disorder had further sub-sections capturing disease characteristics, such as age at onset, phenotype, e.g. CP type/topography, disease severity and current disease status measured using validated tools and disease specific tools where possible, e.g. Cerebral Palsy Gross Motor Function Classification System (128), and medication history including past and current therapies. The third section captured disease aetiology, either confirmed or suspected genetic, structural, metabolic, immune disorders, other not specified or unknown aetiology. This included relevant investigation results. Following detailed phenotyping, disorders were classified using Human Phenotype Ontology terms (129) and ranked from the most dominant phenotypic feature/disorder to other associated features. The final two sections captured the maternal and paternal family history and the pattern of disease onset and trajectory using descriptors i.e. acute illness or chronic disorder with progressive course. To measure the disease severity and impact of treatment in a standardised way I used the Clinical Global Impression scale (130) provided in Appendix 2. This is a clinician-rated global assessment of disease severity and treatment outcomes using seven ordered fixed response categories. Treatment efficacy was also

measured using a matrix to score treatment response in relation to side-effects. I chose this tool as it is not “disease specific”, it can be applied to all individuals and is a widely used scale for clinicians to track progress and response to treatment over time. I completed a standardised medical history using NeuroCONNECT through a 20-minute interview with parents/caregivers at all six visits.

3.3.2.3 Epilepsy Phenotype

Epilepsy phenotype was collated into NeuroCONNECT at all six visits. I collected data from semi-structured parent/caregiver interviews, seizure diaries and review of medical records. At baseline, I recorded in detail any history of epilepsy or febrile seizures, including age at seizure onset, seizure type/s, seizure trigger (fever, infection, vaccination), seizure frequency and duration, episodes of status epilepticus, admission to hospital or intensive care, underlying aetiology, investigations, response to treatment and any co-morbidities. Epilepsy syndromes were classified according to 2022 ILAE operational guidelines. (2, 131, 132)

To accurately phenotype IESS, I confirmed the type of epileptic spasms, defined as per ILAE guidelines as flexor, extensor or mixed by reviewing videos of the event obtained through video-EEG recordings and/or those provided by the caregiver. If/when required a consensus agreement was achieved by a second investigator review (A/Prof Shekeeb Mohammad). Clustering of epileptic spasms was confirmed by the same method and described quantitively by recording the maximum number of epileptic spasms per cluster and number of clusters in the 24-hour period preceding IESS diagnosis. Seizure triggers were noted (sleep, fever, infection, vaccination, other). Time from IESS onset to diagnosis and treatment commencement was calculated in days.

Medications including number, dose range (per milligram/per kilogram/day), side-effects, date of commencement and cessation and reason for cessation (ineffective, side-effects or other) were recorded at each visit. See appendix 3.

At subsequent visits, the IESS/epilepsy phenotype was collected as previously described to capture treatment outcomes over time including change to seizure burden (frequency, medications) and/or seizure types. Other variables recorded included time from treatment commencement to epileptic spasms cessation, duration of treatment, duration of epileptic spasms in days and if participants were “responders” or “non-responders” to treatment at day 14 of prednisolone treatment. For other seizure types, response to treatment was captured by estimating the percentage reduction in total seizures compared to baseline.

3.3.2.4 Neurodevelopmental monitoring including Ages and Stages Questionnaire – Third Edition

Developmental history was obtained at each of the six visits through the semi-structured interview and corroborated with the medical record. At baseline, I screened for the presence of developmental regression and/or plateauing of skills and physician diagnosed isolated or global developmental delay, the latter defined as significant delays in two or more developmental domains including a cognitive component in children younger than six years. (133) Severity of the delay was recorded as per the physician report of mild, moderate or severe. I reviewed formal developmental assessments measuring developmental quotients if they were performed.

To complement this history, I used the Ages and Stages Questionnaire, Third Edition, (ASQ-3)(134) at baseline, six and 12 months as an objective measure to monitor development over time. At baseline, the child’s best developmental performance prior to any regression was also noted. The ASQ-3 is a 10–15-minute parent-completed questionnaire that can be used from one-to 66 months of age. The ASQ-3 measures five developmental domains including communication, gross motor, fine motor, problem solving and personal-social skills. Each domain contains six questions that can be answered yes (10 points), sometimes (five points) or not yet (0 points), with a possible raw score of 60 points per domain and 300 in total. Scores for each domain are plotted in a bar chart that visually discriminates between three groups based on age-matched normative data. The groups are categorised as “doing well” including scores up to one standard deviation below the mean,

"monitoring" where scores fall between one to two standard deviations below the mean and "requires referral" as scores are more than two standard deviations below the mean. The z-scores enabled me to objectively identify typically developing infants from those with or at risk of developmental delay, that required further correlation with the clinical history or discussion with the treating neurologist. The ASQ-3 also has an "overall" section containing 4-10 unscored open response questions that captured parental concerns and promoted discussion.

The ASQ-3 was chosen as it is a quick, cost-effective, and easy to complete tool that did not require training, or extra resources to complete. It is a reliable, validated screening tool with a high sensitivity and specificity (129, 134-136) for identifying children with or at risk of developmental delay with results reproduced in various global populations. (137, 138) Although it is inferior to formal neuropsychological assessments such as the Bayley Scales of Infant and Toddler Development, (139) I used it as serial objective measurement to monitor development over time.

3.3.2.5 Baseline screening questionnaires

At baseline I completed the three screening questionnaires described below with families as part of a detailed medical history. Our research group developed these screeners (127) to evaluate the relationship between NDDs and prenatal, antenatal and postnatal stressors including infections, inflammation, auto-immunity and lifestyle and environmental factors such as obesity, smoking, and exposure to trauma. They were created to capture variables in a standardised way to explore our hypothesis that stress and immune dysregulation influence gene expression in the developing brain.

The screeners included the Family and Pregnancy Screener (Appendix 4), Maternal Current Health Screener (Appendix 5), and the Child Health and Inflammation Screener (Appendix 6).

SCREENING QUESTIONNAIRE 1: The Family and Pregnancy Screener enabled a standardised family history, detailing a three-generation pedigree, presence of

autoimmune, atopic, psychiatric, neurological and other medical conditions in first- or second-degree relatives. Pregnancy history captured age, body-mass index (BMI), complications (e.g. Gestational Diabetes, infections, hypertension), medication use, smoking status, alcohol consumption and presence of stressful life events. Neonatal details included gestational age, mode of delivery, birthweight and complications including infection or hospital admission.

SCREENING QUESTIONNAIRE 2: The Maternal Current Health Screener recorded ethnic and social background (e.g. education, marital status), current use of medication, alcohol and smoking. The Perceived Stress Scale (PSS-10),(140) a validated (141, 142) 10 item psychometric measure of psychological distress was self-completed by mothers to determine the degree to which they perceived life as unpredictable, uncontrollable and were unable to meet current demands. The PSS-10 follows a Likert scale with each response scored 0-4 with a maximum total score of 40. Scores between 0-13 were considered low stress, 14-26 moderate stress and 27-40 high stress. (140) Scores were compared to normative data. High scores are known to be associated with elevated cortisol, suppressed immune function and susceptibility to infections. (143)

SCREENING QUESTIONNAIRE 3: The Child Health and Inflammation Screener captured age, BMI, history of infections, atopy, autoimmune, gastrointestinal disorders, neurodevelopmental symptoms including loss of skills, potential triggers and course of symptoms in addition to stress/trauma exposure.

3.3.2.6 Investigations and Electrophysiology

At each of the six visits, I systematically collated results of investigations performed to accurately phenotype IESS aetiology according to ILAE guidelines as structural, genetic, infectious, metabolic, immune or unknown. (2, 131, 132) It was expected that there would be some variability between IESS participants as the study design stipulated that treating clinicians were free to select the type and timing of investigations, including EEG, imaging, biochemical and genetic testing.

3.3.2.6.1 Laboratory and Imaging studies

Investigations reviewed and recorded included haematology, biochemistry, ammonia, lactate, vitamin B12, folate, vitamin D, biotinidase, copper, ceruloplasmin, amino acids, thyroid function, acylcarnitine profile, transferrin isoforms, very long chain fatty acids, urine metabolic screen, CSF glucose, protein, lactate, cell count, culture, neurotransmitters, folate metabolites, amino acids, genetic testing including microarray, epileptic encephalopathy (EE) panel, exome or genome sequencing, imaging including ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and any other investigations grouped as miscellaneous tests.

3.3.2.6.2 EEG review of interictal and ictal features

As part of this detailed phenotyping I reviewed all EEG recordings performed throughout the 12-month study period. This review was unblinded as I needed to confirm ictal and interictal EEG features were supportive of IESS at diagnosis as per ILAE guidelines (2) rather than an alternate epileptic encephalopathy to be eligible for study inclusion. As there is no uniform consensus statement describing the electrophysiology features of hypsarrhythmia or variants of hypsarrhythmia quantitively, I described the interictal EEG features including using qualitative and categorical descriptions of the background (symmetry, slowing), lateralising features (epileptiform, non-epileptiform activity), the interictal background - either hypsarrhythmia or modified hypsarrhythmia (Table 3.1).

Hypsarrhythmia was used when the background activity appeared “chaotic” or highly disorganised, consisting of continuous high voltage irregular slow wave activity with “random”, independent, multifocal spikes with or without spike-slow or sharp wave morphology. The disorganised background had to be continuously present during wakefulness and sleep, arrhythmic with intrahemispheric and interhemispheric asynchrony. See Table 3.1 for descriptors. Modified Hypsarrhythmia was used when there were variations or modifications to the hypsarrhythmia pattern including asymmetry (“hemi” or unilateral hypsarrhythmia); a consistent focus of abnormal discharges; a predominant slow wave activity with few spike or sharp waves; episodes of voltage attenuation which could be

generalised or regional, the most severe of which termed burst-suppression; and lastly partial preservation of rhythmicity and inter-hemispheric synchronisation.

Table 3.1 Interictal features described in hypsarrhythmia and modified hypsarrhythmia adapted from several sources (57-65)

EEG feature	Description	Hypsarrhythmia	Modified Hypsarrhythmia
High voltage, irregular slow waves	Waves greater in amplitude than background EEG activity	Present across both hemispheres	Present with periods of localised, regional or generalised attenuation AND/OR asymmetry between hemispheres e.g. hemi-hypsarrhythmia
Continuous slow wave activity	Present in wakefulness and sleep	Present	Absent (NB activity often seen in NREM sleep, may be absent or minimal in REM sleep; temporal variations seen during sleep-wake states)
Disorganisation Arrhythmia	Appears chaotic or highly disorganised with no consistent rhythm or pattern	Present continuously (hallmark feature)	Present intermittently - periods alternate with more organised or recognisable awake or sleep rhythms (“relative normalisation”)
Disorganisation - Asynchrony	Sharp, slow and spike waves are arhythmic and do not appear synchronously across regions of the brain	Present	Absent (NB periods of synchronised waveforms between brain regions present)
Spike and sharp waves	Appear random, independent, asynchronous, vary in duration and location	Present. Waveforms are multifocal or may be generalised	Focality to waveforms, may be rhythmic or present in a highly organised pattern suggesting consistent focus, relatively few spike and sharp waves

As part of the EEG review, I confirmed that the ictal recording of the epileptic spasm was consistent with that described in the literature, classically a generalised moderate to high amplitude slow waves (1-2 Hz) with overriding fast activity or lower frequency 14-19 Hz spindle-like waveforms followed by significant electro-decrement or voltage attenuation correlating to the tonic contraction. (4, 38, 39) Variations to this pattern are seen and were qualitatively described.

3.3.2.6.3 Epileptic encephalopathy gene panel testing

Genomic investigations including EE panel were ordered by the treating clinician at their discretion. Sequence analysis was performed at SCHN NATA-accredited laboratories using the TruSight One panel (FC-141–1007) on an Illumina NextSeq550. This panel varied over time capturing up to 300 genes, referenced as "green" genes by PanelApp Australia - Genetic Epilepsies panel (https://panelapp-aus.org/panels/202/, last accessed December, 2024). Genetic testing varied amongst the cohort. Three participants with unknown IESS aetiology (cases 1, 3, 5) only had a microarray and single gene testing for *ARX* variants. Prior to embarking on multi-omics analysis, our study group met and discussed this as a notable limitation. In conjunction with the treating clinicians' support, I consented these three participants to undergo genetic testing on a research basis. Samples were sent for analysis at Australian Genome Research Foundation using Illumina NovaSeq X Plus exome sequence targeting a curated list of 51 genes identified from my literature review in Chapter two, examining genetic aetiology in IESS. These genes were reported as pathogenic/likely pathogenic in two or more individuals from 24 studies including 3000 children with IESS. We chose to perform this rather than a larger genetic epilepsy panel as we felt it would capture known IESS genetic aetiologies and balanced this against our limited bioinformatic resourcing to process larger gene lists. Rare genetic changes were examined using the Sorting Intolerant From Tolerant (SiFT, https://sift.bii.a-star.edu.sg, last accessed December 2024) and PolyPhen scores (http://genetics.bwh.harvard.edu/pph2/, last accessed December 2024) to assign pathogenicity and checked within ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/, last accessed December 2024) and Franklin databases (https://franklin.genoox.com/clinical-db/home, last accessed December 2024). I

reviewed the preliminary results and variants were resolved during a consensus meeting with Prof Russell Dale and A/Prof Shekeeb Mohammad.

3.4 Analysis of clinical variables

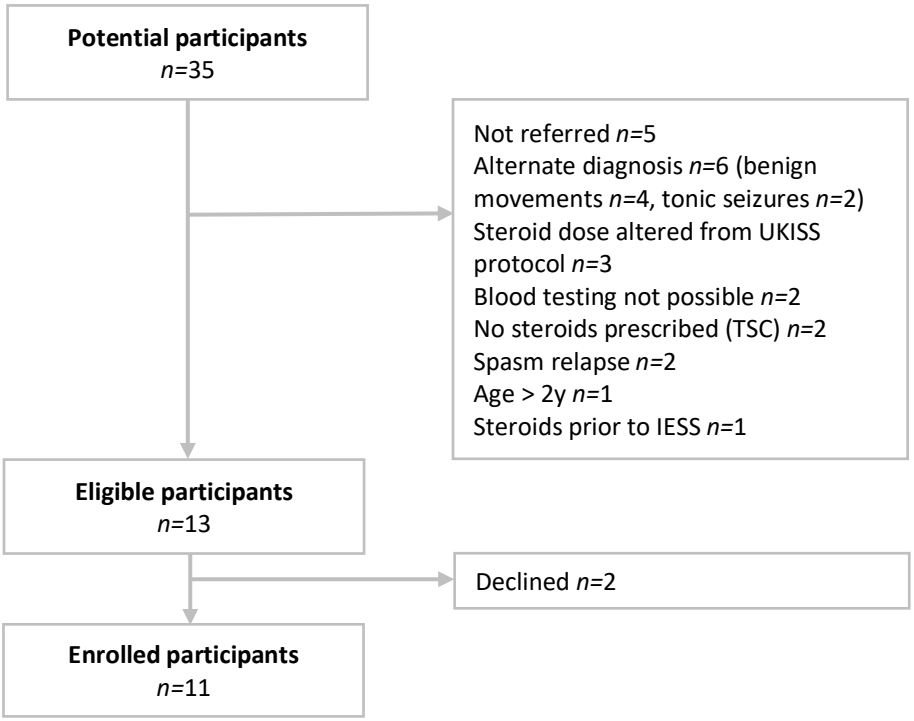
This cohort study was an open label observational study. We did not expect to generate statistically significantly differences for clinical variables, as the research was powered to detect changes in the multi-omics analysis based on earlier studies by our research group. Where possible, I performed statistical analyses and generated graphs using GraphPad Prism (version 10.4.0, 527), San Diego, CA. (144) Data was descriptively presented using mean, median and range. Between group comparisons were performed using the Mann-Whitney U test and statistical significance set at $p < 0.05$. A correction was made for multiple statistical comparisons using the Benjamini Hochberg false discovery rate (FDR).

3.5 Results

3.5.1 IESS participant selection

The selection process of participants included in this study is detailed in Figure 3.2. Thirty-five potential participants were identified over a 22-month period from January 2022 to October 2023. Twenty-two participants were ineligible. Of the 13 eligible, 11 (85%) consented.

Figure 3.2 IESS participant selection flow diagram



Key: IESS=infantile epileptic spasm syndrome, TSC=tuberous sclerosis complex, UKISS=United Kingdom Infantile Spasms Study, y=years

3.5.2 Description of IESS cohort at baseline

The baseline characteristics, including medical background and IESS phenotype of the 11 recruited infants (7F, 4M) are summarised in Tables 3.2 & 3.3. The median age at enrolment was 7.2 months (mean 7.8, range 4.5-11.4 months).

3.5.2.1 Family, pregnancy, and neonatal history of IESS cohort (Table 3.2)

After completion of clinical assessment and investigations, six (55%) of the 11 infants had an unknown IESS aetiology and five (45%) had a structural diagnosis (*n*=2 HIE, *n*=2 arterial ischemic stroke, *n*=1 TSC2). Only 2/11 had a family history of epilepsy (including TSC) and one family had a history of NDDs (*n*=1 ASD). Five families had a history of other medical conditions (Table 3.2). All pregnancies had one or more complications described, including gestational diabetes mellitus (GDM, *n*=3), infection (*n*=5, COVID *n*=3), thyroid disease (*n*=2) and hypertension (*n*=1). Only one infant was born prematurely, at 36 weeks. No infants had low birth weight <2.5kg. Neonatal complications affected five infants, two with HIE (both had neonatal seizures, one with multi-organ failure), two had respiratory distress and three

had jaundice. Epilepsy prior to epileptic spasm onset was present in two infants with HIE, who were taking anti-seizure medications (ASM) at the time of IESS onset. Epilepsy was controlled for one of these patients, whilst the other had ongoing daily seizures (Table 3.2).

3.5.2.2 IESS phenotype (Table 3.3)

Two infants had seizures preceding epileptic spasm onset (cases 7, 8). All infants had developmental concerns before IESS onset, including three with severe GDD (cases 2,7,8). Three infants (27%) had developmental regression at IESS diagnosis.

The median age at epileptic spasm onset was 6.9 months (mean 6.9, range 4-11.3). Flexor spasms were more common (*n*=9/11, 82%) than the extensor type, and epileptic spasms were asymmetric in four infants. Clusters of epileptic spasms occurred in all infants, with a median of 13 epileptic spasms per cluster (mean 15.9, range 5-50) and median of five clusters per day (mean 7.8, range 2-22).

Table 3.2 Aetiology, family, pregnancy, and neonatal history of IESS cohort

Pt	Aetiology	Family Hx (Neurology/ Neurodevelopment)	Family Hx (other medical)	Pregnancy Hx (including complications)	Birth Hx (gestation, delivery)	Neonatal complications
1	Unknown	-	Thalassemia minor (M)	Placenta praevia	36/40 Em. LSCS (placenta praevia) BW 2.8kg	Respiratory distress CPAP 2/7, jaundice -PTx
2	Unknown	-	-	Hyperemesis GDM Gastroenteritis (3rd trimester)	40/40 IOL NVD, BW 3.4kg	-
3	Unknown	-*	MTHFR carrier (M)	Hypertension	38/40 AVD (vacuum), BW 3.1kg	-
4	Unknown	-	-	Hypothyroidism	40/4 NVD, BW 3.14kg	-
5	Unknown	-	-	Hypothyroidism	38/40 LSCS (planned), BW 3.3kg	-
6	Unknown	Generalised genetic epilepsy (F, S)	-	COVID (2nd trimester)	39/40 LSCS (planned) BW 3.6kg	Jaundice-PTx
7	Structural (HIE)	Migraine (M)	-	Post-natal depression	38/40 Em. LSCS (foetal distress), BW 2.8kg Resuscitation required	HIE (seizures, cooled), multiorgan failure (heart, lung, renal), DIC (CNS haemorrhages & thrombosis)
8	Structural (HIE)	ASD (S), migraine (F)	-	IVF GDM	40/40 NVD, BW 3.6kg	HIE (seizures)
9	Structural (R MCA)	-	Factor V Leiden (M)	COVID (2nd trimester) Single umbilical artery	40/40 AVD (vacuum), BW 3.9kg	Respiratory distress (monitored)
10	Structural (L MCA)	-	Crohn's disease (F)	GDM Pneumonia (3rd trimester)	39/40 NVD, BW 3.7kg	-
11	Structural-genetic (TSC2)	TSC2 (F) (epilepsy, ADHD)#	Asthma (M)	COVID (2nd trimester) UTI (3rd trimester) Reduced foetal movements	40/40 LSCS (planned), BW 3.6kg	Jaundice-PTx

Key: ADHD=attention deficit hyperactive disorder, ASD=autism spectrum disorder, AVD=assisted vaginal delivery, BW=birth weight, CNS=central nervous system, CPAP=continuous positive airway pressure, DIC=disseminated intravascular coagulopathy, Em=emergency, F=father, GDM=gestational diabetes mellitus, Hx=history, HIE=hypoxic ischemic encephalopathy, IOL=induction of labour, IVF=in vitro fertilisation, kg=kilogram, L=left, LSCS=lower section caesarean section, MTHFR=methylenetetrahydrofolate reductase, MCA=middle cerebral artery, M=mother, NVD=normal vaginal delivery, Pt=participant, PTx=phototherapy, R=Right, S=sibling, SAH=subarachnoid haemorrhage, TSC=tuberous sclerosis complex, UTI=urinary tract infection,*Neurofibromatosis in 2nd degree relative, #=hydrocephalus in 2nd degree relative

Table 3.3 Baseline characteristics at IESS onset including preceding epilepsy, preceding development and spasm phenotype

Pt	Aetiology	Sex	Epilepsy pre-IESS onset (ASM)	Age at IESS onset (months)	Development pre-IESS onset	Spasm phenotype			Developmental regression with IESS onset
						Type	Frequency /cluster	Clusters /day	
1	Unknown	F	-	6.3	Moderate GM delay	Flexor, symmetric	8-10	6	Y
2	Unknown	F	-	7.0	Severe GDD	Flexor, asymmetric	10-15	10	Y
3	Unknown	F	-	4.0	Mild GDD; Severe speech/vocalisation delay	Flexor, asymmetric	15-50	4	Y
4	Unknown	F	-	7.1	Mild speech/ vocalisation delay	Flexor, symmetric	7	5	N
5	Unknown	F	-	7.0	Mild GDD	Flexor, symmetric	25	5	N
6	Unknown	F	-	6.8	Mild FM delay	Extensor symmetric	25	3	N
7	Structural (HIE)	M	Neonatal tonic Sz (PHB, LEV)	6.2	Severe GDD	Flexor, symmetric	5-10	22	N
8	Structural (HIE)	M	Neonatal apnoeic Sz Focal motor sz (4/12) (LEV)	11.3	Severe GDD	Flexor, asymmetric	10-15	17	N
9	Structural (R MCA)	M	-	8.3	Mild-moderate GDD	Extensor, asymmetric	10-20	5	N
10	Structural (L MCA)	M	-	5.0	Mild FM delay	Flexor, symmetric	8	7	N
11	Structural-genetic (TSC2)	F	-	6.9	Mild-moderate GDD	Flexor, symmetric	35	2	N

Key: ASM=anti-seizure medication, F=Female, FM=fine motor, GDD=global developmental delay, GM=gross motor, HIE=hypoxic ischemic encephalopathy, IESS=infantile epileptic spasm syndrome, L=left, LEV=levetiracetam, M=male, MCA=middle cerebral artery, N=No, PHB=phenobarbitone, Pt=participant, R=right, Sz=seizure, TSC=tuberous sclerosis complex, Y=Yes.

3.5.2.3 EEG (Table 3.4)

EEG at IESS onset demonstrated modified hypsarrhythmia in all 11 infants (see Table 3.4). All had high voltage, chaotic slow wave activity, (see Figure 3.3) yet brief periods of normal awake or sleep rhythms were also seen (*n*=11/11). Four had asymmetrical background rhythms including hemispheric slowing (*n*=2 stroke, *n*=1 unknown) and background attenuation (*n*=1 HIE). All had multifocal epileptiform activity, lateralising features were predominantly seen in those with a structural aetiology.

Figure 3.3 Modified hypsarrhythmia EEG background

Exemplar EEG from participant two, demonstrating a chaotic background comprised of high amplitude slow waves, spike/sharp waves discharges with interhemispheric synchrony as well as independent multifocal spikes and sharp waves.

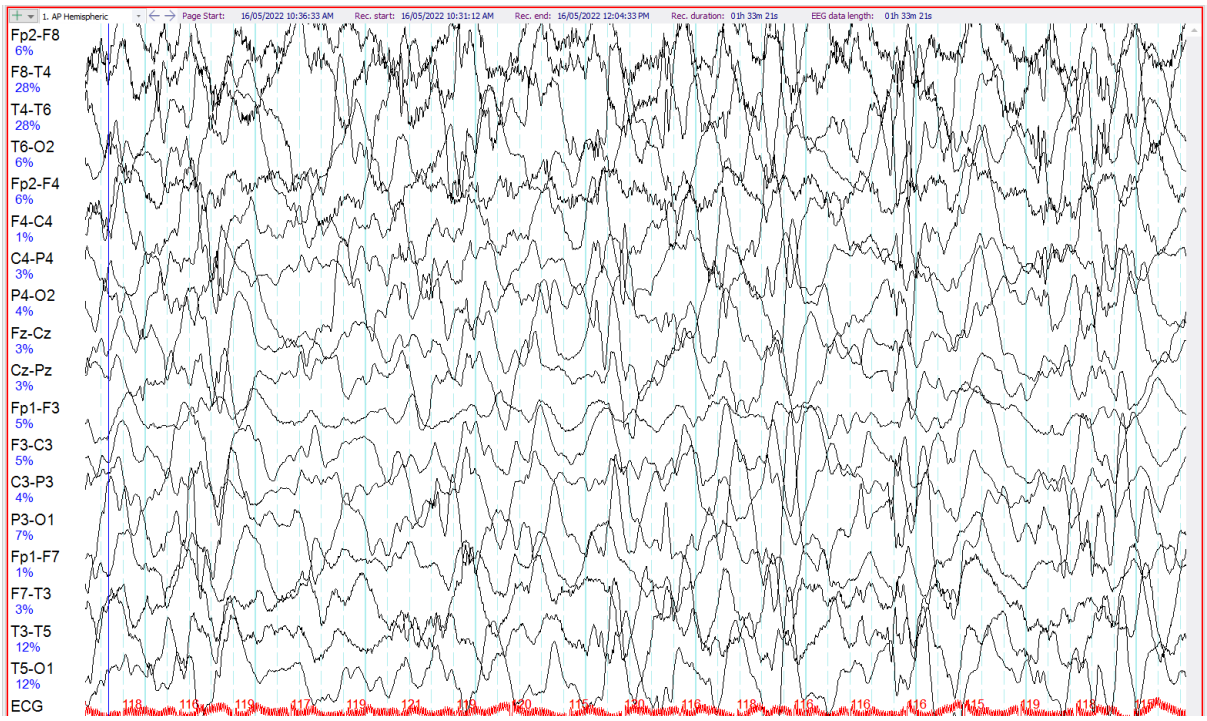


Table 3.4 EEG and MRI brain at IESS onset

Pt	Aetiology	EEG			MRI Brain
		Hyps/Mod Hyps Multifocal/Focal epileptiform activity	Background	Lateralising features	Age performed & findings
1	Unknown	Mod Hyps; multifocal	Symmetric	R post temp/parietal focus	6m: mild thinning corpus callosum
2	Unknown	Mod Hyps; multifocal	Symmetric	L >R L centro/post focus	7m: mild ventriculomegaly (L >R)
3	Unknown	Mod Hyps; multifocal	L hemispheric slowing	Post dominance	4m: grey matter nodular heterotopia at L anterior horn, mild asymmetry of frontal horns (L >R)
4	Unknown	Mod Hyps; multifocal	Symmetric	-	7m: delayed myelination posteriorly
5	Unknown	Mod Hyps; multifocal	Symmetric	Post PFA	7m: normal
6	Unknown	Mod Hyps; multifocal	Symmetric	Fronto-central focus	6m: normal
7	Structural (HIE)	Mod Hyps; multifocal	Post/temporal/parietal attenuation	-	6m: widespread encephalomalacia, ventriculomegaly, thalamic atrophy, resolving changes from past venous sinus clot
8	Structural (HIE)	Mod Hyps; multifocal	Symmetric	R post/temp focus	*Not repeated at IESS onset* Previous MRI brain: D2 - hypoxic changes in frontoparietal regions, internal capsule, motor tracts and thalami; D7: normal MRS Previous MRI brain: 4m - widespread encephalomalacia (posterior sparing), ventriculomegaly
9	Structural (R MCA)	Mod Hyps; multifocal	R hemispheric slowing	L post focus	*Not repeated at IESS onset* Previous MRI brain: 5m- gliosis of right middle cerebral artery territory including basal ganglia, thalamus and motor tracts
10	Structural (L MCA)	Mod Hyps; multifocal	L hemispheric slowing	R frontal sz L temp focus	5m: gliosis of left middle cerebral artery territory, including basal ganglia, thalamus and motor tracts
11	Structural-genetic (TSC2)	Mod Hyps; multifocal	Symmetric	R post temp/parietal focus	6m: tubers in cortical and subcortical regions, subependymal nodules, possible right parietal radial band

Key: D=Days, EEG=electroencephalogram, HIE=hypoxic ischemic encephalopathy, Hyps=hypsarrhythmia, IESS=infantile epileptic spasm syndrome, L=left, MRI=magnetic resonance imaging, MRS=magnetic resonance spectroscopy, MCA=middle cerebral artery, m=months, Mod Hyps=modified hypsarrhythmia, PFA=paroxysmal fast, Pt=participant, post=posterior, R=right, sz=seizure, temp=temporal, TSC=tuberous sclerosis complex

3.5.2.4 Investigations

3.5.2.4.1 MR neuroimaging (Table 3.4)

At epileptic spasm onset, MRI brain confirmed suspected structural diagnoses of TSC2 (case 11) and perinatal stroke (case 10), which were previously undiagnosed. Two infants with HIE (case 8) and stroke (case 9) had imaging six and three months prior to epileptic spasm onset and was imaging not repeated during their admission. Four of the six infants with unknown aetiology had non-specific anatomical variants (see Table 3.4) not considered causative of IESS, including the nodular heterotopia in case 3 with focal EEG findings.

3.5.2.4.2 Blood, urine and CSF testing (Table 3.5)

Other investigations performed were determined by the treating physician, the results are summarised in Table 3.5. Routine blood, urine and CSF testing (when performed) revealed no abnormalities (see Table 3.5). Two infants had a mild dietary B12 deficiency, not considered causally associated with IESS. No underlying thrombophilia was identified for the infants with HIE and strokes.

Table 3.5 Blood, urine, CSF and genetic investigations of IESS cohort

Pt	Aetiology	Normal investigations		Genetic testing			
		Bloods and urine	CSF	Microarray	Single gene testing	EE panel	Exome
1	Unknown	FBC, EUC, LFT, CMP, B12/folate, TSH, T4, lactate, ammonia, amino acids, biotinidase, carnitine, CK, transferrin isoforms UMS	Glucose, protein, lactate, MCS, NT, amino acids	Normal	ARX-negative	Negative*	Not done
2	Unknown	FBC, EUC, LFT, CMP, B12/folate, TSH, T4, lactate, ammonia, amino acids, biotinidase, carnitine, transferrin isoforms	Glucose, protein, lactate, MCS, NT, amino acids	Normal	Not done	Negative	Singleton- negative
3	Unknown	FBC, EUC, LFT, CMP, B12/folate, iron, TSH, T4, lactate, ammonia, amino acids, carnitine, CK, transferrin isoforms, UMS	Glucose, protein, lactate, MCS, NT, amino acids	CNV paternally inherited dup 3q25.3 and del 5q11.2	ARX-negative	Negative*	Not done
4	Unknown	FBC, EUC, LFT, CMP, B12/folate, iron, PTH, TSH, T4, vit D, urate, lactate, ammonia, amino acids, biotinidase, B6, WCE, carnitine, CK, VLCFA transferrin isoforms, copper, ceruloplasmin, lead, UMS	Glucose, protein, lactate, MCS, NT, amino acids, MTHFR	Normal	Not done	Negative	Not done
5	Unknown	FBC, EUC, LFT, CMP, B12/folate, iron, vit D, lactate, ammonia, amino acids, biotinidase, carnitine, VLCFA transferrin isoforms, UMS	Glucose, protein, lactate, MCS, NT, amino acids	Normal	ARX-negative	Negative*	Not done
6	Unknown	FBC, EUC, LFT, CMP, folate, lactate, ammonia, amino acids, biotinidase, carnitine, VLCFA transferrin isoforms, copper, ceruloplasmin, UMS	Glucose, protein, lactate, MCS, NT, amino acids	CNV paternally inherited dup 8q24.22	Not done	Negative	Not done
7	Structural (HIE)	FBC, EUC, LFT, CMP, B12, folate, iron, coags, Protein C/S, prothrombin, factor V, antithrombin III, Factor XII, lupus anticoagulant, homocysteine, beta 2 glycoprotein 1 and anticardiolipin antibodies, vitamin C, Platelet function testing—activated, UMS	Not done	Normal	Not done	Not done	Not done
8	Structural (HIE)	FBC, EUC, LFT, CMP, B12, folate, urate, coags, lactate, ammonia, amino acids, biotinidase, WCE, carnitine, VLCFA, CK, CMV, transferrin isoforms, copper, ceruloplasmin, UMS	Glucose, protein, lactate, MCS, NT, amino acids	CNV maternally inherited dup 16p13.3-p13.2	Not done	Not done	Trio-negative
9	Structural (R MCA)	FBC, EUC, LFT, CMP, B12, folate, lactate, ammonia, coags, Factor V, amino acids, biotinidase, UMS	Not done	Normal	Not done	Not done	Not done
10	Structural (L MCA)	FBC, EUC, LFT, CMP, folate, lactate, ammonia, coags, Protein C/S, prothrombin, factor V, antithrombin III, homocysteine, UMS	Not done	Normal	Not done	Not done	Not done
11	Structural-genetic (TSC2)	FBC, EUC, LFT, CMP, UMS	Not done	Normal	Paternally inherited TSC2 (c 4375C>T, p.ARg459Ter)	Not done	Not done

Key: ARX=aristaless related homeobox, CMP=calcium magnesium phosphate, CNV=copy number variant, CK=creatinine kinase, CMV=cytomegalovirus, del=deletion, dup=duplication, EE=epileptic encephalopathy, EUC=electrolytes urea creatinine, FBC=full blood count, HIE=hypoxic ischemic encephalopathy, L=left, LFT=liver function tests, MTHFR=methyltetrahydrofolate, MCS=microscopy, culture, sensitivity, MCA=middle cerebral artery, NT=neurotransmitters, Pa=paternal, PTH=parathyroid hormone, Pt=participant, R=right, TSH=thyroid stimulating hormone, T4=thyroxine, U=urine metabolic screen, VLCFA=very long chain fatty acids, WCE=white cell enzymes*EE panel performed after 12 months follow-up on research basis.

3.5.2.4.3 Genetic testing (Table 3.5)

All infants had a microarray (*n*=11/11) performed, which was normal or non-pathogenic in all, although three (27%) had parentally inherited copy number variants (see Table 3.5). Single gene testing confirmed the paternally inherited *TSC2*: c 4375C>T, p.ARg459Ter variant (*n*=1/11) and *ARX* testing performed in three infants (*n*=3/11) was negative. EE panel testing (*n*=6/11) and exome studies (*n*=2/11) did not reveal any pathogenic variants.

3.6 Treatment and outcomes

Key treatment timelines and outcomes are presented in Table 3.6.

3.6.1 Treatment details including response to steroids at 14 days

The median time from epileptic spasm onset to hospital admission was 13 days (mean 26.3, range 1-82) and did not differ between aetiological subgroups: unknown vs structural/genetic (17 vs 11, *p*=0.8). Prior to prednisolone, two infants (18%) received nitrazepam (cases 1, 3). Prednisolone doses followed the UKISS protocol of 10mg four times daily for one week and escalated in three infants (27%) to 20mg three times daily due to ongoing epileptic spasms (cases 2, 3, 8). Prednisolone was combined with vigabatrin for infant 11 with TSC.

Prednisolone started after a median of 16 days (mean 27.7, range 2-82) from epileptic spasm onset. All infants were considered prednisolone "responders" as epileptic spasms stopped within one to 12 days of treatment. The median time to epileptic spasm cessation was five days after prednisolone commencement (mean 5.6, range 1-12) with no difference between the unknown or known aetiological groups (5 vs 4, *p*=0.97). The EEG background of modified hypsarrhythmia resolved in all infants, when repeated after one (*n*=9) and/or two weeks of treatment (*n*=5).

The total treatment duration varied from 28-49 days (median 36 days), most infants (*n*=6) had a progress EEG prior to steroids stopping. Side-effects were notable including hunger (*n*=11), irritability (*n*=9), weight gain (*n*=7) sleep (*n*=4) and GIT disturbance (*n*=3). No infants developed hypertension or glycosuria requiring treatment.

Table 3.6 Prednisolone treatment timelines and response at 14 days for IESS cohort

Pt	Aetiology	Spasm onset relative to admission (d)	Spasm onset relative to steroids starting (d)	Steroid dose increased (Y/N)	Duration of steroid treatment (d)	Steroids starting to spasm offset (d)	Total duration of spasms (d)	Responder (Y/N)
1	Unknown	21	22	N	28	1	23	Y
2	Unknown	68	69	Y	49	8	77	Y
3	Unknown	13	16	Y	30	11	27	Y
4	Unknown	57	61	N	49	4	65	Y
5	Unknown	5	6	N	43	5	11	Y
6	Unknown	6	7	N	27	5	12	Y
7	Structural (HIE)	21	21	N	38	8	29	Y
8	Structural (HIE)	4	6	Y	41	12	18	Y
9	Structural (R MCA)	1	2	N	29	3	5	Y
10	Structural (L MCA)	11	13	N	36	4	17	Y
11	Structural-genetic (TSC2)	82	82	N	34	1	83	Y
	Median Mean Range	13 26.3 1-82	16 27.7 2-82	-	36 36.7 28-49	5 5.6 1-12	23 33.4 5-83	-

Key: d=days, HIE=hypoxic ischemic encephalopathy, L=left, MCA=middle cerebral artery, N=no, Pt=participant, R=right, TSC=tuberous sclerosis complex, Y=yes

3.6.2 Spasm recurrence and epilepsy outcomes at 12 months follow-up (Table 3.7)

Epileptic spasms re-occurred in two infants (cases 3, 5) with unknown aetiology, whilst steroids were weaning (case 3) and two months after treatment (case 5). One infant (case 3) had a repeat EEG at epileptic spasm recurrence, demonstrating a disorganised background. Following recurrence, both patients received vigabatrin with epileptic spasm cessation within one to seven days. At 12 months follow-up, both were seizure/spasm free and only one infant remained on vigabatrin (case 3) (see Table 3.7).

In total, all six infants with unknown aetiology were seizure free at 12 months and only one was taking ASM. In contrast, all five infants with structural IESS were on ASM and two had ongoing seizures (cases 8, 11) despite multiple medications.

3.6.3 Developmental outcomes at 12 months follow-up (Table 3.7)

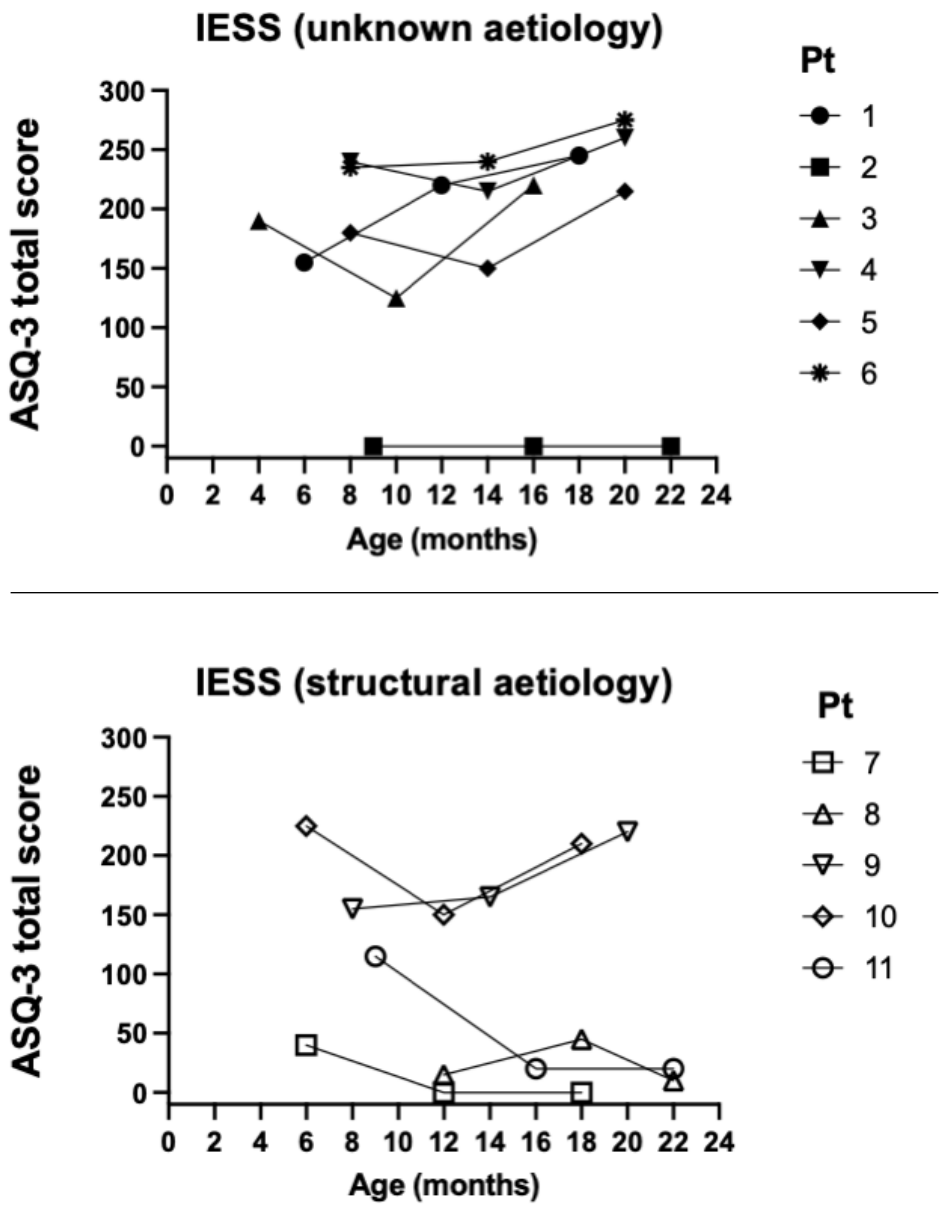
At 12-months follow-up, four infants had severe GDD (cases 2, 7, 8, 11) and three infants had delays in one developmental domain (cases 5, 9, 10) see Table 3.7. ASQ-3 scores confirmed that development had improved for most infants with unknown IESS (83%, *n*=5/6) whereas the majority with structural IESS (60%, *n*=3/5) experienced a decline in their total scores (see Figure 3.4) indicating that their skills had further deviated from age-appropriate norms. Figure 3.5 presents the total and subdomain ASQ-3 scores for each participant.

Table 3.7 Epilepsy and Developmental outcomes at 12 months follow-up

Pt	Aetiology	Spasm Recurrence (Y/N)	12-month follow-up			
			Seizures (Y/N)	ASM	Developmental status	ASQ-3 scores improved (Y/N)
1	Unknown	N	N	-	Appropriate	Y
2	Unknown	N	N	-	Severe GDD	N
3	Unknown	Y	N	VGB	Mild GDD	Y
4	Unknown	N	N	-	Appropriate	Y
5	Unknown	Y	N	-	Mild speech/ vocalisation delay	Y
6	Unknown	N	N	-	Appropriate	Y
7	Structural (HIE)	N	N	LEV	Severe GDD	N
8	Structural (HIE)	N	Y	LEV, CNZ	Severe GDD	N
9	Structural (R MCA)	N	N	LEV	Moderate GM delay	Y
10	Structural (L MCA)	N	N	LEV	Moderate GM delay	Y
11	Structural-genetic (TSC2)	N	Y	OXC, LAC, TOP, VBG,	Severe GDD	N

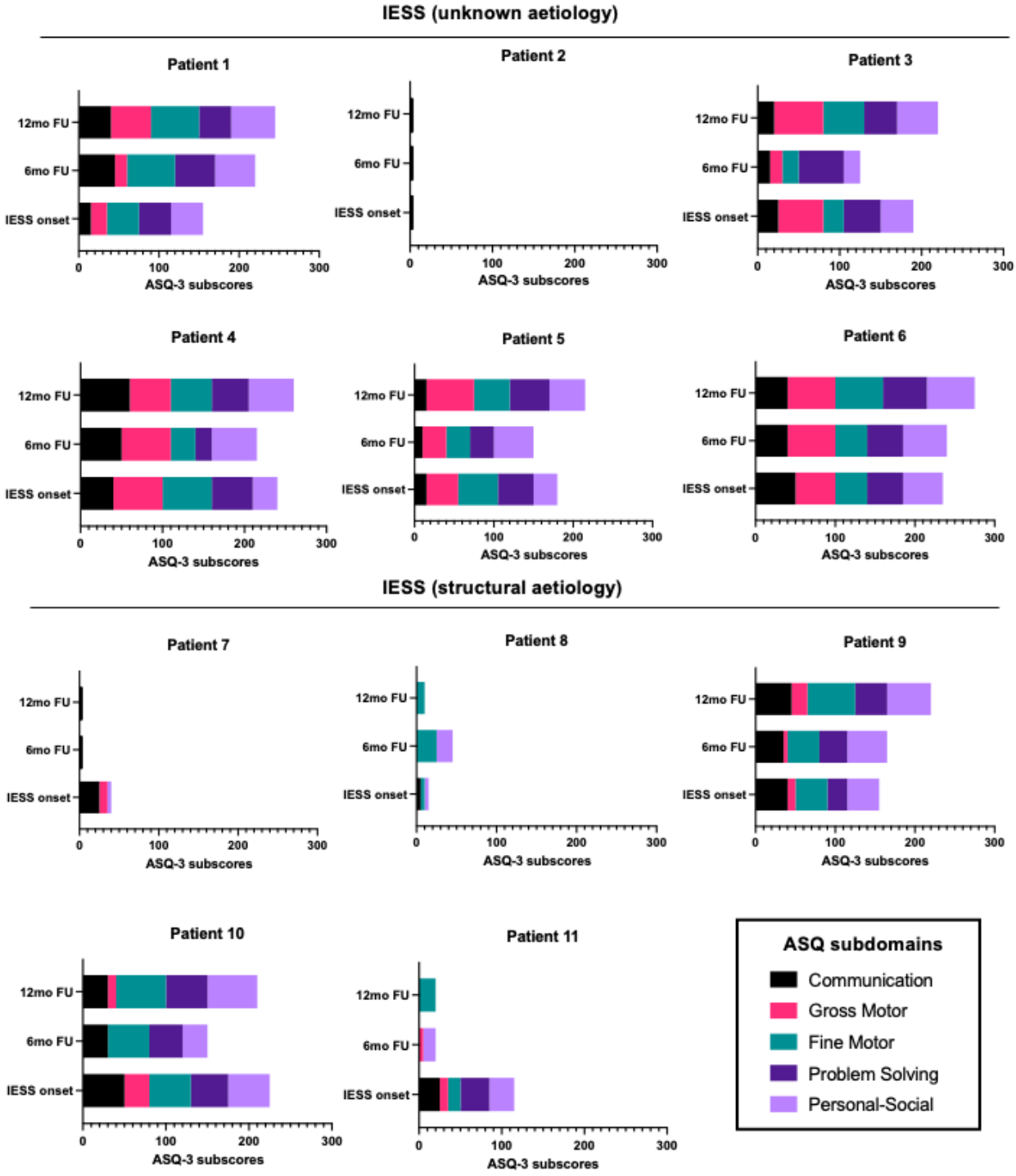
Key: ASQ-3=ages and stages questionnaire, 3rd edition, ASM=anti-seizure medication, CLN=clonazepam, GDD=global developmental delay, GM=gross motor, HIE=hypoxic ischemic encephalopathy, LAC=Lacosamide, L=left, LEV=levetiracetam, MCA=middle cerebral artery, N=no, OXC=oxcarbazepine, Pt=participant, R=right, TOP=Topiramate, TSC=tuberous sclerosis complex, VBG=vigabatrin, Y=yes

Figure 3.4 ASQ-3 total scores by IESS aetiology (unknown vs structural) at IESS onset, 6- and 12-months follow up



Key: ASQ-3=ages and stages questionnaire, 3rd edition, IESS=infantile epileptic spasm syndrome, Pt=participant

Figure 3.5 ASQ-3 total and subdomain scores by IESS aetiology (unknown vs structural) at IESS onset, 6- and 12-months follow-up



Key: ASQ-3=ages and stages questionnaire, 3rd edition, FU=follow up, IESS=infantile epileptic spasm syndrome, mo=months

3.7 Discussion

This longitudinal observational study describes the epilepsy and neurodevelopmental outcomes of 11 infants with IESS managed with prednisolone treatment. Cases of IESS with an unknown aetiology in my study (55%) had a more favourable prognosis at 12 months follow-up than those with structural aetiologies (45%). The unknown aetiology group also had lower rates of epilepsy, developmental delay and other neurological diagnoses.

This study had a high participation rate (85%) for eligible infants with IESS, which is a strength of the design in complementing clinical care. As a study investigator for several research projects I found recruitment was a surprisingly smooth process, especially as it occurred concurrently with a new and serious neurological diagnosis in a child. I encountered families at a difficult time and clinicians determining what, if any, research was in the best interest of the child. I found it incredibly powerful that I could reassure both parties that our research would not alter or interfere with usual care, yet would occur in parallel, and this was the likely explanation of our high participation rate. I would adopt the same approach to future research and recommend this to other clinicians and research groups.

Interestingly, 17% (*n*=6/35) of the cohort referred for study participation received an alternate diagnosis to IESS. The most frequent was “benign movements” (67%) followed by tonic seizures (33%) that were then classified according to their respective epilepsy syndromes. Imitators of epileptic spasms are widely recognised however, very few studies have described the prevalence and characteristics. (4) Previous studies have reported 51-60% of infants referred for suspected IESS receive an alternate diagnosis (29, 40) which is most commonly a non-epileptic phenomena including sleep myoclonus or non-epileptic “benign spasms”. A smaller proportion have epileptic myoclonus or tonic seizures, GORD, spasticity or other medical condition. (29, 40) My study further describes the more common “mimickers” of epileptic spasms and their relative proportion in this referred cohort.

As part of data collection for the IESS cohort, "NeuroCONNECT" proved to be a valuable tool from both a clinician and researcher's perspective, as it provided a reliable, secure and standardised method for collecting and storing data. I learnt the importance of incorporating standardised questionnaires into day-to-day clinical practice to strengthen in-depth phenotyping and objective measurements over time. It also highlighted to me that standardised data collection can help facilitate future robust clinician-led research projects. Table 3.3, the baseline characteristics at IESS onset, is an example of how standardised prospectively collected data can provide detailed quantitative and qualitative information such as frequency of epileptic spasms, clusters and specific timelines.

As part of phenotyping IESS aetiology, it is important to acknowledge that based on our study design clinicians were free to choose the type, timing and frequency of repeat investigations. Although there was no standardised institutional investigation protocol, two clear aetiological groups emerged by 12 months, IESS with unknown aetiology (55%) followed by structural aetiology (45%) with both acquired (36%) and structural-genetic (9%) causes. TSC was the only genetic cause in my small cohort. The 12-month follow-up period allowed sufficient time for the return of results to confirm an unknown rather than rare genetic aetiology. Most investigations (blood, urine, CSF) and genetic tests were negative, however there was significant variability amongst genetic testing completed. As the second part of my study involved a biological multi-omics analysis, we ensured that all IESS cases with unknown aetiology had an exome-based "IESS-gene" panel. Trio exome or genome sequencing would have been more comprehensive, as only 23-25% of IESS cases are explained by monogenic variants. (26, 145-148) However, time and cost considerations were factored into my study. It is hoped that my results will help inform the design of future larger-scale multi-omics analyses.

Another challenge was classifying the EEG data given the lack of international consensus standards distinguishing hypsarrhythmia from its associated variants. Previous publications have also reported that the inter-rater reliability describing hypsarrhythmia and modified hypsarrhythmia is very low. (59) To improve standardisation, I reviewed and classified all EEGs, albeit unblinded, and found all infants presented with a modified hypsarrhythmia pattern and most had epileptic spasms captured on EEG although the

duration of the recordings did vary. It is acknowledged that the 1991 ILAE workshop advocated that the terms “modified” or “atypical” are no longer used, rather substituted for descriptions of the EEG features that are variants of hypsarrhythmia. (47) However, the 2022 ILAE classification paper only broadly defined hypsarrhythmia and did not reference any electrophysiological criteria or define features that are considered “variants”. As such, I reviewed the literature to collate and describe the commonly accepted features of hypsarrhythmia and its variants. There was variability across definitions, and this is an acknowledged limitation of my approach. Future Delphi consensus meetings may reach an agreement on a standardised classification of hypsarrhythmia, in the interim, authors could adopt novel standardised EEG scoring system such as the Burden of AmplitudeS and Epileptiform Discharges (BASED), with high inter-rater reliability. (149-151) I chose not to employ this tool as my study focused on clinical and neurodevelopmental outcomes, rather than EEG phenotyping.

In my study, lead time from onset of epileptic spasms to prednisolone treatment was relatively short (median 16 days), a factor that has been associated with improved outcomes. (77) Infants with pre-existing neurological diagnoses (perinatal HIE/stroke) presented earlier than those without, however the time difference was not statistically different. Two infants (33%) received diagnoses of TSC (case 11) and perinatal stroke (case 10) after the onset of epileptic spasms, despite prior clinical interactions that could have enabled closer monitoring and earlier intervention, notably for TSC given EEG surveillance and early vigabatrin treatment is now standard care. (152, 153) This reinforces the importance of clinicians educating general practitioners, allied health and other care providers regarding early presentation for suspected IESS to improve outcomes.

The response to prednisolone treatment was higher than hypothesised as all infants were considered responders with cessation of epileptic spasms and improvement in the EEG by day 14 of treatment. There were confounders as two infants received nitrazepam prior to prednisolone (cases 1, 3) and one received vigabatrin with prednisolone (case 11) and this heterogeneity is acknowledged. As there were no “non-responders”, no clinical comparison of outcomes was possible between responders and non-responders. This did not affect the biological analysis in Chapter four as although this sub-analysis would have been interesting,

this was not the primary aim. Prednisolone treatment caused notable, but predictable side-effects including mood and behavioural disturbance, increased appetite and weight gain, and one infant later required stress dosing whilst unwell. This is a reminder that prednisolone treatment is not benign, and the side-effects can be challenging for families even though four-six weeks is considered "short-term". Alternative targeted therapeutics with more favourable side-effect profiles would therefore be preferable, if proven to be equally efficacious.

As hypothesised at 12 months follow-up, epilepsy and neurodevelopmental outcomes were more favourable in infants with unknown IESS aetiology. All six infants with unknown IESS were seizure free (100%, *n*=6/6) and the majority were not receiving anti-seizure medication (83%, *n*=5/6) aside from one infant who remained on vigabatrin following epileptic spasm recurrence (case 3). A structural pathology was considered in this infant given the nodular heterotopia and atypical EEG background asymmetry at presentation and later focal discharges, however, after 24 months of extended follow-up, the aetiology was classified as unknown. No identifiable risk factors were present for the second infant with epileptic spasms recurrence (case 5).

Amongst the five infants with structural IESS, prednisolone treatment resulted in epileptic spasms cessation however, neurodevelopmental complications remained at 12 months follow-up. All five infants had epilepsy and neurological co-morbidities, including CP in 80% (*n*=4/5) of cases. This highlights that prednisolone treatment may only be exerting a short-term effect, as UKISS and ICISS demonstrated that long-term outcomes did not significantly differ between treatment groups. It appears that neurodevelopmental outcomes, are not only dependent on epileptic spasm cessation, lending strength to the hypothesis that IESS continues as a longer-term NDD. This raises the question for this subgroup with poor outcomes, whether other medications or therapies should be considered after epileptic spasm cessation.

Developmental outcomes were more favourable in infants with unknown compared to structural IESS. The ASQ-3 identified a clear trend that developmental scores for all infants were lower at six months follow-up compared to baseline. However at 12 months

scores improved beyond baseline for infants with unknown IESS whereas developmental scores further departed from age-appropriate norms in infants with structural IESS. There were two exceptions in each group, one infant with severe GDD and a suspected, yet not confirmed, underlying genetic syndrome (case 2) and one infant with mild CP whose development improved (case 9). The ASQ-3 proved to be a useful tool, objectively demonstrating clear trends and provided structure to the developmental history to elicit parental concerns. In addition, ASQ-3 findings generally correlated to concerns raised by clinicians or allied health therapists and for one infant (case 6), ASQ-3 scores aligned to formal development assessments, the Bayley Scales of Infant and Toddler Development, 4th Edition (139) and Vineland Adaptive Behaviour Scales, Third Edition. Prospective collection of developmental data using this standardized approach enhanced the quality of clinical phenotyping, and our research group will incorporate the ASQ-3 into future studies.

As part of the broader investigation into gene-environment interactions in IESS, the screening questionnaires on child, maternal and family health provided valuable exploratory insights. Our research group developed these screeners to examine mother-child dyads and how maternal health factors such as inflammation and autoimmune disorders may influence the expression of NDDs such as ASD in offspring. (154) In my cohort of infants with IESS, a family history of epilepsy was present in only two infants including one with TSC and seemed comparable to general population rates. (155, 156) However, the pregnancy history unexpectedly identified that most women experienced some form of complication including infection (45%, *n*=5/11), endocrine disorder (45%, *n*=5/11), or pregnancy related condition/placental-cord anomaly (36%, *n*=4/11). In addition, 63% (*n*=7/11) of mothers reported periods of increased or severe stress *during* pregnancy, based on a seven-item ordinal scale. (157) A limitation is that maternal and family histories were not available in controls for comparison. My study adds some preliminary support to the hypothesis and existing evidence that NDDs may be mediated by genetic predeterminants as well as environmental stressors during pregnancy (infection, autoimmune disorders) causing "maternal immune activation" (158, 159) that affects the critical period of CNS development in-utero. (160-162) There are several epidemiological studies confirming an association between maternal immune conditions and higher rates of NDDs and neuropsychiatric conditions in offspring particularly ASD, ADHD and schizophrenia. (163-166)

Maternal autoimmunity has not been explored or confirmed in IESS, however there is a clear aetiological association between congenital infections and IESS and a temporal association between IESS onset occurring in the weeks or months following a post-natal viral or bacterial meningitis. (167) Interestingly in my study one infant who was not eligible to participate and therefore not recruited, experienced epileptic spasms triggered by Influenza A. An EEG confirmed modified hypsarrhythmia, yet given concerns starting steroids whilst unwell, short-term nitrazepam was prescribed and led to cessation of epileptic spasms and resolution of EEG changes. Illness is a recognised trigger for seizures and epileptic spasms, (167) however there are also rare reports of spontaneous remission of IESS following viral illnesses including roseola, rotavirus, measles and chickenpox. (168-170) It is plausible that stress or immune activation at any timepoint from conception and throughout post-natal CNS development may contribute to IESS onset, via complex gene-environment interactions, it may also be paradoxically linked to epileptic spasms cessation. This hypothesis will be further explored in the biological multi-omics study in Chapter four.

3.8 Summary

In my prospective clinical cohort study, prednisolone treatment led to cessation of epileptic spasms in all 11 infants with IESS. The side-effects of prednisolone were notable, although no serious adverse events occurred. Two infants with unknown IESS had recurrence of epileptic spasms, promptly treated with vigabatrin and remained seizure free at follow-up. In my small cohort, neurodevelopmental outcomes at 12 months follow-up appeared to be determined by underlying IESS aetiology, rather than initial response to prednisolone treatment. All infants with structural and structural-genetic IESS aetiologies had epilepsy and neurological co-morbidities. In contrast, all infants with unknown IESS were seizure free and only a minority experienced developmental delays. These findings are consistent with the literature confirming that IESS is a complex NDD characterised by a DEE. It is widely recognised that prednisolone is an effective treatment in IESS however, its mechanisms are unclear and may only be transient given evidence that early cessation of epileptic spasms does not alter long-term epilepsy or developmental outcomes. My small yet well phenotyped clinical cohort served as the IESS cohort in my multi-omics analysis in Chapter four. This is a mechanistic study exploring the effects of gene regulation and

immune abnormalities underlying disease in IESS and possible therapeutic effects of prednisolone in IESS.

CHAPTER 4 - Multi-omics analyses to explore the effects of prednisolone on gene regulation in IESS

4.1 Hypotheses and aims

Hypotheses:

1) It is hypothesised that infants with IESS will have altered gene transcription and expression compared to controls at baseline with distinct epigenetic and immune profiles.
2) It is hypothesised that prednisolone acts to control epileptic spasms by altering gene transcription and expression, through genomic, non-genomic or epigenetic mechanisms and/or exerts immune modulating effects.

Aims:

1) Investigate differences in gene regulation and immune profiles in infants with IESS at baseline before prednisolone treatment (IESS-pre) compared to healthy controls, using
   i) Immunoassay panel of markers of neuroinflammation
   ii) Bulk RNA sequencing
   iii) Proteomic sequencing
   iv) Phosphoproteome sequencing

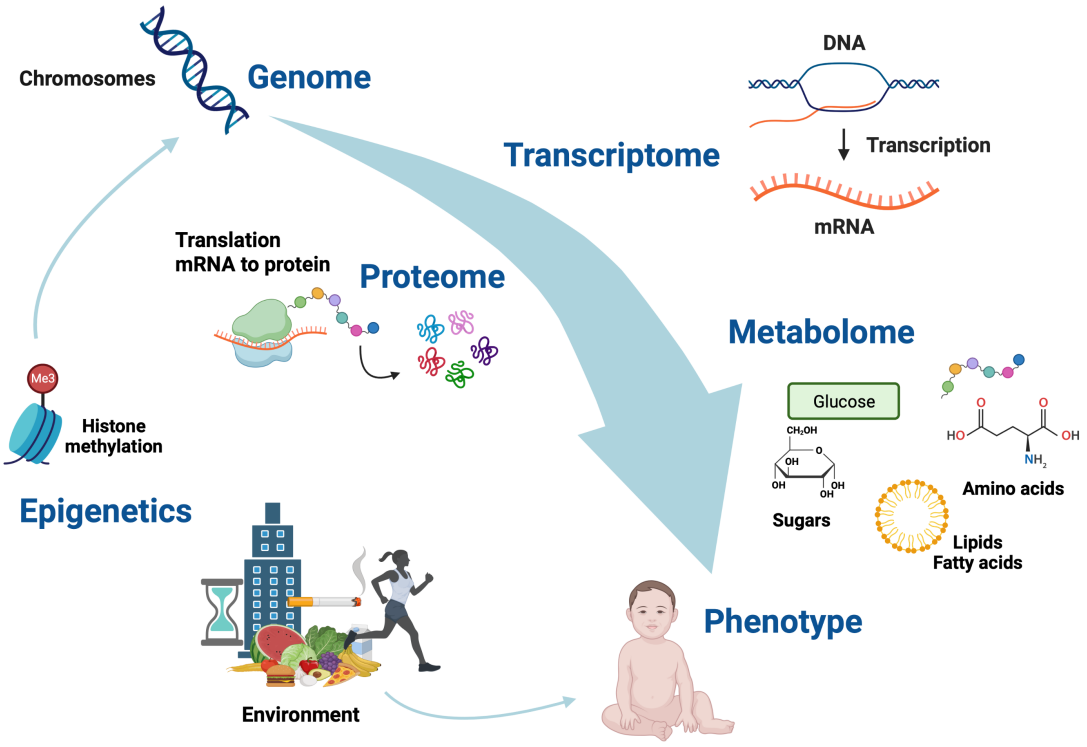
2) Evaluate changes in gene regulation and immune profiles in infants with IESS after seven days of prednisolone (IESS-post) compared to baseline (IESS-pre), using
   i) Immunoassay panel of markers of neuroinflammation
   ii) Bulk RNA sequencing
   iii) Proteome sequencing
   iv) Phosphoproteome sequencing

4.2 Introduction to multi-omics

From my systematic review, it seems plausible that IESS arises from altered gene expression and/or protein abundance resulting in cellular dysfunction across broad regulatory, developmental and immunological pathways. It is possible that the combined dysfunction across these pathways disrupts normal neurodevelopmental processes, thereby contributing to IESS. It is also plausible that in-utero or early life stress and environmental insults may alter expression of vulnerability genes through epigenetic mechanisms.

In this chapter, I will examine gene expression and protein abundance in IESS compared to controls. In addition, I will examine changes following prednisolone treatment utilising techniques of bioinformatic analyses and multi-omics sequencing. Omics involves the study and quantification of biological molecules, that I will refer to as "-omes" in this thesis, see Figure 4.1. Multi-omics determine how changes in DNA (genome) and RNA (transcriptome) expression and protein (proteome) and metabolite (metabolome) abundance influence disease phenotypes. (171) The epigenome refers to the analysis of chemical compounds that change gene and protein expression without altering the DNA sequence. (171) In this study I utilised transcriptomics, proteomics and phosphoproteomics sequencing.

Figure 4.1 Multi-omics approach to understanding how changes in genes, RNA, protein, metabolites and epigenetic control translates to disease phenotypes
Multi-omics refers to the integrative analysis of multiple biological molecules referred to as "-omes" to better understand the complex genotype-phenotype interaction. It is known that changes at the level of the genome, such as DNA variation leads to downstream alterations in RNA expression and protein function that impact biological and cellular processes in a diverse and unpredictable manner, ultimately resulting in phenotypic changes or disease states. The environment may also alter cellular and regulatory process or cause epigenetic modifications further altering disease phenotypes. Multi-omics allows researchers to combine information from multiple omics datasets to identify drivers of disease and potential therapeutic targets. N.B. Authors own figure created with BioRender.com



Genomics, involves the study of an individual's complete set of DNA including approximately 20-25,000 protein coding genes as well as non-coding regions such as regulatory sequences, introns and intergenic regions that may regulate gene expression. (171) A reference human genome was first mapped in 2003, by the Human Genome Project and last updated in 2022 allowing researchers to explore the genetic basis for disease phenotypes. (172) Genomics largely utilises high throughput next generation sequencing to identify DNA mutations or copy number variants contributing to disease states. (173)

Transcriptomics studies the transcriptome, the complete set of RNA molecules transcribed from the genome, including ribosomal RNA (rRNA), messenger RNA (mRNA),

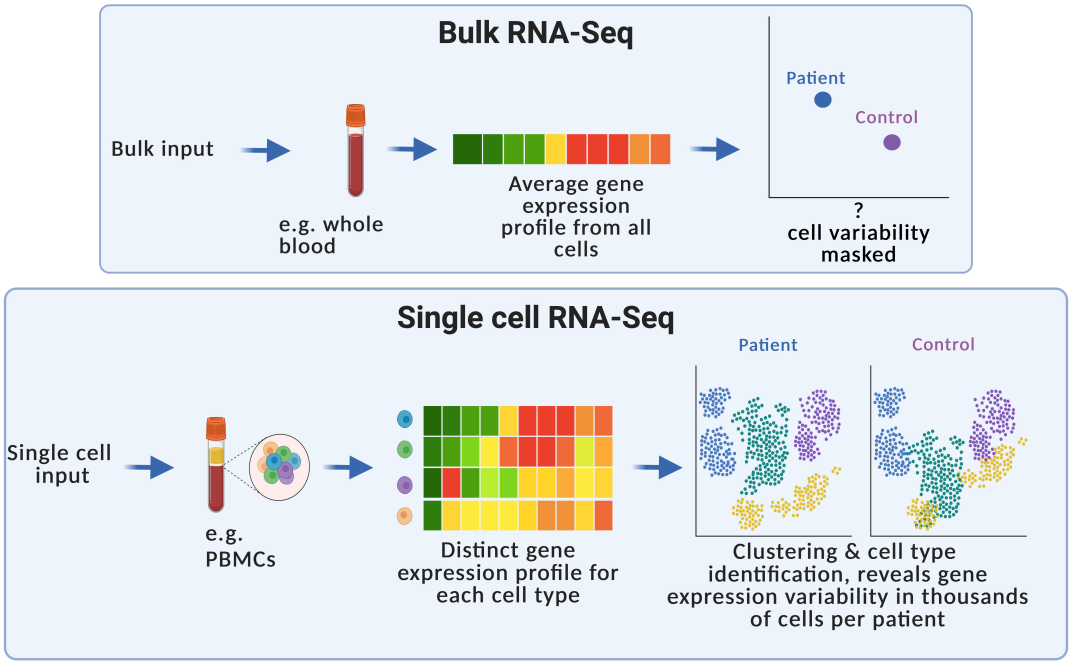
transfer RNA (tRNA), micro RNA (miRNA), and other non-coding RNA (ncRNA). (171) By quantifying the RNA transcripts, we can determine which genes are being actively expressed or are "upregulated" or actively suppressed or "downregulated". (173) This is performed by mapping the transcripts back to the human genome reference assembly (GRCh38.p13). (172) These genes are known as "differentially expressed genes" (DEGs).

Transcriptomics identifies more dynamic or functionally relevant information about changes in gene expression compared to genomics. DNA is relatively static, acting as a blueprint for genetic information which is passed from cell to cell in a relatively constant fashion, but not all genes are expressed in every cell and each gene may produce more than one copy or type of mRNA. (173) The transcriptome, differs between cell types and varies based on disease states or environmental changes. (173) It can be measured by examining the RNA code or the number of transcripts, the latter known as RNA expression, which I will examine. This provides more dynamic information to distinguish between disease and healthy states and pre- versus post-treatment states.

For my study, I used whole blood bulk RNA sequencing which identifies the average gene expression profiles from thousands of cells per individual and provides good between group comparisons, see Figure 4.2. (174) Single cell RNA sequencing, another RNA sequencing technique has the added advantage of discerning gene expression profiles for each cell type, see Figure 4.2. (174) In our research centre, single cell RNA sequencing has traditionally been performed using peripheral blood mononuclear cells (PBMCs), which includes lymphocytes, monocytes, dendritic and natural killer cells. Notably, neutrophils are absent in PBMCs. Due to resource limitations, we focused on bulk RNA sequencing for this study.

Figure 4.2 Transcriptomic analysis using whole blood bulk RNA sequencing or single cell RNA sequencing.

Whole blood bulk RNA sequencing is performed using a microarray or next generation sequencing to generate a "barcode" of RNA transcripts from all cell populations. The RNA transcripts are counted or quantified to determine the level of gene activity/expression from thousands of cells per individual. This technique is useful for group comparisons: for example, between IESS and controls. Single cell RNA sequencing using PBMCs identifies the "barcode" of RNA transcripts for each cell type studied. This provides information about gene expression changes at the individual cell level. One of the limitations of using PBMCs is that only lymphocyte, monocyte, dendritic and natural kill cell types are available for analysis (and not neutrophils). N.B. Authors own figure created using BioRender.com



Proteomics involves the identification and quantification of protein abundance. (171) Like RNA, the proteome is also dynamic, a single gene can produce multiple forms of a protein, the amount and type of proteins produced within cells also varies based on the cell type, disease state and influences from the environment. (173) For my study, I undertook proteomic sequencing using PBMCs and liquid-chromatography (LC) coupled to mass spectroscopy (MS). Similar to transcriptomics, by quantifying protein abundance, we can determine proteins that are differentially abundant -either increased or decreased. Although abundance does not directly represent gene expression, for the purposes of this introductory section, I will refer to these as differentially expressed proteins (DEPs).

Phosphoproteomic sequencing is one technique investigating epigenetic modifications through the identification and quantification of proteins that have differential phosphorylation. Epigenetic regulation is a process of chemical modifications to DNA, RNA or proteins that alter gene expression, without altering the DNA sequence. One example of a post-translational protein modification (PTM) is the addition of a phosphate group to an amino acid side chain, known as phosphorylation, see Figure 4.3. (175) This alters the structure and therefore function of the protein, for example, phosphorylation directly activates or deactivates enzymes and associated cell signalling pathways and it may indirectly affect the function of proteins through altered protein-protein interactions. (173, 175) Phosphorylation is a complex process as there are multiple amino acid sites available for phosphorylation in every protein, the changes to protein function are site-dependent and the process is reversible. (173, 175) Phosphorylated proteins alter gene regulation and expression through multiple mechanisms, including altering the accessibility of DNA for transcription, altering mRNA stability and altering transcription factors that in turn affect transcription and translation. (173, 175) For this study, I undertook phosphoproteomic sequencing to identify proteins and cellular pathways that have differential phosphorylation.

Figure 4.3 Epigenetic modifications altering gene expression

Epigenetic modifications are chemical changes to the DNA, chromatin, RNA and proteins that alter gene expression without altering the DNA sequence. Epigenetic modifications can be induced by stress, environmental and lifestyle factors. Epigenetic modifications to DNA include methylation, adding methyl groups to DNA regions, or histone modification that alters chromatin structure. The epitranscriptome encompasses changes to RNA including altering non-coding RNAs (such as microRNA). Post-translation modifications include the phosphorylation of proteins, whereby protein kinases add a phosphate group to amino acid residues. The three most phosphorylated amino acids, in order are serine (Ser), threonine (Thr), and tyrosine (Tyr). Phosphorylation is a reversible process, phosphatases desphosphorylate proteins. Phosphorylated proteins regulate gene expression and important cellular functions including signalling, metabolism, proliferation, apoptosis and inflammation. N.B. Authors own figure created using BioRender.com

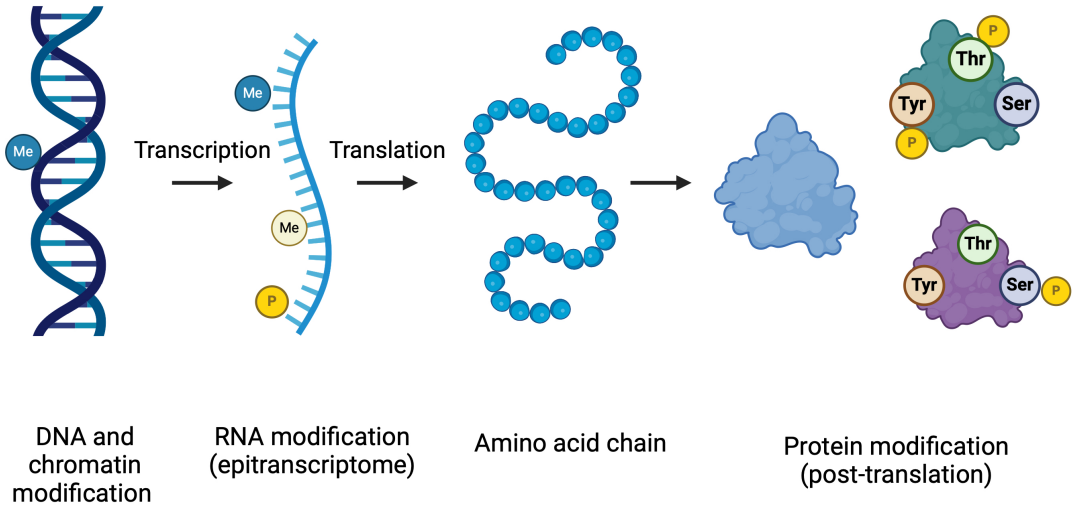
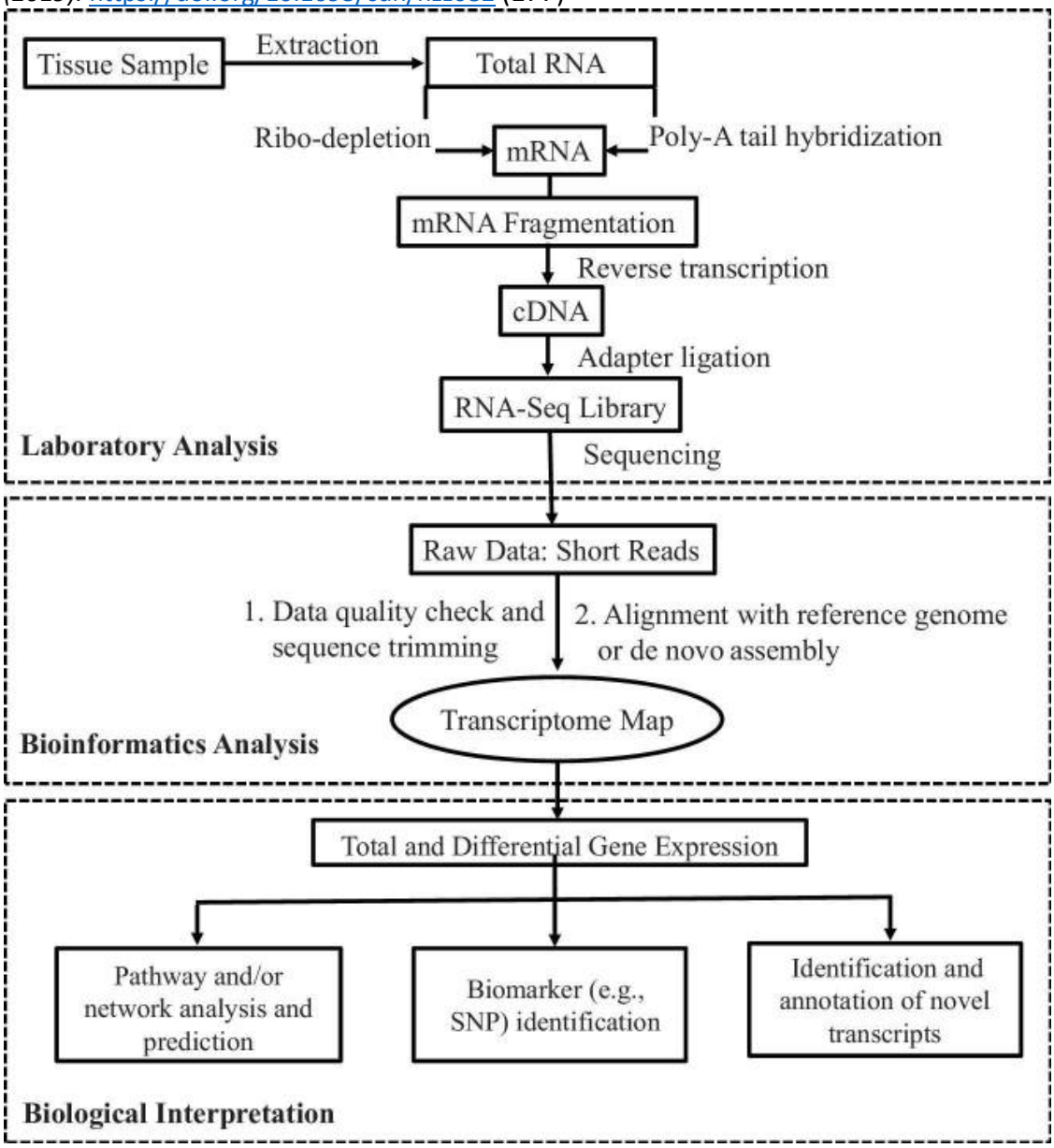


Figure 4.4 summarises the workflow for data generation and analysis. The first step involves laboratory analyses which sequence RNA, protein and phosphoproteome data and generates the multi-omics datasets. This produces an extraordinary volume of high-throughput and high dimensional biological information requiring a bioinformatic analysis. Bioinformatics combines computer science, biology, mathematics and physics to enable the collection, storage and analysis of these high-throughput datasets and compare them to reference genome and proteome. (176) Using pre-defined thresholds defining statistical significance, differentially expressed genes (DEGs), proteins and phosphopeptides/peptides (DEPs) are identified. To interpret the biological and clinical relevance of DEGs and DEPs in the control, pre and post treatment states, a pathway enrichment analysis is performed. This highlights key regulatory processes or functions that are altered in disease states compared to controls and pre and post treatment states.

Figure 4.4 Workflow of multi-omics dataset analysis, using RNA sequencing as example
Three key steps are involved 1) laboratory analysis of tissues to generate sequencing data, 2) bioinformatics analysis of sequencing data to generate differentially expressed genes (DEGs) and 3) biological interpretation of DEGs using pathway enrichment analysis. Figure from Hasan, et al. (2019). https://doi.org/10.1093/cdn/nzz082 (177)



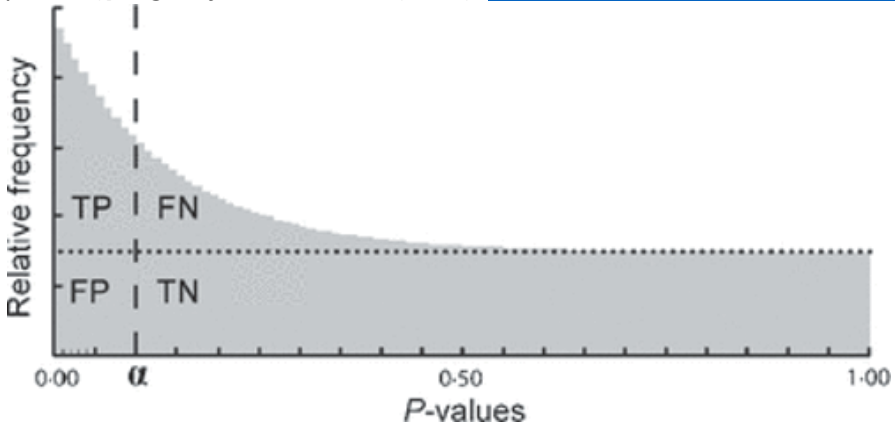
As bioinformatic analyses inherently involve multiple statistical testing, DEGs/DEPs were defined as statistically significant using adjusted p-values to correct for the false discovery rate (FDR). The *a priori* assumption is that p-values <0.05 reject the null hypotheses, meaning there is less than a 5% possibility the result was produced by random chance, and therefore reported as statistically significant. (178, 179) The FDR is the expected proportion of false positives (or type one errors) amongst all positives which reject

the null hypotheses (p<0.05), see Figure 4.5. (178) As multi-omics datasets perform multiple statistical comparisons, the probability of type one errors increases, necessitating a correction. Adjusted p-values use corrections such as the Benjamini-Hochberg method to control for the expected proportion of type one errors. (178)

In this analysis, significant DEGs/DEPs were defined as those with an FDR-adjusted p-value of <0.05, meaning that amongst the 5% results deemed significant, we expect less than 5% to be false positives or false discoveries.

Figure 4.5 False discovery rate

The false discovery rate (FDR), or adjusted p-value, is the proportion of false positives (type 1 errors) among all statistically significant positive findings. FDR= expected [false positive/(false positive +true positive)]. Figure from Pike et al. (2010) https://doi.org/10.1111/j.2041-210X.2010.00061.x (178)



Key: α=alpha line, p<0.05, FN=false negatives, FP=false positives, TN=true negatives, TP=true positives.

After FDR correction, pathway-directed analyses are performed to identify biological pathways enriched by the DEGs/DEPs. Pathway analysis examines the collective effects of the many differentially expressed genes, rather than focusing on single gene effects.

The two common bioinformatic methods for pathway-directed analysis are representation analysis (ORA) and gene set enrichment analysis (GSEA). (180, 181) Both approaches utilise curated gene sets from databases such as Gene Ontology (GO), (182) Reactome, (183) or Kyoto Encyclopaedia of Genes and Genomes (KEGG). (184) Pathway changes are annotated to standardized terms, for example, GO-terms describe biological processes (BP), molecular functions (MF), and cellular components (CC). (182)

ORA identifies biological pathways from gene sets that are statistically over-represented amongst the list of DEGs. (181) ORA relies on a non-ranked list of DEGs, that were defined as significant based on an FDR-adjusted-p value of <0.05. Using a statistical test (often Fisher's exact or hypergeometric test) ORA determines whether there are more DEGs enriching or "over-representing" a gene set or biological pathway, than would be expected by chance. (181) ORA outputs a list of these pathways and their associated *p*- or FDR adjusted *p*-values to indicate significance. (181)

ORA can detect pathways enriched by pronounced changes in gene expression, however there are some limitations which means that ORA may not detect subtle more co-ordinated changes to gene expression resulting in biologically relevant changes at a pathway level. (185) ORA summarises findings that meet statistical significance based on a pre-defined threshold and assumes all significant DEGs contribute to enrichment equally. ORA does not consider the magnitude of the log fold change in gene expression change. (185) As a result, there is no differentiation between gene changes that just met significant thresholds compared to those with more pronounced changes. Also, ORA does not consider the effects of DEGs in relation to the non-significant or "background" genes. (181) Consequently, the subtleties of gene-gene interactions that may contribute to modest pathway-level changes are not detected.

GSEA differs from ORA in that the entire gene list is analysed, yet genes are ranked according to a continuous metric such as log fold change or p-value to reflect the magnitude, direction and significance of the expression change. (180) Genes ranked at the top of the list are "highly upregulated" as they have the largest log fold change with smaller p-values, conversely genes at the bottom are "highly downregulated". GSEA determines whether genes associated with certain pathways are clustered together, either at the top or bottom of the ranked list. (180) An enrichment score (ES) is calculated that reflects the degree to which the pathways cluster and is normalised to create a normalised enrichment score (NES). NES adjusts for differences in the data-set size to allow for between group comparisons. (180) Pathways that are strongly upregulated have a high positive NES, whilst pathways that are strongly downregulated have a high negative NES and those not significantly enriched have a NES around zero. (180) GSEA is advantageous, in that all genes

within an experiment are considered, and the pathways identified are enriched by both pronounced and more modest yet consistent changes in gene expression.

During the process of analysis, for my own learning, I examined the differentially expressed pathways produced by GSEA and ORA using GO and Reactome terms. In this thesis, I will only present GSEA-GO and ORA-GO pathways as the preferred methodology performed by our research group, for reasons explained above.

4.3 Methodology for multi-omics sequencing

4.3.1 IESS and control cohort selection criteria, recruitment and ethics approval

4.3.1.1 IESS cohort

The selection criteria and recruitment of IESS participants from SCHN is described in detail in Chapter 3.3.1. After consent, venous blood samples were drawn and prioritised for clinical testing. The remaining blood volume was then transferred into appropriately labelled collection tubes for research testing. These included PAXgene™ blood RNA tube (Qiagen, Hilden, Germany) for bulk RNA sequencing (minimum 2.5mL required), Acid Citric Dextrose tube (BD Biosciences) for proteomic and phosphoproteomic sequencing (4-6mL), Lithium Heparin tube (BD Biosciences) for neuroimmune enzyme-linked immunosorbent assay (ELISA) panel (0.5mL), and ethylenediaminetetraacetic acid (EDTA) tube (BD Biosciences) for full blood count (FBC) and immunophenotyping flow cytometry (1-2mL).

Samples were collected prior to prednisolone starting and the second collection occurred between days seven-14 of treatment, when it is accepted as standard of care to screen for steroid side effects including blood chemistry for renal impairment and elevated glucose. The exact day of collection was chosen by the treating clinician. The second sample collection time point also correlated with our clinical aim of determining the rate of epileptic spasm cessation by day 14.

Ethics was approved by SCHN HREC-Neuro-Tx: Investigating mechanisms of disease and therapeutics in neurology (HREC 2021/ETH00356). To note, Neuro-Tx is a wider study encompassing several projects by my colleagues/research group at the Kids Research Centre, at the Children's Hospital at Westmead. We are using the same multi-omics workflow and similar sequencing techniques to investigate disease and treatment mechanisms. As a result, sections of methodology described here may have been published by our group in publicly available domains.

4.3.1.2 Control cohort

4.3.1.2.1 Selection criteria

As we required blood from young "healthy" infants for normative control data, we felt the most appropriate controls were infants having planned, elective minor surgical procedures that required cannula insertion under anaesthetic and the opportunity to painlessly draw blood from the cannula.

Controls were matched to IESS participants by median age and gender. Eligible controls had normal neurodevelopment and no significant medical conditions including prematurity (born before 37 weeks), neurological, immune, inflammatory, metabolic or cardiovascular diseases. Suitable procedures included (but were not limited to) hernia repair, orchidopexy or tongue-tie release (Table 4.1).

Table 4.1 Control participants' baseline characteristics, surgical procedures

Pt	Sex	Age (months)	Surgical Procedure
1	F	1.9	Hernia repair
2	F	9.3	Skin tag removal
3	F	5.1	Tongue tie release
4	F	11.5	Tongue tie release
5	F	31.4	Hernia repair
6	F	2.2	Epilus removal
7	F	2.6	Hernia repair
8	M	13.5	Umbilical granuloma excision
9	M	4.7	Orchidopexy
10	M	3.8	Hernia repair
11	M	13.7	Pilomatrixoma excision

Key: F=female, M=male, Pt=participant

Controls were excluded if they were undergoing major operations (heart, deep abdominal surgery) or had a recent history of possible inflammation including an active infection or antibiotic use in the two weeks prior to surgery. Participants were excluded if steroids were a regular medication and if past use of steroids was judged as significant, for example a high dose or intravenous course.

4.3.1.2.2 Recruitment

For this study, controls were recruited through one hospital, the Children's Hospital at Westmead, NSW, Australia in planned outpatient surgical clinics. The paediatric surgeon provided a participant information sheet and referred participants undergoing eligible surgical procedures to the study team.

In the month before the operation, I contacted the parents/carers via phone to discuss participation in the study. Once consented, I screened for eligibility using medical records and a standardised questionnaire (Appendix 7). After obtaining informed written consent, venous blood samples were drawn and transferred into labelled research blood tubes described above in section 4.3.1.2.1.

4.3.1.2.3 Ethics

I made an amendment to the Neuro-Tx protocol (HREC 2021/ETH00356) to allow recruitment from the surgical outpatient department and perioperative ward - Middleton Day Stay Unit, at the Children's Hospital at Westmead.

4.3.2 White cell and lymphocyte subset profiling

4.3.2.1 Method

FBC analysis was performed to measure the effects of prednisolone on white cell and differential counts. In clinical practice, it is commonly observed that steroids produce a neutrophilia and lymphopenia. We wanted to investigate changes to these cell types "pre" and "post" prednisolone. FBC analysis was performed in 33 samples labelled "IESS pre" (*n*=11), "IESS post" (*n*=11) and "control" (*n*=11). IESS samples (7F/4M, median age 7.0 months, range 4-11) were age and sex matched to controls (7F/4M, median age 7.0 months, range 2.5-31).

Immunophenotyping flow cytometry was not able to be performed in all 11 infants with IESS, given blood sampling restrictions. Ten samples from five IESS infants (3F/2M,

median age 9.0 months, range 7-11) were processed for analysis. Samples were labelled "IESS pre" (*n*=5) and "IESS post" (*n*=5). Both analyses were performed according to standard laboratory protocols at the Children's Hospital at Westmead and Sydney Children's Hospital at Randwick.

4.3.2.2 Analysis

I performed the statistical analyses and generated graphs using GraphPad Prism (version 10.4.0, 527), San Diego, CA. (144) Data was descriptively presented using mean, median and range. Between group comparisons were performed using the Mann-Whitney U test and statistical significance set at $p < 0.05$. A correction was made for multiple statistical comparisons using the Benjamini Hochberg false discovery rate (FDR).

4.3.3 Neuroinflammation ELISA panel

4.3.3.1 Method

The LEGENDplex™ Human Neuroinflammation Panel 1 (13-plex) assay was performed to investigate the hypothesis that prednisolone may alter immune-mediated pathways and/or exert an anti-inflammatory effect to control epileptic spasms. Analysis was performed in *n*=33 samples labelled "IESS pre" (*n*=11), "IESS post" (*n*=11) and "control" (*n*=11). Demographics as stated in 4.3.2.1. Processing was performed by Dr Suat Dervish at the Westmead Cytometry Core Facility at the Westmead Research Hub (Westmead, Australia) according to manufacturer's protocol. The 33 plasma samples from *n*=11 IESS participants and *n*=11 healthy controls were thawed and transferred into 1.5ml Eppendorf tubes. Dr Dervish has summarised the method in brief: Samples were centrifuged at 10000g at 4°C for 5 minutes. Samples were then diluted 2-fold with assay buffer in a 96-well plate before being loaded onto a high throughput filter plate (Millipore™) with capture beads. The filter plate was incubated for 120 minutes with shaking at room temperature, washed, and detection antibodies then added for a further 60 minutes. Streptavidin-PE was added to the well for a further 30 minutes, followed by a wash before acquisition using a BD LSRFortessa

with FACSDiva (version 8.0.3) and analysed using the online LEGENDplex data analysis suite (BioLegend, QOGNIT).

4.3.3.2 Analysis

I performed the statistical analyses and generated graphs using GraphPad Prism (version 10.4.0, 527) San Diego, CA. (144) Data was descriptively presented using mean, median and range. Between group comparisons were performed using the Mann-Whitney U test and statistical significance set at $p < 0.05$. A correction was made for multiple statistical comparisons using Benjamini Hochberg false discovery rate (FDR).

4.3.4 Whole blood Bulk RNA sequencing

4.3.4.1 Method

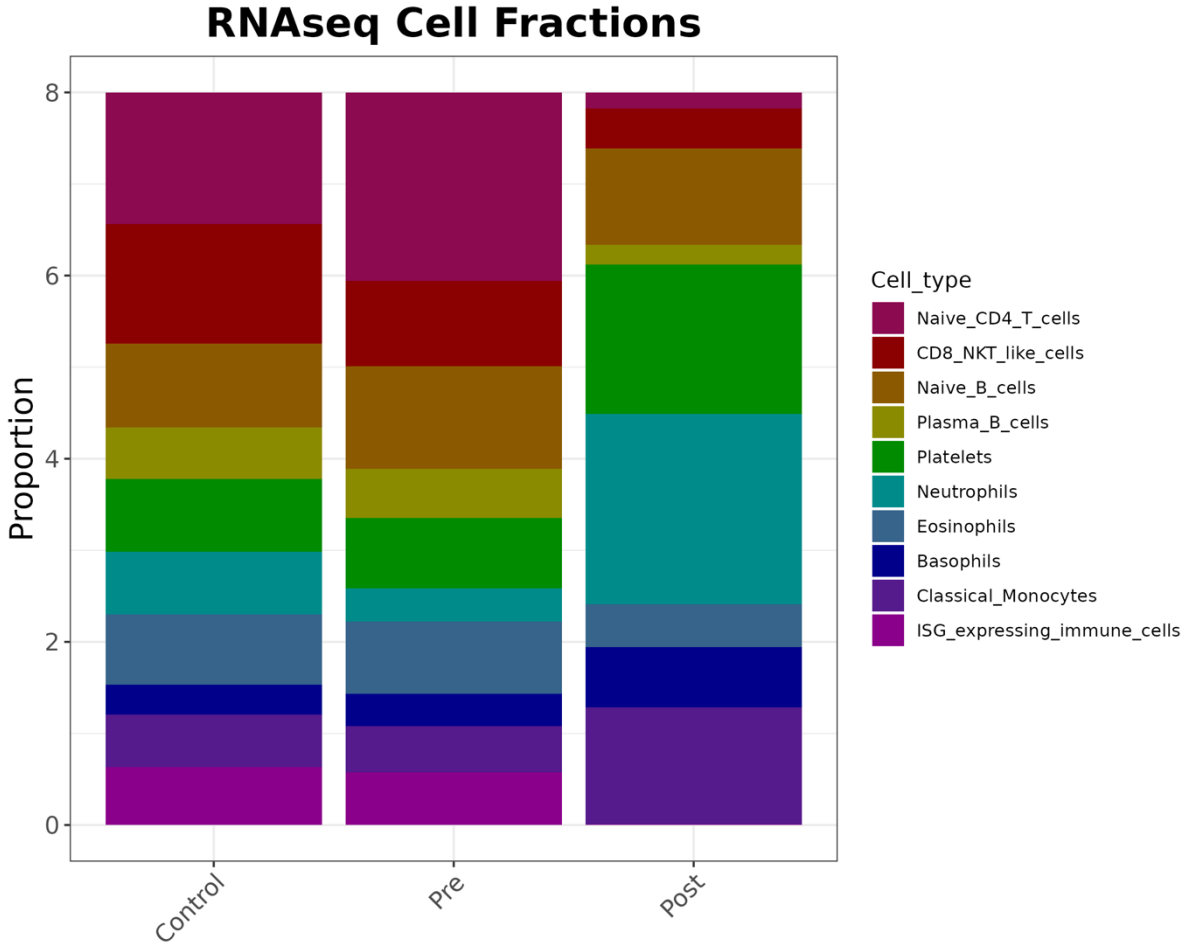
Weight-based volume restrictions limited our ability to collect and perform bulk RNA sequencing in all 11 infants. As such, bulk RNA sequencing was performed in 24 samples, drawn from eight infants with IESS, pre and post prednisolone and compared to eight controls. Samples were labelled "IESS pre" (*n*=8), "IESS post" (*n*=8) and "control" (*n*=8). Infants with IESS (5F, 3M) had a median age of 7.5 months (range 4-11) compared to controls (5F, 3M) with median age 7.0 months (range 2.2-13.7). Whole blood transcriptome sequencing was conducted by the Australian Genome Research Facility (AGRF, Adelaide, Australia). This workflow included RNA extraction from PAXgene™ blood RNA tubes, depletion of rRNA/globin RNA via hybrid capture (Illumina Ribo-Zero), and Illumina TruSeq Stranded Total RNA Library Preparation (input 200-1000 ng of Total RNA). The stranded RNA samples are sequenced on the Illumina NovaSeq 6000 next generation sequencing platform (150 base pair paired end run) for a depth of 50 million paired end reads. The cleaned sequence reads were aligned against the Homo sapiens genome (Build version hg38), and the STAR aligner (v2.5.3a) was used to map reads to the genomic sequences.

For bulk RNA sequencing, a deconvolution analysis was performed to adjust for the significant changes observed in the white cell proportions post prednisolone treatment. As

shown in Figure 4.6, in the IESS-post samples, the neutrophil population significantly increased, and the lymphocyte population significantly reduced. Without deconvolution, the neutrophils would dominate the IESS-post sample and potentially skew the bulk RNA findings.

Figure 4.6 Bulk RNA sequencing deconvolution analysis to identify white cell and lymphocyte proportions

No significant differences in cell proportions were observed between control and IESS pre prednisolone ("Pre") samples. In IESS post prednisolone ("Post") compared to pre-treatment ("Pre") samples the neutrophil population (in aquamarine blue) increased, and the lymphocyte population (in pink and red) reduced. These changes were significant and necessitated "compensation" using a deconvolution analysis.



Key: CD4=cluster of differentiation 4, T Helper cells, CD8=cluster of differentiation 8, cytotoxic T cells, ISG=interferon S genes

The deconvolution analysis identified individual white cell types contributing to the bulk RNA expression data. A statistical adjustment was made so that the cell proportion changes were weighted equally or "normalised" to enable a more representative bulk RNA sequencing comparison between IESS pre and IESS post samples. Herein, bulk RNA sequencing refers to the deconvoluted dataset.

4.3.4.2 Analysis

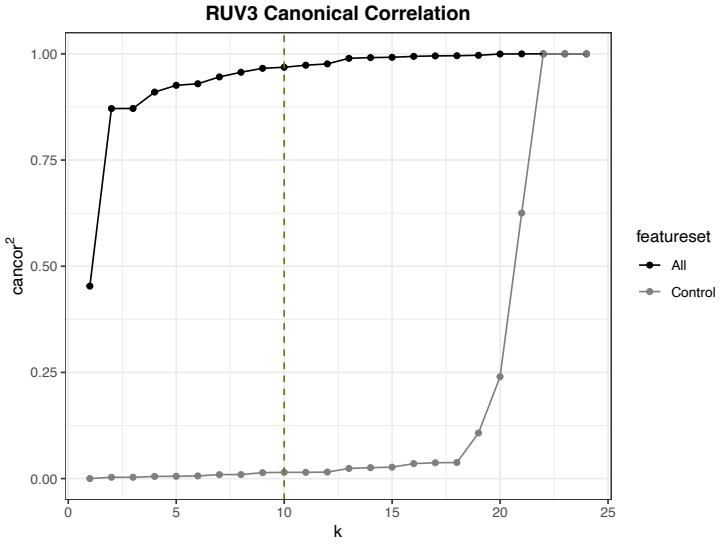
Bulk RNA sequencing was analysed in the R statistical environment with *tidyverse.* (186) Senior bioinformaticians Dr Nader Aryamanesh and Dr Lee Marshall from the Children's Medical Research Institute, Australia performed the initial bioinformatic analysis including deconvolution, quality checks and the removal of unwanted variation (RUV) normalization step. I reviewed these steps and with the assistance and coding expertise of Dr Velda Han, we performed the differential expression and pathways analyses.

Filtering and normalization steps were performed first. Genes that have zero counts or counts <10 across samples were filtered out. The samples counts were transformed to log counts per million, and between sample normalization was performed using the "scaled" normalization from the *limma* R package. (187)

In contrast to cell line or animal models, there is significant variation between human data samples. To better discern relevant biological differences, samples were normalised with removal of unwanted variation, via the remove unwanted variation (RUV)-3 R package.

RUV-3 canonical correlation plot is used to visualize the canonical correlation between the factors of interest and gene expression. This graph shows how the canonical correlation changes with the number of singular vectors (*k*). In this dataset, *k=10* (factors of unwanted variation) was selected to remove genes that had minimal differential expression in IESS samples (black line) compared to control genes (grey line), to the left of the green dotted line (Figure 4.7).

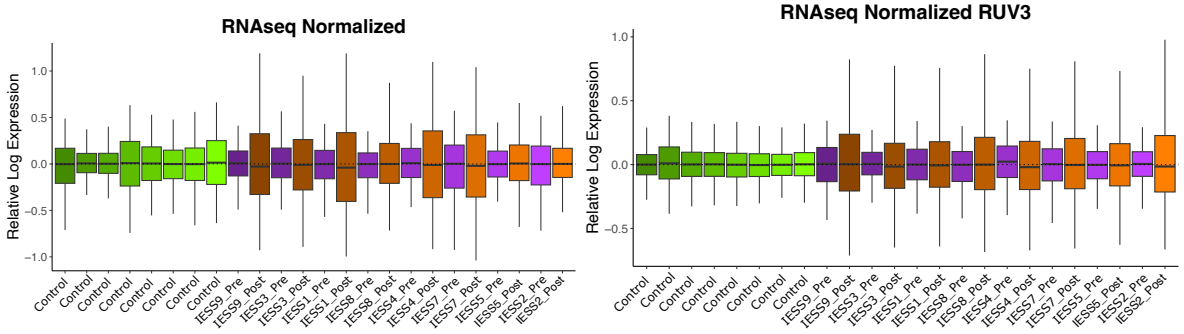
Figure 4.7 Removal of unwanted variation canonical correlation plot. K=10 (factors of unwanted variation) was used (dotted vertical line)



Normalisation was also performed, and the effects of RUV3 can be shown in Figure 4.8.

Figure 4.8 Box and whiskers plots of relative log expression after normalization (RNA seq)
(A)Before removal of unwanted variation (below left) and (B) After removal of unwanted variation (below right). The range of relative log expression is narrower post removal of unwanted variation. (Samples Pre= IESS pre, Post=IESS post, Control).

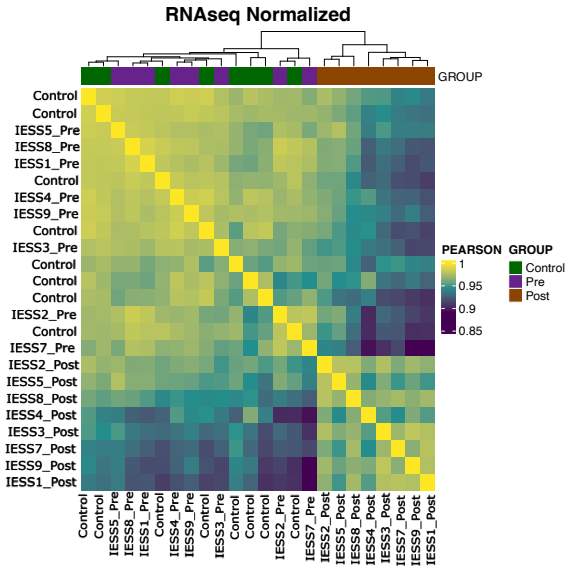
(A) (B)



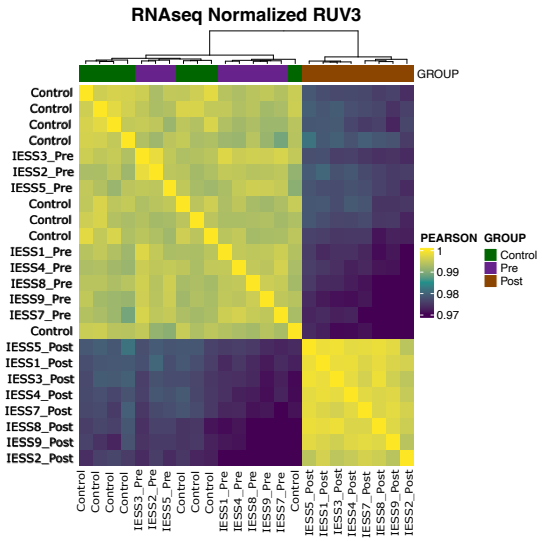
Plotting a Heatmap of Pearson coefficient (Figure 4.9) demonstrates an improved separation of samples (control, Pre, Post) after removal of unwanted variation (RUV-3).

Figure 4.9 Heatmap of Pearson correlation coefficient between samples (RNA seq)
(A)Before and (B) After removal of unwanted variation. There was higher Pearson correlation and better clustering of samples within groups after removal of unwanted variation (Samples Pre= IESS pre, Post=IESS post, Control). Pearson score of more than 1 (yellow) indicates higher linear relationship between samples (i.e. samples are more similar to one another). Pearson score <1 (dark blue) indicates higher differences between samples.

(A)

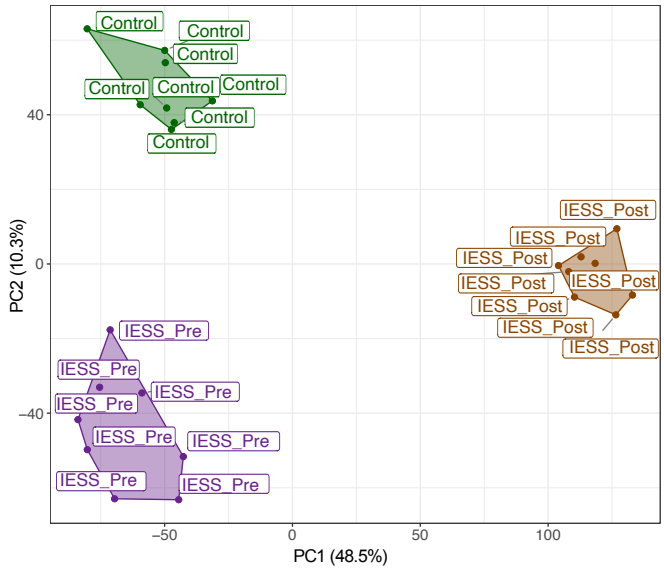


(B)



After normalisation and RUV-3, Principal Component Analysis (PCA) plots enable a visual analysis of the principal causes of variation in a dataset. Figure 4.10 demonstrates segregation in gene expression between controls, IESS-pre and IESS-post treatment groups.

Figure 4.10 Principal Component Analysis plot of sample clustering after normalisation and removal of unwanted variation (RNA seq)
Gene counts are projected onto two dimensions – the x-axis specifies the direction of the data with the largest variability, and the y-axis specifies the direction of the data with the second largest variability. In this dataset, three distinct groups can be appreciated (control, IESS pre and IESS post) with no "outlier" or "overlapping" samples indicating the gene expression in each group are distinctly different.



4.3.4.3 Pathway Enrichment Analysis

For bulk RNA sequencing, pathway enrichment analysis was performed using GSEA. Both GSEA and ORA identify enriched pathways in gene lists. However, they differ in how they evaluate enrichment. ORA assesses if a gene set is over-represented in a list of differentially expressed genes, while GSEA examines the distribution of gene set members within a ranked list of all genes, considering both the direction and magnitude of expression change (see detailed review in method above). We used GSEA as it has the advantage of detecting co-ordinated and consistent changes in gene expression that contribute to modest pathway-level changes.

The genes were ranked based on their sign(logFC) x log10Pvalue scores. (188, 189) Enriched gene sets were identified based on a running sum statistic (adjusted p values) and statistical significance based on the FDR. Significant GSEA-GO pathways (FDR <0.05) were further simplified use the *simplify* function in clusterProfiler, and ranked by NES. (190) NES reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes.

4.3.5 Proteomic sequencing

4.3.5.1 Method

Proteomic sequencing was performed in 18 samples drawn from six infants with IESS pre and post prednisolone and compared to six controls. Samples were labelled "IESS pre" (*n*=6), "IESS-post" (*n*=6) and "control" (*n*=6). Infants with IESS (3F, 3M, median age 7.1 months, range 4.5-9) were age and sex-matched to controls (3F, 3M, median age 7.2 months range 1.9-13.7). Sequencing was conducted by Dr Mark Graham at Children's Medical Research Institute, Australia described in detail in Appendix 8 and summarised in brief here. This workflow involved thawing previously prepared frozen PBMCs, degrading and removing DNA and RNA, unfolding and separating proteins from the solution through precipitation. Dried proteins were then digested/cleaved to produce smaller peptides with a mass suitable for LC-MS. Peptides were labelled with tandem mass tags (TMT) to enable identification and quantification of peptides simultaneously during multiplex (18-plex) analysis.

LC-MS was performed using a Dionex Ultimate 3000 RSLC nano system and Q Exactive Plus hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific). Peptides were separated using hydrophilic interaction liquid chromatography and charged by electrospray ionisation. Ionised peptides were transferred to the mass spectrometer for identification and quantification based on their mass-to-charge ratio and referenced to the *Homo sapiens* reference proteome. (191)

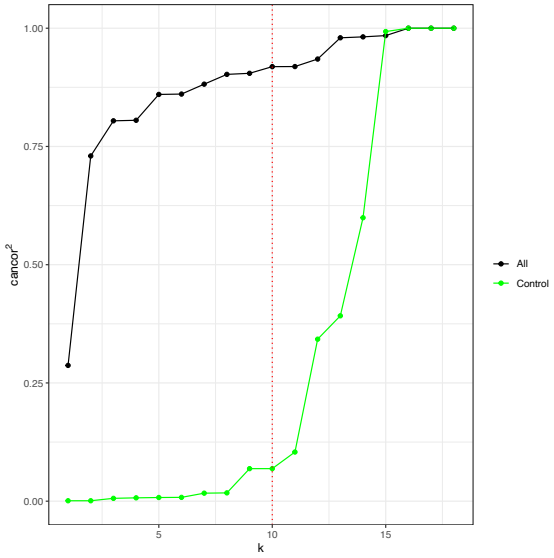
4.3.5.2 Analysis

Proteomic sequencing was analysed in the R statistical environment with *tidyverse.* (186) Senior bioinformatician Dr Nader Aryamanesh performed the initial bioinformatic analysis including quality checks and the RUV normalization step. I reviewed these steps and with the assistance and coding expertise of Dr Velda Han, we performed the differential expression and pathways analyses.

The dataset was filtered and then normalised. Initially, 8,812 proteins were identified, 7,444 were quantifiable and after filtering those with zero counts 7,134 proteins remained. To further discriminate biologically relevant differences, the sample was normalised using the RUV-3 R package.

Figure 4.11 visually demonstrates the canonical correlation between the factors of interest and protein expression.

Figure 4.11 Removal of unwanted variation canonical correlation plot. K=10 (factors of unwanted variation) was used-dotted vertical line (proteomics)
In this dataset, k=10 (factors of unwanted variation) was selected to remove proteins that had minimal differential expression in IESS samples (black line) compared to controls.

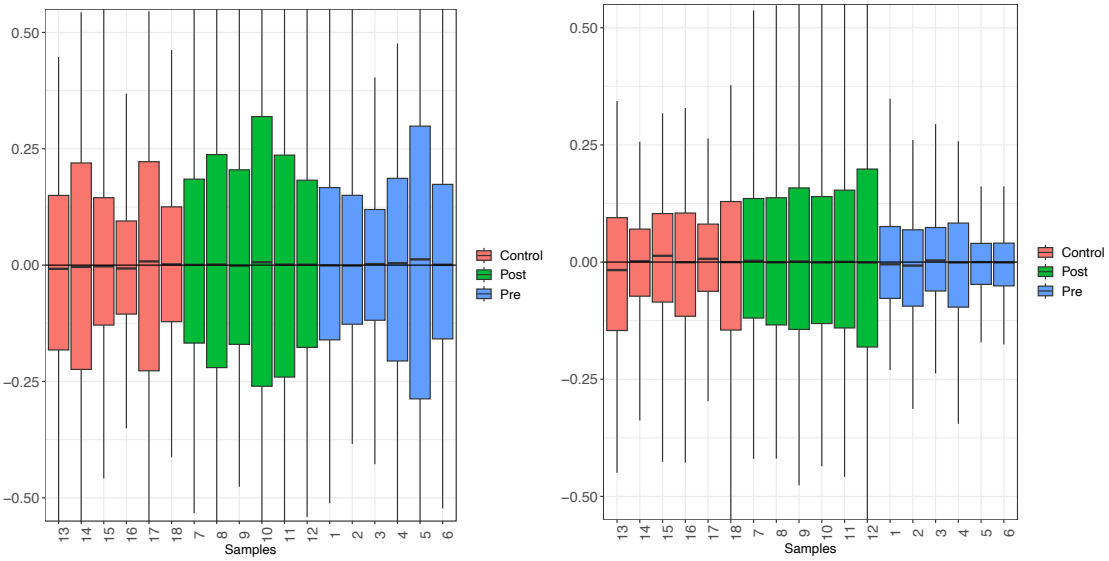


In addition, normalisation was performed as shown in Figure 4.12.

Figure 4.12 Box and whiskers plots of relative log expression after normalization (proteomics)

(A) Before removal of unwanted variation (below left) and (B) After removal of unwanted variation (below right). The range of relative log expression is narrower post removal of unwanted variation.

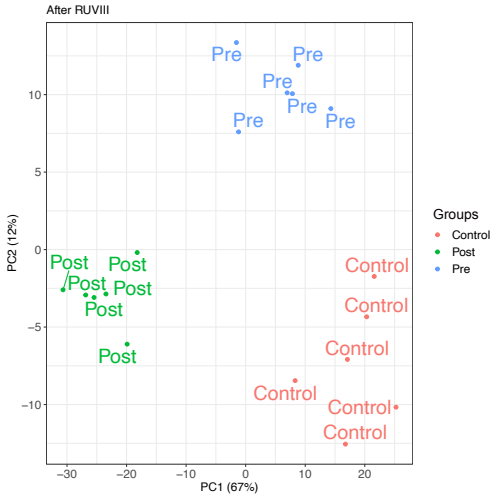
(A) (B)



After normalisation and RUV-3, Figure 4.13 displays the PCA plots which demonstrates good separation in protein abundance between controls, IESS-pre and IESS-post treatment groups.

Figure 4.13 Principal Component Analysis plot of sample clustering after normalisation reveals good separation of the cohorts (proteomics)

The protein abundance counts are projected onto two dimensions – the x-axis specifies the direction of the data with the largest variability and the y-axis specifies the direction with the second largest variability. In this dataset, three distinct groups can be appreciated (control, IESS pre and IESS post) showing good 'separation' of samples.



4.3.5.3 Pathway Enrichment Analysis

Similar to bulk RNA sequencing, GSEA was used for proteomic pathway enrichment. The proteins were ranked based on their sign(logFC) x -log10 p value scores. (188, 189) Enriched protein sets were identified based on a running sum statistic and statistical significance based on the FDR. Significant GSEA-GO pathways (FDR <0.05) were further simplified use the *simplify* function in clusterProfiler, and ranked by NES. (190)

4.3.6 Phosphoproteomic sequencing

4.3.6.1 Method

Phosphoproteomic processing was feasible in the same samples as performed in proteomics, except one of the IESS samples, which was excluded due to insufficient peptide volume. Therefore, phosphoproteomic sequencing was performed in 16 samples labelled "IESS pre" (*n*=5), "IESS post" (*n*=5) and "control" (*n*=6). IESS infants (3F, 2M, median age 7.2 months, range 4-9) were age/sex matched to controls (3F, 3M, median age 7.2 months, range 1.9-13.7). Sequencing was conducted by Dr Mark Graham at CRMI, with their method provided below.

Phosphopeptides were enriched and fractionated prior to LC-MS/MS analysis. Searching of LC-MS/MS data in proteomics and phosphoproteomics: The raw LC-MS/MS data was processed with MaxQuant v2.5.2.0 using the following settings: variable modifications were oxidation (M), acetyl (protein N-terminus), deamidation (NQ) and phospho (STY); carbamidomethyl (C) was a fixed modification; digestion was set to trypsin/P with a maximum of 3 missed cleavages; the TMTpro correction factors were entered for lots XC344112 and XK350589; Minimum reporter peptide ion faction was 0.6; the Homo sapiens reference proteome with canonical and isoform sequences downloaded June 9 2023 with 82,518 entries and 20,590 genes.; the inbuilt contaminants fasta file was also used; minimum peptide length was 6 and maximum peptide mass was 6000 Da; second peptides search and dependent peptides searches were enabled; peptide spectrum matching and protein false discovery rates were set at 1%; all modified peptides and counterpart non-modified peptides were excluded from protein quantification. All other settings were default.

4.3.6.2 Analysis

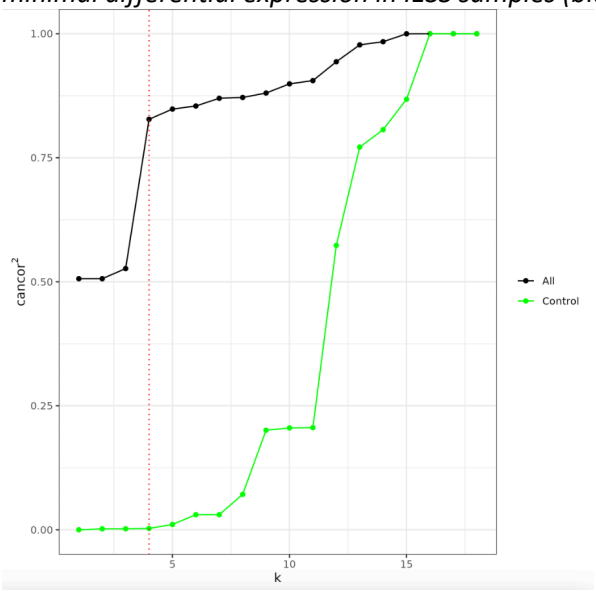
Phosphoproteomic sequencing was analysed in the R statistical environment with *tidyverse.* (186) Senior bioinformatician Dr Nader Aryamanesh performed the initial bioinformatic analysis including quality checks and RUV normalization step. I reviewed these steps and with the assistance and coding expertise of Dr Velda Han, we performed the differential expression and pathways analyses.

The dataset was filtered and then normalised. After filtering those with zero counts 6,592 proteins remained. To further discriminate biologically relevant differences, the sample was normalised using the RUV-3 R package.

Figure 4.14 visually demonstrates the canonical correlation between the factors of interest and protein expression.

Figure 4.14 Removal of unwanted variation canonical correlation plot. K=4 (factors of unwanted variation) was used- dotted vertical line (phosphoproteomics)

In this dataset, k=4 (factors of unwanted variation) was selected to remove proteins that had minimal differential expression in IESS samples (black line) compared to controls.

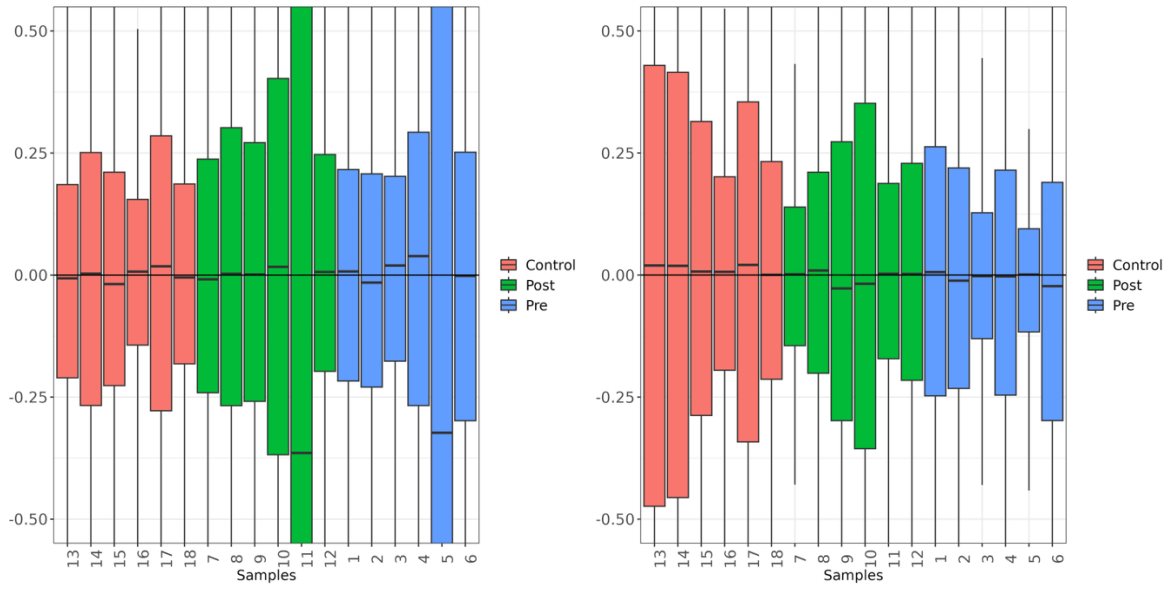


In addition, normalisation was performed as shown in Figure 4.15.

Figure 4.15 Box and whiskers plots of relative log expression after normalization (phosphoproteomics)

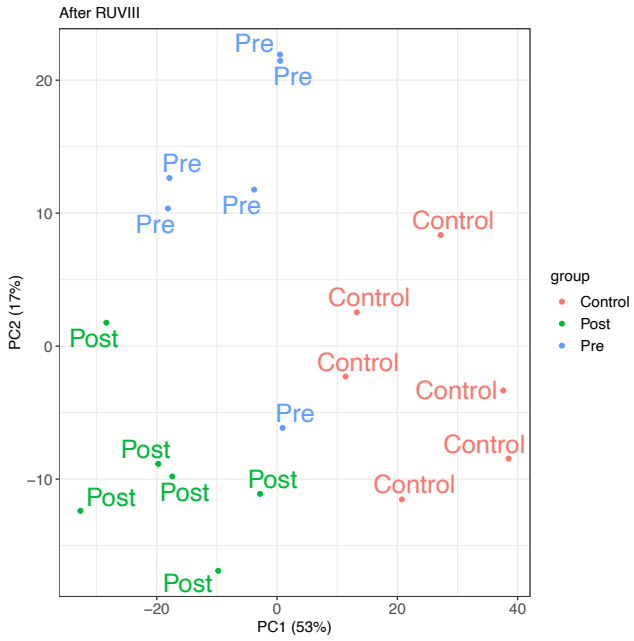
(A) Before removal of unwanted variation (below left) and (B) After removal of unwanted variation (below right). The range of relative log expression is narrower post removal of unwanted variation.

(A) (B)



After normalisation and RUV-3, Figure 4.16 displays the PCA plots which demonstrates good separation in phosphoprotein abundance between controls, IESS-pre and IESS-post treatment groups.

Figure 4.16 Principal Component Analysis plot of sample clustering after normalisation reveals good separation of the cohorts (phosphoproteomics)
The phosphoprotein abundance counts are projected onto two dimensions – the x-axis specifies the direction of the data with the largest variability and the y-axis specifies the direction with the second largest variability. In this dataset, three distinct groups can be appreciated (control, IESS pre and IESS post) showing good 'separation' of samples. There is one outlier "pre" sample.



4.3.6.3 Pathway Enrichment Analysis

For phosphoproteomic sequencing, pathway enrichment analysis was performed using ORA. ORA was used rather than GSEA, as phosphorylation can occur at more than one site on the same protein. It is generally agreed that the effects on protein abundance and gene expression are dependent on the specific phosphosite. The direction of phosphorylation (increased or decreased) is also variable, given protein kinases tend to activate or "turn on" expression whereas phosphatases tend to suppress or "turn off" function. As a result, using GSEA to rank peptide expression to derive enriched pathways seemed less valid, instead we used ORA (with a ranking method within).

We elected to use a previously established method for phosphoproteomics. (192) We took an approach that deliberately reduced the significant pathways identified by

limiting the background to only proteins we could detect by mass spectrometry, and using the ranked list method within gProfiler, which weights enrichment toward highly ranked proteins. We separated up- and down-regulated phosphorylation, assigned the maximum positive and negative quantitative values for phospho-regulation to each protein, and ranked the proteins using both the quantitative value and significance of maximal change.

Proteins were ranked based on their sign(logFC) x -log10 p value scores. (188, 189) Enriched phosphoprotein sets were identified based on a running sum statistic and statistical significance based on the false discovery rate (FDR). Significant ORA pathways (FDR <0.05) were further simplified use the *simplify* function in clusterProfiler. We elected to pathways as “dysregulated” based on -log(FDR) value rather than separate as “up” vs “down” regulated given the variability of phosphoproteomic expression and the uncertain nature of the biological effects of increased versus decreased phosphorylation.

4.4 Results

4.4.1 IESS cohort

Table 4.2 summarises the omics sequencing performed for each IESS participant. Due to limitations in blood volume collected and sample processing requirements, four of 11 IESS participants had all omics analyses performed (grey shade in Table 4.2).

Table 4.2 Post processing analyses performed in IESS cohort

Pt	Aetiology	Sex	Neuro-immune ELISA panel	T/B cell subsets	RNA seq	Proteomics	Phosphoprote-omics
1	Unknown	F	Y	N	Y	N	N
2	Unknown	F	Y	Y	Y	N	N
3*	Unknown	F	Y	N	Y	Y	Y
4*	Unknown	F	Y	Y	Y	Y	Y
5*	Unknown	F	Y	Y	Y	Y	Y
6	Unknown	F	Y	N	N	N	N
7	Structural (HIE)	M	Y	N	Y	Y	N
8	Structural (HIE)	M	Y	Y	Y	N	N
9*	Structural (R MCA)	M	Y	Y	Y	Y	Y
10	Structural (L MCA)	M	Y	N	N	Y	Y
11	Structural-genetic (TSC2)	F	Y	N	N	N	N

Key: F=female, HIE=hypoxic ischemic encephalopathy, L=left, M=male, MCA=middle cerebral artery, N=no, Pt=participant, R=Right, Seq=sequencing, TSC=tuberous sclerosis complex, Y=yes,*=complete battery of omics sequencing

Bulk RNA sequencing was performed in 16 infants (8 IESS, 8 controls), proteomic sequencing in 12 infants (6 IESS, 6 controls), phosphoproteomic sequencing in 11 infants (5 IESS, 6 controls) and neuro-inflammation panel testing in 22 infants (11 IESS, 11 controls).

4.4.2 Multi-omics investigation of IESS at baseline (IESS pre prednisolone) compared to healthy controls

4.4.2.1 Neuroimmune ELISA panel

A neuro-inflammation panel analysing 13 neuro-immune cytokines, chemokines and growth factors was performed in 11 infants with IESS pre prednisolone ("pre") compared to 11 healthy controls, see Table 4.3 (IESS pre vs control). Brain derived neurotrophic factor (BDNF) was significantly higher in IESS pre compared to controls (p=0.01), which remained significant after adjusting for multiple testing (adjusted p value) (grey shade in table 4.3). Other chemokines and cytokines including IL-6 and TNF-α did not significantly differ at baseline (IESS pre vs control).

Table 4.3 Neuroimmune ELISA panel in IESS cohort versus control

Baseline comparisons (IESS pre vs control) as well as effects of prednisolone (IESS post vs pre) are presented for the 13 cytokines, chemokines and growth factors.

	Control (n=11) Median (range)	**IESS pre (n=11) Median (range)**	**IESS post (n=11) Median (range)**	**IESS pre vs Control**		**IESS post vs IESS pre**	
				p-value	adj p-value	p-value	adj p-value
Chemokine/Cytokines							
CCL2 (MCP-1)	404 (106-534)	288 (207-376)	132 (79-221)	0.69	0.87	0.01	0.03
IL-6	25 (5-147)	27 (13-58)	29 (15-47)	0.98	0.98	0.70	0.87
IL-18	316 (223-507)	418 (285-660)	352 (190-539)	0.12	0.24	0.04	0.137
TNF-α	31 (9-164)	32 (16-58)	42 (28-81)	0.98	0.98	0.04	0.137
CX3CL1	3672 (1822-17074)	3973 (1872-6503)	4839 (2901-6947)	0.69	0.87	0.02	0.08
Growth factors							
BDNF	4437 (2552-13519)	7806 (3311-16697)	7743 (1068-19619)	0.01	0.05	0.64	0.87
TGF-β1	42 (26-101)	42 (26-187)	119 (26-343)	0.98	0.98	0.12	0.23
VEGF	208 (85-349)	267 (103-458)	308 (117-475)	0.08	0.20	0.77	0.9
β-NGF	8 (4-16)	9 (6-17)	13 (10-17)	0.54	0.82	0.06	0.05
Other							
VILIP-1	1091 (593-2318)	962 (725-1780)	1280 (640-2180)	0.85	0.96	0.08	0.2
sTREM-1	383 (73-2895)	328 (76-697)	508 (266-856)	0.47	0.76	0.02	0.07
sTREM-2	3920 (2550-6615)	4621 (3121-6362)	3011 (1600-4857)	0.32	0.55	0.01	0.03
sRAGE	6073 (1668-12255)	5441 (1351-11228)	3028 (1581-5903)	0.29	0.54	0.07	0.19

Key: BDNF=Brain-Derived Neurotrophic Factor, CCL2 (MCP-1)=Chemokine (C-C motif) ligand 2, Monocyte Chemoattractant Protein-1, CX3CL1=Fractalkine, Chemokine (C-X3-C motif) ligand 1, IL-6=Interleukin-6, IL-18=Interleukin-18, sRAGE=Soluble Receptor for Advanced Glycation End-products, sTREM-1=Soluble Triggering Receptor Expressed on Myeloid cells-1,

sTREM-2=Soluble Triggering Receptor Expressed on Myeloid cells-2, TGF-β1=Transforming Growth Factor-beta 1, TNF-α=Tumour Necrosis Factor-alpha, VEGF=Vascular Endothelial Growth Factor, VILIP-1=Visinin-like Protein 1, β-NGF=Beta-Nerve Growth Factor

4.4.2.2 Comparison of white cell and lymphocyte populations

White cell populations were compared in 11 infants with IESS pre prednisolone to 11 healthy controls. No significant differences in cell populations between IESS pre and controls were identified.

4.4.2.3 RNA sequencing in IESS at baseline (IESS pre prednisolone) compared to controls

Following deconvolution, data was filtered and normalised using RUV-3. The principal component analysis identified distinct clustering of gene expression separating controls, IESS pre and post treatment groups (See Figure 4.17A, label A). Compared to controls infants with IESS pre-treatment had 2,714 differentially expressed genes (FDR<0.05), 1521 were upregulated and 1103 were downregulated.

Figure 4.17A Multi-omics analyses in infants with IESS at baseline versus healthy controls
(A)Principal component analysis (PCA) of bulk RNA sequencing performed in controls (control), infants with IESS pre (pre) and post prednisolone treatment (post). The x-axis represents Principal Component 1 (PC1), the y-axis represents Principal Component 2 (PC2). Unbiased hierarchical clustering of gene expression showed separation of data and discrimination across the three groups. (B) Exemplar Volcano plot demonstrating differentially expressed genes (FDR <0.05), upregulated in red, downregulated in blue in infants with IESS pre-treatment compared to controls. (C) Bulk RNA analysis: Top five upregulated Gene Set Enrichment Analysis (GSEA)-gene ontology (GO) pathways (in red), included ribosomal pathways. Top five downregulated GSEA GO pathways (in blue) included secretory granule membrane and immune pathways. (D) Connectivity enrichment plot (CNET) of 93 genes downregulated in the RNA "secretory granule membrane pathway". Genes in this pathway were subclustered by GO biological process (BP) terms into regulation of vesicle mediated transport, regulation of cytokine production, regulation of phagocytosis and cell adhesion mediated by integrin pathways.

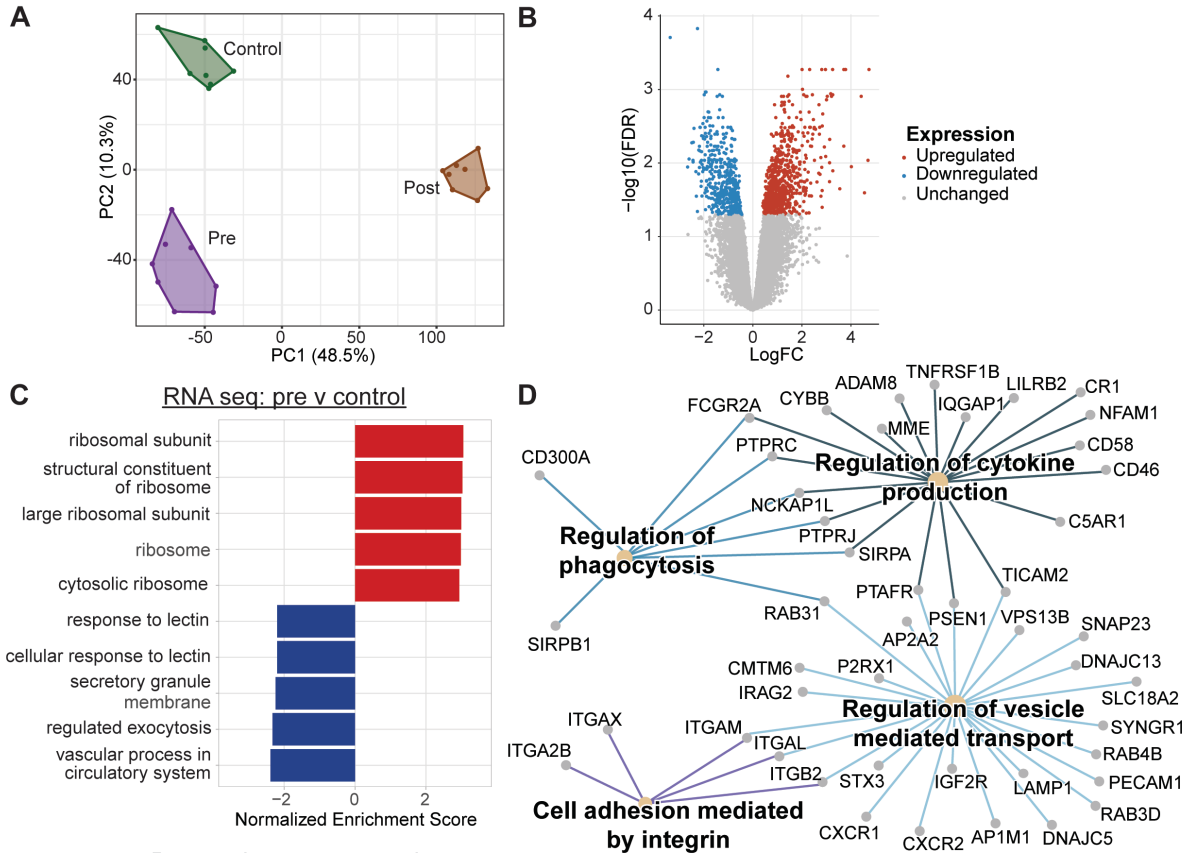
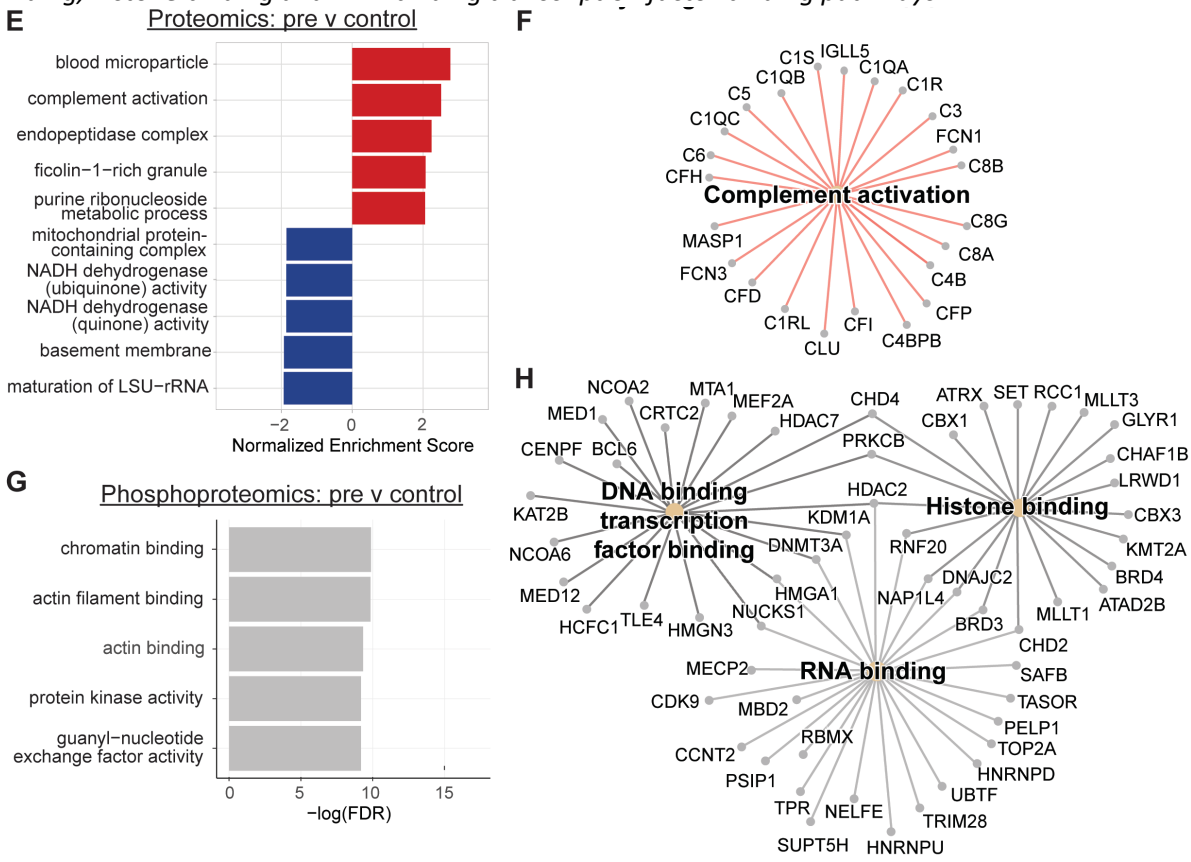


Figure 4.17B Multi-omics analyses in infants with IESS at baseline versus healthy controls
(E) Proteomic analysis: Top five upregulated GSEA GO pathways (in red) included complement activation. Top five downregulated GSEA GO pathways (in blue) related to metabolic/mitochondrial processes. (F) CNET of the 23 genes upregulated in the proteome GO "complement activation" pathway. (G) Phosphoproteomic analysis: Top five Overrepresentation Analysis (ORA) GO pathways that were differentially expressed (in grey) included chromatin binding, actin binding and protein kinase activity. (H) CNET of genes enriching the phosphoproteome "chromatin binding" pathway. Genes in this pathway were subclustered according to GO Molecular Function (MF) terms into RNA binding, histone binding and DNA binding transcription factor binding pathways.



Pathway directed analysis was performed using GSEA. Genes sets were ranked by NES and FDR <0.05 and then mapped to Gene Ontology (GO). In IESS pre compared to controls, the top five upregulated GO terms all involved ribosomal pathways (Figure 4.17A, label C). In contrast, the top five downregulated GO terms related to cellular function and immune response. We focused on secretory granule membrane pathway and created a CNET subcluster using GO biological process (BP) terms (Figure 4.17A, label D). This identified regulation of vesicle mediated transport (enriched by SNAP23, SLC18A1, IGF2R), regulation of cytokine production (enriched by PSEN1, IQGAP1, NFAM1), regulation of phagocytosis (enriched by SIRPB1, SIRPA, PTPRC) and cell adhesion mediated by integrin (enriched by ITGAX, ITGAM ITGAL) pathways.

4.4.2.4 Proteomic and phosphoproteomic sequencing in IESS at baseline (IESS pre prednisolone) compared to controls

Using our proteomic dataset (Figure 4.17B, label E), infants with IESS pre compared to controls had 4038 differentially expressed proteins (FDR<0.05), 1943 were upregulated and 2095 were downregulated. In IESS pre compared to controls, the top five upregulated GSEA-GO terms related to predominantly immune function including complement activation (Figure 4.17B, label E). The top five downregulated GSEA-GO terms related to metabolic/mitochondrial processes. We focused on the upregulated complement activation pathway and plotted a CNET (Figure 4.17B, label F), to highlight the genes enriching this pathway including C1Q, C3, C4.

Using our phosphoproteomic dataset infants with IESS pre compared to controls had 2490 differentially expressed phosphoproteins (FDR<0.05). Pathway directed analysis was performed using ORA-GO to identify dysregulated pathways (rather than up or down-regulated). Dysregulated pathways included chromatin binding, actin binding and protein kinase activity (Figure 4.17B, label G). Focusing on the chromatin binding pathway, genes were subclustered using GO molecular function into RNA binding (enriched by MECP2, HNRNPU, KDM1A), histone binding (enriched by KMT2A) and DNA binding transcription factor binding pathways (enriched by KAT2B, HDAC7) (Figure 4.17B, label H).

4.4.2.5 Summary of multi-omics in IESS at baseline (IESS pre prednisolone) compared to controls

In summary, the multi-omics comparison between IESS pre-treatment and controls identified elevated serum levels of brain-derived neurotrophic factor. The common findings observed in pathway analysis using the multi-omics approach included dysregulated ribosomal, immune and chromatin pathways.

4.4.3 Defining the effects of prednisolone in IESS: Multi-omics comparison of IESS post versus pre prednisolone treatment

4.4.3.1 Comparison of neuro-immune cell types including chemokines, cytokines, growth factors and other biomarkers in IESS post versus pre prednisolone treatment

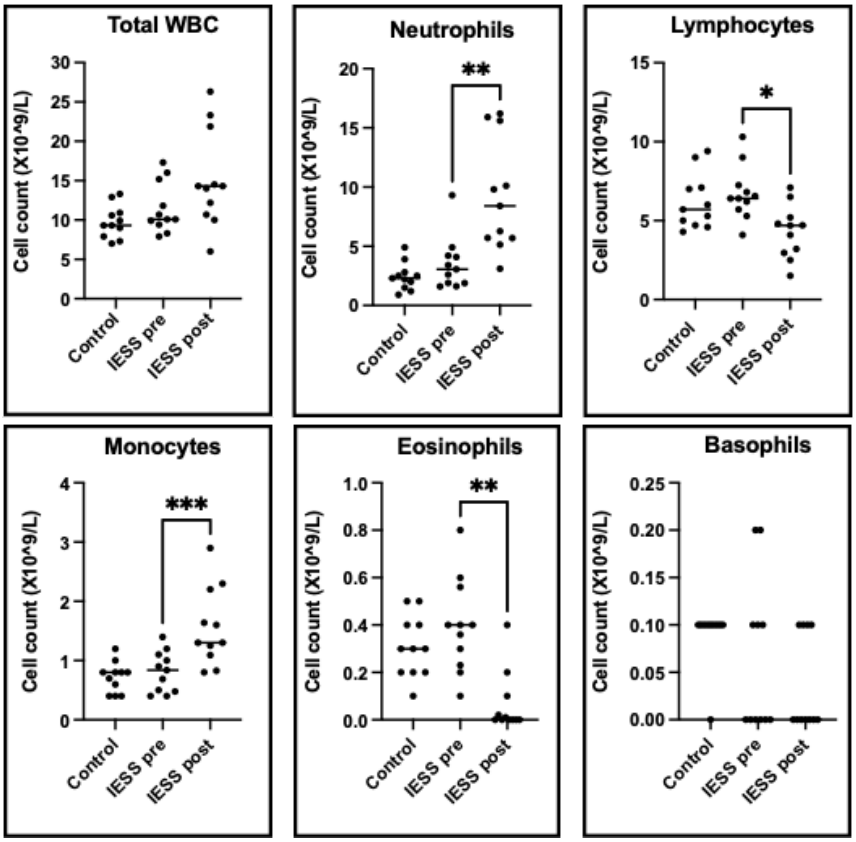
Neuro-inflammation panel testing was performed in all 11 infants with IESS, comparing post prednisolone samples collected after a median of eight days of treatment (mean 9.5, range 7-14 days) to pre prednisolone samples. Prednisolone increased CX3CL1, TNF-α, β-NGF and sTREM-1, and lowered CCL2, IL-18, sTREM-2. After adjusting for multiple statistical testing, the reductions in CCL2 (p=0.03), sTREM-2 (p=0.03) and increase in β-NGF (p=0.05) remained significant. (See Table 4.3)

4.4.3.2 Comparison of white cell and lymphocyte populations in IESS post versus pre prednisolone treatment

White cell populations were compared in 11 infants with IESS post vs pre prednisolone. Post samples were collected after a median of eight days of treatment (mean 9.5, range 7-14 days). Prednisolone caused a significant increase in neutrophil (unadjusted p=0.007) and monocyte (unadj. p=0.001) populations whilst lymphocytes (unadj. p=0.019) and eosinophils (unadj. p=0.002) were suppressed, see Figure 4.18 and table 4.4. After adjusting for multiple statistical testing, the change in lymphocyte population was no longer significant. Due to these changes in cell populations, we performed a deconvolution analysis of the RNA sequencing, as described above.

Figure 4.18 White blood cell and differential counts in IESS participants pre and post prednisolone treatment (*n*=11) compared to controls (*n*=11)

No significant differences in cell proportions were observed between controls and IESS pre prednisolone samples. A statistically significant increase in neutrophils and reduction in lymphocytes was seen in IESS post compared to IESS pre prednisolone samples.



Key: raw p values*=*p*<0.05, **=*p*<0.01, ***=*p*<0.001, IESS=infantile epileptic spasms syndrome, WBC=white cell blood count. N.B. IESS post indicates blood samples taken after one week of prednisolone

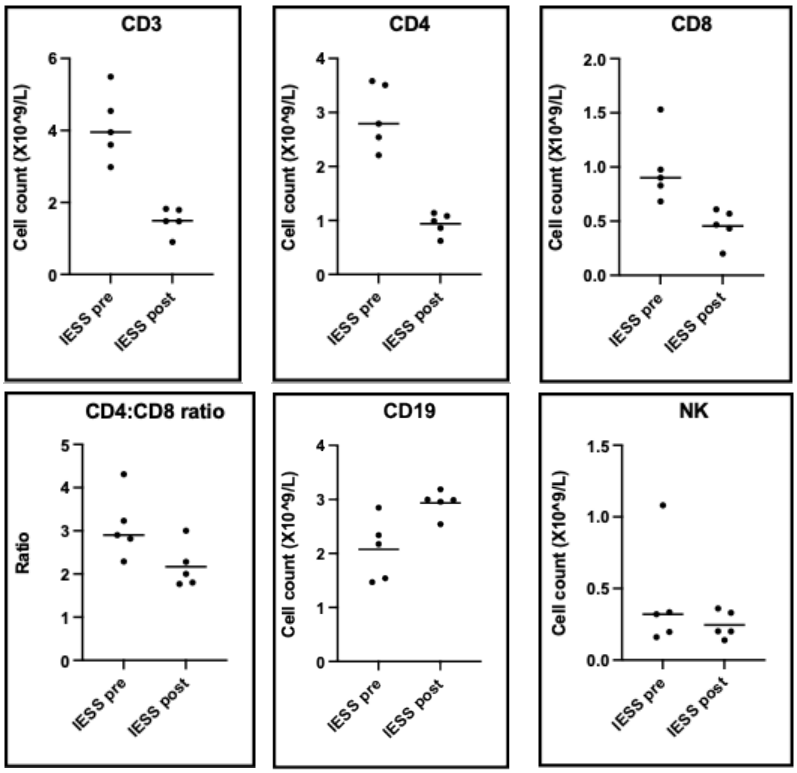
Table 4.4 White blood cell and differential counts in IESS participants pre and post prednisolone treatment (*n*=11) compared to controls (*n*=11)

	Control (*n*=11) Median (range)	IESS pre (*n*=11) Median (range)	IESS post (*n*=11) Median (range)	Control vs IESS pre		IESS post vs IESS pre	
				p-value	adj *p*-value	*p*-value	adj *p*-value
White cells							
Total count	9.3 (7.0-13.3)	10.1 (7.9-17.3)	14.3 (6.0-26.3)	0.17	0.28	0.08	0.22
Neutrophils	2.3 (0.9-4.9)	3.1 (1.6-4.9)	8.4 (3.1-16.2)	0.22	0.33	0.01	0.03
Lymphocytes	5.7 (4.3-9.4)	6.4 (4.1-10.3)	4.7 (1.5-6.5)	0.36	0.43	0.01	0.03
Monocytes	0.8 (0.4-1.2)	0.8 (0.4-1.4)	1.3 (0.8-2.9)	0.46	0.51	0.01	0.01
Eosinophils	0.3 (0.1-0.5)	0.4 (0.1-0.8)	0.0 (0.0-0.4)	0.30	0.40	0.01	0.01
Basophils	0.1 (0-0.1)	0.0 (0.0-0.2)	0.0 (0.0-0.1)	0.17	0.28	0.56	0.56

Key: IESS=infantile epileptic spasms syndrome N.B. IESS post indicates blood samples taken after one week of prednisolone

Five infants with IESS had T and B cell subsets analysed (3F, 2M, median age 8.8 months), no control data was available. No significant differences were found following treatment, however reduction in T cells (CD4 and CD8) were evident, see Figure 4.19 and Table 4.5

Figure 4.19 T & B cell subsets in IESS participants pre and post prednisolone treatment (*n*=5)



Key: raw p values*=*p*<0.05, **=*p*<0.01, ***=*p*<0.001, CD=cluster of differentiation, IESS=infantile epileptic spasms syndrome, NK=natural killer. N.B. IESS post indicates blood samples taken after one week of prednisolone

Table 4.5 T & B cell subsets in IESS participants pre and post prednisolone treatment (*n*=5)

	IESS pre (*n*=5) Median (range)	IESS post (*n*=5) Median (range)	IESS post vs pre
			p-value
T cells			
CD3	4 (3.6-5.5)	1.5 (0.9-1.8)	0.06
CD4	2.8 (2.5-3.6)	1.0 (0.6-1.1)	0.06
CD8	0.9 (0.7-1.5)	0.5 (0.2-0.6)	0.06
CD4:CD8 ratio	2.9 (2.3-4.3)	2.0 (1.8-3.0)	0.06
B cells			
CD19	2.2 (1.5-2.8)	3.0 (2.5-3.2)	0.06
NK cells			
CD16/56	0.3 (0.2-1.1)	0.2 (0.1-0.4)	0.42

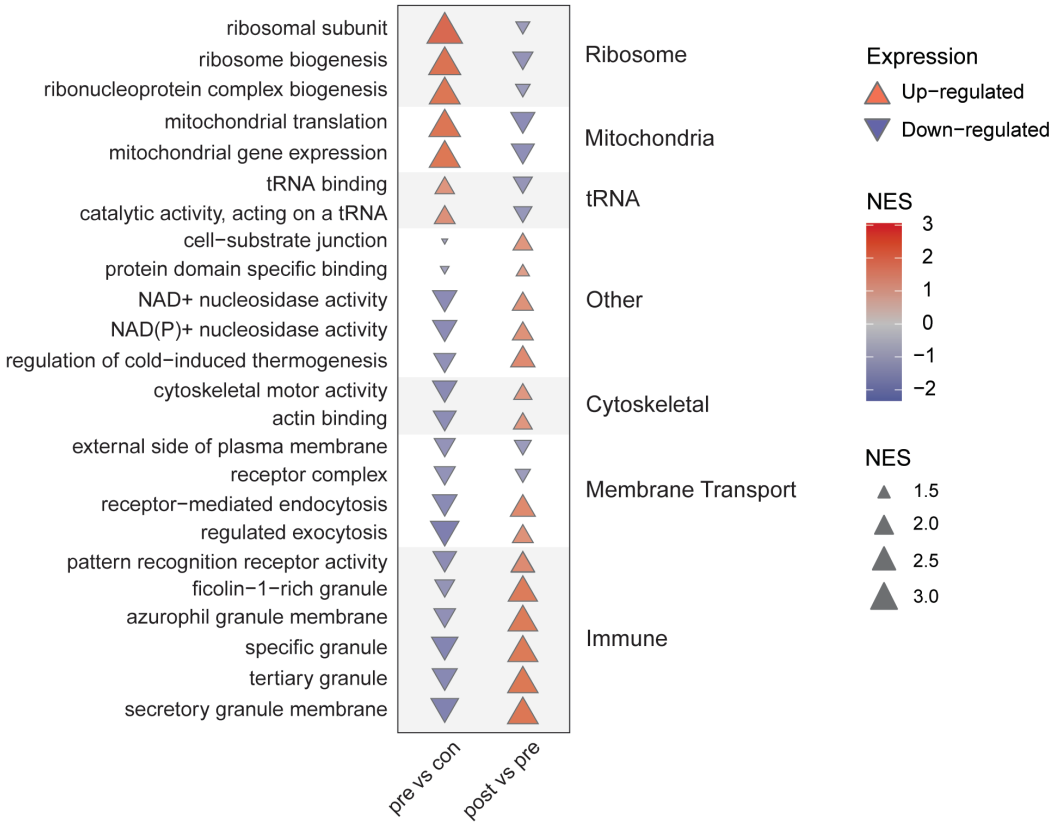
Key: CD=cluster of differentiation, IESS=infantile epileptic spasms syndrome, NK=natural killer, N.B. IESS post indicates blood samples taken after one week of prednisolone

4.4.3.3 Reversal of RNA pathways in IESS post versus pre prednisolone treatment

In IESS post vs pre prednisolone treatment, bulk RNA sequencing identified 9,485 differentially expressed genes (FDR < 0.05), 4500 were upregulated and 4627 were downregulated. We explored the effects of prednisolone and present the data using an arrow plot (Figure 4.20) to examine if there was any significant reversal of pathways following treatment (post vs pre) relative to changes found at baseline between infants with IESS and controls (pre vs con). The top five GSEA-GO pathways were identified for each ontology - Biological Process (BP), Molecular Function (MF), Cellular Component (CC). Only pathways that demonstrated statistically significant reversal were plotted to highlight dysregulated pathways in IESS at baseline that reversed following prednisolone treatment. Ribosomal and mitochondrial translation pathways that were upregulated (in red) in IESS at baseline, were downregulated (in blue) following prednisolone treatment (post vs pre). Immune pathways including secretory granule membrane, pattern recognition receptor activity and cellular metabolism pathways that were downregulated at baseline (pre vs control), were upregulated following prednisolone (post vs pre) (Figure 4.20). Other pathways that were downregulated at baseline and upregulated following prednisolone included nucleosidase activity, cytoskeleton/actin and membrane receptor function (endocytosis/exocytosis).

Figure 4.20 Bulk RNA sequencing analysis highlighting GSEA GO-pathways that reversed in infants with IESS post versus pre prednisolone treatment compared to IESS pre-prednisolone versus controls

Arrow plot highlighting the reversal of statistically significant pathways in infants with IESS following prednisolone treatment (post vs pre) relative to statistically significant changes found at baseline between infants with IESS and controls (pre vs con).

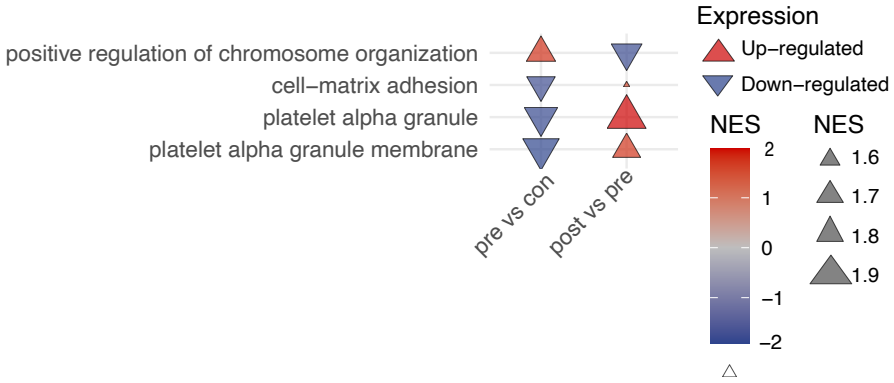


4.4.3.4 Proteomic analysis in IESS post versus pre prednisolone treatment

Proteomic sequencing identified 5996 differentially expressed proteins (FDR < 0.05) in IESS post compared to pre prednisolone treatment, 2981 were upregulated and 3015 were downregulated. An arrow plot identified only four pathways that demonstrated reversal (see Figure 4.21). Similar themes to the RNA dataset were seen including down and then upregulation of “granule” function. Given this commonality we chose to investigate this further.

Figure 4.21 Proteomic sequencing analysis highlighting GSEA GO-pathways that reversed in infants with IESS post versus pre prednisolone treatment compared to IESS pre-prednisolone versus controls

Arrow plot highlighting the reversal of pathways in infants with IESS following prednisolone treatment (post vs pre) relative to changes found at baseline between infants with IESS and controls (pre vs con).

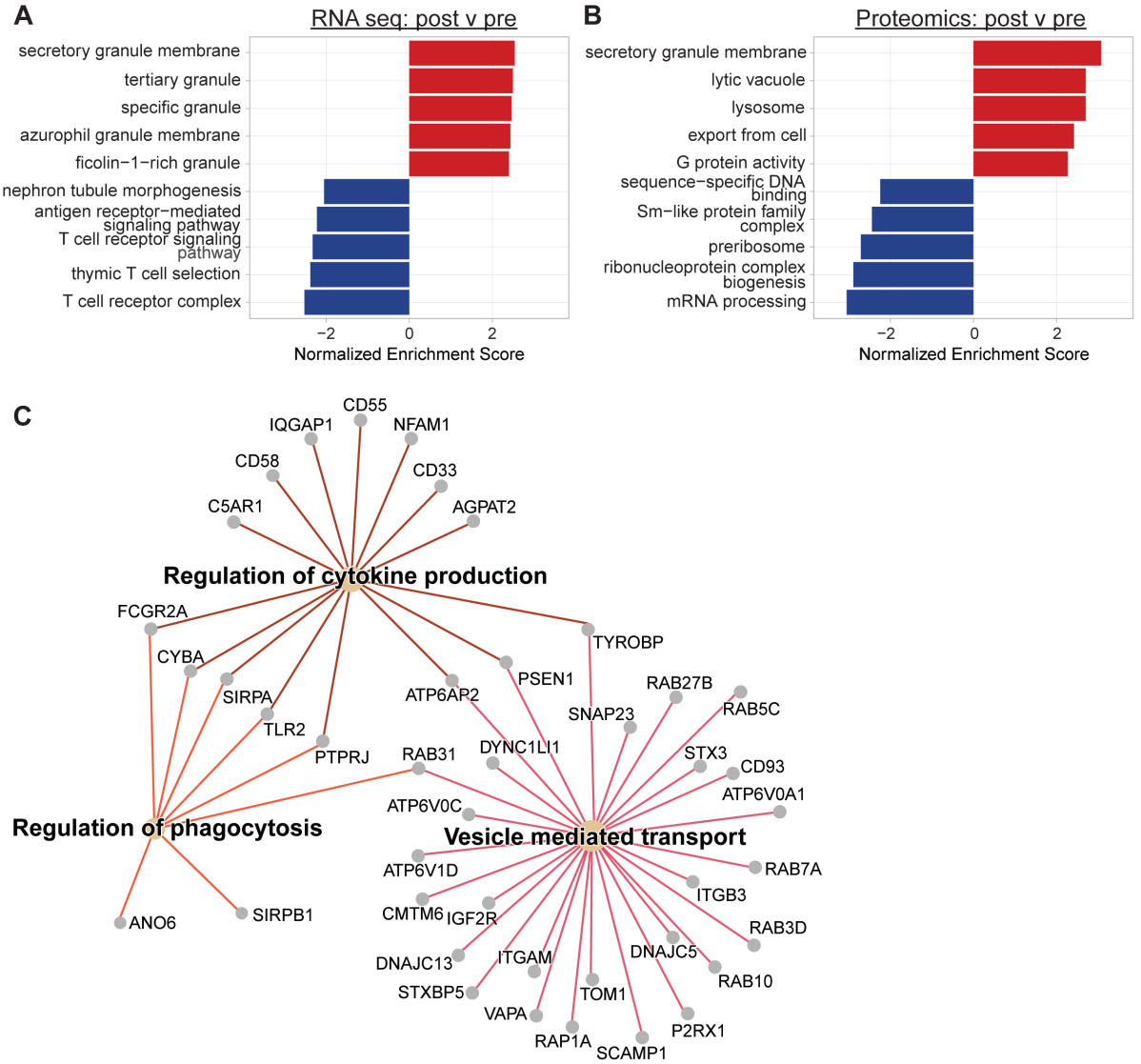


4.4.3.5 Common pathway changes seen at an RNA and protein level in IESS post versus pre prednisolone treatment

We wanted to identify the common pathways that demonstrated changes following prednisolone treatment at both an RNA and protein level. Figure 4.22 presents the top five upregulated and downregulated pathways using GSEA-GO terms for the RNA (Figure 4.22A) and proteomic (Figure 4.22B) datasets. The secretory granule membrane was the most significantly upregulated pathway, which was common to both RNA and proteome datasets. There were 122 genes enriching the secretory granule membrane RNA pathway, and 124 enriching the secretory granule membrane protein pathway, and 89 overlapping genes/proteins which were plotted in a CNET (Figure 4.22C); genes were subclustered by GO biological process (BP) terms into vesicle mediated transport (enriched by IGF2R, ITGAM, RAB genes [GTPases]), regulation of cytokine production (enriched by C5AR1, FCGR2A, TLR2) and regulation of phagocytosis (enriched by SIRPB1, SIRPA) pathways.

Figure 4.22 Significant upregulation of the secretory granule membrane pathway in infants with IESS post versus pre prednisolone treatment, observed in bulk RNA and proteomic datasets

(A)Bulk RNA analysis: Top five upregulated GSEA GO pathways (in red) in IESS post vs pre prednisolone included secretory granule membrane and other granule mediated immune response. Top five downregulated GSEA GO pathways (in blue) were predominantly in T cell signalling pathways. (B) Proteomic analysis: Top five upregulated GSEA GO pathways (in red) included secretory granule membrane and intracellular processes. Top five downregulated GSEA GO pathways (in blue) were related to ribosomal and translation processes (mRNA processing, ribonucleotide complex biogenesis). (C) Connectivity enrichment plot (CNET) of 89 genes upregulated in the "secretory granule membrane" pathway found at both an RNA and protein level. Genes were subclustered by GO biological process (BP) terms into vesicle mediated transport, regulation of cytokine production and regulation of phagocytosis pathways.



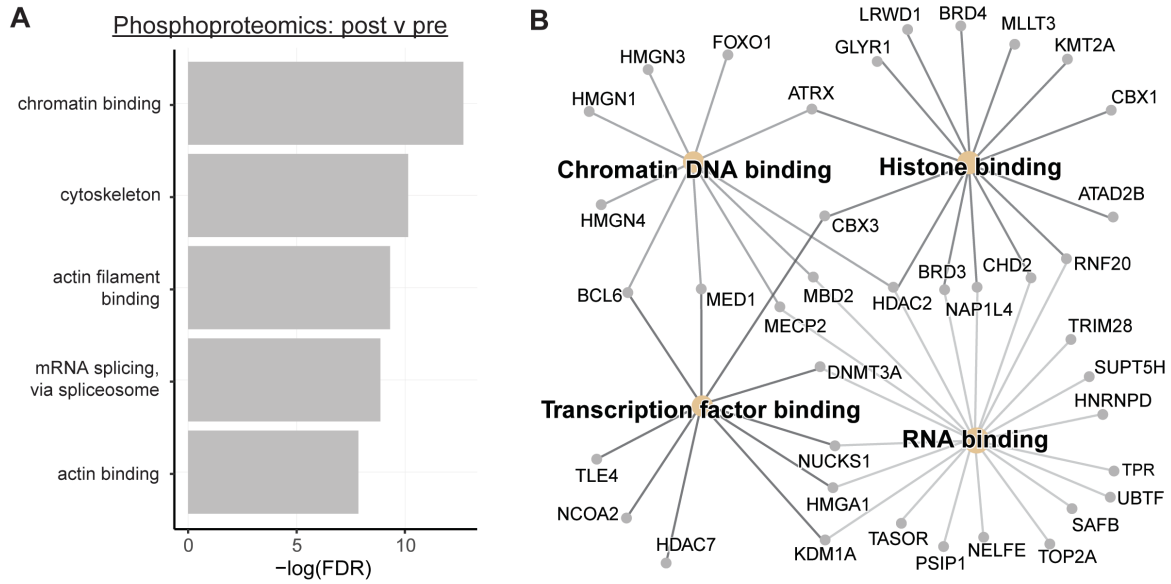
4.4.3.6 Phosphoproteomic analysis in IESS post versus pre prednisolone treatment

In the phosphoproteomic dataset IESS post vs pre prednisolone, there were 1247 differentially expressed phosphoproteins (FDR<0.05). Pathway directed analysis was performed using ORA-GO to identify dysregulated pathways. The top five dysregulated pathways included chromatin binding, cytoskeleton/actin and mRNA splicing (Figure 4.23A).

We focused on genes enriching the chromatin binding pathway that were common to both the IESS pre prednisolone vs control comparison (78 proteins) and IESS post vs pre prednisolone comparison (61 proteins). There were 53 overlapping proteins, which were represented in a CNET (Figure 6B), genes were subclustered by GO Molecular Function (MF) terms into RNA binding (enriched by HDAC and CHD proteins), histone binding (enriched by KMT and BRD proteins), chromatin DNA binding (enriched by HMGN and MECP proteins) and transcription factor binding (enriched by DNMT and HDAC proteins) subclusters.

Figure 4.23 Phosphoproteomic analysis in infants with IESS post versus pre prednisolone treatment

(A)Phosphoproteomic analysis: Top five Overrepresentation Analysis (ORA) GO pathways that were differentially expressed (in grey) in IESS post vs pre prednisolone included chromatin binding, cytoskeleton, actin binding and mRNA splicing. (B) Connectivity enrichment plot (CNET) of 53 genes enriching the "chromatin binding" pathway that were identified in both IESS post vs pre prednisolone and IESS pre prednisolone vs control comparisons. Genes were subclustered according to GO MF terms into RNA binding, histone binding, chromatin DNA binding and transcription factor binding pathways.



4.4.3.7 Summary

In summary, the multi-omics comparison between IESS post compared to pre prednisolone identified several changes to peripheral immune cells including a significant neutrophilia and lymphopenia. β-NGF significantly increased whilst other chemokines were reduced. A key finding identified by multi-omics pathway analysis was that prednisolone treatment in IESS upregulated immune pathways specifically the secretory granule membrane at an RNA and protein level. Genes enriching this pathway were involved in vesicle mediated transport, regulation of cytokine production and regulation of phagocytosis.

RNA pathways that significantly reversed following prednisolone compared to the baseline/control comparison included immune, ribosomal, membrane transport, cytoskeletal and mitochondrial processes. Pathways that reversed at a protein level were less evident yet also included cytoskeletal as well as chromatin pathways. Phosphoproteomic analysis demonstrated similar themes that prednisolone altered chromatin binding, cytoskeletal and mRNA pathways. From this analysis, it seems that steroids may alter gene expression in IESS via immune-mediated and ribosomal/mRNA and chromatin pathways.

4.5 Discussion

IESS is a severe NDD characterised by a complex and heterogenous range of aetiologies including known and unknown causes. (19, 21) My scoping review (Chapter two) highlighted several hypotheses for the pathogenesis of IESS and the responsiveness of this condition to steroids. One of these hypotheses is that IESS may arise from arise altered gene and epigenetic expression in “vulnerability” genes resulting in cellular dysfunction across broad regulatory, developmental and possibly immunological pathways. My multi-omics study in Chapter four lends some support to the hypothesis that infants with IESS have altered gene expression and immune pathways compared to controls. The key findings from RNA, proteome and phosphoproteome sequencing of immune cells from infants with IESS compared to controls included upregulation of ribosomal pathways and dysregulated

immune pathways, specifically downregulation of secretory granule membrane and upregulation of complement activation pathways.

One of the challenges exploring disease mechanisms in IESS and possible therapeutic actions of prednisolone has been the underlying heterogeneity of IESS, including varied aetiological causes encompassing single gene disorders, chromosomal abnormalities and structural insults, as well varied and distinct long-term outcomes. To date, studies have examined biomarkers in serum, CSF or imaging to explore disease mechanisms, some identifying differences between IESS of known and unknown aetiology and associated outcomes, however, most have been limited hypothesis driven analyses and have been unable to explain biological pathways or processes common to IESS, and how prednisolone may be acting. The advent of massively parallel sequencing and bioinformatics have provided a powerful computational tool capable of high-throughput sequencing of multiple "omics-" datasets to identify changes at a cellular, molecular and pathway level, providing detailed insights into biological mechanisms underlying heterogenous disorders such as IESS. Multi-omics is advantageous as it also highlights dynamic changes to gene expression, by sequencing RNA, protein and epigenetic markers, it allows us to understand alterations to transcription and translation and the epigenetic impact of possible stressors, such as infection or inflammation, of relevance to IESS given its established causal associations.

As part of my introduction into bioinformatics, I learnt key principles to consider when analysing multi-omics data. First, it is important to filter genes with low expression counts, as they are not informative. Secondly, it is important to normalise the filtered data to account for differences in the depth sequence or library size, to ensure data is comparable. Thirdly, there is biological variability between individual samples that may contribute to "noise" and confound omics interpretation. Normalisation and RUV are necessary steps to better discern biologically relevant differences. RUV is a statistical method to remove variation across the cohort, without removing genuine differences, and is very pertinent to human studies. Lastly, significant changes to gene expression can be explored by using adjusted p-values to correct for the FDR or proportion of type one errors which increases when performing multiple statistical comparisons in large multi-omics datasets.

By completing this study, I have highlighted several nuisances when handling large multi-omics datasets and that results may reflect the diversity of the dataset and analytical methods employed. For example, we analysed epigenetic regulation using phosphoproteomics, one of several protein post-translational modifications. However, it should be noted there are many types of protein modifications (such as histone modifications) which could be informative. Similarly, as bulk RNA sequencing encompasses all cell types as a homogenate, and neutrophil and lymphocyte proportions varied in the pre and post prednisolone samples, we did not want our post-prednisolone sample to reflect the dominant neutrophil cell type and therefore embarked on a deconvolution analysis to statistically adjust for this proportional change. As multi-omics is a relatively new technology, one of the challenges we faced was establishing an appropriate methodology for deconvolutional analyses, as various centres had adopted different approaches. (193-196) This lack of uniformity also extended to pathway-enrichment analysis as there is no one universally "preferred" or consistent method. In our study we performed GSEA-GO pathway directed analyses for RNA and proteomic sequencing, however, the phosphoproteomic dataset required a different approach and there was a 'ranking based' approach to phosphorylation of peptides is not as meaningful as ranking protein and RNA amounts, which are "quantifiable". The choices made at each step of the multi-omics analyses can focus on different aspects of the results, however as a research group we made these choices stringently using a pragmatic framework. In summary, the diversity of multi-omics data may add richness and depth to our understanding however, it also presents a challenge in the standardisation and reproducibility of results across studies.

This is the first study comparing RNA, protein and phosphoprotein sequencing in IESS to controls. One of our key findings was that ribosomal pathways were upregulated in IESS at baseline. Ribosomes are the key site responsible for translation, a complex process involving ribosomal RNA (rRNA), mRNA, ribosomal proteins and small nucleolar RNAs that translate DNA into functional proteins. (197) Previous studies comparing IESS to other-infantile epilepsies had similar findings that pathways enriched in IESS included regulatory pathways of the CNS as well as organelles including Golgi apparatus and endoplasmic reticulum, involved in protein sorting, post-translation modifications and ribosomal activity. (198) Our findings of upregulated ribosomal activity support the hypothesis of altered gene regulatory

mechanisms in IESS. Additionally, there is preliminary evidence that altered neuronal ribosomal activity may reduce the synthesis of proteins essential for dendritic and synaptic function, leading to altered neuronal connectivity in NDDs. (199-201) This may represent another possible aetiopathogenic mechanism in IESS.

A second key finding from my study was that infants with IESS were found to have dysregulated immune pathways, including downregulated secretory granule membrane pathways at an RNA level and upregulated complement activation pathways at a protein level. Subcluster analysis identified genes in the secretory granule membrane pathway regulate vesicle transport/trafficking, cytokine signalling, phagocytosis and cell adhesion. It is plausible that dysregulation of this pathway may contribute to seizure susceptibility through altered neurotransmitter release and impaired neuronal cell and synaptic signalling, seen in other synaptic-mediated epileptic encephalopathies. (202) Neuronal pathway and circuit formation may also be disrupted by impaired cell adhesion or impaired microglial function, the resident macrophage of the CNS. The role of microglia-mediated synaptic pruning is becoming increasingly recognised as a key contributor in NDDs. (203-206) The complement system is intricately linked to synaptic pruning, C1q tags synapses for elimination, through downstream C3, C4 activation synapses are engulfed by microglia, refining neuronal circuits required for normal brain development. (207, 208) Our study identified complementation activation in IESS at a pathway level, supporting proposals that complement overactivation may cause synaptic dysfunction or promote pro-inflammatory responses. (209) Our targeted ELISA neuroimmune panel testing did not confirm previous findings of elevated cytokines, IL-6 (210-212) and TNF-α (210, 213) in IESS. Neuroinflammation as evidenced by complement and cytokine activation may contribute to IESS onset through altered innate immune function and microglia mediated changes to neuronal networks. These findings support my hypothesis raised in Chapter two, that aetiological mechanisms in IESS such as immune dysregulation and altered gene expression may interact and co-exist.

The key findings from the multi-omics analysis examining prednisolone treatment in IESS was that prednisolone had a significant effect on gene and epigenetic regulation and immune function. Prednisolone reversed and downregulated ribosomal pathways and

upregulated immune, specifically secretory granule pathways. Phosphoproteomic sequencing confirmed prednisolone alters epigenetic regulation in IESS through changes in RNA, histone, chromatin DNA and transcription factor binding pathways.

As this study is the first multi-omics analyses evaluating the effects of prednisolone in infants with IESS, it was not possible to compare these results with other IESS studies. However, the findings of altered ribosomal/translation and transcriptional pathways lends support to the hypothesis that steroids may be exerting their action in IESS by modifying gene expression through indirect non-genomic mechanisms, described in Chapter two. There is evidence in NDDs that altered ribosome biogenesis and translational processes may contribute to the underlying disease-causing state. (199-201) There is additional evidence that similar immune-mediated therapies to steroids, including intravenous immunoglobulin act in Paediatric Acute-Onset Neuropsychiatric Syndrome (PANS), by reversing dysregulated ribosomal, epigenetic, and cell signaling pathways. (214) It may be that therapies, including steroids traditionally thought to act via anti-inflammatory actions, are in fact, exerting their action by altering gene transcription with effects on ribosomal activity being a manifestation of this change in RNA to protein translation. It is also probable that steroids act at a regulatory level rather than altering specific genes/proteins, and this is supported by our phosphoproteomic findings of chromatin dysregulation, highlighting the common theme of altered gene and epigenetic regulation. In our study, reversal of ribosomal pathways was not seen at a protein level although they were downregulated by steroids. It is known that changes in RNA expression do not correlate strongly to protein level changes, with low RNA-protein correlations (0.22) shown in previous studies. (215) This may be because protein synthesis and function are influenced by many factors including ribosomal density and occupancy as well as other epigenetic/post-translational modifications. (215, 216)

In this study, prednisolone altered the quantity of immune cells, causing a significant neutrophilia and lymphopenia. Although suppression of specific lymphocyte subsets was not identified, this was likely a reflection of a low sample size as suppression of T-cell pathways was seen at a gene level in RNA pathway analysis (but not B cells). After deconvolution, pathway analysis of both bulk RNA and protein sequencing identified that prednisolone significantly upregulated secretory granule membrane pathway including vesicle mediated

transport, regulation of cytokine production and regulation of phagocytosis. It is possible these pathways interact with one-another to alter neurotransmission and neuronal networks to achieve epileptic spasms cessation. The role of inflammation in epilepsy is well described (217) particularly cytokine mediated neuronal excitability through altered GABA/glutamate pathways. (218) Brain samples from individuals with drug-resistant epilepsy have demonstrated strong activation of cytokines in the innate immune cells of the brain, including microglia, and that adaptive immune cells were reduced or absent. (219, 220) In IESS, PET scans demonstrated evidence of microglial activation which ACTH reduced or normalised. (221) Our study identified altered phagocytosis in IESS at baseline and post prednisolone treatment, supporting the concept of microglia-mediated synaptic pruning within the broader context of NDDs. It provides plausible evidence that steroids regulate CNS inflammation, neurotransmission and neuronal networks in IESS. From the targeted ELISA neuroimmune panel, we identified that prednisolone significantly increased serum β-Nerve growth factor (β-NGF). No studies have reported pre-post treatment serum β-NGF levels in IESS and those examining CSF β-NGF have lacked statistical comparisons. (222) One study found ACTH response correlated to higher CSF β-NGF levels at baseline. (222, 223) β-NGF may regulate neuronal cell survival and growth and lends some supports to the hypothesis that steroids act in IESS by altering metabolic and neuronal pathways. The ELISA neuroimmune panel found no other significant post treatment changes.

In interpreting these findings, one the key limitations to consider is the low sample size and heterogeneity within the IESS population. Our cohort predominantly included IESS cases with an unknown aetiology followed by structural causes due to stroke, HIE and TSC. Other single gene and chromosomal disorders, metabolic, infectious and immune aetiologies were not represented. Therefore, our findings may only reflect and be applicable to these two aetiological sub-groups. A sub-analysis comparing IESS of known and unknown aetiology was not within the scope of this study, nor was a repeat multi-omics analyses for participants with epileptic spasm recurrence although both may provide important biological insights into disease and therapeutic mechanisms. Repeat omics analyses was considered at the 12-month follow up period to investigate whether the effects of prednisolone were transient, returning participants to their pre-treatment state or if prednisolone normalised gene expression back to control state. The latter seemed unlikely,

given prednisolone and other treatments including vigabatrin induce remission from epileptic spasms yet do not alter the natural history of the disorder including neurodevelopmental outcomes and the risk of future epilepsy. Given our preliminary findings suggest prednisolone alters gene expression and immune function, alternative treatments that also modify these processes, such as the ketogenic diet could be considered as ongoing therapy to optimise future outcomes. For our study, a repeat omics analysis was not feasible, given our ethical approval and initial consent specified that research bloods would be "piggybacked" onto routine clinical tests, which were rarely performed at 12 months follow-up. Future studies longitudinally exploring the disease course are recommended.

A relative limitation of our study was that the complete battery of multi-omics sequencing was not possible for every participant as the blood draw volume was a major limiting factor however, five participants had both RNA and protein sequencing performed. The blood draw volume also limited our ability to perform methylation studies in addition to phosphoproteomic sequencing to evaluate epigenetic changes. There are multiple epigenetic modifications possible to DNA (methylation), chromatin (histone modifications), RNA (microRNA alterations) and proteins, and this study evaluated one type of post-translational protein modification. Similarly, a metabolomic analyses could have been included to investigate kynurenine-pathway metabolites however, the author's chose to focus on the immune/inflammatory hypothesis.

Another limitation of this study was the use of peripheral immune cells for the multi-omics analysis. The neutrophilia noted after prednisolone use may have skewed the bulk RNA sequencing data to reflect more neutrophil-driven functions. Given advances in single cell sequencing including HIVE™ technology which captures neutrophils, unlike PBMC based samples, these methods may better discern pathways of interest from specific cells in future studies. Additionally, it is possible that changes to peripheral immune cells may not reflect changes in the CSF or brain. However, one of the challenges is ethically sourcing these tissue types and getting sequential samples. Previous studies have estimated that 35-80% of transcripts are present in both blood and brain tissue, with the most highly expressed transcripts more likely to be found in both tissue types. (224) This infers, acknowledging

limitations, that immune dysregulation at both an RNA and protein level noted in this study in peripheral immune cells, would reflect a similar signature in CSF or brain tissue.

4.6 Summary

In summary, the key findings from the multi-omics investigation of infants with IESS identified at baseline there is upregulation of ribosomal pathways and dysregulated immune pathways, specifically downregulation of secretory granule membrane and upregulation of complement activation pathways. Prednisolone had a major effect on immune cell types which necessitated a deconvolution analysis in the bulk RNA sequencing dataset to adjust for the significant neutrophilia seen. Prednisolone treatment in IESS reversed and downregulated ribosomal pathways at an RNA level, upregulated immune-secretory granule pathways at both an RNA and protein level and altered chromatin pathways. These results support the hypothesis that IESS is a NDD with altered gene and epigenetic regulation at baseline and dysregulated immune function affecting neurotransmission and neuronal networks. Prednisolone may be exerting its effect by modifying gene expression through indirect non-genomic mechanisms as well as the widely accepted immune-modulating properties. Targeted therapies altering gene and epigenetic expression and immune modifying treatments require further investigation to optimise outcomes in IESS.

CHAPTER 5 – Conclusion

IESS is a complex and severe NDD associated with significant long-term disability including epilepsy, intellectual impairment and neurodivergence including ASD. These major co-morbidities pose a significant burden to the individual, families, and wider healthcare system. Although IESS is the most common epilepsy syndrome in infants and has been the focus of research for the past 180 years, the mechanisms underlying this disorder remain unclear, particularly the biological determinants associated with poor prognosis. ACTH, prednisolone and vigabatrin are the current evidence-based treatments proven to effectively stop epileptic spasms however landmark RCTs have demonstrated that long-term epilepsy and neurodevelopmental outcomes do not differ between treatment regimens. This implies that our current standard of care, despite inducing epileptic spams remission, is not altering or improving the disease trajectory. As part of this thesis, I achieved my key aims of investigating the biological processes contributing to IESS and those modified by treatment. Future replication studies may confirm our findings of altered gene regulation and dysregulated immune pathways. By improving our understanding of disease mechanisms we can reduce the burden of disease in IESS through new disease-modifying treatments and personalised precision-based medicine.

5.1 Summary of findings

In Chapter one I introduced key historical advancements in our understanding of IESS. The complex and heterogenous nature of this disorder was emphasised, including the wide range of known and unknown aetiologies and varied clinical presentations, encompassing different epileptic spasm types, various EEG abnormalities and a spectrum of developmental impairment. The 2022 ILAE definition of IESS captured this heterogeneity (2) which has posed a major challenge to our understanding of underlying disease and therapeutic mechanisms. I summarised findings from the landmark UKISS and ICISS trials. UKISS demonstrated that prednisolone effectively achieves epileptic spasms cessation and was comparable to ACTH yet superior to vigabatrin. (18) ICISS demonstrated that combining ACTH or prednisolone with vigabatrin was more effective than ACTH or prednisolone alone,

achieving higher rates and earlier cessation of epileptic spasms. (14) Both studies demonstrated that treatment response was independent of aetiology, confirming that despite the heterogeneity in IESS, prednisolone exerts an anti-seizure effect across the broader patient population.

Findings from follow-up and ancillary studies highlighted that although first line treatments controlled epileptic spasms, long-term epilepsy and developmental outcomes did not differ between treatment groups and response to treatment remained independent of aetiology. (17, 77, 85, 86) Treatment paradoxes emerged as development was more favourable in infants with unknown-IESS aetiology treated with ACTH or prednisolone (compared to vigabatrin) and long-term epilepsy and development outcomes were improved by shorter lead times to IESS diagnosis and faster clinical responses. (17, 77, 85, 86) This suggested that first-line treatments including prednisolone may transiently improve symptoms in IESS, but do not appear to alter underlying disease mechanisms or the course of this NDD. Reasons for this remain unclear and were explored in my literature review.

In chapter two, my published scoping review was a key achievement. I synthesised the evidence in infants examining biological mechanisms in IESS and how they may be linked to the therapeutic effects of prednisolone. Five key hypotheses were presented that gene regulation, stress, neuroinflammation, altered neuronal networks and metabolic pathways interact and influence neurodevelopmental processes more broadly through altered gene expression. We hypothesised that expression of vulnerability genes or “regulatory” genes due to stress, infection or inflammation may alter biological processes across these five key pathways at a critical timepoint in development, resulting in IESS. The complexity of this interaction may explain the heterogeneity seen in IESS. We proposed that steroids may act at a regulatory level altering gene expression. The evidence exploring gene regulation in IESS was limited and represented a significant gap in our understanding of IESS. To explore our hypotheses and address this gap, we completed a multi-omics analysis to investigate disease and treatment mechanisms of prednisolone in IESS, presented in chapter four.

To perform the multi-omics analysis I conducted a prospective clinical cohort study, presented in chapter three. I recruited 11 infants from two tertiary paediatric hospitals in

NSW, Australia. I completed extensive phenotyping of the IESS cohort at baseline using screeners developed by our research group to identify any genetic, immune, infectious or environmental risk factors including stress that may influence gene expression in IESS. The pregnancy history unexpectedly identified that most women experienced pregnancy complications and periods of increased or severe stress. A limitation was that there was no control comparison available. Future studies could explore maternal-child dyads to determine whether genetic or environmental factors during pregnancy increase vulnerability to IESS as is recognised in other NDDs. (127, 163-166)

The key findings from the prospective clinical cohort study were that all infants responded to prednisolone treatment regardless of aetiology yet at 12 months follow-up, infants with an unknown IESS aetiology had lower rates of epilepsy and developmental delay. Similar to larger cohort studies, most infants with IESS in my cohort had unknown aetiology (55%) followed by a structural diagnosis (45%) including one infant with TSC2 (structural-genetic aetiology). Unknown aetiology was confirmed following brain imaging, CSF and genetic studies, including a microarray and EE panel. A limitation of this study was its observational nature and that further exome/genome sequencing was not performed.

At 12 months follow-up, all six infants with unknown-IESS remained seizure free and the ASQ-3 identified that developmental scores improved beyond baseline for all but one infant. While the ASQ-3 proved useful, formal developmental assessments could have strengthened these results. Overall, the results aligned with earlier studies indicating that individuals without epilepsy have better developmental outcomes. (17, 77, 85, 86) Conversely, five infants with structural-IESS in my cohort had ongoing epilepsy, one considered drug-resistant and most had co-morbid severe GDD. ASQ-3 scores identified the trend for developmental skills to deviate further away from age-appropriate norms over time. My observations aligned with UKISS and ICISS findings; that prednisolone demonstrates short-term efficacy achieving cessation of epileptic spasms, however no treatment improves long-term epilepsy or developmental outcomes. (17, 77, 85, 86) Rather, IESS evolves into a longer-term NDD. To improve outcomes, it is necessary to understand the biological mechanisms leading to IESS and how our current accepted treatments are exerting their effect, albeit in the short-term.

In chapter four, a key achievement was conducting the first multi-omics analysis in IESS comparing gene expression and immune profiles to controls, in addition to pre- and post-prednisolone treatment comparisons. We analysed mRNA gene expression, protein abundance and protein phosphorylation in immune cells to determine dynamic changes to gene/protein expression and common biological pathways enriched by these differentially expressed genes/proteins. A targeted ELISA analysis of serum growth factors, chemokines and cytokines revealed some differences in IESS at baseline (higher BDNF) and following prednisolone treatment (lower β-NGF, unchanged BDNF).

Pathway-directed analysis supported our hypothesis that immune dysregulation is evident in IESS. Dysregulated immune pathways included downregulated secretory granule membrane pathways identified at an RNA level and upregulated complement activation pathways at a protein level. Genes enriching the secretory granule membrane pathway were involved in vesicle transport/trafficking, cytokine signalling, phagocytosis and cell adhesion. As described in other psychiatric and NDDs, (225-229) it is plausible that stress or inflammation during a critical developmental period may disrupt microglial-mediated synaptic pruning and alter neuronal connectivity as a possible aetiopathogenic mechanism in IESS. Microglial-mediated synaptic pruning is a key developmental process, (205, 230, 231) regulated by complement proteins that tag synapses for elimination. (204, 208) In schizophrenia, complement activation has been implicated as a disease mechanism causing excessive synaptic pruning and synapse loss. (228) Animal models have also demonstrated that maternal stress contributes to altered behavioural states in offspring, by altering microglial function (232) or architecture. (233) Maternal stress also causes migration of peripheral blood monocytes to the brain and a dynamic “cross-talk” that activates microglia. (233) This evidence supports a possible complex interaction between stress and neuroinflammation in IESS.

Results from transcriptomic and phosphoproteomic sequencing supported our hypothesis that altered gene, and epigenetic regulation may be another aetiological mechanism underlying IESS. At baseline ribosomal pathways were upregulated at an RNA level, and phosphoproteomic sequencing identified dysregulated chromatin binding, actin binding and protein kinase activity. This suggests that epigenetic modifications may

contribute to altered gene expression at a regulatory level in IESS. There is also literature describing a complex interplay between ribosomal biogenesis, stress and brain development. (199-201) During periods of cellular stress, ribosomes regulate translational processes and immune responses to maintain proteostasis. However, under stress neuronal ribosomal activity reduces the synthesis of proteins essential for dendritic and synaptic function, leading to altered neuronal connectivity. (199-201) Future studies exploring ribosomal profiling and epitranscriptomic signatures may further discern the impact of stress or environmental triggers on gene expression in IESS. Examination of maternal-child dyads may assess the heritability of such epigenetic modifications and/or susceptibility factors such as maternal auto-immunity or "in-utero" stress. (234)

The multi-omics analysis examining prednisolone treatment in IESS identified that prednisolone had a significant effect on gene regulation and immune function. Prednisolone caused a significant neutrophilia, necessitating a deconvolution analysis for bulk RNA sequencing, so as not to skew our results with "neutrophil" driven pathways. Single-cell RNA sequencing would have provided a clearer cell-to-cell transcriptomic comparison, however this was not feasible in my study. In IESS, prednisolone treatment reversed and upregulated immune pathways including secretory granule membrane, pattern recognition receptor activity and cellular metabolism, at both an RNA and protein level. Prednisolone reversed and downregulated ribosomal and mitochondrial translation pathways at an RNA level and phosphoproteomic sequencing further identified dysregulated chromatin/actin pathways. These preliminary findings in my small cohort of infants with IESS suggests that steroids may alter gene expression in IESS via immune-mediated and ribosomal/mRNA and chromatin pathways.

5.2 Limitations

Limitations of my clinic cohort study and multi-omics evaluation were discussed in detail in chapters three and four respectively. To summarise, clinical phenotyping of the IESS cohort, particularly the genomic evaluation, was limited as there was no standardised investigation protocol as part of Neuro-Tx. I was also an unblinded interviewer, however

aspects of clinical phenotyping were strengthened by using standardised data collection tools including NeuroCONNECT and ASQ-3. There was a preliminary exploration of the influence of maternal stress and environment factors during pregnancy in IESS however, comparable data was not available for the control cohort.

The multi-omics analysis was limited by a low sample size and that most, but not all participants had the complete battery of bulk RNA, proteomic and phosphoproteomic sequencing performed. Participants also had both known (structural or structural-genetic) and unknown IESS aetiologies. We acknowledge the heterogeneity of our sampling based on these factors and that results may only reflect these two aetiological groups. However, my aim was specifically examining IESS as a heterogenous cohort to understand the "common" disease and therapeutic mechanisms, before exploring sub-group analyses. As such, our cohort should be considered exploratory, rather than definitive. Future replication studies involving larger prospective cohorts are required to corroborate our results and explore hypotheses further.

A limitation of our multi-omics analyses examining prednisolone treatment in IESS was that prednisolone had a major effect on peripheral immune cells which may have skewed the whole blood bulk RNA sequencing data to reflect more neutrophil-driven functions. A deconvolution analyses was undertaken, however may not completely compensate for this effect. Similarly, proteomic and phosphoproteomic sequencing was performed on neutrophil devoid PBMCs, which again may have influenced the findings. Despite these factors, our findings were quite consistent, indicating ribosomal, immune and chromatin pathways are altered in IESS. Relative confounders included the short-term use of nitrazepam before prednisolone in a minority of patients, which was not expected to significantly alter our findings. In my analysis, blood was the most accessible tissue type to sequence compared to CSF or brain tissue, and it is acknowledged that peripheral processes may not accurately reflect those within the CNS. However, previous studies have reported moderate correlation between blood and brain tissue, particularly for highly expressed transcripts and highly abundant proteins. (224, 235) Repeat omics sequencing was considered at the 12-month follow-up visit, yet given ethical and logistical constraints, this was not performed. We suspected that this comparison would confirm the transient effects

of prednisolone, although we could not predict whether the omics' signature would return to the pre-existing baseline, or a new baseline.

5.3 Future research directions

My preliminary findings were that infants with IESS have altered gene expression including dysregulated ribosomal, immune and chromatin pathways compared to healthy controls. Prednisolone may alter gene expression through altered ribosomal biogenesis, epigenetic modifications or by directly activating or repressing transcription and translation-dependent processes. The latter mechanism is plausible given 10-20% of the genome is directly regulated by glucocorticoids binding to DNA whilst many other genes are indirectly regulated by glucocorticoids and glucocorticoid receptor interactions. (236) My findings suggested that immune-mediated pathways may also influence gene expression or possibly alter neuronal cell signalling and pathways. Future replication studies involving larger cohorts are required.

Further questions regarding disease and therapeutic mechanisms to be explored include:

- Are disease mechanisms including altered gene regulation and immune pathways shared across all IESS aetiologies? Or do they differ between unknown, and known genetic, structural, metabolic, infectious and immune aetiologies?
- What are the risk factors, if any, that contribute to altered gene expression and onset of IESS? Are regulatory genes the "vulnerability genes" that may be expressed in IESS? How does pre, peri or post-natal stress or inflammation contribute to altered gene expression?
- Similarly, in conditions such as HIE or neonatal stroke that may predispose to IESS, can we identify reliable biomarkers in either blood or CSF to predict IESS onset?
- Can we identify reliable biomarkers, either in blood or CSF, that may predict treatment response?

- What mechanisms might explain why some infants are “non-responders” to first-line treatment? Are these pathways also involved in epileptic spasm recurrence after initial seizure freedom?
- If prednisolone exerts its therapeutic effect by altering gene expression and inflammatory pathways, does vigabatrin, a GABA-ergic mediated medication, also exert a similar effect? Could other pathways affecting neurotransmission and neuronal networks more accurately explain how both treatments work?
- If prednisolone affects gene regulation and epigenetics, could interventions with similar mechanisms, such as the ketogenic diet or valproate, be used in combination with or after first-line treatment? Do these interventions influence the same pathways as prednisolone in IESS?
- Which disease-modifying therapies targeting immune dysregulation or ribosomal function could be used or developed?

These questions would be best addressed through prospective longitudinal cohort studies examining multiple treatment regimens (ACTH, prednisolone, vigabatrin, ketogenic diet etc) with stratified sub-analyses investigating aetiological subgroups, treatment responders versus non-responders, and infants with poor outcomes. A multi-centre collaboration would facilitate enrolment of large cohorts to adequately power these analyses.

Future multi-omics comparisons should include appropriate controls and standardised genomic profiling by whole exome or genome sequencing. In addition to whole blood bulk RNA sequencing, single cell RNA sequencing utilising HIVE™ technology would identify neutrophil driven pathways and determine whether immune activation was driven by specific cell types or occurs more broadly. For this reason, future proteomic analyses should also include neutrophils using single cell techniques or platelet-depleted buffy coat proteomics.

The heritability of epigenetic modifications compared to the environmental influence of pre, peri and post-natal stressors should be explored in comparison to

appropriate controls. This would involve genomic, transcriptomics, phosphoproteomics, ribosomal profiling to assess actively translated mRNAs, chromatin-focused assays (ATAC-seq, ChIP-seq) exploring chromatin accessibility and histone modifications or other RNA/mRNA epitranscriptomic signatures. Examining maternal-child dyads and the influence of stressors in the pre and perinatal periods is more complex and may require maternal blood sampling during pregnancy and neonatal sampling at delivery using cord blood samples. Examining post-natal gene-environment interactions for infants at risk of IESS, would require neonatal blood sampling, repeat sampling at IESS onset and/or a comparison to a "positive" control group, that do not develop IESS. This poses several challenges, including weight-based restrictions which limit blood volume drawn and subsequent omics sequencing. Alternative samples such as fibroblasts could be used yet sampling for research purposes would require ethical and carer approval. Rather than investigating biological pathways in infants "at risk" of IESS, a more feasible approach would be identifying infants with IESS at "high risk" of poor outcomes. This would involve longitudinal sampling at key time points pre and post treatment, at recurrence of epileptic spasms and between 18-24 months of age when epilepsy and neurodevelopment outcomes are more established. This approach would provide more informative biological insights into disease prognosis and may facilitate timely adjustments to clinical management.

The relationship between neuroinflammation and neurotransmission warrants further investigation as it may reveal opportunities to provide practical currently available treatments. "Phenomic" profiling may provide further insights by using PET scans to examine microglial activation and/or functional MRI with EEG to study "default" neuronal networks. Blood and CSF samples could be metabolically profiled using ELISA assays or LC-MS quantification to identify biomarkers of neuro-inflammation including complement activation as evidenced in our study. This may prompt consideration of adjunct immune modifying treatment such as complement inhibitors or recombinant biologicals (tocilizumab etc) or re-purposing ASM and mood stabilisers such as valproate known to have anti-inflammatory actions. (237)

Further metabolomic exploration of biomarkers is warranted including targeted analyses of kynurenine pathway disturbances. Reduced CSF kynurenic acid has been

associated with response to prednisolone treatment in IESS (238) however as demonstrated in my cohort study, CSF is not always collected and there is a need for reliable surrogate markers in accessible tissue types such as serum or urine. Biomarker identification may enable the development of personalised prevention and treatment strategies to optimise patient outcomes. As 30-40% of infants in IESS do not respond to ACTH or prednisolone therapy, (90, 239) early identification of non-responders may prompt initiation of alternative and targeted treatments to improve outcomes.

The ketogenic diet is an alternative therapy that has proven efficacy in IESS (240, 241) that may be altering gene expression or exerting epigenetic effects. Ketone bodies are known to function as histone deacetylase inhibitors, thereby modifying chromatin structure and influencing gene expression. (242, 243) Animal studies in IESS have identified that adjuncts to the ketogenic diet including minocycline, and an enzyme inhibitor of the kynurenine pathway, alter gut microbiota and kynurenic acid concentrations. (244) Highlighting a possible role for probiotics and/or antibiotics with anti-inflammatory actions as adjunct targeted treatments. Other epigenetic therapies including histone or RNA modifiers such as butyrate (243, 245) and sodium valproate (246, 247) could also be explored although it may be difficult to examine them in IESS in isolation. Alternative options would be an in vitro exploration of these drug effects on gene regulation using various animal and human cell lines or possibly brain organoids. Although these results may not accurately reflect IESS as a complex NDD.

5.4 Clinical implications and summary

In summary, the current evidence suggests that the first-line treatments in IESS are not altering or improving long-term neurodevelopmental outcomes. There is an urgent, unmet need to investigate disease mechanisms and disease modifying treatments in IESS to improve long-term outcomes. Our study identified dysregulated ribosomal, immune and chromatin pathways which has advanced our understanding of disease and therapeutic mechanisms in IESS. Further work is needed to discern the complex gene and environment interactions underlying this NDD, and potential targets for disease-modifying therapeutics. This could include a biological analysis of treatments such as the ketogenic diet or sodium valproate known to alter gene regulation and epigenetics. Clinicians may also consider these as adjunctive treatments in IESS. Reducing the lead time to IESS diagnosis and treatment is another urgent priority, as it is a clearly established modifiable risk factor. Clinicians should be educating general practitioners, allied health and other care providers regarding recognition and early presentation for suspected IESS to improve long-term outcomes.

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APPENDICES

Appendix 1. NeuroCONNECT

NeuroCONNECT

Record ID

_____

Record ID: [record_id]
NeuroCONNECT Participant ID: [part_id]
DOB: [dob]

Please enter today's date

_____

CLINICIAN INFORMATION

Primary clinician

_____
(Firstname_SURNAME)

Recruiting site

- ◯ CHW Neurology
- ◯ CHW Genetics
- ◯ SCH Neurology
- ◯ SCH Genetics
- ◯ POW Neurology
- ◯ POW Genetics
- ◯ Westmead Neurology
- ◯ Westmead Genetics
- ◯ Other Neurology
- ◯ Other Genetics
- ◯ Other CHW department

Other recruiting site

_____

PARTICIPANT INFORMATION NeuroCONNECT Participant ID - Last 4 digits of MRN + initials (1234XY) _____
Firstname _____ Lastname _____ Full MRN _____
Date of birth _____ Current age _____ Postcode _____
Gender _____ _____

Is the participant deceased?

- ◯ Yes
- ◯ No

Date of death

_____

Age at death (in years)

_____

PRIMARY CONTACT DETAILS Contact name _____ Relationship to participant _____ Mobile phone no. _____ Home phone no. _____ Email _____ Comments regarding contact details _____

CLINICAL PHENOTYPING - NEUROLOGY

Disorder group
Select all disorders that apply

- ☐ Epilepsy or Seizures
- ☐ Movement disorders
- ☐ Neurodevelopmental disorders / Intellectual disabilities
- ☐ Cerebral palsy
- ☐ CNS Infection / Inflammatory / Autoimmune disorders
- ☐ Psychiatric disorders
- ☐ Nerve conditions
- ☐ Muscle and neuromuscular junction disorders
- ☐ Headache
- ☐ Neurosurgery
- ☐ Other acute neurology and stroke
- ☐ Neurofibromatosis Type 1&2 / Schwannomatosis or Legius Syndrome

Involvement of other organs or diseases

- ☐ Microcephaly
- ☐ Macrocephaly
- ☐ Visual impairment
- ☐ Hearing impairment
- ☐ Short stature / growth delay
- ☐ Dysmorphology
- ☐ Cardiac disease
- ☐ Bone / skeleton disease
- ☐ Genito-urinary disorder
- ☐ Gastrointestinal disorder
- ☐ Kidney disease
- ☐ Immune deficiency
- ☐ Autoimmune disease
- ☐ Atopic / allergic diseases

Additional details regarding the involvement of other ograns

_____

EPILEPSY or SEIZURES

Does this child have febrile convulsions ONLY and NOT epilepsy?

◯ Yes ◯ No

Age of onset of epilepsy symptoms
(in years and months e.g. 3 years 6 months)

_____

Select the seizure types

- ☐ Generalised motor
- ☐ Generalised non-motor
- ☐ Focal
- ☐ Unknown
- ☐ Epileptic spasms

Generalised motor

Select applicable seizure types and frequency (e.g. 2 per day, 3 per week)

Leave those that do not apply blank

Seizure type	Number of seizures	Frequency of seizures	Seizure triggers at any time
Tonic-clonic	_____	_____	_____
Clonic	_____	_____	_____
Tonic	_____	_____	_____
Atonic	_____	_____	_____
Myoclonic	_____	_____	_____
Myoclonic-tonic-clonic	_____	_____	_____

Myoclonic-atonic _____ _____ _____

Generalised non-motor

Select applicable seizure types and frequency (e.g. 2 per day, 3 per week)

Leave those that do not apply blank

Seizure type	Number of seizures	Frequency of seizures	Seizure triggers at any time
Typical absence	_____	_____	_____
Atypical absence	_____	_____	_____
Myoclonic absence	_____	_____	_____
Absence with eye lid myoclonia	_____	_____	_____

Focal

Select applicable seizure types and frequency (e.g. 2 per day, 3 per week)

Leave those that do not apply blank

Seizure type	Number of seizures	Frequency of seizures	Seizure triggers at any time
Focal motor	_____	_____	_____
Focal non-motor	_____	_____	_____
Focal with impaired awareness	_____	_____	_____
Focal to bilateral tonic-clonic	_____	_____	_____

Unknown

Select applicable seizure types and frequency (e.g. 2 per day, 3 per week)

Leave those that do not apply blank

Seizure type	Number of seizures	Frequency of seizures	Seizure triggers at any time
Motor	_____	_____	_____
Non-motor	_____	_____	_____

Describe 'other' seizure triggers if applicable
Add notes regarding triggers if applicable

_____

	Number of spasms per cluster	Number of clusters per day	Spasm triggers at any time
Epileptic spasms			

Select frequency

_____ _____ _____

At the worst stage in the epilepsy illness, what proportion of all seizures were triggered by fever/infection or vaccine?

◯ None ◯ Occasional (50%)
◯ Majority (all)

At the current stage in the epilepsy illness, what proportion of all seizures were triggered by fever/infection or vaccine?

◯ None ◯ Occasional (50%)
◯ Majority (all)

No Yes Not known

History of vaccine proximate seizures? (seizures within 48 hours of vaccination)	◯	◯	◯
Any episode of status epilepticus at any stage?	◯	◯	◯
Any episode of febrile status epilepticus at any stage?	◯	◯	◯
Any episode of hemiclonic status epilepticus at any stage?	◯	◯	◯

Admission to hospital with seizures at any time ◯ Yes ◯ No

Admission to ICU with seizures at any time ◯ Yes ◯ No

Type of epilepsy (select one)

◯ Focal Epilepsy
◯ Combined Generalized and Focal Epilepsy
◯ Generalized Epilepsy
◯ Epileptic Encephalopathy
◯ Unknown

Epilepsy syndrome currently (ILAE classification 2017) (select one as appropriate)

Neonatal/infantile

_____

Childhood

_____

Adolescent

_____

Others

_____

If other epilepsy syndrome, describe here

_____

ILAE drug responsive?

◯ Drug responsive
◯ Drug resistant (ongoing seizures despite two well-chosen anti-eplieptics

Medications - current and previous

Tick all that apply

	Current	Previous (at any time)
Cannabidiol	☐	☐
Carbamazepine	☐	☐
Clobazam	☐	☐
Clonazepam	☐	☐
Ethosuximide	☐	☐
Felbamate	☐	☐
Gabapentin	☐	☐
Lacosamide	☐	☐
Lamotrigine	☐	☐
Levetiracetam	☐	☐
Lorazepam	☐	☐
Midazolam	☐	☐
Nitrazepam	☐	☐
Oxcarbazepine	☐	☐
Perampanel	☐	☐
Phenobarbital	☐	☐
Phenytoin	☐	☐
Prednisolone	☐	☐
Pregabalin	☐	☐
Stiripentol	☐	☐
Topiramate	☐	☐
Valproic acid	☐	☐
Vigabatrin	☐	☐
Zonisamide	☐	☐
Others	☐	☐

Specify other medications

_____

Does this patient fulfil responder criteria? (more than 50% reduction in seizures compared to baseline)? ◯ Yes ◯ No

Enter percentage reduction in total seizures compared to baseline

_____

Other phenotypic comments

_____

MOVEMENT DISORDER

Age of onset of movement symptoms
(in years and months e.g. 3 years 6 months) _____

Movement disorder type
- ☐ Dystonia
- ☐ Chorea
- ☐ Athetosis
- ☐ Ataxia
- ☐ Myoclonus
- ☐ Tics
- ☐ Tremor
- ☐ Akinesia
- ☐ Ballismus
- ☐ Stereotypies
- ☐ Oculogyric crisis
- ☐ Spastic paraparesis
- ☐ Paroxysmal dyskinesias
- ☐ Others

(can tick more than one)

Tone
- ☐ Hypotonia
- ☐ Spasticity
- ☐ Rigidity

(can tick more than one)

Movement disorder details
- ☐ Generalized
- ☐ Face
- ☐ Neck
- ☐ Mouth / Tongue
- ☐ Upper limbs
- ☐ Lower limbs
- ☐ Hemi

Movement disorder pattern
- ☐ Paroxysmal / Episodic only
- ☐ Continuous - Awake - Settled in sleep
- ☐ Continuous - Awake and sleep - Worse awake
- ☐ Continuous - Awake and sleep - Worse asleep
- ☐ Continuous with episodic exacerbations

Eye movement disorder
- ☐ Nystagmus horizontal
- ☐ Nystagmus vertical
- ☐ Square wave jerks
- ☐ Vertical gaze palsy
- ☐ Strabismus

Medications - current and previous

Tick all that apply

	Current	Previous (at any time)
Carbamazepine	☐	☐
Clonazepam	☐	☐
Clonidine	☐	☐

Diazepam	☐	☐
Levetiracetam	☐	☐
Levodopa	☐	☐
Tetrabenazine	☐	☐
Trihexyphenidyl	☐	☐
Topiramate	☐	☐
Valproic acid	☐	☐
Risperidone	☐	☐
Aripiprazole	☐	☐
Others	☐	☐

Specify other medications

_____

Other phenotypic comments

_____

NEURODEVELOPMENTAL DISORDER/INTELLECTUAL DISABILITIES

Age of onset of neurodevelopmental symptoms
(in years and months e.g. 3 years 6 months)

_____

Neurodevelopmental disorder/Intellectual disabilities

- ☐ Intellectual Disability (Intellectual Developmental Disorder)
- ☐ Specific learning disorder
- ☐ Global Developmental Delay
- ☐ Language disorder
- ☐ ASD
- ☐ Autistic regression
- ☐ Neurodevelopmental regression
- ☐ ADHD
- ☐ Tourette's disorder
- ☐ Persistent (Chronic) motor or vocal tic disorder
- ☐ Transient tic disorder
- ☐ Dyspraxia (motor coordination disorder)
- ☐ Sensory dysregulation (sensory processing)
- ☐ Motor disorder NOS

Does the child have some features of PANS or PANDAS phenotype?

☐ No ☐ PANS ☐ PANDAS

PANS/PANDAS

	Yes	No
I 1. Abrupt, dramatic onset of obsessive-compulsive disorder	◯	◯
I 2. Abrupt, dramatic onset of severely restricted food intake	◯	◯
II Concurrent presence of additional neuropsychiatric symptoms, (with similarly severe and acute onset), from at least two of the following seven categories: 1. Anxiety	◯	◯
II 2. Emotional lability and/or depression	◯	◯
II 3. Irritability, aggression, and/or severely oppositional behaviors	◯	◯
II 4. Behavioral (developmental) regression	◯	◯
II 5. Deterioration in school performance (related to attention-deficit/hyperactivity disorder [ADHD]-like symptoms, memory deficits, cognitive changes)	◯	◯
II 6. Sensory or motor abnormalities	◯	◯
II 7. Somatic signs and symptoms, including sleep disturbances, enuresis, or urinary frequency	◯	◯
III Symptoms are not better explained by a known neurologic or medical disorder, such as SC	◯	◯

Was there a definite Strep infection (culture or very high titres) that preceded the onset of symptoms by up to 3 weeks?	◯ Yes	◯ No
Was there a subsequent definite Strep infection that triggered a relapse of symptoms by up to 3 weeks.	◯ Yes	◯ No

Medications - current and previous
Tick all that apply

	Current	Previous (at any time)
Atomoxetine	☐	☐
Clonidine	☐	☐
Guanfacine	☐	☐
Lisdexamfetamine (vyvanse)	☐	☐
Methylphenidate	☐	☐
Steroids	☐	☐
IVIG	☐	☐
Risperidone	☐	☐
Aripiprazole	☐	☐
Other neuroleptic	☐	☐
Others	☐	☐

Specify other medications

_____

Other phenotypic comments

_____

CEREBRAL PALSY

Age of onset of CP symptoms
(in years and months e.g. 3 years 6 months)

_____

Motor impairment

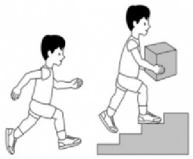
☐ Spastic
☐ Dyskinetic
☐ Ataxic
☐ Hypotonic

Distribution

☐ Diplegia
☐ Quadriplegia
☐ Hemiplegia

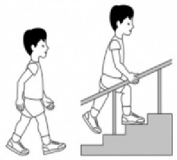
Gross Motor Function Classification System (GMFCS)
(Cerebral Palsy Alliance)

Select GMFCS level _____



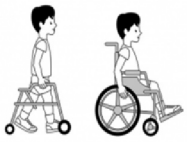
GMFCS Level I

Children walk at home, school, outdoors and in the community. They can climb stairs without the use of a railing. Children perform gross motor skills such as running and jumping, but speed, balance and coordination are limited.



GMFCS Level II

Children walk in most settings and climb stairs holding onto a railing. They may experience difficulty walking long distances and balancing on uneven terrain, inclines, in crowded areas or confined spaces.
Children may walk with physical assistance, a handheld mobilit device or used wheeled mobility over long distances. Children have only minimal ability to perform gross motor skills such as running and jumping.



GMFCS Level III

Children walk using a hand-held mobility device in most indoor settings. They may climb stairs holding onto a railing with supervision or assistance. Children use wheeled mobility when traveling long distances and may self-propel for shorter distances.

GMFCS Level IV

Children use methods of mobility that require physical assistance or powered mobility in most settings. They may walk for short distances at home with physical assistance or use powered mobility or a body support walker when positioned. At school, outdoors and in the community children are transported in a manual wheelchair or use powered mobility.

GMFCS Level V

Children are transported in a manual wheelchair in all settings. Children are limited in their ability to maintain antigravity head and trunk postures and control leg and arm movements.

Medications - current and previous
Tick all that apply

	Current	Previous (at any time)
Baclofen	☐	☐
Trihexyphenidyl	☐	☐
Clonazepam	☐	☐
Diazepam	☐	☐
Botox	☐	☐
Others	☐	☐

Specify other medications

_____

Other phenotypic comments

_____

CNS INFLAMMATORY/AUTOIMMUNE DISORDERS

Age of onset of inflammatory/autoimmune symptoms
(in years and months e.g. 3 years 6 months) _____

CNS disorder type	☐ Encephalitis ☐ Demyelinating ☐ Acute encephalopathies (e.g. FIRES, ANE) ☐ Acute exacerbation of psychiatry ☐ Movement disorder ☐ Autoinflammatory disorder (e.g. Aicardi Goitieres syndrome) ☐ Others
Encephalitis	☐ Cortical ☐ Limbic ☐ Basal ganglia ☐ Cerebellar ☐ NOS ☐ Acute meningitis
Demyelinating	☐ ADEM ☐ Myelitis ☐ Unilateral optic neuritis ☐ Bilateral optic neuritis ☐ Clinically isolated syndrome (CIS) ☐ Multiple sclerosis ☐ Neuromyelitis optica
Encephalopathies	☐ Febrile infection-related epilepsy syndrome (FIRES) ☐ Acute necrotizing encephalopathy (ANE) ☐ Mild encephalitis/encephalopathy with reversible splenial lesion (MERS) ☐ Hemiconvulsion-hemiplegiaepilepsy syndrome (HHE) ☐ Acute encephalopathy with biphasic seizures and reduced diffusion (AESD) ☐ Acute infantile encephalopathy predominantly affecting the frontal lobes (AIEF)
Movement disorder	☐ Immune chorea ☐ Sydenham chorea ☐ Acute cerebellar ataxia ☐ Acute cerebellitis
Others	☐ CNS lupus ☐ CNS vasculitis ☐ Antiphospholipid syndrome ☐ Opsoclonus-myoclonus ataxia syndrome (OMAS) ☐ Rasmussen's syndrome ☐ Paediatric acute-onset neuropsychiatric syndrome (PANS)
Other disorder information/details	_____

Medications - current and previous

Tick all that apply

	Current	Previous (at any time)
Azathioprine	☐	☐
Fingolimod	☐	☐
Interferon beta	☐	☐
IVIG	☐	☐
MMF	☐	☐
Plasma exchange	☐	☐
Steroids	☐	☐
Rituximab	☐	☐
Ruxolitinib	☐	☐
Tofacitinib	☐	☐
Natalizumab	☐	☐
Others	☐	☐

Specify other medications

_____

Other phenotypic comments

_____

PSYCHIATRIC AND SLEEP DISORDERS

Age of onset of psychiatric/sleep symptoms
(in years and months e.g. 3 years 6 months) _____

Psychiatric/Sleep disorder type

- ☐ Brief psychotic disorder
- ☐ Schizophrenia
- ☐ Catatonia
- ☐ Bipolar and related disorders
- ☐ Depressive disorders
- ☐ Separation anxiety disorder
- ☐ Specific phobia
- ☐ Panic disorder
- ☐ Generalized anxiety disorder
- ☐ Obsessive-compulsive disorder
- ☐ Post-traumatic stress disorder
- ☐ Dissociative disorders
- ☐ Somatic symptom disorder
- ☐ Conversion disorder (Functional neurological symptom disorder
- ☐ Avoidant/restrictive food intake disorder
- ☐ Anorexia nervosa
- ☐ Restricted eating disorder NOS
- ☐ Insomnia disorder
- ☐ Hypersomnolence disorder
- ☐ Narcolepsy
- ☐ Parasomnias
- ☐ Disruptive, impulse-control, and conduct disorders

Current exacerbation of psychiatric symptoms? ◯ Yes ◯ No

Medications - current and previous
Tick all that apply

	Current	Previous (at any time)
Escitalopram	☐	☐
Fluoxetine	☐	☐
Fluvoxamine	☐	☐
Lithium	☐	☐
Mirtazepine	☐	☐
Quetiapine	☐	☐
Olanzepine	☐	☐
Sertraline	☐	☐
Steroids	☐	☐
IVIG	☐	☐
Others	☐	☐

Specify other medications

_____

Other phenotypic comments

_____

NERVE CONDITIONS

Nerve condition type

- ☐ Spinal muscular atrophy
- ☐ Charcot Marie Tooth disease
- ☐ Other genetic neuropathy
- ☐ Toxic neuropathy
- ☐ Chronic inflammatory polyneuropathy
- ☐ Traumatic neuropathy
- ☐ Guillain Barre syndrome
- ☐ Others

Specify other conditions

_____

Medications - current and previous
Tick all that apply

	Current	Previous (at any time)
Gabapentin	☐	☐
Pregabalin	☐	☐
IVIG	☐	☐
Plasma exchange	☐	☐
Olanzepine	☐	☐
Gene therapy	☐	☐
Others	☐	☐

If gene therapy, specify details

_____

Specify other medications

_____

Other phenotypic comments

_____

MUSCLE AND NEUROMUSCULAR JUNCTIONS DISORDERS

Muscle and neuromuscular junction disorder type

- ☐ Muscular dystrophy
- ☐ Myotonia
- ☐ Myopathy
- ☐ Neuromuscular junction

Muscular dystrophy type

- ☐ Duchenne Muscular dystrophy
- ☐ Becker muscular dystrophy
- ☐ Limb girdle dystrophy
- ☐ Fascioscapulohumeral dystrophy
- ☐ Oculopharyngeal dystrophy
- ☐ Emery-Dreifuss
- ☐ Myotonic dystrophy

Myotonia type	☐ Congenital myotonia ☐ Paramyotonia congenita

Myopathy type

	Congenital	Non-congenital
Central core myopathy	◯	◯
Nemaline myopathy	◯	◯
Myotubular myopathy	◯	◯
Others	◯	◯

Neuromuscular junction type

	Congenital	Non-congenital
Generalized Myasthenia gravis	◯	◯
Ocular Myasthenia gravis	◯	◯
Myositis	◯	◯

Medications - current and previous
Tick all that apply

	Current	Previous (at any time)
IVIG	☐	☐
MMF	☐	☐
Plasma exchange	☐	☐
Steroids (prednisolone, deflazacort)	☐	☐
Pyridostigmine	☐	☐
Gene therapy	☐	☐
Others	☐	☐

If gene therapy, specify details

_____

Specify other medications

_____

Other phenotypic comments

_____

HEADACHE

Headache type

- ☐ Migraine
- ☐ Idiopathic intracranial hypertension
- ☐ Other

Other phenotypic comments

_____

NEUROSURGERY

Neurosurgery type

- ☐ Hydrocephalus
- ☐ Traumatic brain injury
- ☐ Other

Other phenotypic comments

_____

OTHER ACUTE NEUROLOGY and STROKE

Other acute neurology type

- ☐ Acute stroke syndrome
- ☐ Posterior reversible encephalopathy syndrome (PRES)
- ☐ Other

Other phenotypic comments

_____

NEUROFIBROMATOSIS TYPE 1&2 / SCHWANNOMATOSIS or LEGIUS SYNDROME

Select diagnosis

- ◯ NF1
- ◯ NF2 related Schwannomatosis
- ◯ Schwannomatosis
- ◯ Legius Syndrome (SPRED1)

NEUROFIBROMATOSIS TYPE 1

NF1 diagnosis details

- ◯ Definite NF1
- ◯ Possible NF1
- ◯ Segmental/Mosaic NF1
- ◯ Unlikely NF1
- ◯ Diagnostic Change

NF1 clinical features	☐ Cafe au Lait spots, 6 or more ☐ 1st Degree Relative with NF1 ☐ Axillary/Inguinal Freckling ☐ Bony Dysplasia ☐ Iris Hamartoma/Lisch nodules/choroidal abnormalities ☐ Cutaneous Neuro Fibromas> 2 OR 1 Plexiform Neurofibroma ☐ Optic Pathway Glioma ☐ Comorbidities unrelated to NF1
Comorbidities unrelated to NF1 - details	_____
Bony Dysplasia details	☐ Bowing of long bones (Tibia) ☐ Sphenoid wing dysplasia ☐ Pseudarthrosis of long bone
Segmental/Mosaic NF1 location	_____
Segmental/Mosaic NF1 location photograph upload	
Unlikely NF1 - description	_____
Diagnostic Change - description	_____
NF1 complications	☐ Dermatological ☐ Neurology ☐ Oncology ☐ Behaviour ☐ Endocrinology/Growth ☐ Hypertension ☐ Orthopaedic
Dermatological details	☐ Cutaneous Neurofibroma ☐ Plexiform Neurofibroma
Cutaneous Neurofibroma details	☐ Itch ☐ Pain ☐ Cosmetic/functional
Plexiform Neurofibroma details	☐ Itch ☐ Pain ☐ Cosmetic/functional

Plexiform Neurofibroma Location Details

Select applicable locations _____

Fill in the details below

Leave those that do not apply blank

Location	Size	Complexity?	Complexity details
Facial	_____	_____	_____
Neck	_____	_____	_____

Upper limbs _____ _____ _____

Trunk _____ _____ _____

Spine _____ _____ _____

Lower limbs _____ _____ _____

Internal _____ _____ _____

Neurology details

- ☐ Incoordination/hypotonia
- ☐ Seizures/epilepsy
- ☐ Headache
- ☐ Learning Difficulties
- ☐ Macrocephaly
- ☐ Pain
- ☐ Cerebrovascular/Moya Moya
- ☐ Other

Seizures/epilepsy - details

_____

Learning assessment?

- ◯ Yes
- ◯ No

Pain - details

_____

Cerebrovascular/Moya Moya - details

_____

Other - details

_____

Oncology details

- ☐ OPG
- ☐ MPNST
- ☐ Other Tumour
- ☐ Phaeochromocytoma

OPG vision impaired?

- ◯ Yes
- ◯ No

OPG - location

_____

MPNST - location

_____

Other Tumour - size/location

_____

Behaviour details	☐ ADHD ☐ ASD ☐ Anxiety ☐ Depression ☐ Opposition Defiant Disorder/Conduct Disorder ☐ Other
Behaviour Other details	_____
Endocrinology/Growth details	☐ Precocious Puberty ☐ Short Stature ☐ Tall Stature ☐ Other
Endocrinology/Growth Other details	_____
Hypertension details	☐ Renal Artery Stenosis ☐ Phaeochromocytoma ☐ Other
Hypertension Other details	_____
Orthopaedic details	☐ Scoliosis ☐ Pseudoarthrosis ☐ Long Bone Bowing
Long Bone Bowing details	☐ Upper limb ☐ Lower limb

NF1 Treatment Details

Select applicable treatments _____

Fill in the details below

Leave those that do not apply blank

Treatment Date Details
Neurosurgery _____ _____
Orthopaediac/Scoliosis surgery

_____ _____
Debulking surgery _____ _____
MEK inhibitors _____ _____
Avastin _____ _____
Laser _____ _____
OPG chemotherapy _____ _____
Neuropsychological medications _____ _____
Pain medications _____ _____
Other chemotherapy _____ _____
Other treatment _____ _____

NEUROFIBROMATOSIS TYPE 2

NF2 related Schwannomatosis diagnosis details

◯ Definite NF2 related Schwannomatosis
◯ Possible NF2 related Schwannomatosis

NF2 related Schwannomatosis clinical features

☐ Acoustic Tumour (vestibular Schwannomas)
☐ First Degree Relative with NF2
☐ Meningiomas
☐ Gliomas
☐ Ependymoma
☐ Schwannoma
☐ Cataract
☐ Other features

Acoustic Tumour (vestibular Schwannomas) - location

_____

Meningiomas - location

_____

Gliomas - location

_____

Ependymoma - location

_____

Schwannoma - count

_____

Schwannoma - location

☐ Dermal
☐ Intradermal

Cataract - details

_____

Other features - details

_____

NF2 complications

☐ Hearing loss
☐ Balance Problems/Gait
☐ Tinnitus
☐ Pain
☐ Headache
☐ Cranial Nerve Palsy
☐ Seizures/epilepsy
☐ Weakness/Parasthesia
☐ Tumours
☐ Cataract/visual loss
☐ Psychological - anxiety/depression
☐ Other

Cranial nerve palsy - details

_____

Seizures/epilepsy - details

_____

Tumours - details

- ☐ Intracranial
- ☐ Spinal
- ☐ Peripheral

Other - details

_____

NF2 Treatment Details

Select applicable treatments _____

Fill in the details below

Leave those that do not apply blank

Treatment	Date	Details
Neurosurgery	_____	_____
Orthopaedic/Scoliosis surgery	_____	_____
Biopsy surgery	_____	_____
Avastin	_____	_____
Neuropsychological medications	_____	_____
Pain medications	_____	_____
Other chemotherapy	_____	_____
Hearing aids	_____	_____
Auditory brainstem implant	_____	_____
Other treatment	_____	_____

SCHWANNOMATOSIS

Schwannomatosis treatment

_____

LEGIUS SYNDROME

Legius Syndrome (SPRED1) clinical features

- ☐ Cafe au lait spots
- ☐ Axillary and inguinal freckling
- ☐ Lipomas
- ☐ Macrocephaly
- ☐ Learning Disabilities
- ☐ Behavioral issues
- ☐ Short stature
- ☐ Noonan -like features
- ☐ Other

NEUROFIBROMATOSIS TYPE 1&2 / SCHWANNOMATOSIS or LEGIUS SYNDROME MANAGEMENT

Management

- ☐ Genetic counselling
- ☐ Local GP _____
- ☐ Local pediatrician _____
- ☐ Specialist service _____
- ☐ Learning and behavioural support/early intervention
- ☐ Psychological support
- ☐ Social
- ☐ Transition
- ☐ Complex
- ☐ Close surveillance

Management - Transition details

- ☐ Service/Facility _____
- ☐ Trapeze _____

Management Complex - details

- ☐ Growing Plexiform Neurofibroma
- ☐ OPG
- ☐ CNS/Spinal Cord Tumour
- ☐ Epilepsy
- ☐ Pseudo arthrosis/Scoliosis
- ☐ Heavy Disease load
- ☐ Complex Social /psychological Issues
- ☐ Receiving Advanced treatments

Management details

_____

NF Clinical Trials

- ◯ Yes
- ◯ No

NF Clinical Trials details

_____

DISEASE AETIOLOGY

Select aetiology

- ☐ Genetic
- ☐ Structural, Traumatic, or Brain Malformations
- ☐ Infectious
- ☐ Metabolic/Neurodegeneration
- ☐ Immune
- ☐ Medication/Toxin
- ☐ Complex polygenic-environment suspected
- ☐ Other
- ☐ Unknown

GENETIC AETIOLOGY

Please list all genetic investigations performed in the proband.

FINAL GENETIC DIAGNOSIS (MONDO)

_____
(Choose MONDO number best describing the patient phenotype)

FINAL GENETIC DIAGNOSIS (if MONDO not appropriate)

_____

Which of the following genetic investigations has the participant had?

- ☐ Chromosome microarray (CMA)
- ☐ Fragile X testing
- ☐ Expansion testing (eg. DM1)
- ☐ Panel or single gene testing (including panel analysis on exome backbone)
- ☐ Exome sequencing (ES) - singleton
- ☐ Exome sequencing (ES) - trio
- ☐ Exome sequencing (ES) - other
- ☐ Genome sequencing (GS) - singleton
- ☐ Genomic sequencing (GS) - trio
- ☐ Genome sequencing (GS) - other
- ☐ Methylation studies - Single locus
- ☐ Methylation profiling - Genome-wide (eg. EpiSign)
- ☐ Functional studies
- ☐ Other

CMA Results

Date CMA reported

_____

Age at CMA

_____
(yearsDisplays age deceased if testing performed after death)

Laboratory CMA performed

- ◯ SEALS
- ◯ CHW
- ◯ Concord
- ◯ Exeter
- ◯ Fulgent
- ◯ GeneDX
- ◯ Invitae
- ◯ Mater
- ◯ Monash
- ◯ Path North
- ◯ Pathwest
- ◯ Prevention Genetics
- ◯ SA Pathology
- ◯ UAB
- ◯ VCGS
- ◯ Other

Please detail other lab

_____

Number of variants reported

◯ 0
◯ 1
◯ 2
◯ 3
◯ 4
◯ 5
◯ 6

Variant 1

Variant Classification Variant Reported

_____

_____

eg. arr[GRCh37]15q15.3(43895633_43951301)x1

Inheritance: _____

Variant Type: _____

Variant 2

Variant Classification Variant Reported

_____

_____

eg. arr[GRCh37]15q15.3(43895633_43951301)x1

Inheritance: _____

Variant Type: _____

Variant 3

Variant Classification Variant Reported

_____

_____

eg. arr[GRCh37]15q15.3(43895633_43951301)x1

Inheritance: _____

Variant Type: _____

Variant 4

Variant Classification Variant Reported

_____

_____

eg. arr[GRCh37]15q15.3(43895633_43951301)x1

Inheritance: _____

Variant Type: _____

Variant 5

Variant Classification Variant Reported

_____

_____

eg. arr[GRCh37]15q15.3(43895633_43951301)x1

Inheritance: _____

Variant Type: _____

Variant 6

Variant Classification Variant Reported

_____

_____

eg. arr[GRCh37]15q15.3(43895633_43951301)x1

Inheritance: _____

Variant Type: _____

Was CMA informative?

◯ Informative (test result is diagnostic)
◯ Possibly informative (result may explain presentation)
◯ Uninformative (non-diagnostic, variant(s) reported unlikely related to clinical indication)

Please detail

_____

Other comments on CMA

_____

Upload CMA reportReport upload is optional but is recommended.

Fragile X Testing Results

Date Fragile X testing reported

_____

Age at Fragile X testing

_____
(yearsDisplays age deceased if testing performed after death)

Laboratory Fragile X testing performed

◯ SEALS
◯ CHW
◯ Concord
◯ Exeter
◯ Fulgent
◯ GeneDX
◯ Invitae
◯ Mater
◯ Monash
◯ Path North
◯ Pathwest
◯ Prevention Genetics
◯ SA Pathology
◯ UAB
◯ VCGS
◯ Other

Please detail other lab

_____

Interpretation of CGG repeat number Repeat size on allele 1 Repeat size on allele 2

_____ _____ _____

Was Fragile X testing informative?

◯ Informative (test result is diagnostic)
◯ Possibly informative (result may explain presentation)
◯ Uninformative (non-diagnostic, variant(s) reported unlikely related to clinical indication)

Please detail

_____

Other comments on Fragile X testing

_____

Upload Fragile X study reportReport upload is optional but is recommended.

Expansion Testing Results

Date expansion testing reported

_____

Age at expansion testing

_____
(yearsDisplays age deceased if testing performed after death)

Laboratory expansion testing performed

◯ SEALS
◯ CHW
◯ Concord
◯ Exeter
◯ Fulgent
◯ GeneDX
◯ Invitae
◯ Mater
◯ Monash
◯ Path North
◯ Pathwest
◯ Prevention Genetics
◯ SA Pathology
◯ UAB
◯ VCGS
◯ Other

Laboratory expansion testing performed

_____

Gene Interpretation of repeat number Repeat size on allele 1 Repeat size on allele 2

_____

_____

_____ _____

Was expansion testing informative?

◯ Informative (test result is diagnostic)
◯ Possibly informative (result may explain presentation)
◯ Uninformative (non-diagnostic, variant(s) reported unlikely related to clinical indication)

Please detail

_____

Other comments on expansion testing

_____

Upload expansion testing reportReport upload is optional but is recommended.

Panel or Single Gene Testing Results

Panel performed

_____

Panel type

◯ Multigene panel
◯ Panel on exome backbone
◯ Other _____

Date panel reported

_____

Age at panel testing

_____
(yearsDisplays age deceased if testing performed after death)

Laboratory panel testing performed

◯ SEALS
◯ CHW
◯ Concord
◯ Exeter
◯ Fulgent
◯ GeneDX
◯ Invitae
◯ Mater
◯ Monash
◯ Path North
◯ Pathwest
◯ Prevention Genetics
◯ SA Pathology
◯ UAB
◯ VCGS
◯ Other

Please detail other lab

_____

Number of variants reported

◯ 0
◯ 1
◯ 2
◯ 3
◯ 4
◯ 5
◯ 6

Variant 1

ACMG Classification

_____

Variant

Transcript

_____
eg. NM_000492.3

Gene

_____
eg. CFTR

DNA code

_____
eg. c.3353C>T

Protein code

_____
eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 2

ACMG Classification

_____

Variant

Transcript

_____
eg. NM_000492.3

Gene

_____
eg. CFTR

DNA code

_____
eg. c.3353C>T

Protein code

_____
eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 3

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 4

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 5

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 6

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____
eg. CFTR

DNA code

_____
eg. c.3353C>T

Protein code

_____
eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Was panel testing informative?	◯ Informative (test result is diagnostic) ◯ Possibly informative (result may explain presentation) ◯ Uninformative (non-diagnostic, variant(s) reported unlikely related to clinical indication)
Please detail	_____
Other comments on panel testing	_____
Upload panel reportReport upload is optional but is recommended.	
Exome Sequencing Results	
Date exome sequencing reported	_____
Age at exome sequencing	_____ (yearsDisplays age deceased if testing performed after death)

Laboratory exome sequencing performed	◯ SEALS ◯ CHW ◯ Concord ◯ Exeter ◯ Fulgent ◯ GeneDX ◯ Invitae ◯ Mater ◯ Monash ◯ Path North ◯ Pathwest ◯ Prevention Genetics ◯ SA Pathology ◯ UAB ◯ VCGS ◯ Other
Please detail other lab	_____
Number of variants reported	◯ 0 ◯ 1 ◯ 2 ◯ 3 ◯ 4 ◯ 5 ◯ 6
Variant 1	
ACMG Classification _____	
Variant Transcript _____ eg. NM_000492.3 Gene _____ eg. CFTR DNA code _____ eg. c.3353C>T Protein code _____ eg. p.Arg1459* HGVS nomenclature	
Zygosity _____	
Inheritance	

_____

Variant 2

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 3

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 4

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 5

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 6

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Was exome sequencing informative?	◯ Informative (test result is diagnostic) ◯ Possibly informative (result may explain presentation) ◯ Uninformative (non-diagnostic, variant(s) reported unlikely related to clinical indication)
Please detail	_____
Other comments on exome sequencing	_____
Upload exome sequencing reportReport upload is optional but is recommended.	
Genome Sequencing Results	
Date genome sequencing reported	_____
Age at genome sequencing	_____ (yearsDisplays age deceased if testing performed after death)
Laboratory genome sequencing performed	◯ SEALS ◯ CHW ◯ Concord ◯ Exeter ◯ Fulgent ◯ GeneDX ◯ Invitae ◯ Mater ◯ Monash ◯ Path North ◯ Pathwest ◯ Prevention Genetics ◯ SA Pathology ◯ UAB ◯ VCGS ◯ Other
Please detail other lab	_____
Number of variants reported	◯ 0 ◯ 1 ◯ 2 ◯ 3 ◯ 4 ◯ 5 ◯ 6
Variant 1	
ACMG Classification	_____

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 2

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 3

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 4

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 5

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Variant 6

ACMG Classification

_____

Variant

Transcript

_____

eg. NM_000492.3

Gene

_____

eg. CFTR

DNA code

_____

eg. c.3353C>T

Protein code

_____

eg. p.Arg1459*

HGVS nomenclature

Zygosity

_____

Inheritance

_____

Was genome sequencing informative?	◯ Informative (test result is diagnostic) ◯ Possibly informative (result may explain presentation) ◯ Uninformative (non-diagnostic, variant(s) reported unlikely related to clinical indication)
Other comments on GS	_____
Please detail	_____
Upload genome sequencing reportReport upload is optional but is recommended.	
Methylation Studies (Single locus)	
Date methylation studies performed	_____
Age at methylation studies	_____ (yearsDisplays age deceased if testing performed after death)

Laboratory methylation studies performed	◯ SEALS ◯ CHW ◯ Concord ◯ Exeter ◯ Fulgent ◯ GeneDX ◯ Invitae ◯ Mater ◯ Monash ◯ Path North ◯ Pathwest ◯ Prevention Genetics ◯ SA Pathology ◯ UAB ◯ VCGS ◯ Other
Please detail other lab	_____
Methylation studies performed Results Other comments _____ _____ _____	
Were methylation studies informative?	◯ Informative (test result is diagnostic) ◯ Possibly informative (result may explain presentation) ◯ Uninformative (non-diagnostic, variant(s) reported unlikely related to clinical indication)
Please detail	_____
Upload methylation studies reportReport upload is optional but is recommended.	
Methylation Profiling (Genome-wide)	
Date methylation profiling reported	_____
Age at methylation profiling	_____ (yearsDisplays age deceased if testing performed after death)

Laboratory methylation profiling performed

- ◯ SEALS
- ◯ CHW
- ◯ Concord
- ◯ Exeter
- ◯ Fulgent
- ◯ GeneDX
- ◯ Invitae
- ◯ Mater
- ◯ Monash
- ◯ Path North
- ◯ Pathwest
- ◯ Prevention Genetics
- ◯ SA Pathology
- ◯ UAB
- ◯ VCGS
- ◯ Other

Please detail other lab

_____

Methylation profiling performed

Results

Other comments

_____

_____

_____

Was methylation profiling informative?

- ◯ Informative (test result is diagnostic)
- ◯ Possibly informative (result may explain presentation)
- ◯ Uninformative (non-diagnostic, variant(s) reported unlikely related to clinical indication)

Please detail

_____

Upload methylation profiling reportReport upload is optional but is recommended.

Previous Functional Studies

Laboratory: _____

Date reported Functional study performed Outcomes

_____

Age (years)

_____

_____ _____

_____

Report upload is optional but is recommended.

Other Genetic Investigations

Other Genetic Investigation 1

Laboratory: _____

Date reported Genetic Investigation Outcomes

_____

Age (years)

_____

_____ _____

_____

Report upload is optional but is recommended.

Incidental Findings

Incidental finding?	◯ Yes ◯ No
Variant details of Incidental finding	_____ ((e.g. NM_004006.3. CFTR: c.4375C>T; p.[Arg1459*]))
Clinical diagnosis from Incidental Finding?	◯ Yes ◯ No ◯ Uncertain
Please detail	_____
Management implications from Incidental Finding	◯ Yes ◯ No ◯ Uncertain
Management implications	☐ Prevention ☐ Surveillance ☐ Institute new treatment ☐ Recurrence risk information ☐ Other
Please provide further details	_____
Family history	☐ NO FAMILY HISTORY ☐ AFFECTED FIRST DEGREE RELATIVE ☐ ONLY AFFECTED MALES IN FAMILY ☐ ONLY AFFECTED FEMALES IN FAMILY ☐ MULTIPLE GENERATIONS AFFECTED

Impact of positive genetic result

- ☐ DIAGNOSTIC CLOSURE
- ☐ FAMILY PLANNING - DECISION TO NOT HAVE FURTHER CHILDREN
- ☐ FAMILY PLANNING - PRENATAL TESTING
- ☐ SIBLING GENETIC COUNSELLING
- ☐ PARENTAL GENETIC COUNSELLING
- ☐ SYMPTOMATIC TREATMENT GUIDANCE
- ☐ DISEASE SPECIFIC TREATMENT GUIDANCE
- ☐ MEDICATIONS CHANGED
- ☐ FUTURE INVESTIGATIONS REDUCED
- ☐ FUTURE INVESTIGATIONS CANCELLED
- ☐ OTHER
- ☐ NO CHANGE

Specify other impact

_____

Upload report of genetic investigation/family pedigreeReport upload is optional but is recommended.

STRUCTURAL AETIOLOGY

	Confirmed	Suspected
Hypoxic ischemic encephalopathy	◯	◯
Stroke	◯	◯
Cortical dysplasia	◯	◯
Cortical malformation	◯	◯
Chiari malformations	◯	◯
Hydrocephalus	◯	◯
Vasculopathy	◯	◯
Neoplasms/Tumour	◯	◯
Trauma	◯	◯
Tuberous sclerosis complex	◯	◯
Neurofibromatosis	◯	◯
Others	◯	◯

Specify other structural aetiology

_____

INFECTIOUS AETIOLOGY

Viral or Bacterial? ◯ Viral ◯ Bacterial

Viral

	Confirmed	Suspected
Enterovirus	◯	◯
Herpes Simplex virus	◯	◯
Varicella	◯	◯
Epstein-Barr virus	◯	◯
Influenza	◯	◯
Others	◯	◯

Specify other viral aetiology

_____

Bacterial

	Confirmed	Suspected
Strep Pneumoniae	◯	◯
Hemophilus Influenzae	◯	◯
Group-B Streptococcus	◯	◯
Mycoplasma	◯	◯
Others	◯	◯

Specify other bacterial aetiology

_____

METABOLIC AETIOLOGY

Metabolic aetiology type

◯ Inborn error of metabolism (including storage, leuokodyst etc.)
◯ Mitochondrial
◯ Monoamine neurotransmitter disorders
◯ Neurodegeneration (other)

Inborn error of metabolism

	Confirmed	Suspected
Organic acid disorder	◯	◯
Amino acid disorder	◯	◯
Urea cycle defect	◯	◯
Peroxisomal disorders	◯	◯
Lysosomal disorders	◯	◯
Vitamins and trace elements	◯	◯
Others	◯	◯

Specify other inborn error of metabolism aetiology

_____

Mitochondrial

	Confirmed	Suspected
Leigh syndrome	◯	◯
Pearson syndrome	◯	◯
MERRF	◯	◯
NARP	◯	◯
MELAS	◯	◯
Alpers syndrome	◯	◯
MNGIE	◯	◯
Kearns-Sayre syndrome	◯	◯
MIDD	◯	◯
SANDO	◯	◯
Others	◯	◯

Specify neurodegeneration or other aetiology

_____

IMMUNE AETIOLOGY

Immune aetiology type

◯ Autoimmune (autoantibody)
◯ Immune mediated
◯ Demyelination

Autoimmune (autoantibody)

	Confirmed	Suspected
Anti-N-methyl-Daspartate (NMDA)	◯	◯
Myelin oligodendrocyte glycoprotein (MOG)	◯	◯
Leucine-rich glioma inactivated 1 (LGI1)	◯	◯
Contactin associated protein (CASPR)	◯	◯
Glutamic acid decarboxylase (GAD)	◯	◯
Anti glycine receptor	◯	◯
Dopamine receptor	◯	◯
Hashimoto's encephalopathy	◯	◯
Others	◯	◯

OTHER AETIOLOGY Describe details

_____

UNKNOWN AETIOLOGY

◯ Awaiting testing
◯ Unknown

INDIVIDUAL PHENOTYPIC TERMS - HUMAN PHENOTYPE ONTOLOGY (HPO)

Order these in descending order from dominant phenotypic feature to other associated features, if possible

HPO Term Age of onset HPO symptom HPO symptom progression
(1) _____ _____ _____
(2) _____ _____ _____
(3) _____ _____ _____
(4) _____ _____ _____
(5) _____ _____ _____
(6) _____ _____ _____
(7) _____ _____ _____
(8) _____ _____ _____
(9) _____ _____ _____
(10) _____ _____ _____

Any other phenotypic or aetiology data of importance?

Any missing data or notes to follow-up? _____

DISEASE ONSET AND PROGRESS

Patient disease onset and progress

- ◯ New and acute onset problem in previously well child
- ◯ Acute illness previously, now in remission
- ◯ Acute illness previously, now in relapse
- ◯ Acute illness previously, ongoing problems
- ◯ Acute exacerbation of a chronic disorder
- ◯ Chronic disorder with a progressive deteriorating course
- ◯ Chronic disorder with a relapsing-remitting course
- ◯ Chronic disorder with a static course

Current illness status

- ◯ Ongoing neurological problems - deteriorating
- ◯ Ongoing neurological problems - stable
- ◯ Ongoing neurological problems - improving
- ◯ Previous neurological problems - in remission
- ◯ Previous neurological problem - current relapse
- ◯ Previous neurological problem - current exacerbation of ongoing problem.

Age of onset of INITIAL neurological symptoms (in years and months) _____

Tick procedures/tests done in this admission (admission when this form is filled out)

☐ Lumbar puncture ☐ MRI

How long ago did NEW neurological symptoms appear (prior to LP)?

- ◯ Not relevant
- ◯ < 24 hours
- ◯ 1-2 days
- ◯ 2-7 days
- ◯ 1-4 weeks
- ◯ 4-8 weeks
- ◯ 2-3 months
- ◯ 3-6 months
- ◯ 6-12 months
- ◯ >1 year

Are there any current triggers (or exacerbators of symptoms)?

- ☐ None
- ☐ Fever
- ☐ Viral prodrome
- ☐ Infection
- ☐ Others

Specify other triggers

_____

If yes, how many days ago did the trigger/s occur (prior to LP if done, or prior to this assessment)? Please provide details.

_____

Clinical setting

- ☐ Admission
- ☐ PICU admission
- ☐ Intubation/ventilator
- ☐ Outpatient care
- ☐ Emergency

FAMILY HISTORY

Maternal history

	During pregnancy (with this child)	At any time
Obesity	☐	☐
Diabetes/Gestational diabetes	☐	☐
Pre-eclampsia	☐	☐
Polyhydramnios	☐	☐
Prematurity (delivery)	☐	☐
Smoking	☐	☐
Depression	☐	☐
Autoimmune disease	☐	☐
Asthma	☐	☐
Infection (requiring hospitalisation or treatment)	☐	☐
Epilepsy	☐	☐
Other psychiatry	☐	☐
Other neurology	☐	☐
Any other	☐	☐

Please specify gestation (in weeks) for premature delivery

_____

Please specify autoimmune disease/s

_____

If other psychiatry, please specify

_____

If other neurology, please specify

_____

If any other maternal history, please specify

_____

Paternal history

- ☐ Obesity
- ☐ Diabetes
- ☐ Smoking
- ☐ Depression
- ☐ Autoimmune disease
- ☐ Asthma
- ☐ Epilepsy
- ☐ Other psychiatry
- ☐ Other neurology
- ☐ Any other

Please specify autoimmune disease

_____

If other psychiatry, please specify

_____

If other neurology, please specify

_____

If any other paternal history, please specify

_____

Appendix 2. Clinical Global Impression Scale

Clinical Global Impression

Record ID _____

Clinical Global Impression (CGI)

1. CGI-Severity (CGI-S)

Considering your total clinical experience with this particular population, how mentally ill is the patient at this time?

Rating date: _____

- ◯ 1 = Normal-not at all ill, symptoms of disorder not present past seven days
- ◯ 2 = Borderline mentally ill-subtle or suspected pathology
- ◯ 3 = Mildly ill-clearly established symptoms with minimal, if any, distress or difficulty in social and occupational function
- ◯ 4 = Moderately ill-overt symptoms causing noticeable, but modest, functional impairment or distress; symptom level may warrant medication
- ◯ 5 = Markedly ill-intrusive symptoms that distinctly impair social/occupational function or cause intrusive levels of distress
- ◯ 6 = Severely ill-disruptive pathology, behavior and function are frequently influenced by symptoms, may require assistance from others
- ◯ 7 = Among the most extremely ill patients-pathology drastically interferes in many life functions; may be hospitalized

Select medications to be rated

- ☐ [name_med_1]
- ☐ [name_med_2]
- ☐ [name_med_3]
- ☐ [name_med_4]
- ☐ [name_med_5]
- ☐ [name_med_6]
- ☐ [name_med_7]
- ☐ [name_med_8]
- ☐ [name_med_9]
- ☐ [name_med_10]
- ☐ [name_med_11]
- ☐ [name_med_12]
- ☐ Other: _____

2. CGI-Improvement (CGI-I)

Compared to the patient's condition at admission to the project [prior to medication initiation], this patient's condition is

Rating date: _____

- ◯ 1 = Very much improved-nearly all better; good level of functioning; minimal symptoms; represents a very substantial change
- ◯ 2 = Much improved-notably better with significant reduction of symptoms; increase in the level of functioning but some symptoms remain
- ◯ 3 = Minimally improved-slightly better with little or no clinically meaningful reduction of symptoms. Represents very little change in basic clinical status, level of care, or functional capacity
- ◯ 4 = No change-symptoms remain essentially unchanged
- ◯ 5 = Minimally worse-slightly worse but may not be clinically meaningful; may represent very little change in basic clinical status or functional capacity
- ◯ 6 = Much worse-clinically significant increase in symptoms and diminished functioning
- ◯ 7 = Very much worse-severe exacerbation of symptoms and loss of functioning

3. Efficacy index: Rate this item on the basis of drug effect only.

Select the terms which best describe the degrees of therapeutic effect and side effects and record the number in the box where the two items intersect.

EXAMPLE: If therapeutic effect is rated as 'Moderate' and side effects are judged 'Do not significantly interfere with patient's functioning', select number 06.

Therapeutic effect		**Side effects**			
		None	*Do not significantly interfere with patient's functioning*	*Significantly interferes with patient's functioning*	*Outweighs therapeutic effect*
Marked	Vast improvement. Complete or nearly complete remission of all symptoms	01	02	03	04
Moderate	Decided improvement. Partial remission of symptoms	05	06	07	08
Minimal	Slight improvement which doesn't alter status of care of patient	09	10	11	12
Unchanged or worse		13	14	15	16

Not assessed = 00

Therapeutic effect

- ◯ Marked: Vast improvement. Complete or nearly complete remission of all symptoms.
- ◯ Moderate: Decided improvement. Partial remission of symptoms.
- ◯ Minimal Slight improvement which doesn't alter status of care of patient.
- ◯ Unchanged or worse

Side effects

- ◯ None
- ◯ Do not significantly interfere with patient's functioning
- ◯ Significantly interferes with patient's functioning
- ◯ Outweighs therapeutic effect

Select scoring number from the table above

- ◯ 01
- ◯ 02
- ◯ 03
- ◯ 04
- ◯ 05
- ◯ 06
- ◯ 07
- ◯ 08
- ◯ 09
- ◯ 10
- ◯ 11
- ◯ 12
- ◯ 13
- ◯ 14
- ◯ 15
- ◯ 16

Appendix 3. Medications

Medications

Record ID _____

MEDICATIONS - New, Current, and Previous **Include probiotic and antibiotic use if relevant**

Medication Summary Medication name Type of medication Current dose (if applicable)

1. [name_med_1] [type_med_1] [curr_dose_med_1]
2. [name_med_2] [type_med_2] [curr_dose_med_2]
3. [name_med_3] [type_med_3] [curr_dose_med_3]
4. [name_med_4] [type_med_4] [curr_dose_med_4]
5. [name_med_5] [type_med_5] [curr_dose_med_5]
6. [name_med_6] [type_med_6] [curr_dose_med_6]
7. [name_med_7] [type_med_7] [curr_dose_med_7]
8. [name_med_8] [type_med_8] [curr_dose_med_8]
9. [name_med_9] [type_med_9] [curr_dose_med_9]
10. [name_med_10] [type_med_10] [curr_dose_med_10]
11. [name_med_11] [type_med_11] [curr_dose_med_11]
12. [name_med_12] [type_med_12] [curr_dose_med_12]

Add a medication? ◯ Yes ◯ No

Medication 1

Type of medication

◯ New
◯ Current
◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 2

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 3

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 4

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 5

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 6

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 7

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 8

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 9

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 10

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 11

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____
Add a medication?	◯ Yes ◯ No

Medication 12

Type of medication	◯ New ◯ Current ◯ Previously tried but discontinued

Name of medication	◯ Catapres ◯ Intuniv ◯ Ritalin ◯ Vyvanse ◯ Lovan ◯ Zoloft ◯ Luvox ◯ Risperidone ◯ Abilify ◯ Seroquel ◯ Neurofen ◯ Zyrtec ◯ Piriton ◯ Phenergan ◯ Erythromycin ◯ Azithromycin ◯ Augmentin ◯ Amoxicillin ◯ Penicillin ◯ Steroid ◯ Immunoglobulin ◯ Levetiracetam ◯ Sodium valproate ◯ Carbamazepine ◯ Oxcarbazepine ◯ Clobazam ◯ Lamotrigine ◯ Topiramate ◯ Zonisamide ◯ Lacosamide ◯ Phenytoin ◯ Phenobarbitone ◯ Prednisolone ◯ Vigabatrin ◯ Stiripentol ◯ Cannabidiol ◯ Ketogenic diet ◯ Other
Specify other medication	_____
Date commenced	_____
Current dose (at this visit)	_____
Target dose	_____
Date target dose reached	_____
Dose plan	◯ Decrease dose _____ ◯ No change ◯ Increase dose _____
Date medication ceased	_____

Maximum dose of medication reached	◯ Known, specify dose: _____ ◯ Unknown
Reason for stopping medication	◯ Ineffective ◯ Side effects ◯ Others _____
Side effects?	☐ appetite increase ☐ appetite decrease ☐ weight loss ☐ weight gain ☐ drowsy ☐ insomnia ☐ behavioural change ☐ allergy/rash ☐ headache ☐ stomachache ☐ nausea/vomiting ☐ others _____
Any additional details	_____

Appendix 4. Family and Pregnancy Screener

Family and Pregnancy Screener

Record ID _____

Record ID: [record_id]
NeuroCONNECT Participant ID: [part_id]
DOB: [dob]

FAMILY BACKGROUND OF PROBAND

	0	1	2	3	4	5 or more
Number of biological brothers of proband?	○	○	○	○	○	○
Number of biological sisters of proband?	○	○	○	○	○	○
Number of maternal aunts of proband?	○	○	○	○	○	○
Number of maternal uncles of proband?	○	○	○	○	○	○
Number of paternal aunts of proband?	○	○	○	○	○	○
Number of paternal uncles of proband?	○	○	○	○	○	○

AUTOIMMUNE CONDITIONS

Do any of the child's first degree (parents, siblings) and second degree (aunt, uncle, grandparents) relatives have any of the following conditions? If the child's mother has had any of these conditions, some extra details will be asked. ◯ Yes ◯ No

Hashimoto's thyroiditis
Graves' disease
Thyroid disease not otherwise specified
Type 1 diabetes
Vitiligo
Alopecia
Psoriasis
Celiac disease
Crohn's disease
Ulcerative colitis
Systemic lupus erythematosus
Lupus-like syndrome
Rheumatoid arthritis
Uveitis
Multiple sclerosis
Pernicious anemia
Autoimmune hepatitis
Polymyalgia rheumatica
Others: please specify

Autoimmune condition 1 Select relative _____ Select condition _____

Specify other condition

_____

Mother - extra details Age condition was diagnosed? _____ When was the condition present? _____ Flares or exacerbations during pregnancy? _____

Add another condition? ◯ Yes ◯ No

Autoimmune condition 2 Select relative _____ Select condition _____

Specify other condition

_____

Mother - extra details

Age condition was diagnosed?

_____

When was the condition present?

_____

Flares or exacerbations during pregnancy?

_____

Add another condition? ◯ Yes ◯ No

Autoimmune condition 3 Select relative _____ Select condition _____

Specify other condition _____

Mother - extra details

Age condition was diagnosed?

_____

When was the condition present?

_____

Flares or exacerbations during pregnancy?

_____

Add another condition? ◯ Yes ◯ No

Autoimmune condition 4 Select relative _____ Select condition _____

Specify other condition _____

Mother - extra details

Age condition was diagnosed?

_____

When was the condition present?

_____

Flares or exacerbations during pregnancy?

_____

Add another condition? ◯ Yes ◯ No

Autoimmune condition 5 Select relative _____ Select condition _____

Specify other condition _____

Mother - extra details

Age condition was diagnosed?

_____

When was the condition present?

_____

Flares or exacerbations during pregnancy?

_____

Add another condition? ◯ Yes ◯ No

Autoimmune condition 6 Select relative _____ Select condition _____

Specify other condition _____

Mother - extra details

Age condition was diagnosed?

_____

When was the condition present?

_____

Flares or exacerbations during pregnancy?

_____

Add another condition? ◯ Yes ◯ No

Autoimmune condition 7 Select relative _____ Select condition _____

Specify other condition _____

Mother - extra details

Age condition was diagnosed?

_____

When was the condition present?

_____

Flares or exacerbations during pregnancy?

_____

Add another condition? ◯ Yes ◯ No

Autoimmune condition 8 Select relative _____ Select condition _____

Specify other condition _____

Mother - extra details

Age condition was diagnosed?

_____

When was the condition present?

_____

Flares or exacerbations during pregnancy?

_____

Add another condition? ◯ Yes ◯ No

Autoimmune condition 9 Select relative _____ Select condition _____

Specify other condition _____

Mother - extra details

Age condition was diagnosed?

_____

When was the condition present?

_____

Flares or exacerbations during pregnancy?

_____

Add another condition? ◯ Yes ◯ No

Autoimmune condition 10 Select relative _____ Select condition _____

Specify other condition _____

Mother - extra details

Age condition was diagnosed?

_____

When was the condition present?

_____

Flares or exacerbations during pregnancy?

_____

ASTHMA AND ALLERGIC-TYPE CONDITIONS

Do any of the child's first degree (parents, siblings) and second degree (aunt, uncle, grandparents) relatives have any of the following conditions? If the child's mother has had any of these conditions, some extra details will be asked. ◯ Yes ◯ No

Asthma
Allergy (eg, grass, pollen, food allergies, medication allergies)
Hayfever
Eczema
Other inflammatory conditions: please specify

Asthma/allergic condition 1 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Asthma/allergic condition 2 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Asthma/allergic condition 3 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Asthma/allergic condition 4 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Asthma/allergic condition 5 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Asthma/allergic condition 6 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Asthma/allergic condition 7 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Asthma/allergic condition 8 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Asthma/allergic condition 9 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Asthma/allergic condition 10 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

PSYCHIATRIC CONDITIONS

Do any of the child's first degree (parents, siblings) and second degree (aunt, uncle, grandparents) relatives have any of the following conditions? If the child's mother has had any of these conditions, some extra details will be asked.

◯ Yes ◯ No

Anxiety
Depression: please specify
Post-traumatic stress disorder
Tics/Tourette syndrome
Obsessive-compulsive disorder
Attention-deficit hyperactivity syndrome
Autism spectrum disorder
Schizophrenia
Bipolar disorder
Eating disorder
Substance abuse
Intellectual disability
Learning difficulties
Other: please specify

Psychiatric condition 1 Select relative _____ Select condition _____

Specify depression details

- ☐ Major depressive disorder
- ☐ Perinatal depression
- ☐ Postnatal depression

Specify other condition

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Psychiatric condition 2 Select relative _____ Select condition _____

Specify depression details

- ☐ Major depressive disorder
- ☐ Perinatal depression
- ☐ Postnatal depression

Specify other condition

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Psychiatric condition 3 Select relative _____ Select condition _____

Specify depression details
☐ Major depressive disorder
☐ Perinatal depression
☐ Postnatal depression

Specify other condition
_____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Psychiatric condition 4 Select relative _____ Select condition _____

Specify depression details
☐ Major depressive disorder
☐ Perinatal depression
☐ Postnatal depression

Specify other condition
_____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Psychiatric condition 5 Select relative _____ Select condition _____

Specify depression details
☐ Major depressive disorder
☐ Perinatal depression
☐ Postnatal depression

Specify other condition
_____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Psychiatric condition 6 Select relative _____ Select condition _____

Specify depression details
☐ Major depressive disorder
☐ Perinatal depression
☐ Postnatal depression

Specify other condition

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Psychiatric condition 7 Select relative _____ Select condition _____

Specify depression details

- ☐ Major depressive disorder
- ☐ Perinatal depression
- ☐ Postnatal depression

Specify other condition

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Psychiatric condition 8 Select relative _____ Select condition _____

Specify depression details

- ☐ Major depressive disorder
- ☐ Perinatal depression
- ☐ Postnatal depression

Specify other condition

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Psychiatric condition 9 Select relative _____ Select condition _____

Specify depression details

- ☐ Major depressive disorder
- ☐ Perinatal depression
- ☐ Postnatal depression

Specify other condition

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Psychiatric condition 10 Select relative _____ Select condition _____

Specify depression details
☐ Major depressive disorder
☐ Perinatal depression
☐ Postnatal depression

Specify other condition
_____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

NEUROLOGICAL CONDITIONS

Do any of the child's first degree (parents, siblings) and second degree (aunt, uncle, grandparents) relatives have any of the following conditions? If the child's mother has had any of these conditions, some extra details will be asked.

Cerebral palsy
Migraine
Epilepsy
Others: please specify

◯ Yes ◯ No

Neurological condition 1 Select relative _____ Select condition _____

Specify other condition
_____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Neurological condition 2 Select relative _____ Select condition _____

Specify other condition
_____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Neurological condition 3 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Neurological condition 4 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Neurological condition 5 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Neurological condition 6 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Neurological condition 7 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Neurological condition 8 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Neurological condition 9 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Neurological condition 10 Select relative _____ Select condition _____

Specify other condition _____

For the child's mother, indicate when the condition was present.
Tick all that apply
☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

OTHER MEDICAL CONDITIONS

Do any of the child's first degree (parents, siblings) and second degree (aunt, uncle, grandparents) relatives have any of the following conditions? If the child's mother has had any of these conditions, some extra details will be asked.

◯ Yes ◯ No

High cholesterol
High blood pressure
Type 2 diabetes
Cardiovascular disease (eg. Heart attack, angina, bypass surgery)
Polycystic ovary syndrome
Endometriosis
Fatty liver disease
Periodontitis
Cholestasis
Cancer: specify type of cancer
Chronic fatigue
Fibromyalgia
Others: please specify

Other medical condition 1 Select relative _____ Select condition _____

Specify other condition

_____

Specify type of cancer

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Other medical condition 2 Select relative _____ Select condition _____

Specify other condition

_____

Specify type of cancer

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

☐ Before pregnancy
☐ During pregnancy
☐ After pregnancy
☐ Currently present

Add another condition? ◯ Yes ◯ No

Other medical condition 3 Select relative _____ Select condition _____

Specify other condition

_____

Specify type of cancer

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Other medical condition 4 Select relative _____ Select condition _____

Specify other condition

_____

Specify type of cancer

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Other medical condition 5 Select relative _____ Select condition _____

Specify other condition

_____

Specify type of cancer

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Other medical condition 6 Select relative _____ Select condition _____

Specify other condition

_____

Specify type of cancer

_____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Other medical condition 7 Select relative _____ Select condition _____

Specify other condition _____

Specify type of cancer _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Other medical condition 8 Select relative _____ Select condition _____

Specify other condition _____

Specify type of cancer _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Other medical condition 9 Select relative _____ Select condition _____

Specify other condition _____

Specify type of cancer _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

Add another condition? ◯ Yes ◯ No

Other medical condition 10 Select relative _____ Select condition _____

Specify other condition _____

Specify type of cancer _____

For the child's mother, indicate when the condition was present.
Tick all that apply

- ☐ Before pregnancy
- ☐ During pregnancy
- ☐ After pregnancy
- ☐ Currently present

PREGNANCY HISTORY OF CHILD'S BIOLOGICAL MOTHER

Was your child's conception natural or with assistive technologies such as IVF and ICSI?

◯ Natural
◯ Assisted (Specify type e.g. IVF, ICSI, IUI) _____
(Assisted does not include clomiphene (Clomid))

Did you have any infections during pregnancy? ◯ Yes ◯ No

Influenza
Chest infection
Urinary tract infection
Hepatitis B
Hepatitis C
HIV
Others: please specify

Infection 1 Select infection

_____

Select trimester

_____

Hospitalisation required?

_____

Specify treatments required

_____

If 'Other infection', specify here _____

Add another infection? _____

Infection 2 Select infection _____ Select trimester _____ Hospitalisation required? _____ Specify treatments required _____ If 'Other infection', specify here _____ Add another infection? _____

Infection 3 Select infection _____ Select trimester _____ Hospitalisation required? _____ Specify treatments required _____ If 'Other infection', specify here _____ Add another infection? _____

Infection 4 Select infection _____ Select trimester _____ Hospitalisation required? _____ Specify treatments required _____ If 'Other infection', specify here _____ Add another infection? _____

Infection 5 Select infection

_____

Select trimester

_____

Hospitalisation required?

_____

Specify treatments required

_____

Other details

If 'Other infection', specify here _____

Did you take any medications during pregnancy? ◯ Yes ◯ No

Specify medications used during pregnancy

_____

Did you smoke during pregnancy? ◯ Yes ◯ No

On average, how many cigarettes per day?

E.g., 25 per day

_____

Did you drink any alcohol during pregnancy? ◯ Yes ◯ No

On average, how many standard drinks of alcohol per week? ◯ 1-2 ◯ 3-4 ◯ 5-6 ◯ 7-9 ◯ 10+

Mother's Weight and Height Mother's pre-pregnancy weight (kg)_____ Mother's current weight (kg) _____

Mother's height (cm)_____

Mother's pre-pregnancy BMI _____

Mother's current BMI _____

Father's Weight and Height Father's current weight (kg) _____ Father's height (cm)_____ Father's current BMI _____

Did you experience any complications during pregnancy or delivery?
Please select the answer that best describes the mother's experience of pregnancy with this child.

Some examples of complications:
High blood pressure
Gestational diabetes
Spotting
Threatened preterm labour

◯ Optimal: No complications. Generally free of any discomfort.
◯ Normal: No complications. Occasional periods of discomfort, no physical restrictions.
◯ Mild: Mild complications that require an altered complications life-style but no medical interventions other than increased monitoring.
◯ Moderate: Moderate complications. One or more complications associated with some threats to mother's or foetus's well-being that typically require medical intervention
◯ Severe: Serious threat to health of mother or foetus. Complications such as preeclampsia associated with the need for hospitalisation or extended care.

Were any of the following complications present during pregnancy or delivery? ◯ Yes ◯ No

High blood pressure
Proteinuria
Pre-eclampsia
Seizures
Hyperemesis gravidarum
Gestational diabetes
Placenta praevia
Spotting
Antepartum haemorrhage
Pregnancy-induced cholestasis
Threatened preterm labour
Fetal abnormalities or antenatal scan
Cervical shortening
Group B Streptococcal colonisation
Chorioamnionitis
Others: please specify

Complication 1 Select complication _____ Select trimester _____ If 'Other complication', specify here _____ Add another complication? _____

Complication 2 Select complication _____ Select trimester _____ Other details If 'Other complication', specify here _____ Add another complication? _____

Complication 3 Select complication _____ Select trimester _____ Other details If 'Other complication', specify here _____ Add another complication? _____

Complication 4 Select complication

_____

Select trimester

_____

Other details

If 'Other complication', specify here _____

Add another complication? _____

Complication 5 Select complication

_____

Select trimester

_____

Other details

If 'Other complication', specify here _____

Did you experience any notable or stressful life-events during pregnancy?
Please select the answer that best describes the mother's experience of pregnancy with this child.

- ◯ None
- ◯ Notable events but not stressful.
- ◯ Notable events associated with some increased stress, such as short-term changes in work schedule, brief periods of increased debt, or brief periods of heightened marital conflict.
- ◯ Events are associated with clear periods of increased stress, such as major change in job responsibilities, or illness of a family member.
- ◯ Events are associated with severe stress typically resulting in a disruption of existing patterns of family life such as severe marital conflict with threat of separation, serious injury to a parent, or brief periods of unemployment.
- ◯ Stressful events associated with permanent or extended periods of time such as divorce, unremitting marital discord associated with physical abuse, unexpected loss of a family member, loss of residence, or life-threatening illness

Neonate info Gestational age at delivery (weeks)

_____

Birthweight (kg)

_____

Mode of delivery

_____

Neonatal intubation or ventilation?

_____

If yes, number of days _____

Neonatal lung disease?

_____

Neonatal seizures?

_____

Infection in the first month of life?

_____

If yes, details of infection _____

Admission to NICU or Special Baby Care Unit?

_____

If yes, specify reason for admission _____

Date questionnaire completed _____

Appendix 5. Maternal Current Health Screener

Maternal Current Health Screener

Please complete the questionnaire below.

Thank you!

CHILD'S BIOLOGICAL MOTHER'S CURRENT HEALTH

The following questionnaire is about the current health of the child's biological mother. It is important for us to understand the mother's health and family situation as they contribute to the child's overall well-being. Please try to answer all questions.

Mother's DOB _____

Father's DOB _____

Are you currently taking any medications or receiving any psychological intervention?	◯ Yes ◯ No
If yes, provide details	_____
Do you currently smoke?	◯ Yes ◯ No
On average, how many cigarettes per day?	_____
Do you currently drink alcohol?	◯ Yes ◯ No
On average, how many standard drinks of alcohol per week?	◯ 1-2 ◯ 3-4 ◯ 5-6 ◯ 7-9 ◯ 10+
Did you have any infection in the previous 2 weeks?	◯ No ◯ Yes

CHILD'S BIOLOGICAL MOTHER'S SOCIAL BACKGROUND

Ancestry
(Select up to two options only)

- ☐ Australian
- ☐ Aboriginal
- ☐ Torres Strait Islander
- ☐ European
- ☐ Middle Eastern
- ☐ Indian
- ☐ Chinese
- ☐ South East Asian
- ☐ African
- ☐ Pacific Islander
- ☐ Other _____

What is your postcode?

_____

Postcode at time when child was born?

_____

What is your marital status?

- ◯ Never married
- ◯ Widowed
- ◯ Divorced
- ◯ Separated
- ◯ Married

What is your highest level of education?

- ◯ Primary School
- ◯ High School to end of year 10, 11 or 12
- ◯ TAFE or college certificate or diploma
- ◯ Trade/apprenticeship
- ◯ University degree
- ◯ Postgraduate study
- ◯ Other _____

Which situation best describes your household?

- ◯ Both biological or adoptive parents are still living in the same household
- ◯ Sole parent family after divorce/separation
- ◯ Sole parent family, other parent has never been involved or is deceased
- ◯ Step family (two parents, one being a step parent)
- ◯ Foster family
- ◯ Grandparents
- ◯ Other (please provide additional details below)

Please provide additional detail about household situation if not identified above.

_____

Do you own your own house?

◯ Yes ◯ No

Are you eligible for, or do you have a low-income healthcare card?

◯ Yes ◯ No

STRESSFUL LIFE EVENTS Have you (child's biological mother) ever experienced any stressful life events? This includes any that you experienced personally, witnessed happen to someone else, learnt about happening to a close family member or friend, or been exposed to as part of your job?

Natural disaster
Fire or explosion
Transportation accident
Serious accident at work, home or during recreational activity
Exposure to toxic substance
Physical assault
Assault with a weapon
Sexual assault
Other unwanted or uncomfortable sexual experience
Combat or exposure to war zone
Captivity
Life-threatening illness or injury
Severe human suffering
Sudden violent death of a close friend or relative
Sudden accidental death of a close friend of relative
Serious injury, harm or death you caused to someone else
Any other very stressful event or experience

How many TYPES of these stressful life events have you experienced at any point in your life? _____
How many TYPES of these stressful life events did you experience before you were pregnant? _____
How many TYPES of these stressful life events did you experience during pregnancy? _____

PERCEIVED STRESS SCALE

The questions in this scale ask you (child's biological mother) about your feelings and thoughts during the LAST MONTH. In each case, you will be asked to indicate how often you felt or thought a certain way.

	Never	Almost Never	Sometimes	Fairly Often	Very Often
1. In the last month, how often have you been upset because of something that happened unexpectedly?	○	○	○	○	○
2. In the last month, how often have you felt that you were unable to control the important things in your life?	○	○	○	○	○
3. In the last month, how often have you felt nervous and "stressed"?	○	○	○	○	○
4. In the last month, how often have you felt confident about your ability to handle your personal problems?	○	○	○	○	○
5. In the last month, how often have you felt that things were going your way?	○	○	○	○	○
6. In the last month, how often have you found that you could not cope with all the things that you had to do?	○	○	○	○	○
7. In the last month, how often have you been able to control irritations in your life?	○	○	○	○	○
8. In the last month, how often have you felt that you were on top of things?	○	○	○	○	○
9. In the last month, how often have you been angered because of things that were outside of your control?	○	○	○	○	○
10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	○	○	○	○	○

Date questionnaire completed _____

Appendix 6. Child Health and Inflammation Screener

Child Health and Inflammation Screener

Record ID

_____

Record ID: [record_id]
NeuroCONNECT Participant ID: [part_id]
DOB: [dob]

Child's current weight (kg)?

_____

Child's current height (cm)?

_____

Child's current BMI

_____

GI PROBLEMS

Has your child ever been diagnosed and treated with medication for any of the following gastrointestinal problems?

	Yes	No	Not sure
Gastroesophageal reflux	◯	◯	◯
Constipation	◯	◯	◯
Diarrhoea	◯	◯	◯

INFECTION SCREENER

Has your child experienced any of the following infections or required medical attention for infections in the FIRST 5 YEARS or LAST 12 MONTHS of life? ◯ Yes ◯ No

Clear runny nose
Urinary tract infection
Throat infection/tonsilitis
Ear infection with pain or pus
Sinus infection
Pneumonia
Mouth ulcers
Skin infection (impetigo)
Meningitis or other serious infection (bone, joint, blood)
GP visit for infection
Antibiotic courses
Emergency department visit for infection
Hospitalisation for infection
Other significant infection

Infection/Medication Attention 1 Select infection/medical attention _____
Other infection/other important details _____
Select when present/frequency

First 5 years of life _____
Last 12 months of life _____
Add another infection? _____

Infection/Medication Attention 2 Select infection/medical attention _____
Other infection/other important details _____
Select when present/frequency

First 5 years of life _____
Last 12 months of life _____
Add another infection? _____

Infection/Medication Attention 3 Select infection/medical attention _____
Other infection/other important details _____
Select when present/frequency

First 5 years of life _____
Last 12 months of life _____
Add another infection? _____

Infection/Medication Attention 4 Select infection/medical attention _____
Other infection/other important details _____
Select when present/frequency

First 5 years of life _____
Last 12 months of life _____
Add another infection? _____

Infection/Medication Attention 5 Select infection/medical attention _____
Other infection/other important details _____
Select when present/frequency

First 5 years of life _____
Last 12 months of life _____
Add another infection? _____

Infection/Medication Attention 6 Select infection/medical attention _____
Other infection/other important details _____
Select when present/frequency

First 5 years of life _____
Last 12 months of life _____
Add another infection? _____

Infection/Medication Attention 7 Select infection/medical attention _____
Other infection/other important details _____
Select when present/frequency

First 5 years of life _____
Last 12 months of life _____
Add another infection? _____

Infection/Medication Attention 8 Select infection/medical attention _____
Other infection/other important details _____
Select when present/frequency

First 5 years of life _____
Last 12 months of life _____
Add another infection? _____

Infection/Medication Attention 9 Select infection/medical attention _____
Other infection/other important details _____
Select when present/frequency

First 5 years of life _____
Last 12 months of life _____
Add another infection? _____

Infection/Medication Attention 10 Select infection/medical attention _____
Other infection/other important details _____
Select when present/frequency

First 5 years of life _____
Last 12 months of life _____

ASTHMA AND ALLERGIC-TYPE CONDITIONS

Does your child have any of the following allergic-type conditions?

- ☐ None
- ☐ Asthma
- ☐ Allergy
- ☐ Hayfever
- ☐ Eczema
- ☐ Other inflammatory/immune condition _____

Please specify which allergies your child has

- ☐ Grasses / pollens
- ☐ Dust mite
- ☐ Food allergies
- ☐ Penicillin / medication allergies
- ☐ Other _____

Which of the following treatments have been tried for these condition(s)?

	Currently used (within the last 2 weeks)	Previously used	Never used
Reliever inhaler for asthma	☐	☐	☐
Preventer inhaler for asthma	☐	☐	☐
Oral medication for asthma other than steroid (e.g. montelukast)	☐	☐	☐
Oral steroid for asthma	☐	☐	☐
Emergency department treatment for asthma	☐	☐	☐
Anti-histamines (e.g. Phenergan, Zyrtec) for allergies	☐	☐	☐
Epipen (adrenaline) for allergies	☐	☐	☐
Anti-histamines (e.g. Phenergan, Zyrtec) for hayfever	☐	☐	☐
Nasal spray for hayfever	☐	☐	☐

Moisturising creams for eczema	☐	☐	☐
Steroids creams for eczema	☐	☐	☐

AUTOIMMUNE CONDITIONS

Does your child have any of the following autoimmune diseases?

- ☐ None
- ☐ Graves' disease
- ☐ Hashimoto's disease
- ☐ Thyroid disease not otherwise specified
- ☐ Type 1 diabetes
- ☐ Vitiligo
- ☐ Alopecia
- ☐ Psoriasis
- ☐ Coeliac disease
- ☐ Crohn's disease
- ☐ Ulcerative colitis
- ☐ SLE / Lupus
- ☐ Lupus-like syndrome
- ☐ Rheumatoid arthritis
- ☐ Other inflammatory arthritis _____
- ☐ Uveitis
- ☐ Multiple sclerosis
- ☐ Pernicious anaemia
- ☐ Autoimmune hepatitis
- ☐ Polymyalgia rheumatica
- ☐ Scleroderma
- ☐ Sjogren's syndrome
- ☐ Other _____

Is the autoimmune disease(s) for the child possible or confirmed?
'Confirmed' - tests conducted by a specialist (e.g. blood tests, scans / tissue biopsy) have been done and were diagnostic.
'Possible' - tests are still to be done or are inconclusive.

	Confirmed by specialist	Possible
Hashimoto's disease	◯	◯
Graves' disease	◯	◯
Thyroid disease not otherwise specified	◯	◯
Type 1 diabetes	◯	◯
Vitiligo	◯	◯
Alopecia	◯	◯
Psoriasis	◯	◯
Coeliac disease	◯	◯
Crohn's disease	◯	◯
Ulcerative colitis	◯	◯
Systemic Lupus Erythematosus (SLE) / Lupus	◯	◯
Lupus-like syndrome	◯	◯

Rheumatoid arthritis or other inflammatory arthritis (not osteoarthritis)	◯	◯
Uveitis	◯	◯
Multiple sclerosis	◯	◯
Pernicious anaemia	◯	◯
Autoimmune hepatitis	◯	◯
Polymyalgia rheumatica	◯	◯
Scleroderma	◯	◯
Sjogren's syndrome	◯	◯
[ai_proband_other]	◯	◯

Has your child required treatment for their autoimmune disease(s)?

◯ Yes ◯ No
(e.g. gluten-free diet for coeliac disease, thyroxine for thyroid disease, anti-inflammatory medications, steroid)

Please specify the name or describe the treatment(s)

_____

Is your child currently on treatment for their autoimmune disease(s) (within the last 2 weeks)?

◯ Yes ◯ No

Please specify your child's current treatment(s) (if different from above)

_____

NEURODEVELOPMENTAL SYMPTOMS

What is the reason your child has been referred to neurology clinic?

☐ Tics
☐ Obsessive Compulsive Symptoms
☐ Autism Spectrum Disorder
☐ Other _____

How long did it take for your child's neurodevelopmental symptoms (e.g. tics, OCD, anxiety, ADHD, ASD) to become their most intense?

◯ Less than 24 hours
◯ Less than 1 week
◯ Less than 6 weeks
◯ 6 weeks or longer

Was there any apparent trigger for your child's neurodevelopmental symptoms (e.g. tics, OCD, anxiety, ADHD, ASD) in the four weeks before they started?

◯ Yes ◯ No

Specify the trigger(s)

☐ Infection
☐ Other illness
☐ Medication
☐ Stressful event
☐ Other _____

Did your child lose any skills at the onset of his/her neurodevelopmental symptoms? (e.g. tics, OCD, anxiety, ADHD, ASD)

EXAMPLES
-Learning ability (e.g. attention / concentration)
-Language
-Social skills
-Fine motor skills (e.g. handwriting)
-Gross motor skills (e.g. balance and coordination)

◯ Yes ◯ No

Please check which skills your child lost at the onset of his/her neurodevelopmental symptoms

☐ Learning ability (e.g. attention and concentration)
☐ Language (loss of vocabulary)
☐ Social Skills
☐ Fine motor skills (e.g. handwriting)
☐ Gross motor skills (e.g. balance and coordination)
☐ Other _____

Did your child lose any skills over time?

EXAMPLES
-Learning ability (e.g. attention / concentration)
-Language
-Social skills
-Fine motor skills (e.g. handwriting)
-Gross motor skills (e.g. balance and coordination)

◯ Yes ◯ No

Please check which skills your child has lost over time

☐ Learning ability (e.g. attention and concentration)
☐ Language (loss of vocabulary)
☐ Social skills
☐ Fine Motor Skills (e.g. handwriting)
☐ Gross Motor Skills (e.g. balance and coordination)
☐ Other _____

How do the following triggers affect your child's neurodevelopmental symptoms? Please specify if there are any other factors that make your child's symptoms better or worse.

	No change to symptoms	Worsen symptoms	Improve symptoms
Stress	◯	◯	◯
Infection	◯	◯	◯
Excitement	◯	◯	◯
Other _____	◯	◯	◯

Does your child have any sensory regulation issues?

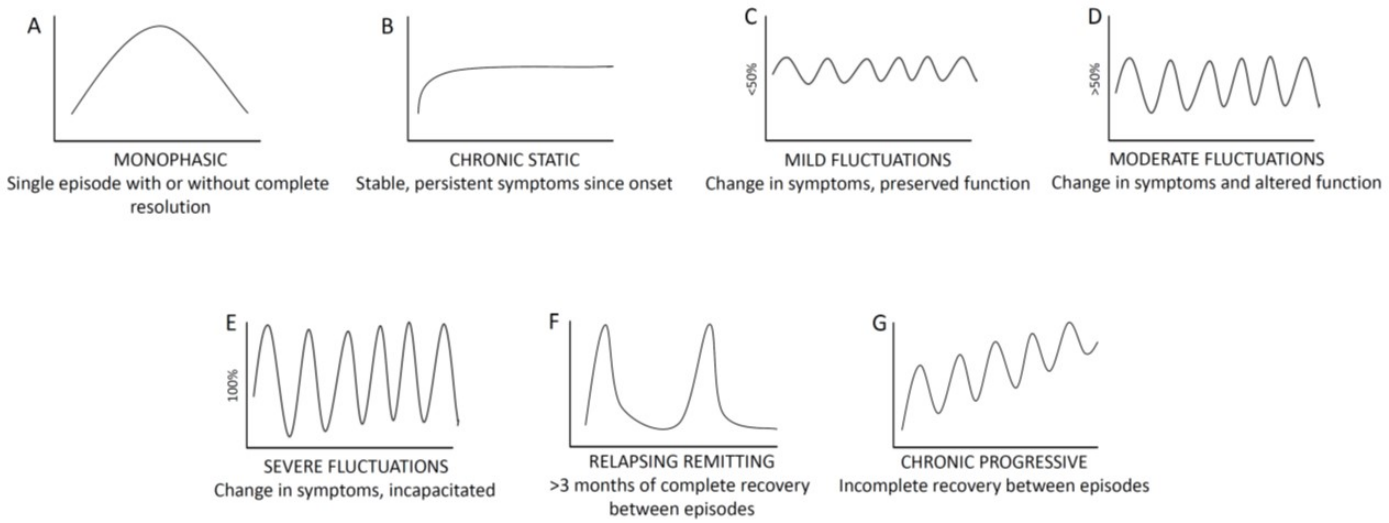
E.g. sensitivity to certain textures/sounds

◯ Yes ◯ No

Select items that your child is sensitive to

☐ Clothing
☐ Sound
☐ Light
☐ Food
☐ Others _____

Which graph best depicts the pattern of your child's illness over time?



Which graph best depicts the pattern of your child's neurodevelopmental symptoms over time?	◯ A ◯ B ◯ C ◯ D ◯ E ◯ F ◯ G ◯ None of the above
Has your child ever missed any school because of their neurodevelopmental symptoms (excluding medical appointments)?	◯ Yes ◯ No
Has your child been unable to attend their usual school for at least 3 months at any time because of their neurodevelopmental symptoms?	◯ Yes ◯ No
Has your child ever presented to the emergency department for their neurodevelopmental symptoms?	◯ Yes ◯ No
Has your child ever been admitted to the hospital because of their neurodevelopmental symptoms?	◯ Yes ◯ No

STRESS/TRAUMA

Has your child ever suffered a stressful event in their life? (e.g. traumatic event, loss of loved one)	◯ Yes ◯ No
If yes, please state the child's age when the stressful event/s occurred	_____
Did the child have a stressful reaction to this event?	◯ Yes ◯ No
Does your child have 'fight and flight' episodes? These are 'fear episodes' which are excessive and cause physical anxiety with dilated pupils, trembling, rapid heart, looking pale or terrified, on edge, or the child makes a bolt for the door (run away), or move restlessly?	◯ Yes ◯ No
If yes, how often do these fight or flight episodes occur?	◯ Occasional (every now and then) ◯ Sometimes (once a month) ◯ Frequently (once a week) ◯ Always (every day)
How do the fight or flight episodes affect the child's function?	◯ No impact on function, and no distress ◯ Some distress, but manageable with reassurance ◯ Significant distress, and sometimes difficult to manage ◯ Major distress and potential danger to safety
Date questionnaire completed	_____

Appendix 7. Neuro-Tx research script template for IESS control group

1. Introduction (confirm consent):

Thank you for consenting to be involved in research run by the neurology and surgery departments. As you know we need healthy children to serve as a comparison group for children with epilepsy – the neurology team are working out why and how epilepsy happens and how treatment helps them. Are you happy for the study researcher (Dr Emily Innes only) to review your child's medical record or discuss your child's health so I can confirm they are eligible to participate?

2. Proceed to medical record review/ screening questions

3. Screening questions:

1. Does your child any have major medical conditions? If yes, please list them
2. Does your child have any neurological conditions (e.g. seizures, tics), any difficulties with their development or learning (delay) or Autism?
3. Does your child have any problems with their immune system (e.g immune system attacking itself – diabetes, coeliac). N.B. (mild asthma/eczema accepted)
4. Has your child had any infections or antibiotics in the last two weeks?
5. Any other medical issues worth mentioning?

Reference for screening clinician/researcher only:
Criteria –Aiming for well infants and children without any neurological/immune/inflammatory/metabolic/genetic diagnoses and normal neurodevelopment i.e. no development delay whether isolated or global.

Exclusion criteria:

- Prematurity (<37 weeks)
- Recurrent febrile seizures
- Epilepsy/Cerebral Palsy/Stroke/other syndrome/ neurological diagnosis
- Developmental delay (Global i.e. two or more developmental domains affected, or single area of delay/receiving therapy)
- Attention Deficit Hyperactivity Disorder
- Autism Spectrum Disorder
- Obsessive compulsive disorder
- Tic disorder
- Genetic or metabolic disorder
- Severe asthma, significant allergy, anaphylaxis
- Immunodeficiency

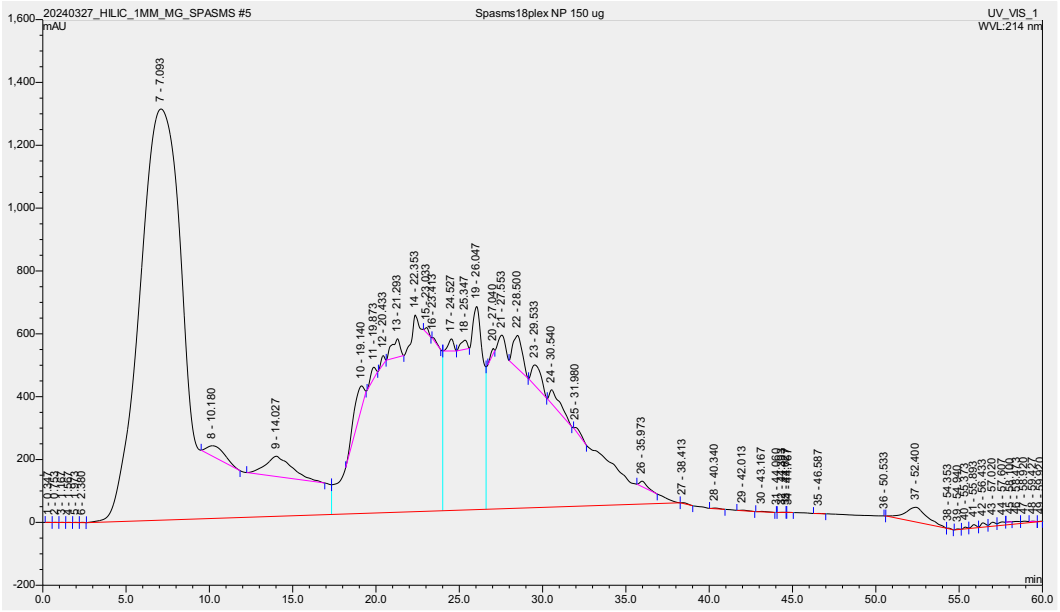
Appendix 8. Proteomic sequencing detailed methodology

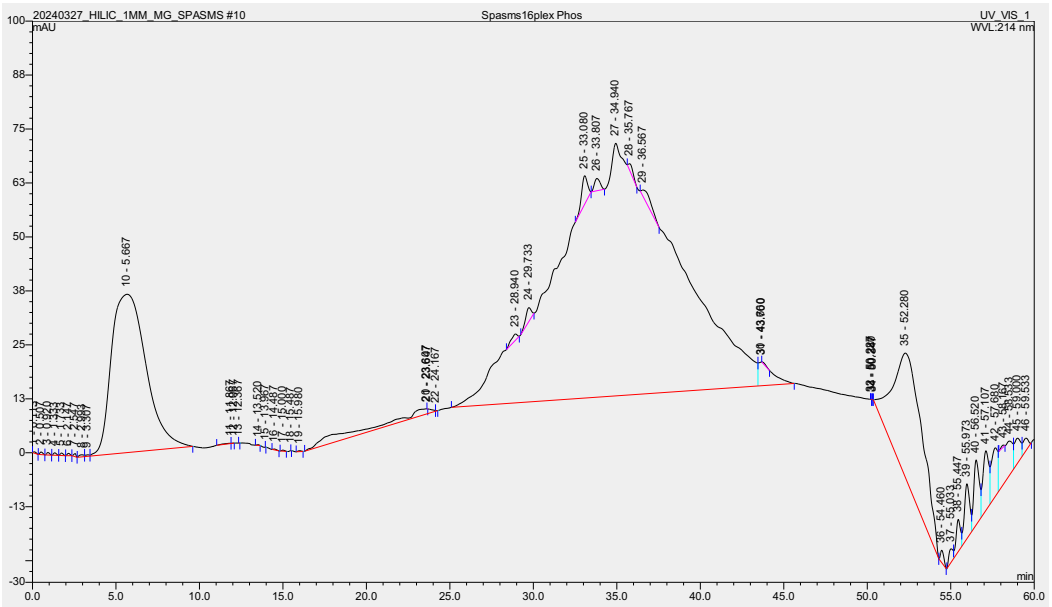
Preparation for LC-MS analysis (provided by Dr Mark Graham):
Samples were provided as frozen lysates (~ 200 uL). Samples were thawed by incubating at 85 °C for 5 min. Samples were cooled and then incubated at 37 °C for 30 min with 10 units of benzonase. 100 µL of 10% SDS was added and enough water to make up to 400 µL. Samples were incubated at 85 °C for 10 min with10 mM TCEP. Samples were sonicated and then cooled. Iodoacetamide (20 mM FC) was added and the samples were incubated at 23 °C for 30 min. The samples were then precipitated using the chloroform-methanol method. Pellets were dried at 37 °C for 1 h. 10 µL of 7.8 M Urea/100 mM HEPES pH 8.0/LysC solution was added (2 ug LysC). LysC digestion was for 12 h at 28 °C. Two typsin digestions were done for 8 h at 28 °C, each with 2 µg of trypsin (TrypZean, Sigma). The approximate amount of protein was determined by UV absorption at 280 nM.

Sample ID	**Group**	**Pre/Post**	**Date**	**mg/mL**	**Volume (uL)**	**Amount (ug)**	TMT label	MQ Header
CH1555	Patient	Pre	12-Oct-23	9.5415	120	1144.98	126	Reporter intensity corrected 1
CH3405	Patient	Pre	23-May-22	9.3705	120	1124.46	127N	Reporter intensity corrected 2
CH9275	Patient	Pre	23-Sep-23	5.9805	120	717.66	127C	Reporter intensity corrected 3
CH6129	Patient	Pre	6-Oct-22	5.124	120	614.88	128N	Reporter intensity corrected 4
CH9006	Patient	Pre	4-Feb-22	0.4485	40	17.94	128C	Reporter intensity corrected 5
CH1436	Patient	Pre	22-Feb-23	2.2485	120	269.82	129N	Reporter intensity corrected 6
CH1555	Patient	Post	19-Oct-23	10.83	120	1299.36	129C	Reporter intensity corrected 7
CH3405	Patient	Post	6-Jun-22	2.49	120	298.8	130N	Reporter intensity corrected 8
CH9275	Patient	Post	29-Sep-23	4.98	120	597.6	130C	Reporter intensity corrected 9
CH6129	Patient	Post	14-Oct-22	13.30	120	1596.48	131N	Reporter intensity corrected 10
CH9006	Patient	Post	16-Feb-22	2.13	40	85.26	131C	Reporter intensity corrected 11
CH1436	Patient	Post	1-Mar-23	5.76	120	691.02	132N	Reporter intensity corrected 12
CH6697	Control	NA	18-Dec-23	6.04	120	724.68	132C	Reporter intensity corrected 13
CH7457	Control	NA	16-Oct-23	12.31	120	1477.08	133N	Reporter intensity corrected 14
CH6637	Control	NA	18-Dec-23	5.41	120	649.26	133C	Reporter intensity corrected 15
CH2700	Control	NA	10-Jul-23	3.52	120	422.46	134N	Reporter intensity corrected 16
CH9367	Control	NA	25-Sep-23	5.12	120	613.8	134C	Reporter intensity corrected 17
CH7100	Control	NA	31-Jul-23	3.54	120	425.16	135N	Reporter intensity corrected 18

Since the peptide amount was low for one patient (CH9006), that patient was excluded from the phosphoproteomics so that a larger amount of sample could be used from each patient/control.
For most samples 220 ug was labelled with TMTpro reagents (Lot: lot VJ313476, XC343801.
I confirmed that the TMT labelling worked by short run LC-MS/MS (45 min/sample).
I used 5% hydroxylamine to quench the TMT then mixed together, acidified and dried to a small volume (~50 µL).
The sample was put through the "TiSH" phosphopeptide enrichment method to produce sample ready for phosphoproteomics.
For proteomics, 150 µg of de-enriched phosphopeptide (mainly peptides) was cleaned/desalted using solid phase extraction (SPE) cartridge (Oasis, Waters).
The SPE eluate was applied to HILIC. The HILIC method was performed as described in published work.
HILIC fractionation was performed on a Dionex Ultimate 3000 HPLC system with a 250 mm long and 1 mm inside diameter TSKgel Amide-80 column (Tosoh Biosciences). The HILIC

gradient used 90% acetonitrile, 0.1% TFA (Buffer A) and a solution of 0.1% TFA (Buffer B). The sample was injected into a 250 µl sample loop. The flow rate was 60 µl/min in Buffer A for 10 min to load the sample. The gradient was from 100% Buffer A to 60% Buffer A for 35 min at a flow rate of 50 µl/min. Fractions were collected into a 96-well plate using a Probot (LC Packings) at 30-second intervals, monitored by absorbance of UV at 214 nm. The UV signal was used to combine selected fractions into similar amounts of peptide. Fractions were dried and reconstituted in 0.1% formic acid for LC-MS/MS analysis.





The LC-MS/MS was performed using a Dionex UltiMate 3000 RSLC nano system and Q Exactive Plus hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific). Each HILIC mono-phosphopeptide, multi-phosphopeptide or non-phosphopeptide enriched fraction was loaded directly onto an in-house 300 mm long 0.075 mm inside diameter column packed with ReproSil Pur C18 AQ 1.9 µm resin (Dr Maisch, Germany). The column was heated to 50 °C using a column oven (PRSO-V1, Sonation lab solutions, Germany)

integrated with the nano flex ion source with an electrospray operating at 2.3 kV. The S lens radio frequency level was 50 and capillary temperature was 250 °C.

For phosphopeptide enriched fractions:
The 5 µL sample was injected into a 20 µL loop and loaded onto the column in 99% reversed phase buffer A (solution of 0.1% formic acid) and 1% buffer B (solution of 0.1% formic acid, 90% acetonitrile). The loading was for 25 min at 300 nL/min using 1% buffer B. The gradient, at 250 nL/min, was from 1% buffer B to 5% buffer B in 1 min, to 25% buffer B in 74 min, to 35% buffer B in 8 min, to 99% buffer B in 1 min, held at 99% buffer B for 2 min, to 99% buffer A in 1 min and held for 8 min as the flow rate increased to 275 nL/min. MS acquisition was performed for the entire 120 min.

For non-phosphopeptide enriched fractions:
The 3.5 µL sample was injected into a 20 µL loop and loaded onto the column in 99% reversed phase buffer A (solution of 0.1% formic acid) and 1% buffer B (solution of 0.1% formic acid, 90% acetonitrile). The loading was for 17.5 min at 300 nL/min using 1% buffer B. The gradient, at 250 nL/min, was from 1% buffer B to 7% buffer B in 1 min, to 29% buffer B in 101.5 min, to 36% buffer B in 8 min, to 99% buffer B in 1 min, held at 99% buffer B for 2 min, to 99% buffer A in 1 min and held for 8 min as the flow rate increased to 300 nL/min. MS acquisition was performed for the entire 140 min.

All samples and fractions were analysed using data-dependent acquisition LC-MS/MS. The MS scans were at a resolution of 70,000 with an automatic gain control target of 1,000,000 for a maximum ion time of 100 ms from m/z 375 to 1500. The MS/MS scans were at a resolution of 35,000 with an automatic gain control target of 200,000 and maximum ion time of 100 ms (115 ms for phosphopeptide enriched fractions). The loop count was 12, the isolation window was 1.1 m/z, the first mass was fixed at m/z 120 and the normalized collision energy was 31 (34 for phosphopeptide enriched fractions. Singly charged ions and those with charge >8 were excluded from MS/MS and dynamic exclusion was for 35 s.

The raw LC-MS/MS data was processed with MaxQuant v1.6.7.0. Variable modifications were oxidation (M), acetyl (protein N-terminus), deamidation (NQ) and phosphorylation (STY). Carbamidomethyl (C) was a fixed modification. Digestion was set to trypsin/P with a maximum of 3 missed cleavages. The TMTpro correction factors were entered. Minimum reporter peptide ion faction was 0.6. The *Homo sapiens* reference proteome with canonical and isoform sequences downloaded March 4, 2024 with 82,485 entries and 20,597 genes. The inbuilt contaminants fasta file was also used. Second peptides search was enabled. The peptide spectrum matching and protein false discovery rates were set at 1%. All modified peptides and counterpart non-modified peptides were excluded from protein quantification. All other settings were default.