NOVEL MARKERS OF NEUROINFLAMMATION IN CEREBROSPINAL FLUID

By

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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.

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Abbreviations

1D LC-MS	One-Dimensional Liquid Chromatography-Mass Spectrometry
2D LC-MS	Two-Dimensional Liquid Chromatography-Mass Spectrometry
Ab	Antibody
AbNAE	Antibody Negative Autoimmune Encephalitis
AbPAE	Antibody Positive Autoimmune Encephalitis
ACE	Angiotensin-Converting Enzyme
ACN	Acetonitrile
AE	Autoimmune Encephalitis
AMPA R	Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor
ANA	Antinuclear Antibody
ANNA-1	Anti-Neuronal Nuclear Antibody Type 1
APC	Antigen Presenting Cell
APC-AF750	Allophycocyanin-Alexa Fluor 750
AUC	Area Under the Curve
BAFF	B-cell Activating Factor
BBB	Blood-Brain Barrier
BCA-1	B Cell-Attracting Chemokine 1
BCB	Blood-CSF Barrier
BCR	B Cell Receptor
BLIMP6	B Lymphocyte-Induced Maturation Protein 6
BoxCar	BoxCar Acquisition Strategy
BV421	Brilliant Violet 421 (fluorophore)
CARTPT	Cocaine- and Amphetamine-Regulated Transcript Protein
CASPR2	Contactin-Associated Protein-Like 2

CCL	C-C Motif Chemokine Ligand (e.g., CCL17)
CD	Cluster of Differentiation (e.g., CD3, CD4, CD8, CD19, CD20)
CEND1	Cell cycle exit and neuronal differentiation 1
CI	Confidence Interval
CHI3L-	Chitinase-3-Like Protein e.g. CHI3L1, CHI3L2
Chk-	Checkpoint kinase-
cGAS	Cyclic Guanosine Monophosphate-Adenosine Monophosphate
cGAMP	Cyclic Guanosine Monophosphate-Adenosine Monophosphate Synthase
CNS	Central Nervous System
CNTNAP4	Contactin Associated Protein Family Member 4
COX-2	Cyclooxygenase-2
CPC	Charles Perkin Centre
CRP	C-Reactive Protein
CS/DS	Chondroitin Sulfate / Dermatan Sulfate
CSF	Cerebrospinal Fluid
CT	Computed Tomography
CXCL	C-X-C Motif Chemokine Ligand (e.g., CXCL9, CXCL10/IP-10, CXCL11)
DAMP	Damage-Associated Molecular Pattern
DDA	Data-Dependent Acquisition
DIA	Data-Independent Acquisition
DNA	Deoxyribonucleic Acid
DPPX	Dipeptidyl-Peptidase-Like Protein 6
dsDNA	Double-Stranded DNA
DTT	Dithiothreitol
EAE	Experimental Autoimmune Encephalomyelitis

EBV	Epstein-Barr Virus
EEG	Electroencephalogram
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunospot (not directly mentioned but implied in cytokine detection context)
ENA	Extractable Nuclear Antigen
ENTV	Enterovirus
EphA5	Ephrin type-A receptor 5
ESR	Erythrocyte Sedimentation Rate
Fc	Fragment Crystallizable Region of Antibody
FABP3	Fatty Acid Binding Protein 3
FDG-PET	Fluorodeoxyglucose Positron Emission Tomography
FDR	False Discovery Rate
FCGR	Fc Gamma Receptor
FGFR	Fibroblast Growth Factor Receptor
FITC	Fluorescein Isothiocyanate
FLC	Free Light Chains
GABA-A/B	Gamma-Aminobutyric Acid Type A/B
GAD	Glutamic Acid Decarboxylase
GCSF	Granulocyte Colony-Stimulating Factor
GFAP	Glial Fibrillary Acidic Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GRIA4	Glutamate Ionotropic Receptor AMPA Type Subunit 4
HEK2	Human Embryonic Kidney 293 Cells (variant)
HLA	Human Leukocyte Antigen

HLH	Hemophagocytic Lymphohistiocytosis
HSV	Herpes Simplex Virus
IAA	Iodoacetamide
ICU	Intensive Care Unit
IFN-γ	Interferon Gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IGF	Insulin-Like Growth Factor
IGFBPs	Insulin-Like Growth Factor Binding Proteins
IgG	Immunoglobulin G
IgG Index	CSF IgG Index (QIgG/Qalb)
IGLON5	Immunoglobulin-Like Cell Adhesion Molecule 5
IgM	Immunoglobulin M
IIF	Indirect Immunofluorescence
IIH	Idiopathic Intracranial Hypertension
IL-	Interleukin (e.g., IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17, IL-21, IL-23)
IP	Intraperitoneal
IP-10	Interferon Gamma-Induced Protein 10
IRF3	Interferon Regulatory Factor 3
ISO	International Organization for Standardization
ITAC	Interferon-inducible T cell alpha chemoattractant (CXCL11)
IVD	In Vitro Diagnostic
IVIg	Intravenous Immunoglobulin
LAIR-1	Leukocyte-Associated Immunoglobulin-Like Receptor-1

LC-MS	Liquid Chromatography-Mass Spectrometry
LGI-1	Leucine-Rich Glioma Inactivated 1
LINGO1	Leucine-Rich Repeat and Immunoglobulin-Like Domain-Containing Protein 1
m/z	Mass-to-Charge Ratio
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MAP2	Microtubule-Associated Protein 2
MFI	Median Fluorescent Intensity
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Imaging
MS	Mass Spectrometry
NETosis	Neutrophil Extracellular Trap Formation
NFk-B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NfL	Neurofilament Light Chain
NI	Non-Inflammatory
NK	Natural Killer (Cell)
NMDAR	N-Methyl-D-Aspartate Receptor
NPAAC	National Pathology Accreditation Advisory Council
NPH	Normal Pressure Hydrocephalus
NPTXR	Neuronal Pentraxin Receptor
NSAID	Non-Steroidal Anti-Inflammatory Drug
OAND	Other Autoimmune Neurological Disease
PAMP	Pathogen-Associated Molecular Pattern
PC	Principal Component e.g. PC1, PC2
PCA	Principal Component Analysis

PCR	Polymerase Chain Reaction
PCSK1N	Proprotein Convertase Subtilisin/Kexin Type 1 Inhibitor
PE	Phycoerythrin
PE-CF594	Phycoerythrin-Cyanine5.5
PET	Positron Emission Tomography
PRR	Pattern Recognition Receptor
Qalb	CSF/Serum Albumin Quotient
QC	Quality Control
QIgG	CSF/Serum IgG Quotient
RBC	Red Blood Cell
RMPI	Roswell Park Memorial Institute (media)
ROC	Receiver Operating Characteristic
RRR	Relative Risk Ratio
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEALS	South Eastern Area Laboratory Services
SIMOA	Single Molecule Array
SLE	Systemic Lupus Erythematosus
SMPDL3A	Sphingomyelin Phosphodiesterase Acid-like 3A
STING	Stimulator of Interferon Genes
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TARC	Thymus and Activation-Regulated Chemokine
TFA	Trifluoroacetic Acid
Tfh	T Follicular Helper Cell
TGF-β	Transforming Growth Factor Beta
Th	T Helper (e.g., Th1, Th2, Th17)

TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor Alpha
TPO	Thyroid Peroxidase (Antibody)
Treg	Regulatory T Cell
UCH-L1	Ubiquitin C-terminal hydrolase L1
VGCC	Voltage-Gated Calcium Channel
VGF	VGF nerve growth factor inducible
VGKC	Voltage-Gated Potassium Channel
VI	Viral Infection
VP	Ventriculoperitoneal
VZV	Varicella Zoster Virus

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Abstract

Diagnosis of autoimmune encephalitis (AE) is challenging due to its diverse clinical manifestations and the limited sensitivity of currently available biomarkers. This is particularly true in antibody-negative cases or those presenting solely with psychiatric symptoms. The recognised overlap between AE and psychiatric disorders has renewed interest in immune contributions to psychiatric disease, highlighting the need for improved diagnostic tools. This thesis aims to identify novel cerebrospinal fluid (CSF) biomarkers to enhance diagnostic precision in AE and to explore immune involvement in psychiatric illness.

To achieve these aims, initial studies examined the utility of conventional disease markers alongside emerging biomarkers, including CSF cytokines and CSF free light chains, in patients with a high clinical suspicion of AE. This cohort was compared with non-inflammatory (NI), viral infection, and inflammatory neurological disease control groups. The analysis confirmed the poor sensitivity of current tests but identified two cytokines, IL-21 and IP-10, as promising candidates for improving diagnostic yield.

Follow-on studies evaluated serum and routine CSF markers of autoimmunity and neuroinflammation in a cohort of patients with psychiatric disease, combined with CSF cytokine profiling. These results were then compared with the AE and NI control groups described above. A subset of psychiatric patients demonstrated high CSF cytokine levels, suggestive of underlying immune dysregulation.

Finally, an unbiased CSF proteomic analysis using mass spectrometry across these cohorts revealed multiple proteins and pathways associated with immune and

neural processes, providing a foundation for future biomarker discovery. Several pre-analytical and post-analytical challenges were also identified, offering important insights for the design of future studies.

Collectively, the findings from these investigations provide new insights into the immune basis of CNS disease and underscore the potential of novel CSF biomarkers to refine diagnosis, guide therapeutic decisions, and improve outcomes. Further validation in larger, independent cohorts will be required to establish their clinical utility.

Chapter 1: Introduction and Literature Review

1.1 Introduction

Over the past two decades, autoimmune encephalitis(AE) has emerged as a critical disorder situated at the intersection of neurology, psychiatry, and immunology. Although diagnostic criteria have been published, diagnosis remains challenging due to the broad spectrum of clinical presentations, and the limited sensitivity and specificity of routinely available biomarkers. Importantly, autoimmune disease may present with primary psychiatric features that are often resistant to conventional treatment. This has reignited interest in the long-standing hypothesis that immune dysregulation can contribute to the pathophysiology of difficult-to-treat psychiatric illness.

Because these conditions are encountered by neurologists, psychiatrists, and immunologists, advancing our understanding requires cross-disciplinary exchange. Each field brings critical insights: neurology and neuroanatomy clarify structural and functional correlates, immunology delineates immune mechanisms, and psychiatry interprets complex behavioural and cognitive phenotypes. Only through integration of these perspectives can I form a holistic view of AE and related neuroimmune disorders.

This review aims to synthesise advances across these domains, with a focus on how they inform the diagnostic challenges of AE, the potential role of neuroinflammation in psychiatric disease, and the evolving diagnostic and therapeutic landscape. By drawing together findings from each discipline, I seek to provide a comprehensive framework for understanding this rapidly developing field.

1.2 The immune and central nervous systems

1.2.1 Introduction

Many of the emerging biomarkers of neuroinflammation are derived from CSF analysis, often reflecting underlying immune system activity. A full exploration of all immunological mechanisms, and the organisation of the central nervous system is beyond the scope of this review. However, to provide essential context for the investigative approach that I have taken and the significance of the results that I have generated, I will outline key concepts in basic immunology and CNS function. This background necessarily touches upon autoimmune encephalitis, immune-mediated psychiatric disease, and novel biomarker discovery.

1.2.2 The Immune System

The immune system plays a vital role in maintaining tissue integrity by protecting from harmful pathogens and resolving injury[4]. However, when its function becomes dysregulated, the very mechanisms designed to defend us can mistakenly attack the body's own tissues. This misdirected immune response can lead to autoimmune disorders, such as AE, where the immune system targets the brain and nervous system.

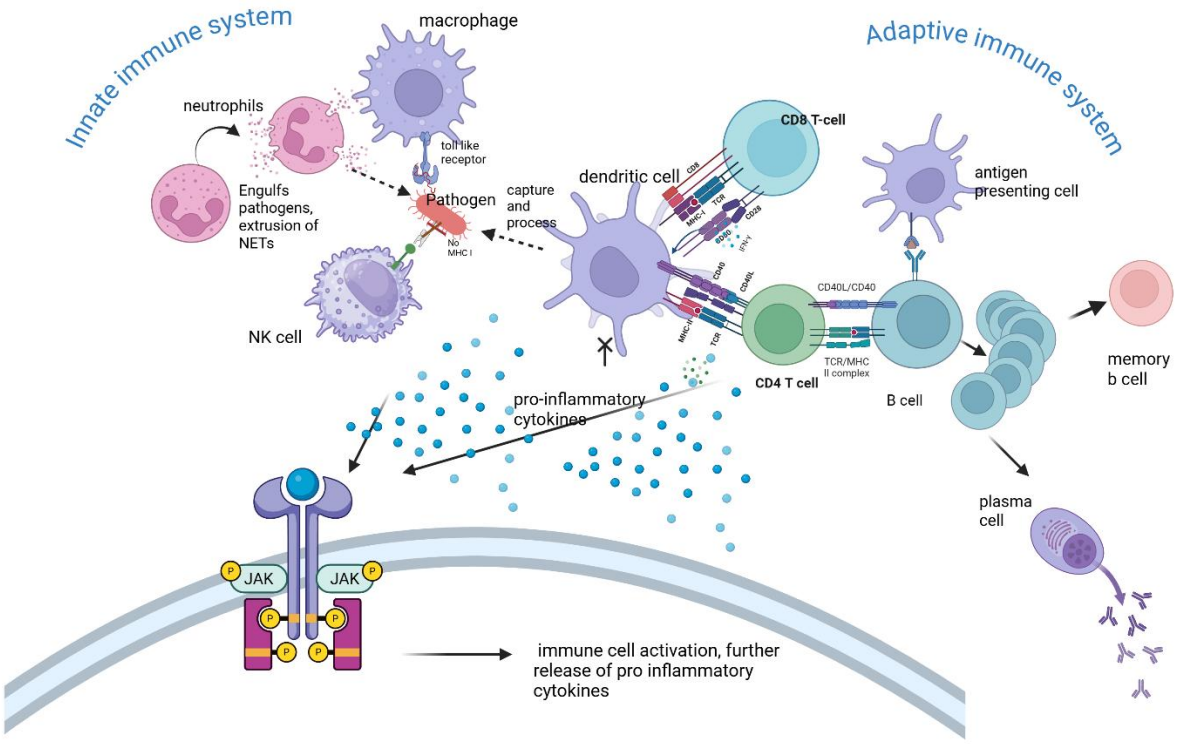

Figure 1: A representation of the host response to infection

This illustrates the immediate response of the innate immune system, antigen presentation and interaction with the adaptive immune system and responses by the activated adaptive immune system [4,12].

Adapted from: Knowledgebase R. Image for Immune System [Pathway R-HSA-168256]: Reactome; 2025 [13 May 2025]. Available from: https://reactome.org/content/detail/R-HSA-168256

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1.2.2.1 Innate Immune Responses

1.2.2.1.1 Local Innate Immune cell response

For a pathogen to enter the human body, it must first breach barrier epithelia such as skin and mucous membranes[4].

Innate immune cells act as first responders, with macrophages, mast cells, and dendritic cells surveying tissues via pattern-recognition receptors (PRRs). These receptors detect pathogen-associated molecular patterns (PAMPs) from microbes and danger-associated molecular patterns (DAMPs) from cellular damage. Pattern-recognition receptor activation triggers cytokine and chemokine release, which recruits additional innate cells including monocytes, dendritic cells, myeloid cells, natural killer cells, and innate lymphoid cells, initiating the first wave of a highly coordinated immune response. [4,13,14].

1.2.2.1.2 Effector responses of the activated innate immune system

Monocytes activate and differentiate into macrophages which, alongside phagocytic myeloid cells such as neutrophils, engulf microbes and ingest them into vesicles which fuse with intracellular lysosomes with microcidal properties and destroy them[13]. Activated neutrophils also trap and kill microbes by extruding their deoxyribonucleic acid (DNA) and granule contents into neutrophil extracellular traps (NETosis)[15]. This process can stimulate further NETosis in neighbouring neutrophils, perpetuating the inflammatory response[4].

Natural killer (NK) cells eliminate virally infected or damaged host cells through release of granzymes and perforin. Here, perforin forms pores in the affected host cell, through which granzyme gains entry to destroy reservoirs of infected cells. Activated NK cells also secrete the cytokine interferon-gamma (IFNγ), further supporting activation of nearby inflammatory cells like macrophages.

Innate lymphoid cells, including NK cells as a subgroup, also secrete cytokines depending on the local activated immune environment, to further recruit and activate other immune cells[4,16].

1.2.2.1.3 Antigen presentation and the bridge to adaptive immune response

Dendritic cells, resident and recruited by activated local innate immune cells, also capture and process microbes. They then migrate into draining lymph nodes to present captured antigens to naive T cells. After recognition of foreign antigen, T cells become activated, proliferate and enact their specific effector roles. Antigen presenting cells, with the assistance of activated CD4+ T positive cells also activate B cells to form germinal centres in the lymph node and differentiate into antibody-producing plasma cells, as part of the humoral adaptive immune response[4,17].

1.2.2.2 Adaptive Immune Responses

The adaptive immune system is so named because it can adapt and tailor its responses to specific pathogens that the body recognises during its lifetime. Through antigen presentation, dendritic cells activate the primary cellular members of the adaptive immune system: the B and T lymphocytes[17,18]. This results in a cascade of further immune responses including activation of cytotoxic T cells for cellular killing, activation of CD4+ T cells for further cytokine release (cellular immunity)[17] and activation of B cells leading to production of antibodies specific to the target antigen (humoral immunity) which will be detailed below[18]. These cells once activated are also able to develop into long lived memory cells which are primed for a faster response if the same pathogen is encountered again[4].

1.2.2.2.1 T lymphocytes

T-lymphocytes can be broadly divided into CD4+ T helper cells and CD8+ cytotoxic T cells with a numerically smaller third group that is CD4 and CD8 negative: gamma delta T cells[16,19]. T lymphocytes can only recognise antigen that is presented on the major histocompatibility complex (MHC)[4], aside from some receptors such as CD1 that present of lipid antigens[20].

1.2.2.2.1 Antigen presentation and T cell activation

The MHC are a collection of cell surface proteins that bind peptide fragments produced during normal cellular turnover or fragments of antigens that have been captured and processed by an antigen presenting cell[4,16].

There are two classes of MHC molecules. Class I is present on all cells, recognised by cytotoxic CD8+ T cells and consist of a heavy chain (α chain) with three domains: α1, α2 and α3 along with a light chain called β2 microglobulin. Class II MHC is present only on antigen presenting cells (APC) like dendritic cells and is recognised by CD4+ T cells. Class II MHC has two chains: an α chain and β chain each with 2 domains. While antigen presenting cells can encompass many cell types including macrophages, B-lymphocytes and dendritic cells. Dendritic cells are the most efficient at initiating primary responses, via T-cell activation[4,16,21].

Each MHC binds a single peptide with binding specificity determined by the highly polymorphic variations in the human leukocyte antigen (HLA) locus and properties of the presented peptide. [4,16,22]. As MHC can present both self and non-self-

antigens, one of the mechanisms for the development of autoimmunity is through MHC alleles with greater efficiency at presenting self-antigens.[23] .

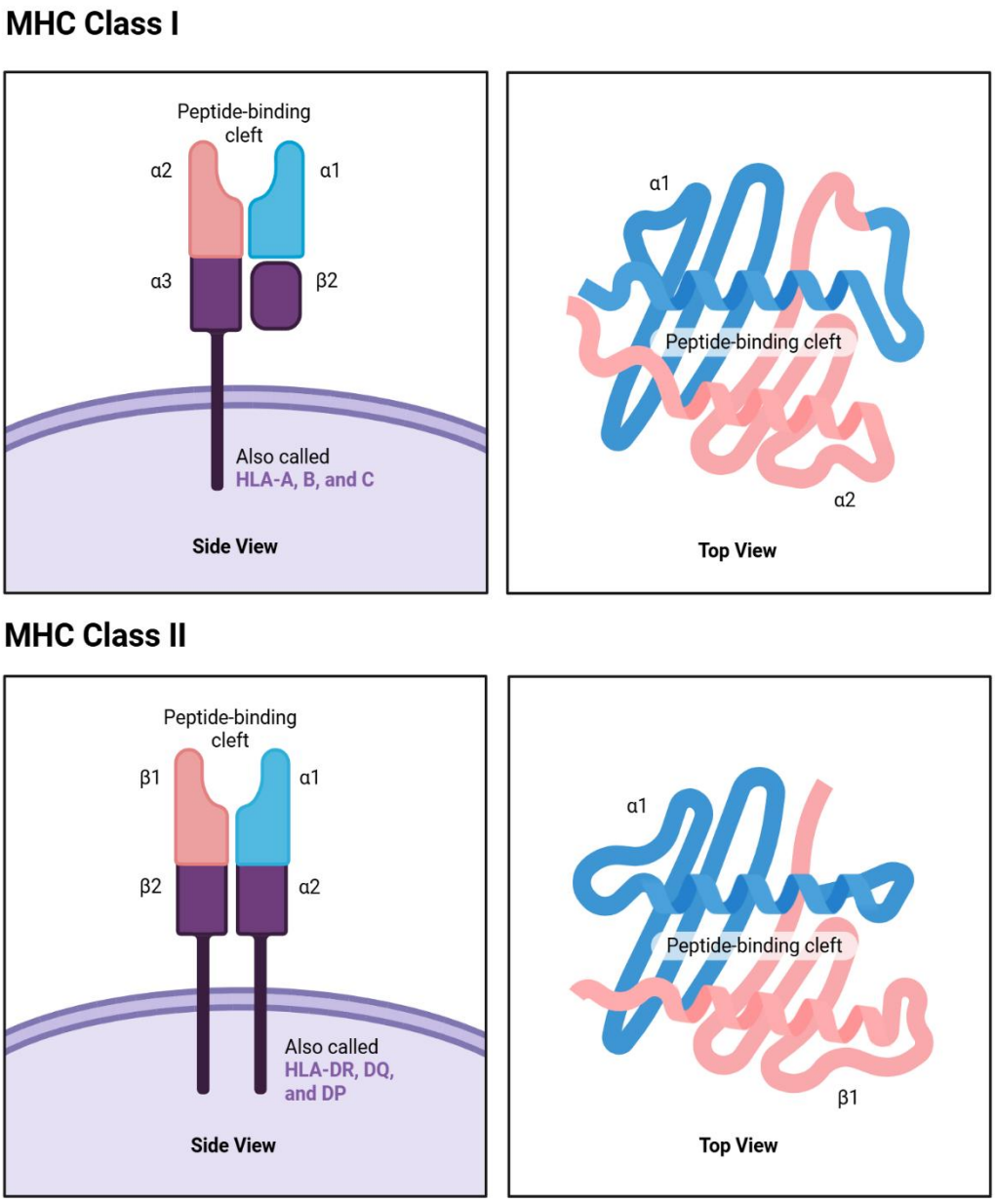

Figure 2: Structure of the MHC Class I and Class II molecules demonstrating the peptide binding groove.

Adapted from [4]: Abbas AK, Lichtman AH, Pillai S, Baker DL, Abbas AK. Cellular and molecular immunology. Tenth edition ed. Philadelphia, PA: Elsevier; 2021.

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When a naive antigen-specific T cell recognises the peptide being presented by an APC, and simultaneously receives a co-stimulatory signal, often dictated by the surrounding environment the APC is exposed to, it becomes activated[24,25]. The activated T cells undergo proliferation and clonal expansion under further stimulation from autocrine growth factors such as the cytokine IL-2, generating the lymphocytes required to eliminate the pathogen. They differentiate into effector cells and memory cells[4,16,26]. Conversely, if the antigen presenting cell is not activated (i.e. in the absence of PAMP or DAMP signalling or absence of activating cytokines), it will not express a co-stimulatory signal and the T cell will become anergic[4,27], leading to no significant T-cell response.

Usually, an antigen can only activate a small number of antigen specific T-cells. The exception are superantigens: a protein toxin, usually produced by a pathogen, that can activate 20-30% of the body's CD4 and CD8 T cells at once. These superantigens bind to MHC II molecules outside the usual antigen recognition site and trigger cross linkage of the T-cell receptor, resulting in extensive T cell proliferation and an unregulated surge of proinflammatory factors referred to as a "cytokine storm". This causes severe inflammation and life-threatening conditions like toxic-shock syndrome[21,28,29].

1.2.2.2.2 CD4 T helper lymphocytes

CD4+ T helper (Th) cells have important roles in cytokine production, recruitment and activation of other leukocytes, and assist in B-lymphocyte activation. The types of cytokines/chemokines produced by the CD4+ T cell, and associated innate immune cells, will influence type of leukocytes recruited. This in turn depends on the local

environment including cytokines that have already been produced by the “first responder” innate immune cells[4,30].

There are several classes of T helper cells which are responsible for responses against different pathogens and have a differing associated “signature” cytokines, outlined in Table 1[30,31]. However, there are additional T helper cell subsets including Th3, Th9, Th22, that have been described in the literature but are less universally accepted as distinct lineages[4].

Table 1: CD4+ T helper cell subsets [4,16,30]

Subset	Cytokines produced	Transcription Factor	Role
Th1 Stimulated by intracellular pathogens and cytokines IL-12, IL-18 and type 1 interferons	Interferon-gamma (IFN-γ) axis cytokines	T-bet	Défense against viral and other intracellular pathogens; macrophage activation; role in autoimmunity
Th2 Stimulated by parasitic infections and cytokine IL-4	IL-4, IL-5, IL-13	GATA3	Defense against parasites Allergy IgE production
TH17 Stimulated by IL-6, IL-1, IL23 and TGF- β,	IL-17 IL-22 IL-21	RORγt	Defense against extracellular bacteria and fungi
Tfh (T follicular helper cell)	IL-21 IFN-γ IL-4	Bcl6	B cell activation and antigen production
T reg cells	FOXP3	IL-10 TGF-beta	Suppress immune responses Maintain peripheral tolerance

Interestingly, a subset of CD4+ T cells also have cytotoxic effects, able to kill infected target cells presenting antigens via MHC class II in addition to their cytokine producing T helper cell function. They use similar mechanisms as CD8 cytotoxic cells (discussed in section 1.2.2.2.3) and have been observed in chronic infections. They can compensate if CD8+ T cell function is impaired, contributing further to antiviral and antitumour immunity[32,33].

1.2.2.2.3 CD8 cytotoxic T cell responses

APCs historically were thought to present intracellular derived proteins, such as their own or viral peptides only on MHC 1, while ingested pathogen derived peptides were thought to only be presented on MHC Class II. However, it is now recognised that most pathogen derived peptides are able to be presented on MHC class I and class II molecules simultaneously, called cross presentation[34]. This allows engagement of CD8 lymphocytes by peptides from both intra- and extracellular origin. CD8+ naive T cells also require co stimulation (from activated CD4+ helper cells) alongside recognition of the foreign antigen to become activated and proliferate into a large enough pool of cytotoxic cells to destroy cells that express the HLA bound antigen[4,35,36]. Several cytokines are important for this step: IL-2[37,38] and IL12[39] have roles in the differentiation and proliferation of CD8 effector lymphocytes whereas IL-15[40] and IL-21 are important for the induction and survival of CD8+ memory T cells.

Activated CD8+ lymphocytes recognise infected cells or tumour cells via antigens presented within their class I MHC and initiate cell death through the delivery of cytotoxic proteins including granzymes and perforin stored within the CD8 lymphocyte secretory lysosomes to trigger apoptosis[41].

CD8+ T cells also produce the cytokine interferon-γ[42], which as discussed in the context of Th1 CD4+ helper cells, has an important role in macrophage activation and phagocyte clearance of ingested microbes[4].

1.2.2.2.4 Gamma delta (γδ) T cells

Gamma delta (γδ) T-cells are unconventional lymphocytes with features of innate and adaptive immunity. Whilst they form a minority of all T cells, they have

important roles in immune surveillance at barrier sites as well as contribute to tissue homeostasis and repair[43].

Gamma delta T cells lack CD4 or CD8 expression and are distinguished by their antigen receptor composed of γδ chains, unlike the αβ chains of the T cell receptors of CD4 and CD8+ T cells[4]. They do not need MHC presentation to recognise a broad range of antigens and are able to directly kill infected or tumour cells. Gamma delta T cells have been described as both innate-like or adaptive-like depending on the context. While they express toll like receptors and can act quickly (like innate cells), they can also develop memory like features (like adaptive lymphocytes)[4,43-45].

Gamma delta T-cells can also assume features similar to CD4+ T helper cells subsets and can secret a variety of cytokines, depending on the context**.** These cytokines include interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), interleukin-17 (IL-17) and regulatory cytokines such as TGF-β, interleukin-10 (IL-10), alongside others[46]. IL-21 is also involved: it shapes γδ T cell functions by promoting their ability to help B cells[47], enhancing cytotoxicity, and driving regulatory or immunosuppressive capacities[48].

1.2.2.3 B cells and antibody production

1.2.2.3.1 Introduction

Antibodies are proteins produced in response to antigen exposure and are the mediators of humoral immunity. After B cells are activated (via their receptor, which incorporates its unique antibody) they proliferate and clonally expand then differentiate either into antibody secreting plasma cells or memory B cells.

1.2.2.3.2 The structure of the immunoglobulin

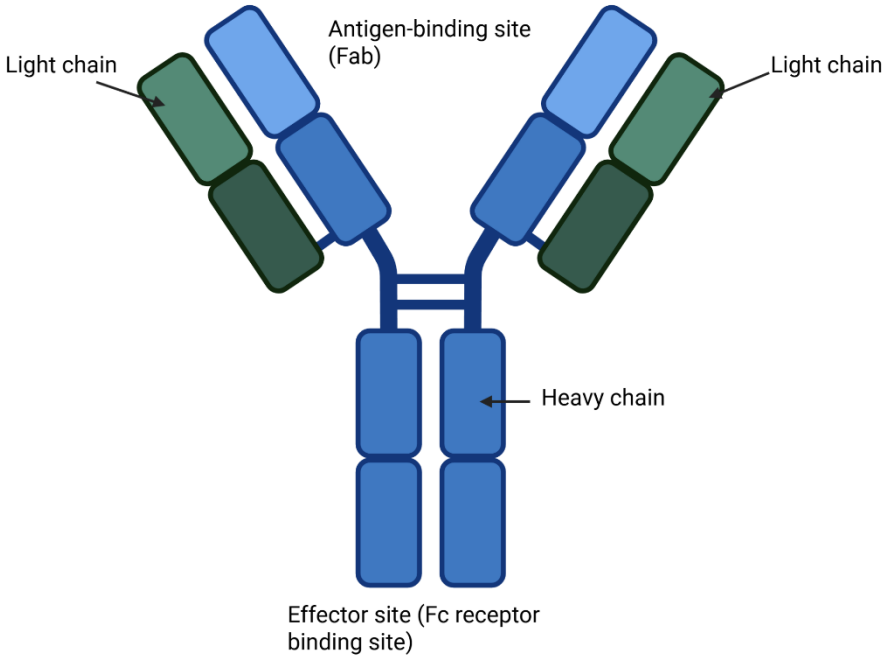

Figure 3: Structure of antibody demonstrating heavy and light chains.

Adapted from[3]*: Malek A. Role of IgG antibodies in association with placental function and immunologic diseases in human pregnancy. Expert Rev Clin Immunol. 2013;9(8):735-747.*

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Immunoglobulins or antibodies are circulating proteins and the mediators of humoral immunity. Each antibody will bind to a specific antigen. The basic immunoglobulin structure is Y-shaped, composed of heavy and light chains (kappa or lambda) covalently linked by disulfide bonds formed between cysteine residues in the carboxy terminus of the light chain and the C_H1 domain of the heavy chain[4]. The heavy and light chains of an immunoglobulin have variable and constant regions with the

variable region being diverse and forming part of the antibody binding site. The diversity of variable regions, which are determined in B cell development and in affinity maturation (described below) gives antibodies differing affinities to different antigens. All antibodies contain an antigen binding site and an Fc region that is involved in its effector functions[4].

1.2.2.3.3 Activation of B cells

Most B cells are follicular B cells that move between secondary lymphoid organs (lymph nodes, spleen and mucosal lymphoid tissues) in search of antigen. B cells can recognise soluble antigen that has diffused directly into the lymphoid tissue, but the most common way they encounter antigen is via presentation by APCs[49].

Naive B cells express IgM on their cell membrane. IgM is a large pentameric protein with high avidity (functional affinity mediated by having 5 antibodies linked together) but lower antigen affinity of individual antibody components and can cross react with a greater range of antigens than other immunoglobulins. If surface-bound IgM recognises and binds to an antigen, usually in combination with stimulation from CD4+ helper T cells, B cells become activated[49].

In lymphoid tissue, activated B cells divide, forming a germinal centre. It is during this process that they undergo isotype switching where they change the constant region of the heavy chain to produce an IgG, IgA or IgE isotype. This process and the isotype of antibody they produce can be heavily influenced by the cytokine environment in which they are activated[4,50].

Rapidly dividing B cells move to the dark zone of the germinal centre. During this antibody class switching they also undergo affinity maturation: proliferating B cells rearrange the V genes encoding the variable parts of the immunoglobulin heavy chain. The rearranged immunoglobulin v genes undergo point mutations at a very high rate (somatic hypermutation)[4]. These new B cells move to the light zone of the germinal centre where they interact with antigen. If there is binding with high affinity, the B cell survives. B cells with mutations that generate lower, or a loss of antigen binding die by apoptosis[4]. This process produces antibodies that have higher affinities to the antigens being presented. These class-switched B cells now produce antibodies of high affinity to the presented antigen. The B cells differentiate into antibody secreting plasma cells or become long-lived memory B cells which are capable of continuous antibody expression or a larger, more rapid expansion if the same antigen is encountered[4]. [49,51].

Not all B cell activation requires T cell help. Marginal zone B cells, located in the marginal sinus of the spleen respond independently to polysaccharide capsules and lipopolysaccharides present in bacteria. These independently activated B cells still undergo clonal expansion and differentiate into plasma cells but little isotype switching occurs and there is no generation of memory B cells[4,52]. This is why patients prior to splenectomy require vaccination against bacteria with polysaccharide cell wall content producing long lived antibody memory[53].

1.2.2.3.4 Humoral Immunity

Antibodies are produced by plasma cells residing in secondary lymphoid organs or bone marrow, but they may also be produced in peripheral non-lymphoid tissues including at sites of infection or chronic inflammation. The plasma cells producing the

antibodies can be short or long lived. Plasma cells generated earlier in an immune response or from T-cell independent B cell responses tend to be shorter lived while plasma cells generated in germinal centres tend to be longer lived[4,16].

The main function of antibodies is to neutralise and eliminate infectious microbes and microbial toxins. Leukocytes express Fc receptors that can bind to the constant regions of antibodies that are bound to their antigen. IgG antibodies can coat microbes and promote phagocytosis by binding to Fc receptors on phagocytes[4]. NK cells, neutrophils and other leukocytes can also induce killing of antibody coated cells via Fc receptors they express. IgE antibodies that target helminths can bind to Fc receptors on eosinophils, facilitating degranulation of these cells. IgG antibodies are also able to form complexes that activate the classical complement cascade. This both results in creation of further pro-inflammatory proteins as well as facilitates lysis of antibody coated foreign cells via the formation of the complement MAC attack complex[4,54].

If an individual encounters the same antigen that stimulated the original antibody production then circulating antibodies can provide an immediate, highly targeted first response[4,54].

1.2.3.3.5 B cells as antigen presenting cells and other "innate" functions

In addition to their role in antibody production, B cells also function as antigen presenting cells in activating CD4+ T cells. B cells, like innate cells, express PPRs including TLRs. Activation of B cells through PPR/TLR signalling leads to B cell proliferation, antibody secretion, cytokine production and becoming a more effective APC[55]. It has been demonstrated that B cells present antigen and activate CD4+ T

cells[56]. How much of a role B cells have in antigen presentation: whether they act similarly to "professional" APCs like dendritic cells or if their role is to provide an ongoing stimulus for Th follicular cells is still being debated with evidence found for and against both these hypotheses. B cell antigen presentation has been implicated in autoimmune disease such as systemic lupus erythematosus (SLE) and some studies have reported B cell depletion reduces the CD4 T cell response to pathogen[56,57].

1.2.2.4 Resolution of the immune response

Once the pathogen is cleared, ongoing inflammatory signals (PAMPS and DAMPS) dissipate, reducing ongoing immune cell activation. Neutrophils and other short lived immune cells undergo apoptosis[58]. Macrophages recognise and engulf these apoptotic cells, signalling their transition from a pro-inflammatory (M1) state to a predominantly anti-inflammatory/tissue remodelling (M2) state secreting anti-inflammatory cytokines.[4,58].

Regulatory CD4+ T cells and myeloid derived suppressor cells also secrete immunosuppressive cytokines leading to further downregulation of inflammatory chemokines and cytokines and recruitment of inflammatory cells. This transitions into a resolution phase of tissue remodelling and repair.[4]

1.2.2.5 Cytokines and immune activation

Cytokines are small signalling proteins that act as key intercellular messengers, orchestrating and regulating innate and adaptive immune responses. They are secreted from a variety of cells: from the innate immune system in response to danger and pathogen-associated signals or from the adaptive immune system in an immunostimulatory or regulatory fashion. They can also be secreted from cells that are

not part of the immune system, usually cells that have barrier functions including epithelial cells, stromal cells and fibroblasts, to attract inflammatory cells when the initial barrier defence mechanisms are breached [4,16,59].

Cytokines are necessary to orchestrate immune responses and can be considered the 'hormones' of the immune system. They play a key role immune cell development, maturation, and survival with trophic support for lymphocytes by IL-7[60], IL-5 for eosinophils[61] and BAFF/IL4 for B-cells[62,63]. Cytokines can be classified in several different ways. Some cytokines are important for initiation and regulation of inflammation: e.g.IL1, TNF-α. Others are important for downregulation and resolution of the immune response: e.g. IL10, IL-4 to prevent excessive tissue damage and promote healing. Some cytokines such as IL-6 and TGFβ may have both pro- and anti-inflammatory actions depending on the environmental context they are acting in and the expression of target cell receptors which they bind[59].

Some cytokines regulate the adaptive immune response. Cytokines produced and secreted by $CD4^+$ helper T cells are determined by at least a four-way "conversation". This involves the cytokine environment shaped by innate and barrier cells, the activation state of previously recruited adaptive immune cells, and the receptor repertoire expressed by the $CD4^+$ helper T cell and neighbouring immune cells[4,16]. Any perturbation of this tightly regulated process can result in the dominance of an immune compartment's "voice," leading to autoinflammatory and autoimmune conditions, some of which can be mediated by these cytokines. Type 1 cytokines (IL-2, IL12, interferon-gamma and TNF-beta) are associated with Th-1 CD4 cells and type 2 cytokines (IL-4, IL5, Il-6, IL10 and IL-13) are associated with Th2 CD4 cells. A summary

of associations of a range of different cytokines is given in Table 2[4,16,64]. Hence, the cytokine profile within a tissue environment can give information about the immune system status at the time: whether in a proinflammatory or resolution phase and if there is dysregulation present (if cytokines are unexpectedly present or levels are unexpectedly low or high).

Profiling of cytokines disease states will give information about differences in immune system involvement and may give insight into disease pathogenesis as well as highlight areas of possible immune dysfunction. In CNS viral infections, Th-1 axis related cytokines are often elevated (IFN gamma, TNF alpha, IP-10, CXCL9) along with other cytokines IL-1ra, IL1β, IL6 and IL-8[4]. However, the profile of cytokine elevation may differ, even in different CNS viral infections (IL1β elevated in enterovirus infection, TNF alpha in herpes simplex, and interferon gamma not being elevated in influenza encephalopathy)[65]. Cytokine profiling in stroke [66] has led to understanding of how the neuroinflammatory responses in this setting are perpetuated. While in neurodegenerative diseases such as Alzheimer's has found an association with pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α and has led to insights into the role of neuroinflammation in its pathogenesis[67].

Understanding the role of cytokines in disease pathogenesis has also led to novel targets for therapy: anti-IL-17 for psoriasis[68]; TNF-α inhibitors in rheumatoid arthritis[69] and inflammatory bowel disease[70]; and type 1 interferon targeting in systemic lupus erythematosus[71] being some examples.

Table 2: Cytokines and their associations[4,16,59]

Cytokine Group	Associated Cytokines
Th1	IFN-gamma, ITAC (CXCL11), IL-12p70, TNF-α, CXCL9, CXCL10/IP-10

Th2	IL-13, IL4, IL5, TARC (CCL17), Eotaxin, IL-21
Th17	IL-17a, IL-23, IL-21
Other	
B-cell	BCA-1 (CXCL-13), BAFF
Acute inflammation and immune activation	IL-1, IL-2, IL-8, IL-6, TNF-α, IL-21
Immune cell production and development	G-CSF, GM-CSF, IL-7, IL-2
Immune regulation	IL-10

1.2.2.6 Development of Autoimmunity

1.2.2.6.1 Innate Immune dysregulation

Dysregulation of innate immunity can result in autoimmune responses. This can occur systemically or be organ-confined such as restricted to the CNS[72-74]. In the innate immune responses, dysregulation of PPRs pathway activation can lead to excessive cytokine production and activation of autoreactive adaptive immunity[75]. Similarly, defective clearance of apoptotic cells (as described in section 1.2.2.4) may result in these self-antigens being presented to the adaptive immune system resulting in auto antibody production and inflammation. Neutrophil NET traps may also be excessive or defective, releasing self-DNA and proteins which thereby trigger autoimmunity and chronic inflammation[75].

Defective or abnormal NK cell function is not only associated with immune deficiency but also with autoimmunity and autoinflammatory disorders which at their most severe manifest as macrophage activation syndromes/hemophagocytic lymphohistiocytosis(HLH). This is due to a highly stimulated but ineffective immune response leading to uncontrolled activation of other immune cells and production of

pro-inflammatory cytokines[76,77]. Again, this can be systemic but CNS confined HLH has been reported[78].

Dysregulation of other innate lymphoid cells are increasingly reported in autoimmune disease. Dysregulated γδT cells are implicated in excess cytokine production and potential barrier dysfunction. [79].

1.2.2.6.2 Adaptive immune system dysregulation

Developmental processes and the environmental cues that B and T cells encounter during their interactions with various cell types in the bone marrow, thymus, peripheral tissues, and secondary lymphoid organs (including the spleen and lymph nodes) play critical immunoregulatory roles in maintaining self-tolerance[4,16,80,81]. Dysregulation at any of these sites, whether through germline or somatic mutations, infectious agents, or environmental triggers in a polygenic permissive background, can disrupt self-tolerance and result in autoimmunity[82,83]. Conversely, in the context of other pathologies affecting these sites, via defects in the same systems, excessive down regulation of immune cells can instead lead to immunodeficiency or maladaptive immune responses[4,16]. A particularly well-recognised example is the cancer microenvironment, where checkpoint molecules promote tolerance of malignant cells[84]. Therapeutic inhibition of these molecules can restore immune attack against tumours but carries the unwanted consequence of breaking self-tolerance, thereby precipitating autoimmunity[85]. A comprehensive description of the mechanisms underlying these regulatory events is beyond the scope of this review but can be found in recent authoritative texts [4,16].

1.2.3 The Central Nervous System and Neuroinflammation

1.2.3.1 Structure of the nervous system

The nervous system is broadly organised into two sections: the central nervous system (CNS) which includes the brain and spinal cord and the peripheral nervous system [86,87]. The brain, housed in the skull, is the largest component of the CNS[88]. It is a specialised organ responsible for input and integrating of information provided through the spinal cord and the 12 brainstem/cranial nerves[89]. The organism's response to this information is relayed by the same routes [86,87,89] .

The brain and spinal cord are encased by the three-layered meninges (dura mater, arachnoid mater and pia mater) and surrounded by the cerebral spinal fluid (CSF). Within the brain are several fluid-filled spaces called ventricles, filled with CSF[87,89], contributed to by the choroid plexus. A further layer: the glial limitans is a thin layer of astrocyte endfeet that lines the CNS just underneath the pia mater[90].

1.2.3.2 Major spaces of the CNS

There are several key anatomical spaces within the CNS defined by the layers of the meninges and the cavities within the brain and spinal cord. There are spaces between each layer of the meninges, with the CSF circulating in the subarachnoid pace between the arachnoid layer and the pia mater. This area also contains major blood vessels and connective tissue trabeculae. The subpial space is a potential space between the pia mater and glial limitans that lines the brain surface and is part of the blood brain barrier[87,89].

There are fluid-filled perivascular spaces surrounding blood vessels termed "Virchow-Robin spaces" (including arteries, arterioles, veins and venules) as they

penetrate the brain parenchyma from the subarachnoid space. These channels are important for fluid exchange, communicating with the CNS' glymphatic system (the brain's interstitial fluid circulating system). Depending on the type of vessel they surround, they may be continuous with the subpial or sub arachnoid space. Thus, they may play a key role in detection of antigen and immune response to infection[89,91,92].

Interstitial spaces are the microscopic spaces between neural cells filled with interstitial fluid and extracellular matrix. They are an area for nutrient and waste exchange [93]. The CSF flows from the perivascular spaces into the interstitial space, modulated by aquaporin 4 channels. This allows interchange of metabolites and waste substances between the interstitial fluid and CSF. The fluid then drains into the perivenous space as part of the glymphatic clearance pathway [1,92,93,94 ,95,96].

1.2.3.3 The Cerebrospinal Fluid (CSF)

The central nervous system is bathed in clear, water like, CSF. It is a shock absorber, provides buoyance to the lipid rich brain and assists in the delivery of nutrients and in the removal of waste products[87,97]. It is also important for CNS immune functions: it contains a small population of immune cells that patrol the subarachnoid space for pathogens. The CSF allows for immune cell trafficking through the CNS as well as diffusion and/or active transport of immune proteins like immunoglobulins cytokines[98].

CSF is continuously secreted from the choroid plexus in the lateral, third and fourth ventricles of the brain. It is classically absorbed by the arachnoid villi, with homeostasis of volume and hence, pressure, being tightly regulated under normal circumstances[87]. However, there are also pathways that allow CSF absorption via the

spinal nerves[97,99], explaining increased protein concentration if CSF circulation is blocked higher up in the spinal column.

The CSF drains out through multiple avenues: through arachnoid granulations that extend into the venous sinuses, through the cribriform plate via perineural pathways with cranial nerves (the olfactory nerve via the cribriform plate and optic nerve) but also via the meningeal lymphatic vessels of the dura mater. [91,100].

The central nervous system has been viewed as an "immune privileged" site with the blood brain barrier restricting the passage of proteins and other molecules from the periphery[98]. The constituents of CSF differ vastly from extracellular, interstitial fluid elsewhere, with the passage of large molecules in and out of the CSF being tightly controlled by the blood-brain barrier[87,98] and by structures in the arachnoid space, explaining why CSF indices may not reflect what is present in the CNS interstitial fluid[101].

Analysis of cerebral spinal fluid (CSF) while being essential in the evaluation of various disorders of the central nervous system, has limitations. The procedure used to obtain CSF, a lumbar puncture (LP), is invasive. It involves the insertion of a needle between the vertebrae into the subarachnoid space. The procedure also requires considerable technical skill, and a limited volume of CSF can be safely removed at a time; repeating the procedure exposes individuals to further risk. Hence, it requires more consideration than and is more invasive than a simple blood draw. There are several complications possible, including headache, bleeding and introduction of infection into the CNS space. CSF collected during lumbar puncture is often of limited volumes, and recollection exposes the patient to another invasive procedure with

associated complication risks[102,103]. It is thus also more ethically difficult to subject healthy volunteers to this invasive technique.

However, a study only of patient's blood and serum may not accurately reflect activity within the central nervous system. An example of this can be seen where antibodies in AE may be detected in the CSF but not in the serum or antibodies detected in the serum can be less specific[104]. Additionally, there are significant unknowns, such as the role of the foetal Fc gamma receptor in CNS and how it regulates immunoglobulin level in the CNS interstitial fluid and CSF[105]. While the considerations above necessitate caution in interpreting CSF analysis, the identification of the cellular and soluble components may provide more insights into the status of the CNS and has been a focus leading to the identification of CSF biomarkers for diagnosis (section 5).

1.2.3.4 The Blood Brain Barrier (BBB)

The blood-brain and blood-CSF barriers (BCB) regulates and restricts the exchange of materials between the blood and CSF, keeping the blood separate from the brain parenchyma and CSF[1]. The blood brain barrier (BBB) is formed by the endothelial cells that line the cerebral blood vessels which are fastened by both tight junctions and adherends junctions. These endothelial cells also have no fenestrations, thus the free diffusion of molecules between blood and brain parenchyma is greatly limited. Astrocyte end feet appose the vascular basement membrane, with aquaporin-4

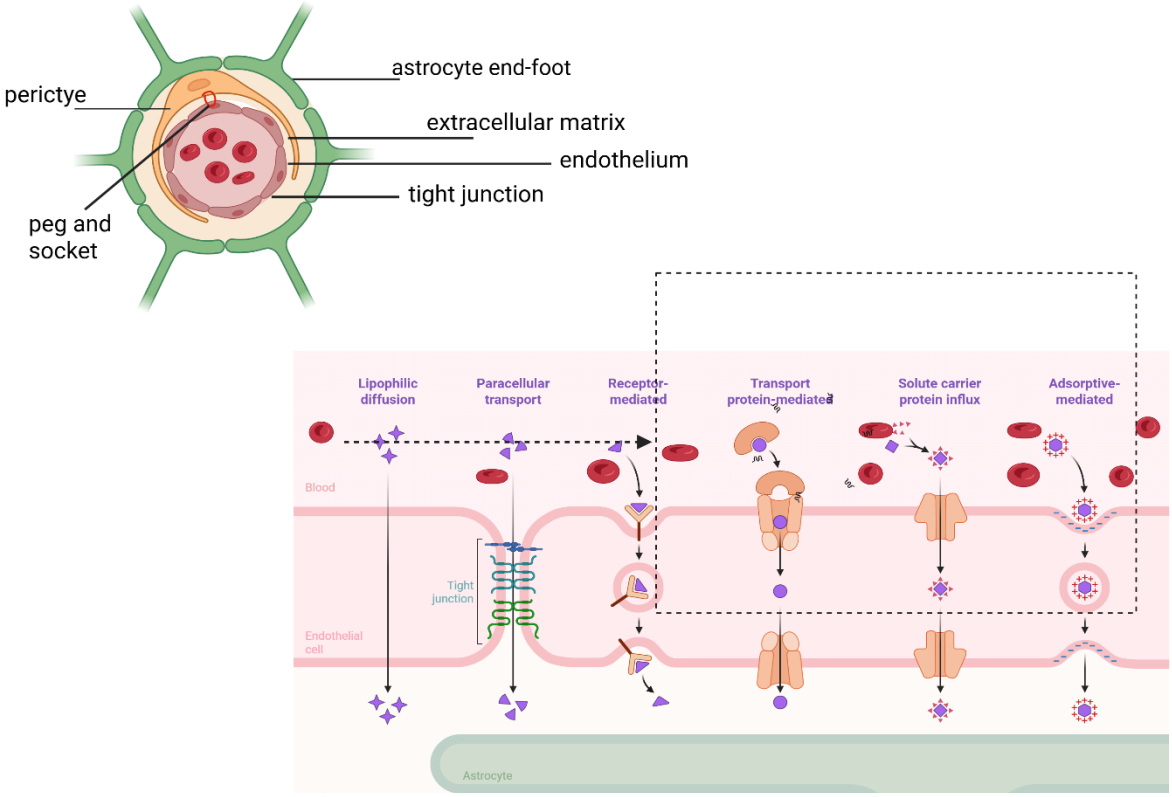

Figure 4: The blood brain barrier tightly regulates exchange of materials between the blood and CSF.

Adapted from[5]: Xiao M, Xiao ZJ, Yang B, Lan Z, Fang F. Blood-brain barrier: more contributor to disruption of central nervous system homeostasis than victim in neurological disorders. Frontiers in neuroscience. 2020 Aug 6;14:764.

Created in BioRender. Jiang, J. (2026) https://BioRender.com/ejhb7cw

concentrated at the perivascular endfeet and tethered by the dystroglycan–dystrophin complex. Pericytes, mural cells present along the walls of capillary blood vessels, are also embedded in the vascular basement membrane and modulate the integrity of the BBB[106] (Figure 4). This barrier exists at the choroid plexus and tissue capillary membranes in all areas of the brain with only very few exceptions, predominantly being the circumventricular organs that regulate whole organism homeostasis. [87].

The BBB is highly permeable to water, carbon dioxide, oxygen and lipid soluble substances, slightly permeable to electrolytes and almost totally impermeable to plasma proteins and non-lipid soluble large organic molecules, unless there are active or passive transport systems. This low permeability is due to the presence of tight junctions between the cells of the endothelial layer of brain tissue capillaries. Thus, the presence of this barrier has role in maintaining a stable environment for optimum neural function. It shields the CNS from potentially damaging neurotoxins, macromolecules and neurotransmitters in the peripheral blood but can also retain beneficial molecules within the CNS interstitial fluid. [87,107].

However, in disease states, the endothelial cells of the BBB and their intercellular tight junctions can be disrupted [5,108]. Comparison of the levels of proteins in the CSF (which are normally tightly regulated by the BBB) with those in the serum provides insight into BBB integrity and helps distinguish intrathecal versus systemic immune activation. Hence, the analysis of the content of CSF will not only given information about intrathecal production of immune proteins such as immunoglobulin but also changes of the blood brain barrier function.

1.2.3.5 CNS Glymphatic and Lymphatic systems

Historically, the CNS was not thought to have lymphatic vasculature, as seen in other tissues. This led to a presumption that there was a lack of drainage of CNS-associated antigens to lymph nodes, preventing immune cells from detecting CNS antigens[109]. However, in the last decade multiple routes of CNS derived antigens to the periphery have been defined.

A unique brain-wide clearance pathway has been described in the CNS. Termed the glymphatic system, it utilises perivascular spaces formed by astrocytes[1,92]. Continuous CSF production, together with blood vessel pulsations generated by the rhythmic nature of blood flow, drives CSF movement from the ventricular system into the subarachnoid space and subsequently into the Virchow-Robin spaces[1]. This inflow is facilitated by aquaporin 4 present on astrocyte foot processes that line outer wall of the Virchow-Robin spaces[92] . There, the CSF mixes with the interstitial fluid carrying metabolic waste into the perivascular space. This system helps clear waste products from the CNS, regulates fluid homeostasis and is also a mechanism in which CNS antigens are delivered to the peripheral immune system[1,92,94,96,110,111].

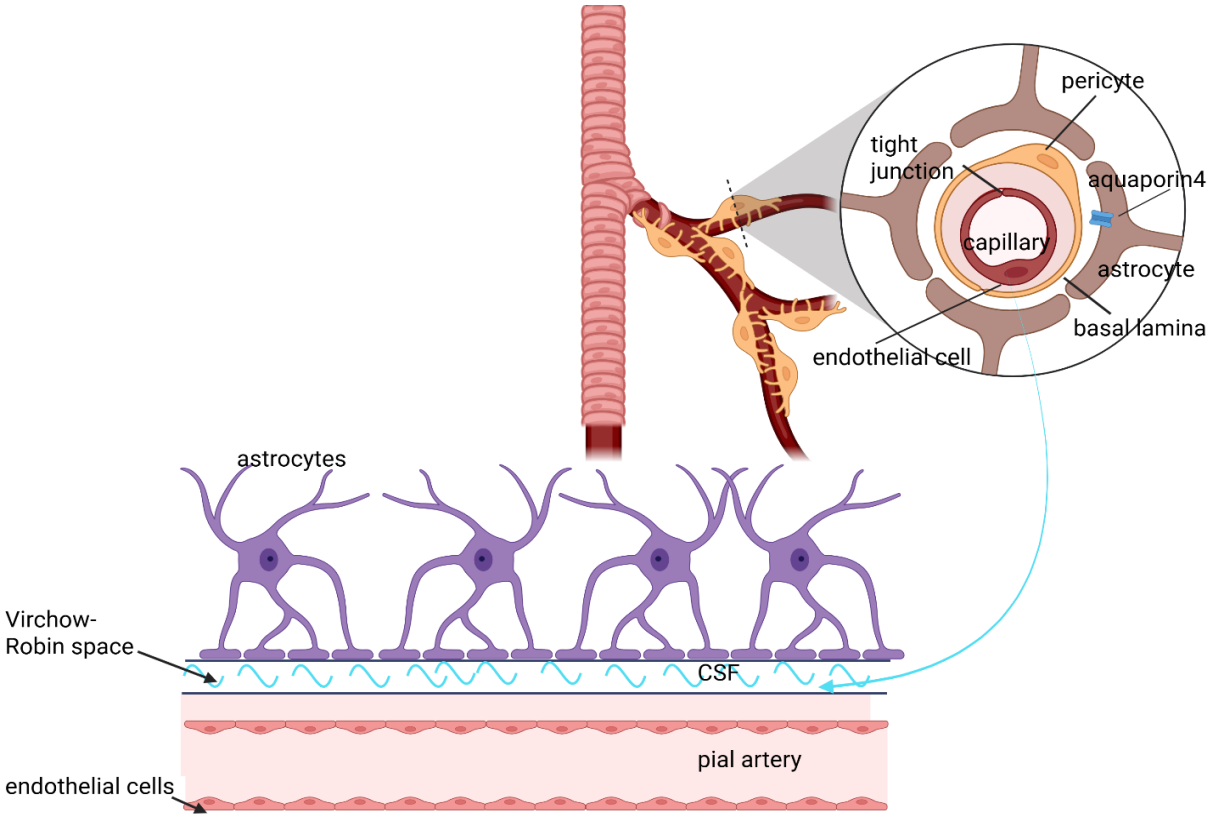

Figure 5: The neurovascular unit allows two-way signalling between micro vessels and neurones with astrocytes serving as key intermediaries.

As penetrating arteries divide into smaller arterioles and capillaries, the cerebrospinal fluid (CSF)-filled Virchow–Robin spaces progressively constrict and eventually disappear. Nevertheless, a form of perivascular continuity persists along these smaller vessels, extending from arterioles through to venules. In this region, the space is represented by the extracellular matrix of the basal lamina, which maintains a continuous fluid pathway between arteriolar and venular segments[1].

Adapted from: Jessen NA, Munk AS, Lundgaard I, Nedergaard M. The Glymphatic System: A Beginner's Guide. Neurochem Res. 2015 Dec;40(12):2583-99. doi: 10.1007/s11064-015-1581-6. Epub 2015 May 7.

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Recently an extensive lymphatic vascular network has been rediscovered. These are located in the dura mater and run alongside arteries, major venous sinuses and cranial nerves including through the cribriform plate with the olfactory nerve into the nasal mucosa[92,112]. Recent studies have suggested that this may be an additional way CSF is transported out of the CNS compartment. [1,92,111,113,114]. The deep cervical nodes

primarily drain the meningeal lymphatics whereas the superficial lymphatics drain nasal tissue[109,115-117].

The dura mater, where CNS lymphatics are located, is also rich in both innate and adaptive immune cells, ready to fortify immune responses within the CNS if needed[118]. Hence, whilst the CNS was once believed to be isolated from the immune system, growing evidence now demonstrates that it possesses specialised pathways for immune surveillance and active immunoregulation

1.2.3.5 The CNS as an immune privileged site

The CNS, located in the closed vault of the skull and vertebral column, is particularly vulnerable to the consequences of unchecked immune activation. Oedema resulting from inflammation can lead to devastating increases in intracranial pressure that can result in brainstem herniation and death [89].

However, the CNS also needs the immune system for defence against infection, tissue repair and trophic support. Hence, it has developed unique anatomical and physiological features that limit and tightly regulate immune responses.

Immune responses with the CNS are compartmentalised, with much of the immune surveillance being at the border tissue sites[109,118]. The unique aspects of the BBB and BCB as well as the glymphatic system have already been described[1,111].

The CNS also possesses a unique cellular immune environment, which will be described below alongside its response to potential pathogens.

1.2.3.6 The unique aspects of CNS cellular immunity

The CNS has long been regarded as exhibiting reduced immune activity and fewer immune elements compared to peripheral tissues[4,119]. It was thought that classic antigen presenting cells were absent with low MHC I and MHC II expression[120]. B cells are generally absent and T cell numbers are low: consisting of mostly a late-differentiated tissue resident memory CD4 and CD8 phenotype[121-123]. However, this traditional view has now evolved, with research now demonstrating that the CNS is not simply immune-isolated but rather immunological specialised[4,119].

The CNS has its own resident macrophages: microglial cells. Microglia have several functions: they are phagocytes, responsible for elimination of microbes and debris from dead cells[124-126]. They can function as antigen presenting cells and initiate and modulate immune responses[124,126]. Like macrophages, their actions are categorised as an M1 (classical) or M2 (alternative phenotype)[126]. The M1 phenotype is pro-inflammatory and neurotoxic. They produce several proinflammatory cytokines and proinflammatory mediators including NADPH oxidase and matrix metallopeptidase. Inflammation further activates microglial phagocytosis, and this has can induce apoptotic neuronal death[124-126].

Conversely, M2 macrophages are anti-inflammatory and are induced by cytokines such as IL-2, IL-13 and IL-10, detection of apoptotic cells and litigation of Fc receptors by immunocomplexes[126]. M2 macrophages secrete additional anti-inflammatory cytokines, growth factors and neurotrophic factors. Increasingly, it is evident that the type of phenotype microglia exhibit is dependent on context and local environment, and this binary phenotype is not sufficient to describe all its roles. [124,126].

Microglia respond to neurotransmitters and play important roles in synaptic pruning and plasticity, in the organisation of neuronal network during brain development and participate in remodelling and elimination of synapses in the mature healthy brain[124,126]. Microglia have been implicated in neuroinflammatory disorders such as multiple sclerosis[126,127] but also seem to have a role in neurodegenerative disorders such as Alzheimer's disease, Parkinsons's disease[128] as well as psychiatric disease[129,130].

Interestingly, astrocytes which have been traditionally thought to be cells that support the structural and metabolic requirements for the CNS also have immunological roles. Astrocytes are capable of processing exogenous antigens, although their efficiency may differ from classical antigen-presenting cells like microglia and dendritic cells[131]. They have inducible expression of MHC II in inflammatory conditions and can produce cytokines and chemokines[132,133]. Indeed, under normal conditions, astrocytes and glial cells express the immunoregulatory cytokine, TGF-β which alongside immune regulating neuropeptides, maintain a more trophic, or anti-inflammatory environment[120,134]

In addition to these antigen presenting cells within the CNS, border-associated macrophages (meningeal and vascular associated) present in CNS border tissues also have immune surveillance roles[135].

Many cells of the adaptive immune system are absent from the healthy CNS. However, during infection or autoimmune inflammation. both T and B lymphocytes, as well as innate immune cells such as NK cells, can be detected[136-139]. Some of this infiltration results from blood–brain barrier disruption, while other activity reflects

immune surveillance, which may also provide trophic support[140]. Peripheral antibodies that gain access to the CNS can also be pathogenic[141]. In addition, intrathecal immunoglobulin production by resident B cells and plasma cells has been clearly demonstrated, and in some cases these populations persist despite systemic B-cell–depleting therapies such as rituximab [142,143].

1.2.3.7 The CNS and autoimmunity

As in the periphery, autoimmunity can develop in the CNS when there is inappropriate or prolonged immune system activation. Here, genetic predisposition interacting with environmental and epigenetic factors also play a key role[144]. Infections can also trigger autoimmunity. [145,146].

Much of the understanding of autoimmunity in the CNS comes from multiple sclerosis and the EAE mouse model based on multiple sclerosis. Invasion of the CNS by peripheral lymphocytes that gain access to the subarachnoid spaces via normal patrolling precede and perhaps trigger inflammatory cell recruitment and breach of the BBB[147]. T-lymphocytes are major players in the pathogenesis of this disease: particularly cytokine producing CD4+ helper Th1 and Th17 cells. However, CD8+ cytotoxic T cels are also prominent in the EAE model for multiple sclerosis[148].

While antibodies are not thought to have a prominent role in multiple sclerosis, the success in treating multiple sclerosis with B cell depletion suggests that B cells also have a significant role[149]. This likely includes the role of B-cells in presenting antigen to T-cells and producing the pro-inflammatory cytokines that activate them[149]. This contrasts with antibody associated neuroinflammatory diseases such as AE where some antibodies such as NMDAR-Ab are directly pathogenic[150].

The innate immune system also plays a crucial role in the development of autoimmunity. Activated microglia are the hallmark of multiple sclerosis[127,151] and their pro-inflammatory role has been described in 2.3.6. In the context of multiple sclerosis, they phagocytose cellular debris including myelin debris which can then stimulate release of reactive oxygen species which are neurotoxic. [127,151]. In addition to microglia, infiltrating macrophages recruited by the inflammatory response have a role in the proinflammatory lesions of multiple sclerosis[126,151,152 ,153]. Outside of multiple sclerosis, microglia have been implicated in other CNS autoimmune and neurodegenerative diseases such as Rasmussen's encephalitis[151] and Parkinson's disease[128].

CNS autoimmunity may also be perpetuated by loss of tolerance in the peripheral immune system. Anti-tumour responses such as those that produced onconeural antibodies result in encephalitis and iatrogenic disruption of immune checkpoints such as in cancer therapy can also result in CNS autoimmune complications[144]. In antibody mediated diseases, anti-NDMAR antibody (associated with AE) [154] and anti-aquaporin-4 antibody (associated with neuromyelitis optica) producing B-lineage cells are observed in reactive germinal centres[155] of the deep cervical lymph nodes, which as discussed, drain the meningeal lymphatics.[154,155].

In conclusion, like systemic autoimmunity, CNS autoimmunity arises from a complex interplay of genetic predisposition, environmental triggers disturbed barrier function, and immune dysregulation. Both adaptive and innate immune responses: particularly the roles of T cells, B cells, and microglia, contribute to disease pathogenesis. However, the clinical and pathological diversity of CNS autoimmune processes, as well as the specialised border and immune function in the CNS are at

least in part responsible for the limitations of current diagnostic tools. This underscores the urgent need for biomarkers that can better discriminate patients with predominantly organic verses psychiatric disease, as well as indicate disease activity, guide treatment decisions, and improve outcomes for patients with CNS immune disorders

1.3 Autoimmune Encephalitis

1.3.1 Introduction

Autoimmune encephalitis is the most common cause of non-infectious encephalitis[156,157]. This section provides a detailed examination of AE, including its history, clinical manifestations, diagnostic criteria, and treatment options.

1.3.2 History of Autoimmune Encephalitis

The first cases of an autoimmune mediated encephalitis syndrome were published in 1960[158,159], describing a limbic encephalitis associated with malignancy and onconeural antibodies (Table 3)[160]. Since then, the understanding of the clinical breadth of this condition has evolved. Significantly, the discovery of autoantibodies against neuronal surface and synaptic proteins has led to a more refined diagnostic criteria, and new treatment strategies.

When AE was first described, treatment of the underlying associated malignancy was more effective than attempts at immunomodulation and most patients had poor neurological outcomes[161]. However, advances in the understanding of pathological pathways and the development of targeted immunotherapies have reached a stage where many syndromes that may initially present as a pure immune induced syndrome will be reclassified as paraneoplastic, as successful immune targeted treatment may be

associated with progression of occult to overt malignancy. In this setting, the use of immune modulation by check point inhibitors in cancer management may be contraindicated[162-166].

The prognostic considerations in the broad spectrum of AE were difficult to define; however, this changed when in the 1990s case reports emerged of a reversible neuropsychiatric disease associated with ovarian teratomas[167-169]. Antibodies to the cell surface antigen, N-methyl D-aspartate receptor were soon detected in these patients and described in others with similar clinical phenotypes[170-174]. Then, in the early 2000s, further case series of patients presenting with limbic encephalitis, neuromyotonia or psychiatric symptoms were reported, associated with voltage gated potassium channel antibodies but also without onconeural antibodies[173,175,176].

Importantly, these patients responded to immunomodulatory modalities including plasmapheresis, steroids and intravenous immunoglobulin with response correlating with reduced serum antibody levels[172,174,176]. It was later found that these patients do not have antibodies to potassium channels but to associated proteins such as anti-LG-1 and anti-CASPR2[177].

These reports heralded a second group of autoimmune encephalitides[178], associated with antibodies directed against cell-surface or synaptic antigens that respond well to immune suppression, with patients being able to return to premorbid levels of functioning after treatment. Although this was initially thought to be a very rare phenomenon, studies on the incidence of AE indicate a similar incidence to infectious encephalitis[156,157]. Increasing awareness of this clinical syndrome and the potential response to treatment led to expansion of the clinical spectrum with an antibody-

negative group now also well described. This led to the publication of clinical guidelines for diagnosis[179].

However, like any disease diagnosed with reference to criteria, there remain uncertainties in the diagnostic process which has led to concern about both under and over diagnosis of AE.

1.3.3 Clinical Presentation of Autoimmune Encephalitis

AE typically presents with a subacute onset of neuropsychiatric symptoms, including memory deficits, psychosis, seizures, and movement disorders[171,173,174,177-179]. Features associated with either onconeural antibodies (as described in earlier case reports) and with cell surface antibodies (described more recently) are listed in Tables 3 and 4 respectively. Recognition of these features is critical for early diagnosis and intervention.

Patients presenting with AE sometimes report a prodrome of a flu-like illness with behavioural changes that can appear subtle at first. This can progress to frank psychosis or catatonia over a period of days to weeks[178,179]. Indeed, psychiatric symptoms may be the first and only presenting feature[180,181], although most cases described to date rapidly progressed with the development of other focal neurological symptoms including new onset seizures (often resistant to antiepileptic therapy) and cognitive disturbances including profound memory loss. Movement disorders can also develop with a wide range of antibody specific stereotypic ways, and the current phenotype of these motor symptoms are evolving. Some of these antibodies may be

associated with autonomic nervous system disturbances ranging from postural hypotension to apnoea and temperature dysregulation[178,179].

AE may also be triggered by herpes simplex virus encephalitis and up to 27% of patients suffering this infection develop it in the following 6 months[146]. Immune modulating therapies such as TNFa-inhibitors or immune checkpoint inhibitors have also been reported as triggers.[182-186].

The clinical syndrome can differ based on the associated antibody (Tables 3 and 4). In antibody negative AE, the patients present similarly to antibody positive patients, but no associated antibody is identified. Hence a high degree of clinical suspicion is required for identification of patients, supported by some investigation results which will be detailed further below.

Many patients require intensive care unit (ICU) admission, often requiring sedation and ventilation. Patients with antibodies against cell surface or synaptic antigens (Table 4) or patients who fit the clinical phenotype of AE but without associated identified antibody do respond well to immune suppression despite the severity of initial presentation.

Table 3: Autoimmune encephalitis associated with onconeuronal antibodies/antibodies with intracellular targets[160,187-189]

Antibody	Associated Clinical features	Cancer associations
Hu	Encephalomyelitis	Small cell lung cancer
Ri	Encephalomyelitis, opsoclonus-myoclonus syndrome	Breast, small cell lung cancer
Yo	Cerebellar degeneration	Ovary, breast
Ma2	Encephalomyelitis	Testicular
CV2/CRMP5	Encephalomyelitis, chorea, neuropathy, cerebellar degeneration	Small cell lung cancer, thymoma
SOX1	Lambert-Eaton syndrome, cerebellar degeneration	Small cell lung cancer, gynaecological, breast cancer
Amphiphysin	Encephalomyelitis, stiff person syndrome	Breast, small cell lung cancer
Tr	Cerebellar degeneration	Hodgkin's lymphoma
GAD-65	Stiff person syndrome, cerebellar ataxia, seizures, limbic encephalitis	

Table 4: Autoimmune encephalitis associated with cell surface and synaptic antibodies [171,177,189-200]

Antibody	Associated Clinical features	Cancer associations
NMDAR	Prodromal flu-like illness, psychiatric symptoms, short term memory loss, social withdrawal, stereotypical behaviours, agitation, catatonia, movement abnormalities and autonomic instability, seizures	Ovarian teratoma, testicular germ cell tumours Also: pancreatic cancer, breast cancer
LGI-1	Limbic encephalitis, hyponatremia, faciobrachial dystonic seizures. Associated with thymoma and lung cancer.	Small cell lung cancer, prostate, colon cancer, thymoma

CASPR-2	Limbic encephalitis, Morvan's syndrome including autonomic dysfunction and insomnia, peripheral nervous system hyper excitability with fasciculations and cramps.	Lung cancer, thymoma
IGLON-5	Sleep disturbance with parasomnias, bulbar dysfunction, gait abnormalities, a progressive supranuclear palsy like disease, cognitive decline, excessive daytime sleepiness	Rarely associated with cancer
GFAP Associated disease	Meningitis, encephalitis, myelitis, optic disc papillitis, seizures, encephalopathy, psychiatric symptoms, tremors, typically very responsive to steroids, often proceeded by flu-like symptoms	Ovarian teratoma, other solid organ malignancies
GABA-A	Seizures and refractory status epilepticus, encephalopathy, behaviour and cognitive change	Thymoma and lymphoma
GABA-B	Seizures, cognitive impairment, behavioural change, unsteady gait, dysphasia, proceeded by flu-like symptoms, associated with lung cancer,	Small cell lung cancer
AMPA R	Psychiatric symptoms, encephalopathy, cognitive impairment, limbic encephalitis, involuntary movements, autonomic dysfunction	Breast and other solid organ malignancies
Glycine R	Stiff person syndrome, seizures, brain stem/spinal hyperexcitability disorders	Breast, lung, lymphoma
DPPX	Prodromal fever, diarrhoea, weight loss, encephalopathy, cognitive impairment, sleep disorder	Lymphoma

1.3.4 Diagnosis of Autoimmune Encephalitis

Diagnosis of AE is based on the application of the appropriate clinical presentation with supportive confirmatory testing. Currently much of the diagnosis of AE is based on the Graus criteria, published in 2016[179]. These criteria include identification of "possible autoimmune encephalitis" based on the clinical picture of a subacute onset (less than three months) of working memory deficits, altered mental status or subacute psychiatric findings with focal neurological disease and/or new onset seizures. Cases which are more chronic in onset are thought usually due to neurodegenerative causes and hyperacute presentations are thought more likely due to vascular aetiology[201,202]. Since publication of these guidelines, some clinicians have found that there are cases (particularly associated with the antibodies anti-LGI-1, anti-CASPR2, anti-DPP, X and anti-GAD65 and anti-IgLon5) where the presentation of encephalitis can be more chronic, involving 1 to five years of symptom development, which can make clinical identification difficult and increase time to commencement of efficacious therapy[203].

Differentials that need to be considered with these patients include infection, temporal lobe tumours and prion diseases that can cause rapidly progressive dementia (although the latter is usually easily distinguished on cerebrospinal fluid (CSF) analysis and imaging), metabolic encephalopathies and encephalopathies due to other systemic autoimmune disease, such as Hashimoto's thyroiditis. Hence, patients who present with a clinical picture suspicious of AE undergo investigations including CSF analysis, neuroimaging including magnetic resonance imaging (MRI), positron emission

tomography (PET) to look at brain and possible associated malignancy, electroencephalogram and blood tests which will all be discussed below.

1.3.4.1 The use of CSF in diagnosis

CSF analyses are considered some of the most important tests in evaluation of AE. CSF analyses can identify AE associated antibodies, exclude infectious encephalitis and define the presence of other markers of neuroinflammation (including pleocytosis and intrathecal IgG synthesis) which support a diagnosis of AE[179,202]. However, disease can exist with no findings on CSF examination using our current tests.

1.3.4.1.1 Auto-antibody detection

Auto-antibodies indicative of systemic autoimmunity, onconeural and cell surface and synaptic proteins can be detected in blood(serum) and CSF, but CSF is thought to be generally more and specific for disease caused by most synaptic protein autoantibodies[104].

Autoantibodies can be detected by indirect immunofluorescence where the patient's serum or CSF is applied to commercially fixed rat or primate brain tissue and a detection antibody is added to detect any bound human immunoglobulin G. For many autoantibodies, there are specific patterns associated with the different onconeural and cell surface/synaptic antibodies[204].

Presence of onconeural antibodies can also be confirmed by using immunoblot.[205]. More recently, commercially prepared HEK2 cells transfected with the DNA of the antigens of interest have become available to detect IgG autoantibodies to cell surface or synaptic antigens [204,206]. The detection of IgA and IgM autoantibodies is possible as well but their utility for diagnosis is uncertain. [207].

1.3.4.1.2 Routinely available CSF markers for autoimmune encephalitis diagnosis

1.3.4.1.2.1 CSF Cell count

When CSF is acquired from lumbar puncture, it routinely undergoes several biochemical and microscopic analyses. Of these, the CSF cell count is often considered the most fundamental. This includes a red cell count (RBC) which is most often raised in the setting of a traumatic tap leading to contamination of CSF with blood, and non-CNS derived red and white cells[208].

The white cell count is divided into polymorphs (neutrophils) and mononuclear cells (monocytes and lymphocytes)[208,209]. An isolated raised polymorph count is most often associated with bacterial CNS infection but can occur in AE.

CSF pleocytosis or a white cell count of greater than or equal to 5/mm^3 has been proposed to be indicative of a neuroinflammatory state.[179]. A raised mononuclear cell count is commonly described in viral infections but can also been seen in fungal infections and may also be more indicative of autoimmune encephalitis[179,208].

The white cell count may increase when there is blood contamination in the CSF (usually confirmed with an increased red cell count), and a CSF mononuclear cell count can be corrected subtracting 1 white cell for every 500 red cells[210].

Eosinophils are not normally present in CSF. Presence of 10 or more eosinophils/microlitre, or eosinophils composing of at least 10% of CSF leukocyte count is associated with parasites, coccidioidomycosis, malignancies or due to adverse reactions to medication or ventriculoperitoneal shunts[211-213].

1.3.4.1.2.2 Glucose

CSF glucose should be 60-70% of plasma levels, usually 2.8-4.4mmol/L[208,209]. Low CSF glucose generally thought to be a marker of meningitis caused by bacteria, mycobacteria, fungi and parasites but not viruses, probably due to consumption by organisms[214]. However, low CSF glucose can also be seen outside of infection particularly in the setting inflammatory conditions such as neurosarcoidosis, neuromyelitis optica and CNS neoplasms[215].

1.3.4.1.2.3 Protein

The normal reference range for CSF protein for an adult is 0.15-0.45g/L[208]. In newborns, CSF protein concentrations are higher (up to 1.7g/L[208]) but decrease gradually during first year of life and are maintained at low levels in childhood. In adults, CSF protein concentration increases with age[216].

Elevated CSF protein can be due to infectious and non-infectious causes including infectious encephalitis, Guillain-Barre syndrome[217], neuroinflammatory conditions such as multiple sclerosis with local immunoglobulin production and disruptions of the BBB, protein release by cells within the CNS [208,218,219]. CSF protein may also be altered by contaminating blood[220] and may be due to other diseases like diabetes[221].

1.3.4.1.2.4 Oligoclonal Bands

CSF oligoclonal bands are a marker of intrathecal immunoglobulin production and is part of the diagnostic workup for multiple sclerosis, which includes identification of lesions on brain MRI [222]. However, their presence is not specific for multiple sclerosis,

and they can be seen in infection and other neuroinflammatory states. In the context of AE, the presence of oligoclonal bands forms part of the criteria to confirm diagnosis[223].

In the diagnostic laboratory, oligoclonal bands are detected by protein isoelectric focusing where an electric current is passed through a protein membrane embedded with CSF and the immunoglobulin moves according to charge[224,225].

In normal CSF, IgG will migrate towards the negative pole indicating low levels of many antibody types. If there is an increased representation of some antibody types, they will be seen as more strongly stained bands. The CSF pattern of band staining is compared to that of a matching serum sample. If the bands are present in both samples, their presence in the CSF are due to diffusion from serum. If bands are absent in the serum, their presence is thought due to intrathecal production[225].

Two or more bands confined to the CSF is considered abnormal, but three or more bands has optimal sensitivity and specificity for multiple sclerosis (and considered to be required for a positive result). The finding of one band is also occasionally seen and whilst it can be associated with some neuroinflammatory conditions may have less clinical significance[226].

In addition to this, the quality of the CSF bands may also impact on their diagnostic specificity. One study has reported a strong, distinct pattern being associated with multiple sclerosis and a weaker, more subtle pattern of banding being associated with other neurodegenerative and some autoimmune causes but never multiple sclerosis[225].

1.3.4.1.2.5 Assessment for blood brain barrier dysfunction

1.3.4.1.2.5.1 IgG Albumin ratio and CSF IgG albumin index

The blood-brain barrier is a physical barrier consisting of different anatomical structures for diffusion and filtration of macromolecules from blood to CSF. Importantly for diagnostic evaluation, it excludes most proteins present in the blood from the CSF. Proteins gain access to the CSF using absorptive or receptor mediated transcytosis. The integrity of the blood brain barriers and the CSF bulk flow determine the protein content of CSF[227,228]. Hence assessment of protein levels in the CSF compared to serum levels gives an assessment of blood brain barrier integrity and whether raised levels of proteins of interest such as immunoglobulin are derived from intrathecal or systemic synthesis[229].

A comparison of CSF IgG versus CSF albumin, albumin being the most abundant protein in the CSF as a CSF IgG/albumin ratio can give an indication if CSF IgG is disproportionately high and an indication of intrathecal production of CSF IgG. However, this may be significantly impacted by systemic changes in immunoglobulin and albumin concentrations. The CSF/serum quotient of albumin known as Qalb can be used to evaluate blood-CSF barrier integrity. It is not influenced by intrathecal protein synthesis and is corrected for plasma concentration of albumin. The CSF/serum quotient of immunoglobulin (QIgG) reflects the amount of IgG derived from the serum normally and any additional IgG due to intrathecal synthesis[230].

A comparison of QIgG against Qalbumin evaluated as QIgG/Qalbumin determines the CSF IgG index. An elevated CSF IgG index is suggestive intrathecal synthesis of IgG, even if oligoclonal bands are absent. This index may be a more

sensitive measure than a CSF IgG/albumin ratio[230-232]. However, oligoclonal bands are a more specific indicator of intrathecal immunoglobulin synthesis even when they are seen in the absence of a raised CSF IgG index.[233]

1.3.4.1.2.5.2 Reibergram

While the Qalbumin can give some indication of blood brain barrier dysfunction, it is not overly sensitive. The use of a Reibergram: a graphical representation of Qalbumin and QIgG may be a more sensitive method to detect blood brain barrier dysfunction. In this, the CSF IgG quotient is located on the Y-axis, and the CSF albumin quotient is located on the X-axis. The intersection of these results, plotted on the diagram gives an indication of whether the patient falls into normal range or if there is BBB dysfunction or intrathecal IgG production present[230,234].

The use of the Reibergram to visualise blood brain barrier dysfunction has been plotted in a range of disorders[230]. However, it is not always used in routine clinical practice in Australia.

1.3.4.1.2 Exclusion of infection

CSF analysis is an essential step in excluding an infectious encephalitis. As already discussed, increased CSF white cell count may indication infection with an

This image was removed due to copyright

Figure 6: The Reibergram

The use of a Reibergram can assist in assessing blood brain barrier dysfunction or intrathecal production of immunoglobulin [10].

Images taken from: Smith AJ, Jin BJ, Verkman AS. Muddy waters in brain oedema. Fluids Barriers CNS. 2012;9:14. doi:10.1186/2045-8118-9-14

increase in polymorphs suggestive of bacterial infection and mononuclear viral infection[208].

Microscopic evaluation can detect micro-organisms, and culture is often undertaken for bacteria, mycobacteria and fungi. Fungal antigen analysis of CSF can also detect potential fungal infections, e.g. cryptococcal antigen[208], Often these tests are performed in parallel with molecular testing to detect pathogen derived nucleic acid. This is mostly performed by PCR, and sequencing of nucleic acid can also be performed. These molecular tests are the preferred testing platforms for viral detection and other pathogen classes that may not be able to be cultures[235-237].

1.3.4.2 Other investigations for Autoimmune Encephalitis

1.3.4.2.1 Imaging modalities

1.3.4.2.1.1 Magnetic-resonance imaging (MRI Brain)[238]

In additional to CSF analysis, magnetic-resonance imaging (MRI) brain is critical for the diagnosis of AE. MRI may highlight a picture of inflammation where the anatomical distribution is supportive of one of the AE syndromes. An MRI result that is supportive of AE form part of the clinical criteria for diagnosis[179,239].

Importantly, MRI may also help rule out alternative causes such as macro or micro vascular, mass lesions, extensive demyelination or leptomeningeal enhancement from tumours, bleeding or other inflammatory diseases such as sarcoidosis[239].

1.3.4.2.1.2 Other imaging modalities

Brain fluorodeoxyglucose positron emission tomography (FDG-PET) can give additional information regarding multifocal brain abnormalities[202] but may be difficult to access because of availability and cost.

Computed Tomography scan (CT scan) is quick to access in the emergency department setting and may help exclude differential diagnosis such as mass lesions, vascular occlusion or bleeding[240]. However, it has less sensitivity compared to MRI for inflammatory brain conditions and is not useful to confirm a diagnosis of AE, as is the case for other neuroinflammatory diseases[241].

1.3.4.2.2 Other investigations

Other supportive investigations for diagnosis include electroencephalogram (EEG) to exclude subclinical status epilepticus although other findings such as diffuse slowing or a delta brush pattern may also be supportive of AE[179].

Blood tests look for presence of AE associated antibodies, and autoantibodies or markers of systemic autoimmune diseases that may affect the CNS. These include antiphospholipid antibodies, antithyroid antibodies, anti-nuclear antibodies or serum ACE, serology for infection such as HIV or syphilis, toxicology if appropriate, general markers of inflammation such as ESR and CRP and other tests to exclude metabolic and nutritional deficiencies (ammonia, B group vitamins and certain hypervitaminoses[202]). As already discussed, AE antibodies may be ordered in serum, but these are generally viewed as not as specific as CSF examination.

Rarely, if ever, in Australia, is a brain biopsy required, though this is more commonly done where there is diagnostic uncertainty and drug funding criteria need to be complied with. In patients where a diagnosis of AE is established, screening for a potential neoplasm is often undertaken with full body CT scan with or without full body FDG-PET because of the high association of neoplasms with some AE subtypes[202].

1.3.4 Treatment of Autoimmune encephalitis

The various treatment modalities for AE involve significant and aggressive immune suppression with early immunomodulation associated with better outcomes. While a comprehensive review of these therapies is beyond the scope of this review, it should be noted that serum testing often detect transferred antibodies from intravenous immunoglobulin (IVIg) and therefore is ideally performed before commencement of this treatment.[202,242-248]

The decision to treat is difficult if the patient does not meet classic clinical or investigational parameters[249-253]. This is because such extensive immunosuppression carries risks. This includes infection, including the difficult to treat John Cunningham virus; long term risk of malignancy; and disturbances to metabolic status, blood count, liver and renal function, amongst other potential side effects[249-253]..

1.3.5 Current Limitations of Diagnostic Criteria

Current diagnostic criteria for probable AE requires an appropriate clinical phenotype supported by appropriate findings on MRI, CSF (pleocytosis, oligoclonal bands, elevated CSF IgG index) or brain biopsy showing inflammatory infiltrates[179]. The finding of a positive antibody in a recognised clinical setting supports a more definite diagnosis. However, as discussed, MRI and CSF analysis can appear normal in the setting of AE and negative investigation findings in the right clinical context do not necessarily exclude diagnosis[179].

Some in the literature argue that the current diagnostic criteria are of suboptimal sensitivity to detect all patients with true AE, particularly in early stages of the

disease[254]. Certainly, when AE was first described the main concern was potentially missing antibody-negative cases where treatment would greatly change outcome. Hence, there is general agreement that treatment for AE can be started if the clinical syndrome is suspicious, before all investigations are complete.

However, recent reports have also emerged where there has been a misdiagnosis AE based on over-reliance on positive antibody results, particularly positive results in serum. The patients described in these case series do not meet the criteria for AE and in many cases an alternative diagnosis was eventually found[255,256]. With the broad spectrum of clinical presentations, the overlap with other non-immune mediated differentials and lack of consistently reliable biomarkers, it is possible these patients may be exposed to the harms of unnecessary immune suppression[238,255,257]. Conversely, false negative biomarkers (because of lack of diagnostic sensitivity) may lead to delays in therapy which has been reported to then impact on final outcomes[258,259]. Hence the diagnostic process for AE can certainly be refined, particularly with the incorporation of emerging and novel biomarkers to assist diagnostic certainty.

1.4. The Immune System and Psychiatric Disease

1.4.1 History of the Link Between Psychiatric Disease and Immunity

That the immune system could be involved in the pathogenesis of psychiatric disease was first postulated in the late 19th century. In 1887, Julius Wagner-Jauregg published a paper where patients were intentionally infected to induce a fever as a therapeutic intervention for psychiatric illness[260]. In 1890, Emil Kraepelin described a

case series of psychiatric disorders that developed during an influenza epidemic associated with the infection[261] and Karle A. Menniger also reported this association in the later Spanish Influenza epidemic[262]. In 1913, the infective organism Treponema pallidum was first described to cause neurosyphilis, which also has pleomorphic psychiatric manifestations[263]. In 1937, Lehmann-Faucis hypothesised that schizophrenia was due to an autoimmune disease and further association between specific autoimmune diseases and psychiatric disease were later found[264].

The recently renewed interest in the role of immunomodulation in treatment-resistant cases of psychiatric disease has led to publication of a consensus document that aims to aid identification of cases for further investigative interrogation[265]. The criteria in this consensus statement were then further applied to a cohort of patients with psychosis and a significant proportion were diagnosed as having AE based on further investigations, and improved with immune suppression[266].

1.4.2 Infection and Psychiatric Disease

There are strong associations between psychiatric disease and infection, which may indicate a role for immune responses in the development of psychiatric disease. A large population-based study reported severe infections in children and adolescents increasing the risk of diagnosis of a psychiatric illness later in life. Risk of psychiatric illness increased in a dose-response manner with the number of treated infections and temporal proximity to last infection[267]. Maternal infections are also a risk factor for affected foetuses for the development of schizophrenia in adult life[268] with more severe infections leading to increased risk[269].

Interestingly, like the early reports associating the influenza pandemic with development of psychiatric disease, it has also been observed during the recent COVID19 pandemic that the risk of psychiatric disease increased in the year following infection. Again, risk increased with severity of illness [270].

Other specific infections that increase the development of psychiatric disease include herpes simplex virus[271] and toxoplasmosis[272], both associated with schizophrenia. Maternal infections with both these pathogens also increased risk of schizophrenia in offspring[273,274]. Infections with cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) have been associated with schizophrenia and mood disorders[275-277].

The immune system's response to infection, as detailed above, is a pro-inflammatory activated state. The association between infection and later development of psychiatric disease, therefore, is not just suggestive of pathogens being a causative agent but that pro-inflammatory immune dysfunction may play a role in the pathophysiology of mental illness[278]. Further evidence of this is presented in the association of psychiatric disease with other autoimmune disease and the shared genetic loci between psychiatric disease, propensity to infection and autoimmunity. This will be discussed further below.

1.4.3 Genetic Susceptibility Loci of Psychiatric Disease, Infection and Autoimmunity

Genetic studies have shown that there is overlap in susceptibility loci between psychiatric disease, susceptibility to infection and development of autoimmune disease. One study found 138 possible associations between infection susceptibility

and psychiatric disease, suggesting that immune dysfunction may be involved in the pathogenesis of either[279].

As discussed in section 1.2.2.2, the MHC genes encode the proteins that bind to peptide fragments for presentation to the adaptive immune system. Hence, certain MHC variations have higher bias for presenting self-antigens. Psychiatric disease shares significant genetic susceptibility loci with autoimmune disease, with MHC genes being the most prominent[280]. The association of specific MHC genes with schizophrenia, suggest that certain MHC interactions with the adaptive immune system may influence the development of psychotic disease[22,281]. Genome wide association studies in schizophrenia identified several major histocompatibility complex (MHC) loci associations with immune function, particularly with B cell linages involved in acquired immunity[281].

Non-MHC related shared genetic loci include those common to systemic lupus erythematosus (SLE), Sjogren's syndrome, scleroderma, ankylosing spondylitis, inflammatory bowel disease and autoimmune thyroid disease[280,282,283]. The identification of shared genetic susceptibility may give some explanation for the association of psychiatric disease and autoimmunity. It may also suggest that the shared genetic susceptibility leading to immune dysfunction may cause the pathology in both instances.

1.4.4 Psychological Trauma and Neuroinflammation

Psychosocial stress, trauma, bullying or a history of abuse are all well-reported risk factors for later development of psychiatric illness[284]. However, trauma and stress

may be also neuroinflammatory: this has been demonstrated in animal models and in human studies as discussed below.

In mouse models, trauma or exposure to stress itself leads to increased immune activation in the brain with an increase in CNS levels of the proinflammatory cytokines IL1, IL6 and TNF-α also being reported, with an influx of innate inflammatory cells into the CNS[285]. Longitudinal studies in humans have found associations between stress in childhood with a doubling of the risk of chronic inflammation[286,287]. Acute psychological stress has been reported to increase circulating proinflammatory cytokines[288], with evidence of CNS immune activation seen in humans undergoing trauma or stress with chronic PTSD [289]. Hence, it can be reasonably hypothesised that a mechanism by which psychosocial stress or trauma leads to the development of psychiatric disease may be due, at least in part, to activation of inflammatory pathways and neuroinflammatory responses. A further question that stems from this hypothesis is whether ongoing inflammation is still a contributory factor at the time of clinical presentation and if treating any ongoing inflammation will be effective in alleviating symptoms.

1.4.5 Evidence for Immune Activation in Primary Psychiatric Disease

Microglia (the resident macrophages of the CNS) activation, which have been implicated in neuroinflammatory diseases like multiple sclerosis[127], also play a role in the development of psychiatric disease[129,130,285]. Microglia also play important roles in synaptic pruning and hence in both the organisation of neuronal network during brain development as well as in remodelling and elimination of synapses in the mature healthy brain[124,285], which also requires MHC I expression. Synaptic loss is thought to be an important part of the pathophysiology of schizophrenia[290]. Postmortem studies on

brains from patients with schizophrenia or patients who have died from suicide have also confirmed evidence of microglial and other immune cell activation in relevant brain regions[291].

Increased markers of inflammation and immune activation have been reported in psychiatric patients. Associations between the rise in systemic markers of inflammation such as CRP and ESR have been associated with flares of psychiatric, particularly psychotic illnesses[292-294]. In some studies, the rise in these inflammatory markers seem to resolve with treatment[295].

Disturbances in blood-CSF barrier permeability has been described[296,297], with an increase in Qalb being associated with development of psychosis[297,298] and reports an increase of CSF:serum IgG indices in schizophrenia[299] (although this has not been well replicated).

Increases in serum cytokines such as IL1 and IL6 have also been associated with active schizophrenia, whilst IL-6 and TNF alpha have been increased in the serum of patients with depression[300-303]. Indeed, depressive-like behaviours have been observed in studies where healthy participants are given endotoxin infusions to trigger cytokine release, demonstrating reduced mood can be a direct consequence of immune activation[304]. Depression has been reported in up to 25% of patients treated with interferon therapy for Hepatitis C infection[305] with some authors advocating for antidepressant medications to commencement of interferon therapy[306]. Other psychiatric disease has also been associated with interferon-based therapy: mania, anxiety and psychotic disorders have all been reported though to a lesser incidence[307,308].

Studies of CSF have consistently found evidence of mild inflammatory diseases in a subset of patients with psychiatric disease. This includes changes in CSF protein, mild pleocytosis and the presence of oligoclonal bands[309-312]. More recent studies have found disturbances of cytokines and other novel biomarkers in the CSF of patients with psychiatric diseases suggesting evidence of immune dysfunction[310,313]. CSF cytokine disturbances in psychiatric disease will be further discussed in Chapter 5.

1.4.6 Immune System Effects of Psychotropic Medication

It is commonly understood that psychotropic medications work by altering the balance of neurotransmitters allowing communication between neurones such as the increase of serotonin in depression and dopamine blockade in psychosis[314,315]. However, it is increasingly recognised that the conventionally used psychotropic medications also have effects on the immune system.

Most of the available evidence has been with antipsychotics. Clozapine is an atypical antipsychotic medication usually used treat schizophrenia in patients who have not adequately responded to other antipsychotics. While its main mechanism of action is through to be its effects on multiple neurotransmitter receptors, it has well documented effects on the immune system. Clozapine is associated with secondary antibody deficiency[316] and well known to cause neutropenia and rarely, agranulocytosis[317]. Clozapine can suppress lymphocyte proliferation and alter cytokine profiles[318-320], leading to patients taking clozapine being more at risk for bacterial infections[321], like patients on immunosuppressive medication. Clozapine significantly attenuated experimental autoimmune encephalomyelitis(EAE), a mouse model of neuroinflammation used to study multiple sclerosis[322]. However, whether clozapine's

immunomodulatory actions contribute to its efficacy as an antipsychotic is still not established.

Other antipsychotics, both typical and atypical have also been demonstrated to have potential immunomodulatory actions including upregulation of anti-inflammatory cytokines (for example IL-4 and IL-10)[323,324], suppression of pro inflammatory cytokines (such as interferon-γ, TNF-α and IL-6)[324 325,326]and alteration of leucocyte counts[327-329]. Quetiapine has also been reported to attenuate EAE[330]. Similarly, antidepressants such as fluoxetine and mirtazapine may have immunomodulatory effects including altering cytokine expression and T-cell proliferation[331-333]. Fluoxetine may have antiviral effects[334,335] and whether these are due to immune changes or not is not understood.

How different psychotropic medications modulate the immune system is complex and likely differs from drug to drug [336]. Interestingly, it has been noted that T and B lymphocytes do carry neurotransmitter receptors[337,338] and may even produce neurotransmitters such as acheylcholine[339]. This may be a pathway for psychotropic to have immunomodulatory effects.

With increasing evidence for the role of the immune system in the pathogenesis of psychiatric disease, it has been postulated that the therapeutic effects of psychotropic mediations may also be due to their immunomodulatory effects[340,341]. However, this is still an emerging concept and more research into this area psychotropic medications contribute to their therapeutic effects is needed.

1.4.7 Psychiatric Disease and Response to Anti-Inflammatory Medication

Not only have psychotropic medications demonstrated immunomodulatory effects, but immunomodulatory medications have also demonstrated some efficacy in treating psychiatric disease, primarily as an adjunct. The selective COX-2 inhibitor non-steroidal anti-inflammatory (NSAID), celecoxib[342], non-COX-2 selective NSAIDs such as aspirin[343] and minocycline[344] have all been shown to improve outcomes in patients with schizophrenia. Celecoxib has also been demonstrated to improve outcomes in depression[342].

Canakinumab, a monoclonal antibody targeting IL-1β a pro-inflammatory, acute phase cytokine was recently shown to reduce symptom severity in patients with schizophrenia alongside reducing systemic CRP levels. While this was a randomised control trial, the patient numbers were only 27 and results need to be replicated in a larger study to be considered robust[345]. However, inhibition of other pro-inflammatory cytokines such as IL-6 blockade with tocilizumab has not been reported to improve outcomes[346]. There has also been promising preliminary results for Rituximab, an anti-CD-20 monoclonal antibody that targets B-lymphocytes use in treatment resistant schizophrenia, but again larger studies are needed to confirm results[347].

1.4.8 Autoimmune Disease and Psychiatric disease

Autoimmune disease can present with psychiatric symptoms (such as in AE or neuropsychiatric lupus) but has also been associated with an increased risk of primary psychiatric disease[348]. The chronic elevation of cytokines in autoimmunity is thought to contribute to the development of depression[349]; conversely, there is also an increased

prevalence of autoimmunity in patients with psychiatric disease[350]. Table 5 presents manifestations of psychiatric disease in a range of autoimmune diseases.

Table 5: Autoimmune Disease with psychiatric symptoms as part of its presentation

Condition	Psychiatric manifestations
Autoimmune Encephalitis	• Disorientation, behaviour change, hallucinations, suicidality and psychosis; often treatment resistant[351,352]. • Antibodies to the NDMAR receptor have been demonstrated to cause symptoms of psychosis[353] • 5% of patients with NMDAR receptor antibody associated autoimmune encephalitis will present with only psychiatric symptoms [352]
Multiple Sclerosis	• The most common association is depression with a lifetime prevalence of 50% compared with 10-15% in the general population[354,355] • Bipolar disorder is also twice as common general population[354,355] • Structural brain damage could be contributory [355] • Increased psychiatric disease prevalence before presentation with multiple sclerosis[356] • Intrathecal inflammation can lead to mood alterations that herald multiple scelrosisrelapse[357].
Systemic lupus erythematosus (SLE)	• Neuropsychiatric SLE can present with frank psychosis[358] and may be the only feature of activity [359,360]. • >39% of patients report comorbid depression and anxiety; risk of suicide was also higher [361].
Sjogren's Syndrome	• CNS involvement includes cognitive dysfunction, psychiatric symptoms and sleep disorders[362-365] • Associations with bipolar disorder [366]and psychosis or schizophrenia[367,368] have also been reported
Hashimoto's Encephalitis	• Presents with seizures, confusion, cognitive decline and a wide range of psychiatric symptoms, including depression, mania and psychosis • High levels of anti-thyroglobulin or thyroid peroxidase antibody but normal or minimally disrupted thyroid function[179,369,370]. • Up to 50% of patients will show some response to corticosteroids alone; will go into complete remission[179,369,371]. • Controversial diagnosis: thyroid autoantibodies are common in normal population including at high levels [372]

Graves' Disease	• Antibodies stimulating thyroid receptor leading to hyperthyroidism[373] • Mood changes can be symptoms of hyperthyroidism but mood changes in Graves' disease can persist even after euthyroid state acheived[374] • Increased risk of bipolar disorder and psychosis[374]
Anti-GAD65, Stiff Person's syndrome and Diabetes Mellitus	• GAD65 antibodies is a defining feature of type 1 diabetes mellitus[375]. • GAD65 antibodies also associated with Stiff Person's syndrome and limbic encephalitis (can present with psychosis) [376,377] . • No clear association between type 1 diabetes and psychosis[378].
Coeliac Disease	• Autoimmune disease affecting small intestine due to immune reaction to gluten[379]. • Most manifestations are gut-restricted by some systemic features e.g. dermatitis herpetiformis[379], and rarely, vasculitis[380-382]. • Observed association with psychiatric disease[383] • Twice the risk of developing schizophrenia compared to a healthy control[384,385] • Manifestations may be due to nutritional deficiencies[386] • Genetic associations also reported[387].

1.4.9 Psychiatric Disease and Immune Deficiency

An increased odds ratio for any psychiatric disorder has also been observed in patients with primary humoral deficiency, even after adjusting for coexistence of autoimmune disease, suggesting that overall immune dysfunction may have a role in the development of psychiatric disease[388]. This is in keeping with the observation that some classes of humoral immunodeficiency have a significantly higher risk of autoimmunity because of altered immunoregulatory capacity.[389] Patients with a primary immune deficiency and autoimmunity had the highest risk of psychiatric disorders and suicide, which suggests a potential additive effect[388].

1.4.10 Identification of Patients with Autoimmune Contribution to Psychiatric Disease

As discussed so far, there is evidence for a role of the immune system in psychiatric disease with associations with both infection and autoimmunity as well as standard treatments being immunomodulatory medications.

Many patients with psychiatric disease will respond to conventional psychotropic medication[390]. The challenge is to identify those patients who would benefit from immunomodulatory therapy: either because there is a different underlying causative process or because there is a significant immune contribution to their disease pathophysiology. Current diagnostic criteria for AE require other neurological features beyond a subacute onset of psychiatric and behavioural changes to make a possible case[179]. However, in a published case series of 91 patients where psychiatric disease meeting criteria was the only presenting feature of suspect AE (patients with other neurological features were excluded), more than 80% patients benefited from immunomodulatory therapy[391]. Antibody positivity was found in 39% in CSF and 65% of serum of the investigated patients.

A recent consensus statement has attempted to create a set of criteria clinically identify patients who may have an autoimmune cause of their psychoses[265]. This included patients with an abrupt or subacute onset (rapid progression over less than three months) with certain clinical "red flag" features. This includes currently or recently diagnosed patients with a tumour; movement disorder; adverse responses to antipsychotics raising suspicions of neuroleptic malignant syndrome; severe or disproportionate cognitive disfunction; decreased level of consciousness; seizures not

explained by a previously known seizure disorder or clinically significant autonomic dysfunction. Diagnosis of probable autoimmune psychosis could be considered if there are positive investigation findings similar to that of AE. This would then justify a trial of immunomodulation.

The authors also acknowledge that these criteria are conservative and may miss patients who for instance may have an autoimmune cause to their symptoms but have been unwell for longer than three months. Further clinical "red flag" features were also proposed to further raise suspicion of a possible autoimmune cause beyond the initial diagnostic criteria including infectious prodrome; new onset headache or change in headache pattern; rapid progression; insufficient response to antipsychotics; focal neurological disease; aphasia, mutism or dysarthria; history of other autoimmune disorders or paraesthesia.

The above criteria proposed by the consensus statement has been recently applied to a population of 195 psychotic patients[266]. Of these 164 were diagnosed as having psychosis of possible autoimmune origin. Interestingly 118 of these patients were identified to have anti-NDMAR antibodies and significant improvements were seen with immune suppression. Another study reported a cohort of 564 patients with psychiatric disease where 12% of patients received a new neurological diagnosis after CSF analysis[392].

The difficulty in relying on investigations similar to those in AE for the diagnosis of autoimmune psychosis is that these investigations may be normal even when there is an autoimmune cause present[179]. The current consensus paper advocates for consideration of immunomodulatory therapy on a case-by-case basis. More sensitive

and specific biomarkers beyond what is currently available in the diagnostic laboratory or radiology would help further in identifying patients who may benefit from immunomodulation, while possibly assisting others avoid unnecessary immunosuppression.

1.5 Emerging biomarkers of Neuroinflammation

1.5.1 Introduction

In the search for better biomarkers for neuroinflammation, there has been interest in older diagnostic markers not currently routinely utilised for diagnosis of neuroinflammation as well as newly described biomarkers. While some biomarkers may be available on serum testing, the CSF can give unique insights into the immune status of the CNS because it is a site of immune privilege. CSF cytokines have been an of particular interest in biomarker discovery, although each pose their unique challenges.

This section will discuss the currently available and emerging novel biomarkers that may be utilised in investigation of neuroinflammatory disease.

1.5.2 Markers Available in Diagnostic Laboratories but Not Routinely Utilised for Assessment of Encephalitis

1.5.2.1 CSF β-2 microglobulin

Beta-2 microglobulin is a small membrane protein, belonging to the immunoglobulin superfamily and constitutes the light chains of class I MHC. It is present on the surface of all nucleated cells, but its prevailing expression is on lymphocytes and macrophages. Beta-2 microglobulin is noncovalently bound to the

MHC Class-I heavy chain and disassociates from MHC I when the T-cell is activated and viral infected cells endocytose the MHC complex[393].

Concentration of β-2 microglobulin reflects a rate of cell membrane renovation and turnover as all cells express MHC I. Serum and plasma β-2 microglobulin is a marker of cellular immune system activation as well as a marker of immune cell neoplasm in multiple myeloma, Hodgkin's disease and non-Hodgkin lymphoma[394]. Increased CSF values are attributed to immune activation and lymphoid cell turnover. CSF β-2 microglobulin is currently utilised for investigation of CNS involvement in lymphoma[395]. However, it has also been proposed to be a reliable marker in infection and different autoimmune CNS disorders. CSF values of β-2 microglobulin are higher in CNS infections including patients with neurological complications of HIV infections, multiple sclerosis, neuromyelitis optical spectrum disorders and Gullian-Barre syndrome[396,397,398,399].

1.5.2.2 CSF neopterin

Neopterin is produced by macrophages activated by IFN-γ, a type II interferon cytokine[400,401]. As well as being a marker for Th1 activation, neopterin also has biochemical and physiological functions in host defence[402]. Neopterin appears to promote prooxidative stress, which may amplify the cytotoxic effects of reactive oxygen and nitrogen species against invading pathogens[402]. Additionally, it is potentially a cytoprotective molecule for nonimmune resident cells[403,404].

Neopterin in the brain is independently produced; there is no correlation between the concentrations of neopterin in the serum and CSF of patients with immune-inflammatory disorders[405]. Hence, increased CSF neopterin may indicate

activation of the IFN-γ pathways in the CNS, independent of systemic immune processes[402]. Microglia and astrocytes are the cells proposed in the CNS that produce neopterin. Biogenic amine producing cells may also synthesise neopterin (including dopaminergic and serotoninergic neurones) in inflammatory diseases[405].

Normal CSF adult neopterin are less than 20nm/L in adults and older children, levels can be much higher infants[406]. Neopterin may be elevated in the CSF of viral (acute and chronic) and bacterial infections, AIDS dementia[405,407], and autoimmune and neuroinflammatory disorders: multiple sclerosis[408] and other CNS demyelinating diseases[409] as well sarcoidosis[410] but is not thought to be able to discriminate amongst different inflammatory pathologies[411-413]. CSF neopterin has been reported to be elevated in a cohort of 24 patients with possible AE as defined by the Graus criteria[414].

CSF neopterin are also elevated in delirium[415], suggesting a role of neuroinflammation in its pathogenesis. It's also present after severe traumatic brain injury and subarachnoid haemorrhage. Interestingly, CSF analysis in patients with affective or psychotic disease may demonstrate neopterin from intrathecal, not systemic origin, raising the possibly of a role for neuroinflammation in the pathogenesis of psychiatric disease[407]. Hence whilst raised CSF neopterin may indicate a state of neuroinflammation, they are a non-specific marker and not diagnostic of any one disease.

1.5.2.3 CSF free light chains (FLC)

There is increasing interest in using CSF FLC (kappa and lambda) as biomarkers in neuroinflammation. Kappa and Lambda light chains form part of the immunoglobulin protein when paired with their heavy chain counterparts and are produced by plasma

cells as part of the humoral immune response[4]. Under normal physiological conditions, most light chains are bound to heavy chains; unbound free light chain concentrations are low. However, in disease states (e.g. multiple myeloma; significant systemic inflammation) FLC levels are abnormally high[416].

CSF FLC (kappa and lambda) are raised in several neuroinflammatory processes such as multiple sclerosis without concurrent serum elevation of light chains[417]. Kappa FLC were elevated in a cohort of patients with AE when compared with a cohort of patients with non-inflammatory neurological disease[418]. In a paediatric population, elevation of kappa and lambda FLC was found in a cohort with inflammatory and infective conditions compared to non-inflammatory controls[419].

There is interest in kappa FLC specifically, in multiple sclerosis. Use of a ratio of kappa FLC in CSF serum combined with the CSF:serum albumin quotient enables a calculation of a kappa index[417]. The kappa index correlates highly with the presence of CSF oligoclonal bands in multiple sclerosis[420]. An argument for using the kappa index in place of oligoclonal bands for the diagnosis of multiple sclerosis has been proposed: kappa FLC are an automated assay and may have a faster turnaround time than oligoclonal bands which need to be manually read[421].

1.5.3 Novel Biomarkers Not Yet Routinely Available in Diagnostic Laboratories

1.5.3.1 Neurofilament Light

Biomarkers of neuronal damage have been increasingly recognised as a potential marker of the consequences of neuroinflammation, neural damage. The most promising of these markers is neurofilament light.

Neurofilaments are neuron-specific, filamentous structures about 10 nanometres in diameter[422]. They play a vital role in maintaining axonal shape and supporting the extension of myelinated fibers[423]. These filaments are built as heteropolymers from four main protein subunits: neurofilament light (NfL), medium (NfM), heavy (NfH) chains, and α-internexin. Among them, NfL is the most prevalent and the most soluble[424]. In healthy neurons, small amounts of NfL are continually released from axons; however, in the event of central nervous system injury, there is a marked increase in NfL release[422]. There is good correlation between serum and CSF levels of neurofilament in active neuroinflammatory disease[425] marking it a potential peripheral blood biomarker of disease activity.

As a marker of neuronal damage, NfL is not specific to neuroinflammation per se, but is a highly specific biomarker for neuronal death and axonal degeneration[426]. Increased neurofilament levels in serum and CSF have been seen in CNS infections[427] and exacerbations of multiple sclerosis[422,428]. It is thought that high CSF (and possibly serum) neurofilament is predictive of long-term clinical outcomes with multiple sclerosis[429]. However, NfL is also elevated in conditions not traditionally thought to be

inflammatory: including dementias, amyotrophic lateral sclerosis, atypical parkinsonian disorders[427,430] and traumatic brain injury[431].

In AE, increased CSF neurofilament has also been reported in the acute stage and decreased with clinical improvement[432]. Aside from indicating neuronal damage, high levels of CSF NfL have been associated with worse outcomes and hence has been proposed to be a measure used to stratify patients in whom early more aggressive combined therapy may be entertained[433,434]. However, this finding has not been consistently replicated, and other studies show no correlation between serum and CSF neurofilament levels with disease activity or clinical features[435,436].

While commercial kits have been available for measurement of NfL using ELISA for some time, this method is only sensitive enough to detect CSF NfL levels. Serum NfL levels may be up to 100-fold lower than CSF levels, and traditional ELISAs lack the required sensitivity to detect this. However, with the advent of highly sensitive technique such as the SIMOA (single molecular array) analyser which uses paramagnetic beads in femtolitre sized wells to capture single protein molecules, the far lower serum NfL levels can be accurately measured and the assay has suitable characteristics to reliably use it as a biomarker[422,429].

1.5.3.2 CH13L1

CHI3L1 is a glycoprotein secreted by a variety of cells including macrophages, neutrophils, chondrocytes and in the CNS, by neurotoxic astrocytes induced by microglia[437]. CHI3L1 has roles in tissue injury, inflammation, repair and remodelling responses and is another marker of neuronal damage.

CSF CHI3L1 is increased in multiple sclerosis[438], NMDAR and LG-1 antibody associated AE[439,440], stroke and Alzheimer's dementia[437,441]. Hence, while it may be present as a consequence of neuroinflammation, it is not specific to diseases traditionally considered to be neuroinflammatory.

1.5.3.3 Osteopontin

Osteopontin is another secreted glycoprotein, originally isolated in bone but also now found in many other tissues including kidney, epithelial linings and bodily fluids such as milk, blood and urine. It tends to be upregulated at sites of inflammation and remodelling and has many functions on the immune system including inducing B cell proliferation, antibody production and Th17 cell differentiation[442].

In neuroinflammatory disease, osteopontin is elevated in the CSF of multiple sclerosis[443] and NMDAR-antibody associated AE[440]. However, like other markers of neuronal damage it has also been reported to be a good biomarker for neurodegenerative disease: mild cognitive impairment, dementias and Parkinson's disease.

1.5.3.4 CSF Cytokines

Cytokines are intracellular messengers with different cytokines being associated with activation of different parts of the immune system. Elevation in different cytokines will indicate the activation status of different immune cells. Hence cytokine profiling can give an overall picture of the state of the immune system in a local tissue environment at the time of sampling.

However, one single cytokine is not necessarily specific for any given disease process. Interferon gamma which is associated with the Th1 response to infection has

also been implicated in autoimmune disease such as rheumatoid arthritis and multiple sclerosis[444-446]. Th-2 cytokines are largely associated with allergy but may also have a role in SLE[447]. Th-17 associated cytokines have been associated with psoriatic arthritis[448] and inflammatory bowel disease[449]. TNF-α is associated with rheumatoid arthritis[450]. IL-21 is increasingly recognised as a role of immune activation in autoimmunity[451]. There has been some interest in looking at the CSF cytokine profile of patients with AE to better characterise a range of cytokines that could give better diagnostic precision and gain a better understanding of pathological processes.

Further discussion on findings of CSF cytokines in AE will be in the introduction and discussion of results in Chapter 4.

1.6. Conclusion

The diagnosis of AE, particularly with isolated psychiatric symptoms, is challenging, both because of its expanding spectrum of clinical presentations, meaning some treatable cases may be missed if they are not "classic", and because of the potential for overdiagnosis and inappropriate immunosuppression. Although published diagnostic criteria exist, the limited sensitivity of routinely available investigations for neuroinflammation means that, in some cases, the diagnosis cannot be definitively excluded.

Patients with atypical or treatment-resistant psychiatric disease face an equally difficult clinical dilemma, as poor response to standard therapies leads to substantial morbidity and a high risk of mortality: particularly in younger patients, where suicide

remains a leading cause of death. The recognised association between autoimmunity and psychiatric disease, together with the clinical overlap between AE and certain psychiatric presentations, has driven growing interest in the immune system's role in the pathophysiology of psychiatric illness.

In both contexts, a deeper understanding of pathogenic pathways and improved biomarkers are needed to clarify the contribution of immune mechanisms to disease. While promising research has emerged, particularly in the investigation of novel CSF markers, cytokines, and CSF proteomics via mass spectrometry, available data remain limited. Further studies in these areas will strengthen the current evidence base and may ultimately support the development of more effective diagnostic pathways.

1.7. Aims of studies

In patients with antibody-positive and antibody-negative AE:

- Determine the diagnostic characteristics of routine diagnostic CSF markers.
- Identify novel CSF biomarkers that can aid in the diagnosis of AE with non-diagnostic clinical presentation and antibody negative AE

In patients with new-onset or chronic atypical psychiatric disease:

- Ascertain additional standard neurological and immune investigations to exclude an organic cause for the presentation.
- Identify CSF biomarkers that may indicate a predominant immune contribution to psychiatric symptoms.

For both patient cohorts:

- Apply mass spectrometry and proteomic analysis to elucidate differentiating CSF protein biomarkers to aid diagnosis and identify possible pathogenic pathways of disease.

Chapter 2: Methods

2.1 Introduction

This chapter describes the general methodology employed by the studies in the current thesis. Any variations to methodology will be identified and discussed in relevant chapters.

All sections of this research were approved by the Ethics Committee of Westmead Hospital (LNR/16/WMED/192) and written informed consent was obtained by all participants.

2.2 Patient Recruitment

Patients were prospectively recruited over 18 months between 2016-2020 from two hospital sites within a local health district in Western Sydney, Australia (Figure 7)

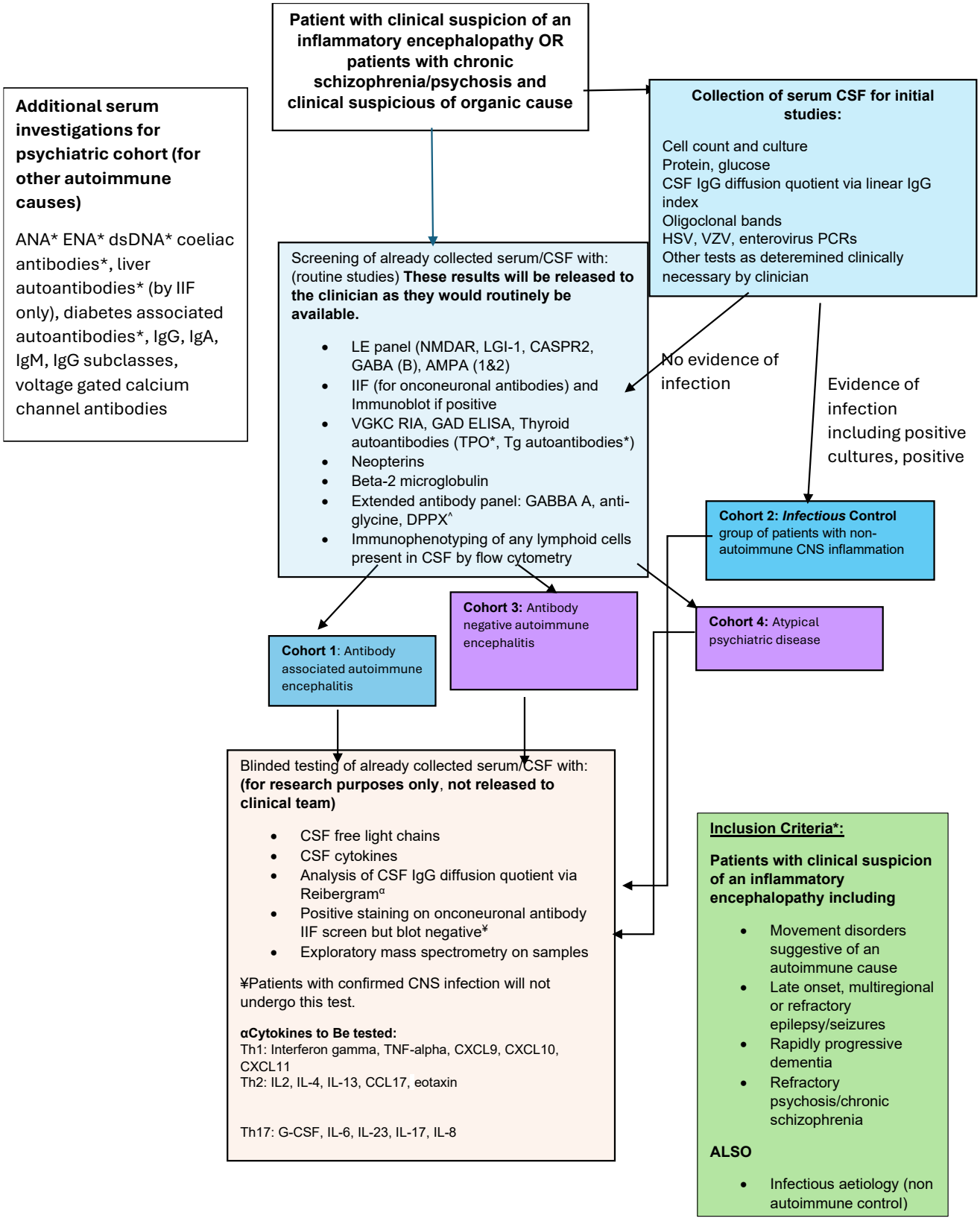


Figure 7: Algorithm for inclusion into study and investigations performed

**Positivity in ANA, ENA, dsDNA, coeliac antibodies, thyroid autoantibodies, liver autoantibodies, diabetes associated antibodies did not constitute a diagnosis of antibody positive AE*[7]

2.2.1 Patients with Autoimmune Encephalitis

All adult patients recruited (15 or older) had a high clinical suspicion of an AE, as assessed by a neurologist, were serially enrolled into the study. Patient inclusion was based on the current clinical criteria for definite and probable AE [178,179,452-454]:

- Subacute onset of symptoms
- Refractory or multiregional seizures/epilepsy
- Rapidly progressive cognitive decline
- Unexplained movement disorders
- Behavioural disturbance including psychiatric symtptoms if presenting with features of 1-5

Included patients, then had extensive serological and CSF evaluation with currently available testing. List of the optimal investigations performed on each patient are detailed in the algorithm in Figure 7 and in the list of assays described in section 2.6.

However, sample collection relied on normal clinical testing procedures and prioritised what was clinically necessary for the patient undergoing investigation. Hence, whilst patients were recruited prospectively, sometimes the CSF had already been collected (if there was a clinical urgency for this to be performed) and collected volumes were variable, leading to insufficient CSF for some testing.

These investigations were used to classify patients into three groups:

1. Viral encephalitis, based on viral detection by PCR
2. Antibody positive AE: AE antibody detected.

3. Antibody negative AE: Fulfil criteria for probable AE, no specific encephalitis detected

Patients were excluded from the study if investigations revealed a clear alterative diagnosis e.g. brain tumour. To prevent information bias, negative results from CSF analysis did not exclude enrolled patients.

2.2.2 Patients with Psychiatric Disease

Patients identified as clinically requiring further evaluation for a possible organic or autoimmune cause by their treating psychiatrist were referred for immunological assessment. All patients referred were reviewed by a clinical immunologist.

A published international consensus paper outlined a list of potential "red flag" clinical features that may increase suspicion/prompt investigation for potential autoimmune contribution to psychiatric disease [265].

Patients were assessed for any "red flag" features that formed the basis of the clinical inclusion criteria into this study[265]:

- Acute or subacute onset
- Treatment resistance
- Personal or family history of autoimmunity
- Increased incidence of psychotropic treatment related side effects
- Presence or history of recent tumor
- Physical symptoms/signs suggestive of organic disease including:

- Focal neurological disease
- Movement disorder
- Decreased consciousness
- Seizures and aphasia
- Mutism or dysarthria.

Patients with a more chronic duration of disease were also invited to partake in the study if they had other "red flags".

All psychiatric patients underwent extensive autoimmune serological evaluation after their historical assessment for the "red flags" outlined above. Those who had "red flag" [265] features underwent further investigation with extensive serological testing, Magnetic Resonance Imaging (MRI) of the brain, lumbar puncture, and electroencephalography (EEG).

Exclusion criteria for this study were:

- Absence of "red flag" features
- Refusal of consent for lumbar puncture as CSF analysis could not be performed

2.2.3 CSF Control Cohorts

Stored CSF samples classified as viral infection (VI) based on positive PCR results were obtained from the microbiology department and included as infectious controls. These samples were supplied as deidentified aliquots. Any patient recruited

into the study who was subsequently diagnosed with an infectious aetiology by microscopy and culture or by PCR were also included in the infectious control cohort.

Non-inflammatory (NI) control CSF samples were obtained from patients undergoing large-volume lumbar puncture for “non-inflammatory” neurological disease including simple headache, normal pressure hydrocephalus (NPH) and idiopathic intracranial hypertension (IIH) as well as from patients undergoing routine spinal anaesthesia.

A disease control group consisting of patients with neuropsychiatric lupus, cerebral vasculitis, and multiple sclerosis were also included (OAND group) in the analysis for Chapter 4.

2.3 Collection of Clinical Details

Clinical details for AE patients, NI and OAND cohorts were collected by interviewing treating clinicians and verified through medical records.

Patients with psychiatric disease were assessed at the immunology clinic and clinical details were collected through history and examination at that time. If any further clinical details were required, this was collected by interviewing their treating psychiatrist and verified through medical records.

Clinical data for VI samples were not available as they were supplied as deidentified samples.

2.4 Sample Collection and Storage

CSF samples for all patients were collected in standard 10mL CSF tubes. All conventional investigations were collected according to current clinical practice and performed according to the usual procedures available at the receiving diagnostic laboratory: Institute of Clinical Pathology and Medical Research (ICMPR), NSW Health Pathology, Australia.

Samples for CSF cytokine and light chain analysis were aliquoted from CSF samples collected and stored at -80 degrees Celsius. Assays for CSF FLC and CSF cytokines were batched for analysis to minimise analytical variation.

NI control patients were recruited from patients undergoing lumbar punctures for other investigations, or as part of spinal anaesthesia.

2.5 Documentation of Clinical Information

Clinical details in the psychiatric cohort, AE patients and NI and cohorts were collected during patient assessment, interviewing treating clinicians and verified through medical records. The data collected included symptoms, seizures, headaches, pain, paraesthesia, movement disorders, sleep disturbance, family or personal history of autoimmunity and cancer as well as a past history of cancer and any current medications, past medications, lack of efficacy and adverse effects

As the clinical history and examination were not included in the study protocol, some clinical details were obtained and verified retrospectively. Additional clinical details were collected from the treating clinician for the purposes of interpretation of any positive diagnostic test results.

2.6 Assays

2.6.1 Routine Assays Available with the Diagnostic Laboratory

All investigations, unless otherwise stated, were performed at New South Wales Health Pathology's Institute for Clinical Pathology and Medical Research (NSW Health Pathology- ICPMR-Westmead, Australia). All conventional investigations were collected according to current practice and performed according to the usual procedures available at the receiving diagnostic laboratory regulated as per ISO 15189 and NPAAC guidelines, according to manufacturers' instructions or register with the therapeutic goods administration as an inhouse invitro diagnostic assay (IVD) as per (Regulatory requirements for in-house IVDs, most recently Version 3.0, May 2024 for individual variation).

Serum and CSF studies Included: isoelectric focussing for oligoclonal bands (Sebia Paris, France); indirect immunofluorescence (IIF) on primate brain (Inova San Diego, USA) and line blot (PCA-1, PCA-2, ANNA-1, ANNA-2, Ma-1, Ma2, Amphiphysin, CV2, CRMP5) for onconeural antibodies (Ravo Bettlach Switzerland) and a limbic encephalitis panel (NMDAR, LGI-1, CASPR2, GABA(B), AMPA1 and AMPA2) on HEK2 transfected cells (Euroimmun Lubeck, Germany), as well as voltage-gated potassium antibodies (VGKC) (performed by radioimmunoassay; Queensland Pathology, Royal Brisbane Hospital, Australia; kits from RSR Cardiff, United Kingdom). Confirmation of IgLON5 antibody was initially performed at Euroimmun and then incorporated into the encephalitis HEK2 transfected CBA workflow.

Additional CSF studies included microscopy; culture; protein (Siemens Vista Erlangen, Germany), anti-glutamic acid decarboxylase (GAD) antibodies (ELISA, South Eastern Area Laboratory Services (SEALS) Pathology, Prince of Wales Hospital, NSW Australia; RSR Cardiff, UK) and polymerase chain reaction (PCR) for HSV (Artus Hamburg, Germany), VZV (in-house PCR assay), ENTV (IVD PCR assay) and EBV (Ellitech Paris, France).

Characterisation of CSF cell populations was performed by routine microscopic quantification of red cells and white cells (including monocytes, neutrophils and other possible cells), this was confirmed by cytological examination and flow cytometric characterisation of cellular content. The panel of antibodies used for flow cytometric examination 1-2 ml of CSF was centrifuged and isolated cells labelled with monoclonal antibodies conjugated with fluorophores included in an ISO 15189 compliant assay. The antibody cocktail included: CD19 (BV421) and CD20 (APC-AF750) to enumerate B cells; CD3(PE-CF594) to identify T cells along with CD4 (FITC) and CD8 (PE) for CD4:8 ratios: and side scatter with CD3-CD4+ was used to identify monocytes.

CSF studies performed at the ICMPR diagnostic immunology laboratory on a research basis were CSF FLC (Freelite assay; Binding Site, Birmingham United Kingdom). CSF cytokine detection will be described in section 2.6.3. Samples for CSF FLC and CSF cytokines were batched for analysis to minimise analytical variation.

The following tests were also performed on serum and CSF: anti-thyroid antibody (Siemens Munich, Germany) and thyroglobulin antibody (Siemens Munich, Germany); and CSF: microscopy and culture; protein (Siemens Vista Erlangen, Germany), anti-glutamic acid decarboxylase (GAD) antibodies (ELISA, SEALS Pathology, Prince of Wales

Hospital, NSW Australia; RSR Cardiff, UK) and polymerase chain reaction (PCR) for viral infections: HSV (Artus Hamburg, Germany), VZV (in-house PCR assay), ENTV (in house PCR assay) and EBV by PCR (Ellitech Paris, France).

Patients with psychiatric disease had addition serum testing to establish evidence of systemic autoimmunity: ANA (Euroimmun Lubeck, Germany), ENA (Orgenteck ELISA), dsDNA (Bioflash Werfen Barcelona, Spain), coeliac autoantibodies (ELISA and indirect immunofluorescence (for anti-endomysial antibodies), Inova San Diego, USA), EPG/IFE (Sebia, Lisses, France), ESR (Starrsed), liver autoantibodies by indirect immunofluorescence (multiblock) on rat liver kidney stomach tissue substrate (Aesku), anti-GAD antibodies(RSR ELISA), anti IA2 (RSA ELISA, and anti-ZNT8 antibodies (Euroimmun), IgG, IgA, IgM (Abbot Architect,), C3, C4 (Abbot Architect Illinois, USA), IgG subclasses (Siemens BNII, Erlangen Germany) and voltage gated calcium channel antibodies (VGCC) radioimmunoassay; Queensland Pathology, Royal Brisbane Hospital, Australia (kits from RSR Cardiff, United Kingdom)

2.6.2 Additional Investigation

Any additional necessary investigations for clinical diagnosis or management including MRI were performed. MRI features supportive of a possible neuroinflammatory cause included features of hyperintensity, hippocampal or other evidence of oedema and hippocampal asymmetry. MRI results were not available for normal and infectious controls.

2.6.3 CSF Cytokines

2.5.3.1 Introduction to CSF Cytokine Detection Methods

Detection of cytokines in body fluids can be performed by a number of different methods as outlined in Table 6, with different assay platforms and manufacturers having different sensitivity and lower limit of detection.

Table 6: Comparison of different methods for cytokine detection

Method	**Description**
ELISA[455,456]	Solid phase immunoassay utilising capture and detection antibodies to quantify cytokines. Generally, only one cytokine detected per well although multiplex assays available.
Bead Arrays[457,458]	Multiplex assays where specialised beads conjugated with various dyes that fluoresces strongly within a specific channel filter. Each bead is coated with antibody detecting a cytokine of interest and multiple beads can be incubated with a particular sample
SIMOA[459-461]	A new technology using magnetic beads capable of detection one protein molecule per bead with very high sensitivity. However, there is limited availability of access to this platform and currently assay kits only offer detection of a limited number of cytokines.
Western Blot[457].	Involves separating denatured proteins in a polyacrylamide gels followed by protein transfer to nitrocellulose or polyvinylidene difluoride membranes. Detection of the presence of specific cytokines is then made by the addition of specific antibodies and staining. Western blotting is not as sensitive as ELISA-based methods but gives additional information about protein molecular weights.
Mass spectrometry[457,462].	Mass spectrometry can be used for identification and quantification of different proteins and hence can detect a multitude of cytokines. I performed discovery mass spectrometry to examine for biomarkers including, potentially, cytokines. This is discussed in chapter 6.

Based on the available literature, the sample sizes I had and the limit of detection of assays, I selected the Milliplex Millipore High Sensitivity T Cell Magnetic

panel, which had multiplexed bead assays that represented pathways of immunity that were likely to be represented (Chapter 1, section 1.2.2.5 and Chapter 4, section 4.1) and high sensitivity/ low limit of detection. Given that the immune response in antibody-negative AE may encompass pathways other than those relative to B cell antibody production, I aimed to examine a broad range of CSF cytokines including those associated with CD4+ T cell subsets, B cell function and proinflammatory responses (Table 7) [16 4 ,463 ,464]. Where cytokines of interest were not available on the High Sensitivity T Cell Magnetic panel, other cytokine panels from the same manufacturer were used to avoid potential issues with manufacturer-manufacturer assay variation.

Table 7: Cytokines and related immune cells/function [4,16]

Related immune cells/function	**Cytokines examined**
Innate immunity	GM-CSF, GCSF, IL1β
Th1	ITAC, ITAC (CXCL11), IL-12p70, CXCL9, CXCL10/IP-10
Th2	IL-13, IL4, IL5, TARC (CCL17), Eotaxin
Th17	IL17a, IL-6, IL-8, IL23
B cell	BCA-1 (CXCL13)
Immunoregulatory	IL-10
Other/multiple immune actions	IL-21, IL-2, IL-7, TNF-α

2.5.3.2 Multiplex CSF cytokine Assay

CSF cytokines analysis was performed using a bead-based multiplex Luminex assay on a research base only (Milliplex; Merck Millipore Darmstadt Germany; Table 7)

using the magnetic multi bead array kits (Milliplex; Merck Millipore Darmstadt Germany; Kits MPHSCTMAG28SK17; MPHCYP3MAG63K0; MPHCYTOMAG60K02; MPHCYP2MAG62K01). CSF cytokines were run in duplicate except when the sample amount was insufficient when it was run in singlicate as per manufacturer's instructions[465-468]. The serum matrix supplied in each commercial kit was intended for use in preparation of serum samples. Under the instructions of Milliplex's scientific liaison, serum matrix was substituted with assay buffer which was more appropriate for use with CSF samples.

Prior to commencement of each assay reagents were allowed to come to room temperature. All standards buffers and quality controls were reconstituted and diluted according to kit instructions. Individual vials of beads for cytokine detection were mixed according to kit instructions. All CSF samples were used neat (undiluted) in the assay as per the protocols.

The protocol for the Human High Sensitivity T-cell Magnetic Bead Panel is outlined below as representative for the cytokine kits used. Protocols for all other cytokine kits followed similar steps and were performed as per manufacturer instructions. Lower limits of detection of each cytokine are outlined in Tables 8-11. Standard curves were appropriate for all cytokine assays performed. Figure 8 illustrates a standard curve for the Human High Sensitivity T-cell Magnetic Panel representative across the assay kits. R^2 for all standard curves ranged from 0.9654-0.9994. Table 12 describes the expected concentration of standards across the different cytokine kits. The majority of results fell between standards 0-2. Supplied kit quality controls were within appropriate levels.

2.5.3.2.2.1 Immunoassay procedures

Immunoassay procedure was followed as per the supplied protocols cytokine assay kits which were similar between kits. For example the #HSCTMAG-28SK kit insert[466] is as follows:

1. 200uL of wash buffer was added to each well of a 96 well plate. This was sealed and mixed on a plate shaker for 10 minutes at room temperature
2. Wash Buffer was decanted and residual amount removed from all wells but tapping it onto absorbent towels
3. 50uL of diluted standard or quality controls added to appropriate vials. Assay buffer was used for the 0pg/mL standard (background)
4. 25uL of assay buffer was added to sample wells
5. 25uL of CSF was added neat to sample wells
6. The mixed cytokine detection beads were vortexed. 25uL of mixed beads were added to each well
7. The plate was sealed, wrapped in foil and incubated with agitation on a plate shaker overnight (16 hours) at 4 degrees Celsius.
8. Well contents were removed after incubation and plate was washed 3 times according to washing instructions:
    a. The plate was rested on a magnet for 60seconds prior to removal of contents to ensure beads were not inadvertently removed
    b. Washing was performed by adding 200uL of wash buffer, shaking for 30 seconds on a plate shaker and well contents removed again
9. 50uL of detection antibodies were added to each well

10. The plate was sealed, covered with foil and incubated with agitation on a plate shaker for 1 hour at room temperature
11. 50uL of Streptavidin-Phycoerythrin were added to each well
12. The plate was sealed, covered with foil and incubated with agitation on a plate shaker for 30minutes
13. Well contents were removed and wash plate washed 3 times
14. 150uL sheath fluid was added to all wells, the beads were resuspended by agitation with a plate shaker for 5 minutes
15. The plate was analysed by the Luminex® 200™

Table 8: Lowest limits of detection for the Milliplex Map Kit Human High Sensitivity T Cell Magnetic Bead Panel[466]

Analyte	**Lower limit of detection (pg/mL)**	**Lower limit of detection +2SD (pg/mL)**
ITAC	1.25	1.98
GM-CSF	0.35	0.60
IFN-γ	0.48	0.94
IL-10	0.56	0.93
IL-1β	0.14	0.24
IL-6	0.11	0.17
IL-8	0.13	0.2
TNF-α	0.16	0.21
IL-12p70	0.15	0.27
IL-13	0.23	0.34
IL-17	0.33	0.52
IL-2	0.19	0.3
IL-21	0.14	0.20
IL-23	3.25	5.11
IL-4	1.12	1.84
IL-5	0.12	0.22
IL-7	0.42	0.6

Table 9: Lower limits of detection for the Milliplex Human Cytokine/Chemokine Magnetic Bead Panel[468]

Analyte	**Lower limit of detection (pg/mL)**	**Lower limit of detection +2SD (pg//mL)**
Eotaxin	4.0	6.8
GCSF	1.8	3.3
IP-10	8.6	14.0

Table 10: Lower limits of detection for the Milliplex Map Kit Human High Sensitivity T Cell Magnetic Bead Panel II[466]

Analyte	**Lower limit of detection +2SD (pg//mL)**
BCA-1	1.3
TARC/CCL17	0.4

Table 11: Lower limits of detection for the Milliplex Map Kit Human High Sensitivity T Cell Magnetic Bead Panel II[466]

Analyte	**Lower limit of detection +2SD (pg//mL)**
CXCL9	19.2

Table 12: Range of expected concentration for standards (pg/mL)

Standard	**Human High sensitivity cell Magnetic Panel**	**Human cytokine/ chemokine Magnetic panel**	**Human cytokine/ chemokine Magnetic panel II**	**Human cytokine/ chemokine Magnetic panel III**
1	0.18-7.9	3.2	1	48.83
2	0.73-31.7	16	3.9	195.31
3	2.9-126	80	15.6	781.25

4	11.7-507	400	62.5	3125
5	46.87-2031.25	2000	250	12500
6	187.5-8125	10000	1000	50000
7	750-32500	n/a	n/a	n/a

*NB: Ranges are provided as concentrations may vary across the different cytokines detected if many cytokines are being examined in one multiplex assay

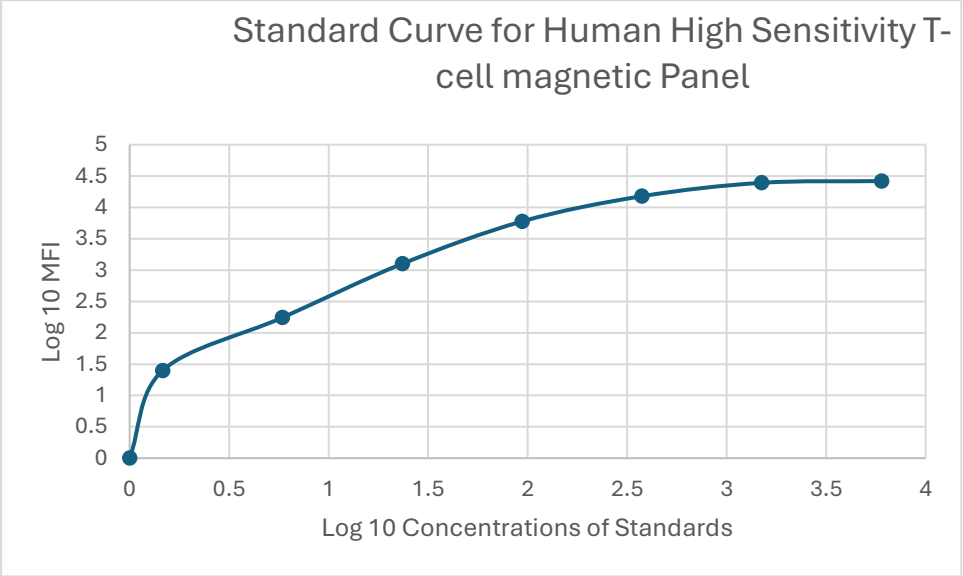


Figure 8: Standard curve for Human High Sensitivity T cell magnetic Panel

This standard curve is representative of all standard curves across the different cytokine detection assays. The first point at 0,0 represents background (blank), each subsequent point represents standards 1-7.

**MFI = Median Fluorescent Intensity*

2.7 Proteomics Analysis of CSF by Mass Spectrometry

2.7.1 Inclusion of Patients

The aim of this pilot study was to see if there was any indication of differences in proteomic signatures in patients with AE (antibody positive and negative) and psychiatric disease. Patients included for mass spectrometry analysis were previously recruited as described in chapters 2 and 3[7]. They were compared with the NI control cohort, consisting of patients with idiopathic intracranial hypertension and normal pressure hydrocephalus as described in chapter 2 and 3.

Patients who were selected for mass spectrometry were patients who had already been included in the analysis of other biomarkers of neuroinflammation as described in chapters 2 and 3. To be included patients and controls needed to have sufficient (at least 200uL) available CSF.

2.7.2 Albumin Depletion

Albumin depletion of pooled human CSF samples was conducted with Abcam Albumin depletion kit (ab241023) according to kit instructions[469]. Samples were processed according to manufacturer instructions and applied to the binding columns in 1.5 mL Eppendorf tubes then incubated on a rotatory spinner for 30mins. The columns were then centrifuged at 1,500 x g for 1 minute at room temperature to collect albumin depleted samples.

Albumin depletion was confirmed by checking CSF protein and albumin (Siemens BNII Nephelometer) levels of the sample pre and post depletion.

Confirmation of albumin depletion was also checked by use of polyacrylamide gel electrophoresis.

2.7.3 Confirmation of Albumin Depletion

Confirmation of albumin depletion was performed by quantifying albumin using a commercially available diagnostic assay at a diagnostic laboratory (SEALS Pathology; Siemens BNII Nephelometer), and also quantifying CSF total protein (ICPMR Pathology; Roche Cobas Turbidimeter)

Albumin depletion was also examined using polyacrylamide gel electrophoresis. Briefly, 30 microlitres of sample was mixed with 10microlitres of 4x Laemmli buffer containing 10% 2-mercaptoethanol. Samples were incubated at 95 degrees Celsius for 10 minutes. Samples pre and post dilution were pipetted into individual wells at volumes of 5-20 microlitres.

Gels were run at 80V for an hour, after an initial 120V for 3 minutes. Gels were removed and washed with tap water. Gels were fixed in 20mLs of 50% methanol containing 10% acetic acid. Gels were stained in Coomassie blue for 1 hour then washed with tap water and destained over several days with 5% acetic acid.

Results for this are discussed in section 3.2.3 and in Figure 13.

2.7.4 Sample Preparation for Mass Spectrometric Analysis

CSF samples were examined in 200-270uL aliquots, depending on sample availability. All samples were checked for total protein content using Qubit Fluorometric Quantification (Thermo-Fisher) prior to analysis.

10ug of protein from the CSF sample was taken and made up and made up to 208uL with 25mM ammonium bicarbonate (Sigma Aldrich, Burlighton MA) (ph7.8). Dithiothreitol (Sigma Aldrich, Burlighton MA) (DTT) was then added to give a final concentration of 10mM. Iodoacetamide (Sigma Aldrich, Burlighton MA) (IAA) was then added to a final concentration of 55mM. Then 0.2ug of sequencing grade, modified trypsin (Promega, Madison WI) was added, and the samples were incubated overnight at 37C.

On the following day, 100% trifluoroacetic acid (Sigma Aldrich, Burlington MA) (TFA) was added to give a final TFA concentration of 1%. The samples were spun down and cleaned up using an OASIS Hydrophilic-Lipophobic Balanced cartridge 1cc/10mg (Waters, Taunton MA, USA)elution plate. The samples were eluted in 200ul 50% Acetonitrile (ACN) and 0.1TFA, dried down and reconstituted in 8uL using a mass spectrometry (MS) loading buffer. Three microlitres of the resulting peptide solutions from the single digest procedure were then analysed using the MS acquisition strategy of choice.

Sample preparation for mass spectrometry and analysis with the mass spectrometer as outlined in this section (2.7.4) was performed by Angela Connolly from the Charles Perkin Centre (CPC) whose technical expertise ensured reliability of the results.

Chapter 3: Mass Spectrometry Method Evaluation and Selection

The first step of my analysis was to try to identify the most efficient method for discovery proteomics of my CSF samples. I wanted to compare the protein recovery using various mass spectrometry techniques in unaltered CSF samples to determine the optimal approach. Method selection was performed with discussion with my collaborators at the CPC who processed the supplied CSF samples as outlined in section 2.7.4.

3.1 Introduction to Mass Spectrometry Methods for Biomarker Discovery

Mass spectrometry is a powerful analytical technique that measures the mass to charge ratio of ions. Mass spectrometers consist of an ion source that converts analyte molecules into gas-phase ions which are then separated based on the mass-to-charge ratio. A detector then records the number of ions at each mass-to charge ratio[470]. As the analytes travel through the mass spectrometer, fragmentation may occur. Fragmentation occurs at specific bonds depending on the chemical nature and strength of the bond and this produces a unique mass spectral signature related to the analyte's molecular structure[471].

Mass spectrometry has applications across a wide range of field with both qualitative and quantitative uses. It is used in the diagnostic laboratory for toxicology, therapeutic drug monitoring and identification of micro-organisms by matching ionised

proteins and peptides that are specific to the organisms, matched within a validated spectral library[471]. Indeed, the ability of mass spectrometry to accurately identify a wide variety of analytes in very low concentrations has meant that it is a powerful tool in lipidomic, metabolomic and proteomic research, particularly in the identification of novel biomarkers in a variety of specimen types[472,473].

There are two main approaches in the use of mass spectrometry: quantitative and discovery. In the diagnostic laboratory, quantitative mass spectrometry allows detection of an absolute concentration of an analyte by measuring the signal of the analyte's mass relative to a known amount of an internal mass standard[474]. In contrast, in discovery proteomics, mass spectrometry enables comprehensive analysis of the entire proteome [475]. There are several different mass spectrometry methods available and selection of them depends on the required sensitivity, and area of application. Techniques are being continuously refined with newer instruments and analytical techniques being developed that offer greater sensitivity and accuracy.

Liquid chromatography-tandem mass spectrometry (LC-MS) is current the dominant method used for discovery biomarker proteomics in biological fluids[476]. In this technique, proteins from the biological sample are isolated and enzymically digested into peptides, usually with trypsin. Liquid chromatography then separates these peptides based on their chemical properties and mass spectrometry is used to analyse the separated components by ionising them and measuring their mass-to-charge ratio. Protein data is then inferred from analysis of the peptide data[476]. LC-MS has advantages of high sensitivity and selectivity including the ability to detect proteins present at low abundances in complex biological samples. It does not require prior knowledge of

proteins present and thus allows for simultaneous analysis of a vast number of proteins in an unbiased manner[477,478]. It has high throughput and reproducibility and a broad dynamic range[479], being able to quantify proteins spanning a wide range of concentrations [476].

In the diagnostic laboratory, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is used for microbiology applications[480]. However, LC-MS is also commonly employed in analysis of biological fluids including in biochemistry and toxicology due to its high sensitivity, specificity and ability to quantify low abundance molecules in complex biological samples[480] [476]. There are also advanced acquisition techniques used in LC-MS including BoxCar and data-independent acquisition (DIA) which further increases sensitivity of analysis.

Image removed due to copyright

Figure 9: The process of mass spectrometry

Images taken from[2]: Biotechnology N. Traditional means to study proteins – Mass spectrometry. (https://www.nautilus.bio/blog/traditional-means-to-study-proteins-mass-spectrometry/).

3.1.1 1D vs 2D LCMS

There are two main LC-MS strategies that can be employed in discovery mass spectrometry. In one-dimensional (1D) LCMS, the sample components are separated by a single chromatographic column and then analysed by mass spectrometry. In 2D-

LCMS, the sample is separated by one liquid chromatography method into multiple fractions in the first dimension. The fractions from this separation are further separated by a second, orthogonal liquid chromatography method. Thus, compounds that may co-elute in one dimension may be resolved in the other and the overall separation power is increased. This increases the sensitivity of the method to detect low abundance peptides[9,481,482].

Image removed due to copyright

Figure 10: 2D-LCMS involves 2 dimension of separation of the sample using liquid chromatography before mass spectrometry analysis. This increases overall separation power

Images taken from[9]: Stoll DR, Carr PW. Two-Dimensional Liquid Chromatography: A State of the Art Tutorial. Analytical Chemistry 2017;89(1):519-531. DOI: 10.1021/acs.analchem.6b03506.

Available at: https://pubs.acs.org/doi/10.1021/acs.analchem.6b03506

When deciding between 2D-LCMS and 1D-LCMS for analysing biological fluids, several additional factors should be considered. Adjustments such as varying gradient erlengths, peptide loading amounts, analytical column dimensions, and specific sample preparation steps, especially when proteins are pre-fractionated using gel electrophoresis, can significantly improve protein coverage in 1D LC-MS[483]. These optimisations can make 1D LC-MS results comparable to those of 2D LC-MS, though they often require more extensive preparation and higher consumable [483]. As a result,

the typical advantages of 1D LC-MS in terms of cost and time efficiency may be diminished.

3.1.2 BoxCar Liquid Chromatography-Mass Spectrometry

BoxCar LC-MS is an advanced acquisition strategy which may enhance the detection of low-abundance proteins. In conventional LC-MS, during MS1 (the first level of mass spectrometry that surveys all the molecules in a sample), all the ions are captured simultaneously which may lead to dominance by highly abundant ions and reduced sensitivity for low abundant species. However, BoxCar LC-MS divides the MS1 spectrum into small mass:charge segments and then selectively accumulates ions within each box. This then allows for longer injection times for low-abundance regions which enhances sensitivity and dynamic range[484].

The BoxCar method also reduces missing data that can be a problem in conventional LC-MS because of preferential selection of abundant ions, by providing more consistent quantification across replicate injections and deeper sampling of low abundance proteins[485]. Hence, it is also a technique that can enhance protein discovery in in evaluating clinical and biological samples.

3.1.3 DIA LC-MS

Data-independent acquisition (DIA) LC-MS is another acquisition strategy in LC-MS that offers detection of high and low abundance molecules across complex biological samples. This technique differs from data-dependent acquisition (DDA), which is commonly used in traditional 1D and 2D LC-MS. In DDA, peptide signals that rise above the noise in a full-scan mass spectrum are selected for fragmentation which then produces tandem mass spectra for database matching. However, this process is biased to pick peptides with the strongest signal and hence there is a weakness in the detection of low-abundance peptides[486,487,488].

Image removed due to copyright

Figure 11: BoxCar LC-MCS acquisition

Box Car LC-MS acquisition involves divide the mass spectrum into "boxes": narrow m/z windows, accumulation ions within each box and then analysing accumulated ions in a single scan which increases sensitivity and dynamic range[11].

Images taken from[11]: Wu J, Wang H, Zhao X, Qiu H, Li N. High sensitivity and high-confidence compound identification with a flexible BoxCar acquisition method. Journal of Pharmaceutical and Biomedical Analysis. 2022 Sep 20;219:114973.

In contrast, DIA overcomes these limitations by systematically fragmenting all ions within predefined mass-to-charge (m/z) windows. The analysis is repeated until the full mass-to-charge range is covered. This results in detection of low abundance peptides with greater reproducibility and reduced missing values[486,489,488]. Additionally, the workflow of DIA using 1D LC-MS potentially avoids the dilution issues in some 2D-

LC-MS setups as analytes do not need to undergo two rounds of separation. As a result, DIA may offer increased sensitivity and a more simplified workflow, with analyses completed faster than traditional 2D-LC-MS methods.[9,489-491].

Disadvantages of DIA mass spectrometry include that it generates massive datasets due to systematic fragmentation of all ions within the predefined m/z ratios. Specialised, commercial software is often required for effective data deconvolution[488,492] . DIA methods have traditionally depended on first collecting exhaustive, sample-specific spectrum libraries[490 493]. However, methods for DIA analysis that do not require spectrum libraries (termed library-free), where peptides are detected directly from DIA experiments, are now also described and used.[493 ,494 ,495].

Despite these complexities, DIA has become a viable alternative to traditional DDA-based 2D-LC-MS for discovery-driven mass spectrometry analysis of complex biological samples, such as cerebrospinal fluid, which presents its own unique analytical challenges.

3.1.4 Challenges in Discovery (Shotgun) Mass Spectrometry of CSF

CSF itself is a challenging biological fluid to analyse using mass spectrometry. The total protein concentration in adult CSF is low (0.15-0.45 g/L[208]), especially when compared to blood (60-80g/L in adults[496]). Furthermore, there is a large dynamic range of individual protein concentrations in CSF, making detection and quantification of low abundance proteins more challenging[497].

Image removed due to copyright

Figure 12: DDA vs DIA LC-MS techniques

Traditional data dependant acquisition (DDA) mass spectrometry vs data independent acquisition (DIA) mass spectrometry. In DDA, the mass spectrometer selects a fixed number of the most intense precursor ions which are fragmented and analysed. In DIA, all peptides within a defined mass to charge (m/z) window are fragmented and analysed. This can provide a more complete view of the proteome.[8]

Images taken from: Crown Bioscience. 4D-DIA quantitative proteomics [Internet]. 2025 [cited 2025 Aug 22]. Available from: https://www.crownbio.com/technologies/biomarker-

Biological fluids with high dynamic range may result in failure of detection of low abundance proteins. Trypsin preferentially digests abundant proteins, which can lead to higher abundance proteins dominating the enzyme's activity and less abundant proteins are likely to be reached less effectively. This feature of trypsin, however, can be exploited in 2 step digestion protocols to increase low abundance protein recovery[498]. In addition, samples with high dynamic range can exceed the dynamic range of the mass spectrometry detectors. High abundance proteins are hence preferentially detected,

leading to under sampling of low abundance proteins or missing their detection all together.

High abundance proteins in CSF include albumin, immunoglobulins, transthyretin, transferrin, alpha 1 antitrypsin, alpha-1-acid glycoprotein, haptoglobin, alpha2-macroglobulin, complement C3 and, and apolipoproteins)[499]. In contrast less abundant proteins in CSF include neurofilament, light chain[500], cytokines[7,463] and orexin[501]. However, these low abundance proteins (as discussed in chapter 1) do have biological roles and serve as biomarkers in disease.

Hence, strategies to overcome the challenge of low protein concentrations, wide dynamic range and dominance of high-abundance proteins are essential in mass spectrometry based proteomic analysis. These approaches are designed to enhance both the detection and quantification of low-abundance proteins, addressing the obstacles that complicate comprehensive biomarker discovery[499 ,502].

Depletion of high abundance proteins, including depletion of albumin alone or depletion of other high abundance proteins, reduces the dynamic range required for detection and improves identification of low abundance proteins[499 ,503,504]. Other enrichment strategies include using ligand libraries to enrich medium and low abundance proteins [499 ,504] and fractionation methods to better map the global CSF proteome[502]. One common enrichment strategy is to deplete albumin, the most common protein in CSF[503]. This process allows for better detection of low-abundance proteins, increases the number of proteins detectable in CSF and hence improves biomarker discovery.

3.2 Which method is best for proteomic CSF examination?

3.2.1 Introduction

The process of deciding which mass spectrometry analysis to undertake was to determine if mass spectrometry techniques available with my collaborators at the CPC was appropriate for CSF analysis and for decision of which method enabled the best protein identification for use on study samples. The aim as a first step was to compare Box Car LC-MS, 1D-LC-MS and 2D-LC-MS initially on raw CSF samples. For these initial steps, samples of patients and controls included in the study were not used as these were available in very limited volumes.

3.2.2 Mass Spectrometry Method Selection

4 samples of de-identified human CSF were pooled to a total volume of 6mL (1.5mL each). These de-identified samples were samples from the diagnostic laboratory where all diagnostic testing requested had been performed, and they were to be discarded. For this first step, no depletion of high abundance proteins was performed.

The 6mL of pool human CSF was vortexed and split into 250uL aliquots for processing, which is similar to the standard volumes available for testing in routine diagnostic workflows, and submitted to the CPC. Samples were prepared for mass spectrometry analysis using the routine procedure outlined in chapter 2, section 2.7.4.

1D LC-MS identified 383 protein groups, 2D LC-MS identified 1573 protein groups and BoxCar 1D LC-MS identified 1648 protein groups (Table 13).

The most abundantly available protein was albumin which consisted of 20% of the peptide spectral matches. the other five most abundant proteins were

serotransferrin, complement C3, apolipoprotein E and haptoglobin. These findings were consistent with most common protein groups identified previously in CSF[499].

Table 13: Initial comparison of identifications across different mass spectrometry techniques with pre-depletion samples only

Method	Protein groups identified (pre albumin depletion)
1D LC-MS	383
2D LC-MS	1573
Box Car	1648

Surprisingly, a 1D LC-MS method, albeit using the more advanced BoxCar acquisition technique, was able to identify more protein groups than 2D LC-MS. As discussed in the introduction to this section, 2D LC-MS generally has better sensitivity than 1D LC-MS methods. However, it is to be noted that the processing of data for 2D LC-MS employed a software package called Proteome Discoverer whereas the BoxCar method used a software package called MaxQuant. MaxQuant was the only known available software package available for BoxCar LC-MS at the time of analysis. Hence, the differences in protein identification could have been contributed by differences in how the different software programs identified proteins(e.g. data processing and filtering of results) for 2D LC-MS versus BoxCar.

It was hence decided that any further comparisons or decision between methods should be made between BoxCar and 2D LC-MS given their clear advantages in being able to identify more protein groups over 1D LC-MS.

3.2.3 Albumin Depletion to Improve Proteomic Identification

The aim of protein enrichment was to determine 1. if albumin depletion would increase protein discovery (as discussed in section 3.1.4) and 2. Which mass spectrometric method post depletion allowed for the best identification of protein groups.

As all the previous pooled human CSF had been submitted to the CPC, a new batch of 6mL of pooled CSF was made using the method outlined in 2.7 and 3.2.2. This was again split into 250uL aliquots.

Albumin depletion of pooled human CSF samples was conducted using instructions from the Abcam Albumin depletion kit (ab241023) as outlined in Chapter 2, section 2.7.2. This was performed on six of the aliquots (1.5mL of pooled human CSF in total).

Albumin depletion was confirmed by submitting pre and post depleted CSF aliquots to check CSF protein and albumin (Siemens BNII Nephelometer). The level of protein in the pooled CSF pre depletion was 0.52g/L and post depletion was 0.1g/L. Level of albumin pre depletion was 0.18g/L in samples post depletion were <0.02g/L. The discrepancy between protein and albumin levels was noted but this may have been due to different instruments and assays measuring protein and albumin respectively, alternatively, it may represent some non-albumin proteins were also depleted.

The albumin depletion procedure was also performed with the Pierce™ Albumin Depletion Kit (Thermo Fisher Scientific) as a comparator (using another 6 x 250

microlitre aliquots), following manufacturer instructions. Results of albumin and protein levels pre and post depletion was equivalent to the Abcam kit.

Confirmation of albumin depletion was also checked by use of polyacrylamide gel electrophoresis (Figure 13). The process of this was detailed in section 2.7.3.

At the time of analysis, the focus was ensuring adequate albumin depletion, which there was evidence of. However, when reviewing this data (after study samples had been analysed), it was noticed that albumin was not the only protein depleted, consistent, in part, to the discrepancy between protein concentration reduction being greater than the albumin reduction. To confirm and quantify this observation, pre and post depletion bands were compared using standard densitometry analysis of the scanned imaged using ImageJ (https://imagej.net/). Briefly, the image was uploaded to imagej.net and densitometry of each protein band was obtained. Comparison of

densitometry peaks gave data about relative reduction in protein across the non-depleted versus depleted aliquots.

After albumin depletion, there was a 41–44% relative reduction in non-albumin protein bands, indicating that the depletion process was not entirely specific for albumin and led to the loss of other proteins as well. This conclusion was further supported by nephelometry and turbidimetry: the measured pre- vs. post-depletion

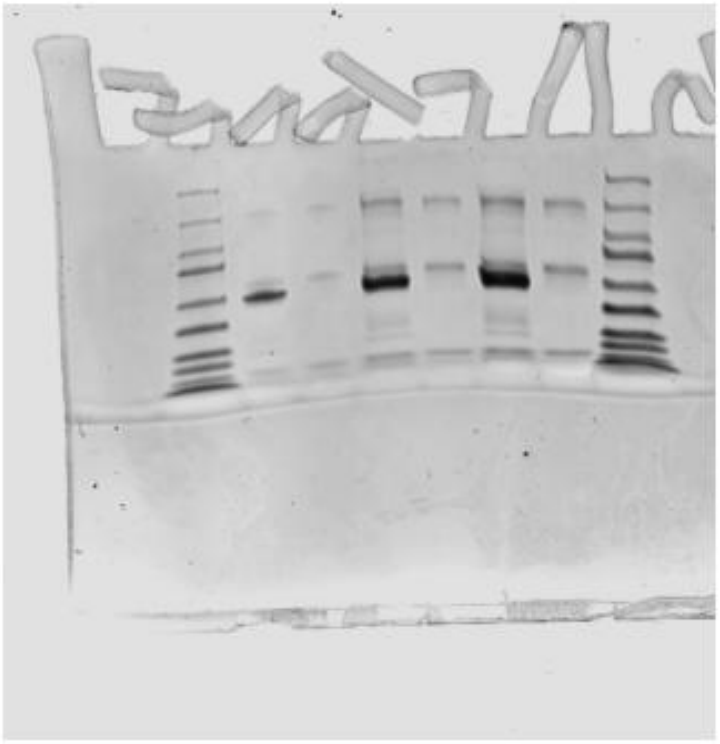


Figure 13: Polyacrylamide Gel electrophoresis of CSF pre and post albumin depletion.

Wells from right to left are as follows: Well1: No sample; Well 2: 5 microlitres Protein standards: 250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa (15 kDa and 10 kDa partially resolved); Well 3: 5 microliters CSF (30 microliters of sample with 10 microlitres of Laemmli buffer); Well 4: 5 micro litres Albumin depleted CSF (30 microliters of sample with 10 microlitres of Laemmli buffer); Well 5: 10 microliters CSF (30 microliters of sample with 10 microlitres of Laemmli buffer); Well 6: 10 microlitres Albumin depleted CSF (30 microliters of sample with 10 microlitres of Laemmli buffer); Well 7: 15 microliters CSF (30 microliters of sample with 10 microlitres of Laemmli buffer); Well 8:15 microlitres Albumin depleted CSF (30

differences in albumin concentration were smaller than the total protein concentration differences, confirming that proteins other than albumin were also depleted.

3.2.3 Comparison of Techniques Post Albumin Depletion

Samples following albumin depletion underwent mass spectrometry with comparison of methods. As a fresh pool of human CSF was prepared for this step of the analysis, depleted and non-depleted aliquots of the same pooled human CSF was supplied to the CPC. Both depleted and non-depleted aliquots were analysed to determine how much additional increased identification of low abundance proteins was achieved with albumin depletion. At this stage, after discussion with my collaborators, I decided to also compare DIA LC-MS with BoxCar and 2D LC-MS. Aliquots from the same pool of human CSF were used across all analysis methods for consistency in comparison.

Depletion of albumin increased protein recovery across all methods, even in the face of protein lost due to albumin depletion (Table 14). As discussed in section 3.1.4, this was an expected outcome as depletion of high abundance proteins such as albumin improves identification of low abundance proteins [499,502].

Table 14: Second comparison of identifications across different mass spectrometry techniques with pre and post albumin depletion samples

Sample/Method	Peptide sequences identified	Protein groups
DIA pre albumin depletion	3856	627
DIA albumin depleted	10391	1430
BoxCar pre albumin depletion	4152	646

BoxCar albumin depleted	10200	1393
2D LC-MS pre albumin depletion	4055	730
2D LC-MS albumin depletion	7310	1264

3.2.4 Decision About Choice of LC-MS Technique For Sample Analysis

As detailed above (Table 14), BoxCar identified fewer peptide sequences and protein groups than DIA. 2D LC-MS appeared to find the fewest peptides (with the caveats of differences in software packages as discussed in section 3.2.2). DIA analysis offered advantages compared to BoxCar because the data could be analysed by a range of software packages and hence, there was more flexibility in analysis of results. In comparing DIA versus 2D LC-MS, it was decided that exploratory analysis with 1D-LCMS utilising DIA would be the best option: to give the optimum amount of data analysable.

3.2.5 Concentration Dependent Detection of CSF Proteins

Given that patient diagnostic CSF samples have varying protein concentration, I analysed sequential dilutions to the same sample volume of my pooled human CSF to determine at what protein level the diversity of peptides detected by mass spectrometry would start to reduce. This analysis was performed on a fresh sample of pooled human CSF (as not enough volume was available after the previous experiment comparing different techniques). This sample of pooled human CSF had a protein concentration of 0.2mg/mL pre albumin depletion. Protein concentration post albumin depletion was 0.11g/L (22ug of protein in a 200uL aliquot).

Different sample volumes were tested post depletion ranging from a total sample protein content of 11ng to 10ug of protein. The number of protein species detected peaked at 5ug and gave similar results to 10ug of protein. A sample with total protein content of 1ug resulted in the detection of 70-90% of protein species than the 5ug total protein.

Chapter 4: Novel Surrogate Markers of CNS Inflammation in CSF in Autoimmune Encephalitis

4.0 Preface

This chapter is based on a manuscript that has been accepted for publication:

Jiang JX, Fewings N, Dervish S, Fois AF, Duma SR, Silsby M, et al. Novel surrogate *markers of CNS inflammation in CSF in the diagnosis of autoimmune encephalitis*. Frontiers in neurology. 2020;10:1390.

Avaliable at:
https://www.frontiersin.org/journals/neurology/articles/10.3389/fneur.2019.01390/full

I was the primary author, responsible for study design, patient recruitment, data collection, specimen analysis for cytokine assays, data analysis and manuscription drafting, preparation and revision.

Other author contributions are as listed and detailed in Acknowledgements:

Nicole Fewings for running some of the cytokine assays and assistance with preparation of the heat map; Suat Dervish for operating the Luminex platform for cytokine analysis; Sushil Bandodkar for expertise in running and interpretation of the CSF neopterin assay; Alessandro Fois, Stephen Dua, Matthew Silsb, Sudarshini Ramanathan, Andrew Bleasel for patient recruitment; Bryne John for recruitment of non-inflammatory controls; David Brown and Ming-Wei Lin for assistance with refining the conceptualisation of the study design and supervision of research undertaken.

All co-authors contributed to review and revision of the published manuscript.

4.1 Introduction

Autoimmune encephalitis has emerged in the last decade as an important differential in the clinical presentation of patients with new-onset encephalopathy[156,157]. However, diagnosis remains challenging due to the varying range of clinical presentations and overlapping symptoms with other neurological and psychiatric conditions[238,257,505]. Biomarkers, including autoantibodies in CSF and serum, and inflammatory markers, play critical roles in differentiating autoimmune encephalitis from other differential diagnosis[179]. However, there are limitations in what is currently available, and many routinely used investigations are not specific[179,238].

Diagnosis of any medical condition is not based solely on an investigation result but requires clinical phenotyping based on a patient's clinical history and relevant examination as well as exclusion of other causes. In real world clinical practice, there is a constantly evolving clinical spectrum for antibodies associated with AE, and there are many situations where the clinical presentation may not be classic. Available testing modalities may also not be sensitive nor specific enough to give clinical certainty[179,238,506].

Patients with AE respond to treatment with immunosuppression and delays in treatment may worsen outcome[202,258,259]. However, the clinical conundrum of whether and how aggressively to treat patients when the clinical picture is unclear and investigations are negative or within normal parameters remains. Incorrect diagnosis has the potential to expose a patient to the harmful effects of immune suppression with little benefit[238].

Thus, whilst a published guideline for the clinical identification and diagnosis of AE is available[179], the need for more reliable biomarkers of autoimmune neuroinflammation persist.

4.1.1 Cytokines as Biomarkers For AE

Investigations routinely used for diagnosis of autoimmune encephalitis and some novel biomarkers of neuroinflammation have already been discussed in Chapter 1. CSF cytokines have also emerged as a novel potential marker of interest in AE as cytokine profiling of CSF can give us information about the immune pathways involved in certain disease states.

Most of the studies examining CSF cytokine profiles in AE have been in the context of antibody-positive AE. Unfortunately, studies in the literature have been difficult to directly compare with each other because of differences in cytokine detection methods used and differences in definition of patient cohorts. Published studies have used various detection methods (the majority being either Luminex bead-based assays or multiplex assays but some also using ELISA for cytokine quantitation). Even when a Luminex-based assay was used, the choice of commercial kits differed between studies. Hence, the studies have reported a range of cytokines associated with autoimmune encephalitis and many need to be more extensively examined with other methods such as proteomics.

NMDAR-ab AE is the highest prevalent antibody associated AE, one of the first described, and the condition with the greatest number of studies. IL17a and CXCL13 were the most consistently reported increased cytokine associated with NMDAR ab-AE. One study, using the Bio-Plex Cytokine Assay System (Bio-Rad) found increases in IL17

and found concurrent elevations in IL1β, IL6 (associated with acute inflammation) and CXCL13 (associated with B cell activation and chemotaxis)[507]. This study also found infiltration of Th17 cells in the CSF of patients which further confirmed the associated with IL17. [508]. A second study looked at both patients with NMDAR-antibody and LG-1 antibody associated encephalitis and found that of the cytokines they examined (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IL-23, GM-CSF, IFN-gamma, TNF-α, and CXCL13 by Biorad Luminex based assay) that IL-17A, IL-6 and CXCL13 were elevated in the CSF of patients with NDMAR-antibody AE[509]. However, there were no significant differences between the CSF cytokine profiles of LG-1 AE and controls. Elevation of BAFF and APRIL (both important for B cell development and activation[4]) have also been reported in NDMAR-AE[510]. However, this study did not examine other cytokines in their analysis. A third study, using a Luminex based method from a different manufacturer (ProcartaPlex) looked at changes in CSF cytokines in patients with NMDAR-ab AE during their disease. They found elevations in IP-10 and CXCL13 to be associated to be associated with early-stage disease progression and IL17a, TNFα and, IL15 and INFγ to be slightly increased at all disease periods examined[508].

Studies of non-NMDAR antibody encephalitis has also shown CSF IL-17a to be associated with AE, although not all studies are concordant. A in study adult patients with a mix of different non-NMDAR-associated but antibody-positive AE subtypes, using a Biorad Luminex-based assay, reported higher CSF IL17a associated with AE [511]. High CSF IL-17a was also reported in a study looking at LGI-1 antibody AE, alongside increases in serum and CSF IL8 and serum IL-6 when compared to non-inflammatory controls (patients with NPH, IIH and functional neurological disorders)[512].This study used a multiplex assay (ELLA by Bio-Techne). However, a different study looking only at

patients with LG-1 associated encephalitis (7 patients) and CASPR-2 associated encephalitis (9 patients) found that CXCL13, ICAM (associated with B cells and acute inflammation respectively) and serum IP-10 may be candidate markers for AE in their cohorts[513].

In paediatric populations, one paper found that in a combined cohort of patients with inflammatory neurological disease: NMDAR-ab receptor encephalitis but also demyelinating disease, enteroviral encephalitis and acute disseminated encephalomyelitis (ADEM), it was found that TNF-a, IL10, IFN-a, CXCL13 and IP-10 had the highest associated with any encephalitis. Patients with ADEM had higher Th-17 related cytokines but cytokine levels between enterovirus encephalitis and NMDAR-ab encephalitis were similar[463].

As seen from the above discussion, most of the data available focuses on NMDAR-ab associated AE. Of the few non-NMDAR ab studies, the majority focus on LGI-1 encephalitis. Whilst these studies have shown some similarities with CSF cytokine signatures in NMDAR-ab AE, there are also differences reported. As we increasingly discover dual antibody mediated disease and antibody negative disease, it is possible that these will have significantly differing cytokine signatures. The presence of an antibody negative group where the pathogenesis is not well-defined adds further heterogeneity in the patient group.

Studies on the antibody-negative AE group were particularly lacking in the literature. This is the group where there is the most diagnostic difficulty. The lack of detectable antibodies in this clinical group may be due to the presence of an antibody

that has not yet been described or may point to a different autoimmune/inflammatory pathological mechanism.

4.2 Methods

4.2.1 Patient Enrolment

Patients were enrolled as described in Chapter 2 (Methods), section 2.1. Briefly, patients who had a high clinical suspicion of autoimmune encephalitis were recruited sequentially into the study. These patients were compared against a cohort of viral infection (VI), other autoimmune neurological disease (OAND) and non-inflammation (NI) controls. Inclusion criteria for control cohorts are also detailed in Chapter 2, section 2.1.

4.2.3 Laboratory Tests Available in the Diagnostic Laboratory

Patients recruited into the study underwent laboratory tests routinely available in the diagnostic laboratory. List of what diagnostic tests were performed is described in Chapter 2, section 2.6.

All AE patients underwent the listed assays. Where specimen sample was very limited (the VI cohort and the NI cohort, where the CSF was obtained during spinal anaesthesia), these samples were reserved for novel assays where the reference range for CSF was not established (CSF FLC and cytokines). This is because diagnostic assays available routinely in the diagnostic laboratory have a standard reference range derived from healthy, normal populations.

4.2.2 Cytokine Measurement

Assay selection and cytokine measurement was performed as described in Chapter 2 section 2.6.3. All assays were performed according to manufacturer instructions.

4.2.4 Statistical Analysis

Analysis of the surrogate markers examined in this study was performed using StataMP 13 and scatterplot figures of results were prepared using GraphPad Prism. Heat map analysis of cytokines was performed using Morpheus (Broad Institute) to find cytokines of potential interest.

For continuous independent variables, univariate analysis using the Mann-Whitney U test or Kruskal-Wallis test was performed to compare the various disease groups. Univariate logistic regression was performed for binomial and categorical variables. Significant findings were then combined in a multivariate binary logistic regression modelling to determine markers that were significantly and independently associated with disease group classification (AE vs VI, NI and OAND groups). These markers were then fitted to a predictive model and a receiver operating characteristic (ROC) curve created.

4.3 Results

4.3.1 Patients Details

A total of 32 patients with a high clinical probability of AE (AE) were recruited. These were subdivided into nine antibody positive (AbPAE) patients and 23 antibody

negative (AbNAE) patients. Only one of these patients had an associated malignancy (Table 16). Ten NI controls, 24 VI controls, and five OAND (2 cerebral lupus, 2 multiple sclerosis, 1 cerebral vasculitis) were also included in the analysis. Demographic details of recruited patients are described in Table 15 and were not available for de identified VI controls. The antibodies detected in the AbPAE group were NMDA-R, GFAP, IgLon5, LGI-1, CASPR2, Anti-ANNA1(Hu) and Anti-GAD. Clinical details about these patients are summarised in Table 16.

Of the 10 NI controls, five were perioperative patients where only 1mL of CSF was able to be collected. These samples were reserved for assessment of novel markers. Of the 10 normal/ NI controls, one of the NI patients presented with headache which self-resolved with no apparent cause found. All other patients included as a NI control were diagnosed with either NPH or IIH by a neurologist, had corresponding appropriate investigation findings and demonstrated of improvement with appropriate treatment. Only one NPH patient had a VP shunt insertion prior to his CSF collection for this study.

Two of the OAND controls had commenced immunosuppression at time of lumbar puncture: one cerebral lupus (methotrexate and mycophenolate) and one with cerebral vasculitis (pulsed methylprednisolone). However, both these patients required intensification of immunosuppression prior to remission.

Twenty-five PCR positive de-identified VI controls were included in this study. One was a recruited patient diagnosed with herpes simplex virus (HSV) positive on PCR. Twenty-four others were obtained from aliquoted stored samples and consisted of nine enterovirus (ENTV) positive samples, four HSV positive, three Epstein-Barr virus (EBV)

positive and eight varicella-zoster virus (VZV) positive samples. All ENTV samples were 500 microliters in volume and were used for both CSF cytokine and CSF light chain analysis. There were only 200 microliters of CSF for VZV, EBV and HSV samples. Therefore, 5 HSV, 3 EBV, and 4 VZV samples were used for cytokine analysis and a further 4 VZV samples were used CSF light chain analysis. Thirteen VI samples also underwent IIF on primate brain.

Most investigations were analysed in over 90% of samples from the AE groups. Exceptions were CSF GAD (78%) and serum VGKC (59%). Volumes of samples collected from NI control patients were limited (1-2mL), hence novel investigations without a defined reference range such as CSF cytokines were prioritised for comparison over routine investigations where a reference range for clinical abnormality was already established.

Table 15: Demographic details of included patients for surrogate markers of AE[7]

	NI Controls	OAND controls	AbPAE		AbNAE		
				P (vs NI)		P (vs NI)	P (vs AbPAE)
Number of patients	10	4	9	n/a	23	n/a	n/a
Median Age	54	46.5	37	0.98	44	0.4	0.2
Age Range	17-81	19-60	15-58	n/a	18-73	n/a	n/a
Gender (M:F)	4:6	1:3	3:6	0.4	16:7	0.1	0.02

Table 16: Clinical details of autoimmune encephalitis patients

a) AbPAE: Clinical details and associated antibodies

Patient number	Clinical Presentation	Antibody associated
1	33F Refractory seizures requiring ICU admission	NMDAR

2	15F Severe headaches, atypical depression with psychosis, intermittent scotoma, cognitive decline	NMAD-R
3	38F Transverse myelitis and encephalomeningial myelitis	GFAP
4	18F Recurrent seizures, cognitive decline, optic neuritis	NMDAR and Anti-MOG
5	49M Insomnia, cold sensitivity, cerebellar ataxia	IgLON5
6	48F New temporal lobe seizures	LGI-1
7	55F New seizures, difficult to control	CASPR2
8	23M Difficult to control seizures, involuntary facial movements	Anti-ANNA 1 (Hu)
9	58F Stiff Person's syndrome	Anti-GAD

a) Clinical Details of AbNAE group

Patient	Clinical presentation
1	69F, treatment resistant seizures
2	18F recurrent seizures, facial twitching
3	38M headache, recurrent/refractory seizures
4	23M refractory seizures
5	25M refractory seizures, psychosis, cognitive decline
6	25M refractory seizures
7	36F confusion, seizures, fatigue, short term memory loss
8	22M Unusual behaviour, refractory seizures
9	48M refractory seizures
10	70M recurrent treatment resistant seizures, short term memory loss
11	21F status epilepticus, acute memory decline, agitation, fevers, facial twitching, dysautonomia
12	46F new onset treatment resistant seizures

13	73M Cerebellar ataxia
14	67M cerebelar ataxia, tremor, vitiligo
15	32M rapidly progressive cerebellar syndrome
16	66M rapid onset opsoclonus myoclonus; base of tongue tumour
17	59M cerebellar ataxia
18	26M movement disorder, facial dystonia
19	70M cerebellar ataxia
20	51M, rapid onset cognitive decline and movement disorder
21	69M rapid cognitive decline, myoclonus
22	69F rapid cognitive decline and psychosis
23	38F agitation, rapid cognitive decline, lip smacking, unusual behaviour

4.3.2 Conventional Diagnostic Tests

There was a trend for increased proportions of positive results in some conventional CSF markers of CNS inflammation in patients with AE and OAND compared to NI controls (Table 17), but this was not seen in all patients. Comparison of these markers with VI controls was not available.

Results for conventional CSF markers are summarised here: two of 9 (22%) of AbPAE and 4/23 (17%) of AbNAE patients had evidence of CSF pleocytosis > 5 mononuclear cells. Six of 9 (67%) of the APAE and 4/23 (17%) of AbNAE high-risk patients had evidence of CSF oligoclonal bands. None of the NI group had CSF pleocytosis >5 or oligoclonal bands and these markers could not be statistically

analysed. Raised CSF protein (>0.45g/L) was seen in 4/9 (44%) of the AbPAE and 9/23 (32%) of AbNAE groups but also in 3/5 (60%) of the NI group.

CSF neuronal IIF staining on primate brain was observed in 7/23 (30%) of the AbNAE group but was also observed in 1/5 of the NI group, 1/13 (8%) of the VI controls with sufficient sample for testing (enterovirus only) and 3/4 (75%) of OAND controls. The VI sample with nonspecific IIF staining was EBV positive on PCR however this was supplied as a deidentified aliquot and further clinical details could not be verified.

Results of MRI were only available for patients who were in the combined AbPAE and AbNAE group as determined by their neurologist. Four of 9 (44%) of the AbPAE group and 5/23 (22%) of the AbNAE group had nonspecific changes on MRI indicative of neuroinflammation. No MRI results were available for VI or NI controls.

Receiver operator curve (ROC) analysis was performed using the univariate analysis for CSF protein, CSF pleocytosis and neuronal IIF results. All area under the curve (AUC) results for these conventional markers of AE were less than 0.7. ROC analysis could not be performed on MRI changes due to no control comparator group available, or on CSF oligoclonal bands due to perfect separation (as no abnormalities were found in the NI cohort).

Therefore, whilst markers such as CSF oligoclonal bands, pleocytosis or presence of MRI changes may indicate an autoimmune process, the findings from this study are that they are not sensitive or specific alone [514,515] enough for a reliable diagnosis.

Table 17: Conventional Markers of CNS inflammation[7]

	NI N=5	OAND N=5	AbAE N=9	AbNAE N=23		
	n(%)	n(%)	n(%)	p	n(%)	P
CSF mononuclear cells ≥ 5 (2)	0 (0%)	1(20%)	2(22%)	n/a	4(17%)	n/a
CSF oligoclonal bands	0 (0%)	3(60%)	6(67%)	n/a	5(22%)	n/a
CSF protein > 0.45g/L	3 (60%)	2(40%)	4(44%)	0.5	9(32%)	0.6
Neuronal IIF (any)	1 (20%)	2(40%)	n/a	n/a	8(35%)	0.4
MRI changes	n/a	4(80%)	3(33%)	n/a	5(22%)	n/a

P-values are calculated as AE vs all other control groups (NI, VI and OAND)

4.3.3 CSF Light Chains

Only CSF lambda FLC were higher both AE (p=0.03),) and VI control (p=0.03) groups compared to NI controls. Kappa and lambda were both significant raised in OAND controls (p=0.03 and 0.003 respectively). However, when the AE group were compared to VI controls there was no statistical difference in CSF (kappa or lambda) FLC. Therefore, whilst CSF lambda may be a nonspecific marker of neuroinflammation, it cannot be relied on to differentiate between AE from other differentials, such as viral infection.

4.3.4 CSF Cytokines

Heatmap cluster analysis revealed differential profiles of cytokine concentrations in VI and NI controls compared to the combined AbPAE, AbNAE group and OAND groups (Figure 14a).

On univariate analysis, were no significant differences in cytokine levels between the AbPAE and AbNAE groups. Therefore, for statistical analysis, the AE patients were analysed as one group when compared to NI and VI. There were also no statistically significant differences in cytokine levels between the combined AE versus the OAND groups of patients.

When the combined encephalitis autoimmune group was compared with NI controls, IL-21, IL-12p70, IL-5, IL-4, IL-1β, GCSF, IL-13, IL-2 and GM-CSF were associated with the combined AE group. When compared with VI, IP-10, IFN-γ, IL-10, IL6, IL-8, TNF-α, BCA1/CXCL13, TAC/CXCL11, GCSF, CXCL9, IL-2 and ITAC were higher in VI and IL7, IL13, IL12p70 and IL21 were higher in the AE group (Table 18). Elevation of IP-10, IFN-γ, IL-10, IL-6, IL-8, TNF-α, BCA1/CXCL13, TARC/CXCL11, CXCL9, IL-5, GCSF, IL-2, ITAC and GM-CSF were associated with patients with viral infection vs normal/ NI controls.

Due to the predominance of presentations with seizures in my AbNAE groups, I also examined cytokines in the CSF of all combined AE patients presenting with seizures vs those without, in case that seizures themselves or this disease subgroup was associated with altered CSF cytokines. IL4 was associated with patients presenting with seizures (p=0.01) but there were no other significant differences in cytokine expression.

A multivariate binary logistic regression model was used to compare the combined AE patients with a combined group of, normal, viral and OAND controls[7]. Only IL-21 (p=0.002) and CXCL10/IP-10 (p=0.003) independently contributed to the model. A ROC constructed using this multivariate logistic regression model had an area under the curve (AUC) of 0.90 (Figure 15a). A ratio of IL21/IP-10, in a univariate logistic regression model was also significant when compared a combined group of normal and VI (p=0.01) with an AUC of 0.84 (Figure 15b).

Table 18: Cytokines that may differentiate autoimmune encephalitis from NI and VI[7]

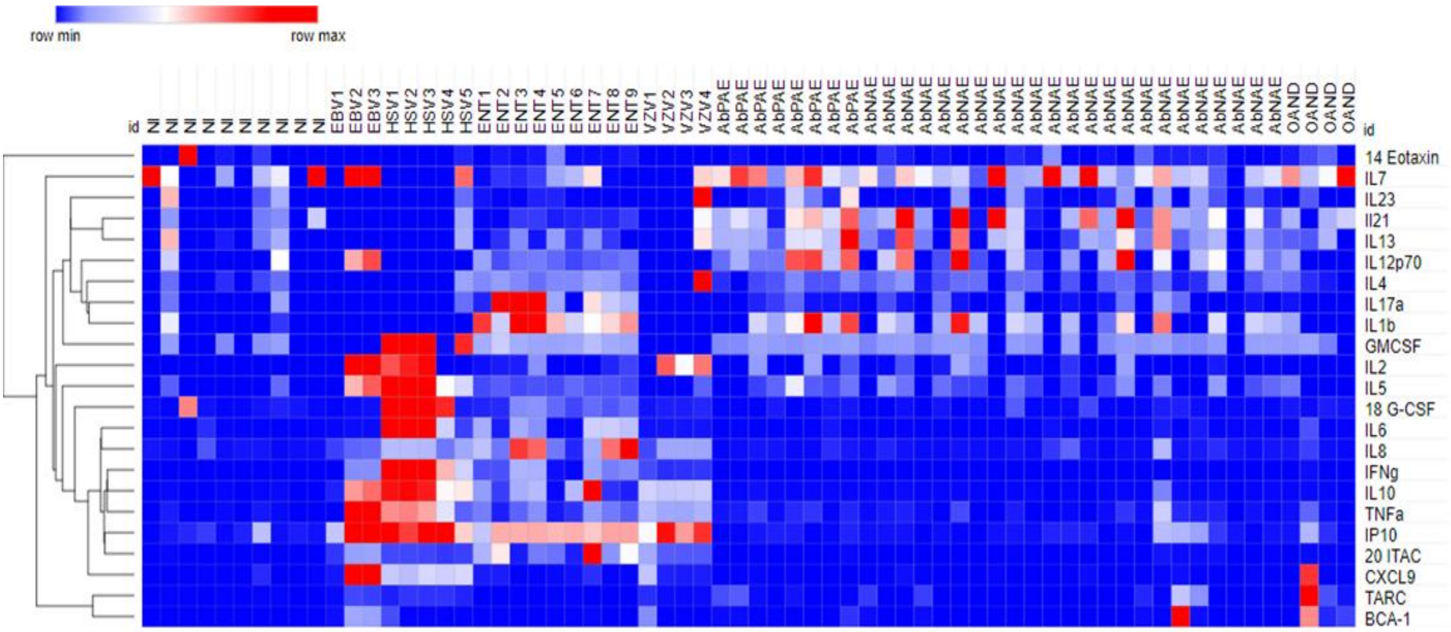
	AE vs Viral	AE vs NI	Viral vs NI	AE vs OAND	OAND vs NI

	Significant?	P-value	Significant?	P-value	Significant?	P-value	Significant?	P-value	Significant?	P-value
IL21	AE	0.0001	Y	0.0002	N	0.8	N	0.49	N	0.097
IL12p70	AE	0.009	Y	0.0164	N	0.2	AE	0.03	N	0.86
IL13	AE	0.0005	Y	0.0145	N	0.89	N	0.3	N	0.25
IL23	AE	0.0004	N	0.30	N	0.16	N	0.12	N	0.33
IL7	AE	0.001	N	0.2145	N	0.51	N	0.59	N	0.32
IP-10	V	0.0001	N	0.2	Y	0.0001	N	0.37	N	0.9
IFN-γ	V	0.0001	N	0.36	Y	0.0001	N	0.7	N	0.86
IL10	V	0.0001	N	0.05	Y	0.0001	N	0.72	N	0.3
IL6	V	0.0001	N	0.7	Y	0.0001	N	0.66	N	0.33
IL8	V	0.0001	N	0.84	Y	0.0001	N	0.63	N	0.71
TNF-α	V	0.0001	Y	0.0093	Y	0.0001	N	0.96	N	0.093
BCA1/CXCL13	V	0.0007	N	0.3995	Y	0.0053	N	0.1	N	0.18
TARC/CXCL11	V	0.013	N	0.77	N	0.09	N	0.23	N	0.32
CXCL9	V	0.0001	N	0.86	Y	0.0001	N	0.8	N	0.54
IL5	N	0.12	Y	0.02	Y	0.006	N	0.26	N	0.86
IL17α	N	0.44	N	0.3	N	0.16	N	0.14	N	0.3
IL1β	N	0.5	Y	0.013	N	0.13	AE	0.038	N	1
GCSF	V	0.0001	Y	0.03	Y	0.042	N	0.7	N	0.27
IL4	N	0.9	Y	0.019	N	0.13	N	0.12	N	0.68
IL2	V	0.012	Y	0.019	Y	0.0012	N	0.09	Y	0.0001
ITAC	V	0.0001	N	0.49	Y	0.0001	N	0.49	N	0.21
Eotaxin	N	0.64	N	0.77	N	0.94	N	0.29	N	0.33
GM-CSF	N	0.13	Y	0.018	Y	0.022	N	0.56	N	0.25

Key: N = not significant; Y = significant; AE = significant and higher in the autoimmune encephalitis group V = significant and higher in the VI group; AO= significant and higher

in the OAND group. *Levels of IL2 in OAND and Normal controls were both undetectable and therefore unable to be statistically compared.

a)



b)

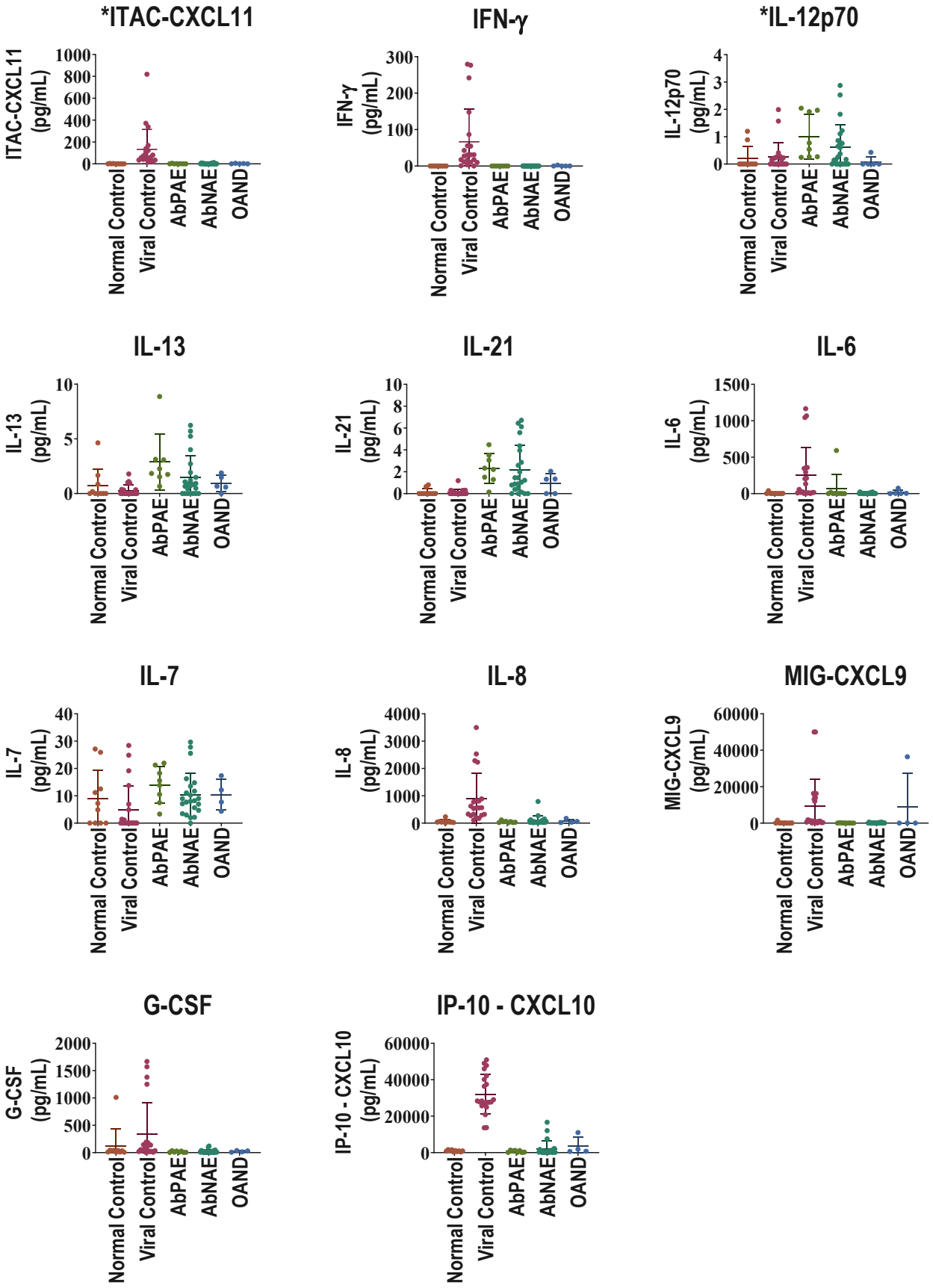

*ITAC-CXCL11
ITAC-CXCL11 (pg/mL)
1000
800
600
400
200
0
IFN-γ
IFN-γ (pg/mL)
300
200
100
0
*IL-12p70
IL-12p70 (pg/mL)
4
3
2
1
0
IL-13
IL-13 (pg/mL)
10
5
0
IL-21
IL-21 (pg/mL)
10
8
6
4
2
0
IL-6
IL-6 (pg/mL)
1500
1000
500
0
IL-7
IL-7 (pg/mL)
40
30
20
10
0
IL-8
IL-8 (pg/mL)
4000
3000
2000
1000
0
MIG-CXCL9
MIG-CXCL9 (pg/mL)
60000
40000
20000
0
G-CSF
G-CSF (pg/mL)
2000
1500
1000
500
0
IP-10 - CXCL10
IP-10 - CXCL10 (pg/mL)
60000
40000
20000
0
Normal Control
Viral Control
AbPAE
AbNAE
OAND

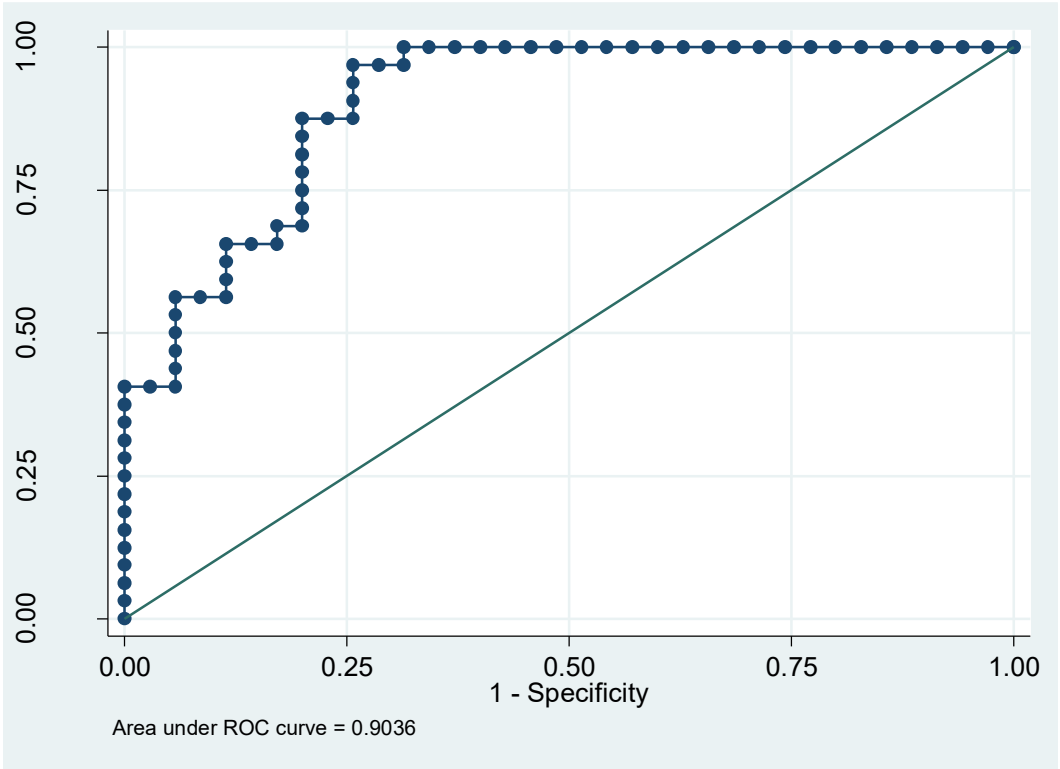
Figure 14: CSF cytokines in cohort groups

(A) Heat map[7] cluster distinguishes viral from AbPAE and AbNAE encephalitis. Each column represents a patient. The X-axis identifies the cohort each patient belongs to, while the right-handed Y axis describes the corresponding cytokine. Increasing expression is depicted as increasing shades of red. Th1 cytokines appear to be associated with viral infections, with the addition of IL1b and IL4 associated with enterovirus infections in this group.

(B) Univariate analysis of cytokines discriminating the autoimmune encephalitis group from a combined pool of NI, viral and other autoimmune disease controls

Abbreviations: AbPAE = patients clinically high risk for autoimmune encephalitis who had identified associated antibodies; AbNAE = patients clinically high risk for autoimmune encephalitis without identified associated antibodies; normal controls = samples from patients either undergoing perioperative anaesthesia or diagnosed with non-inflammatory neurological diseases; OAND = patients with other neurological disease; viral control: CSF samples with PCR detected EBV, VZV, HSV or enterovirus infection

a)



b)

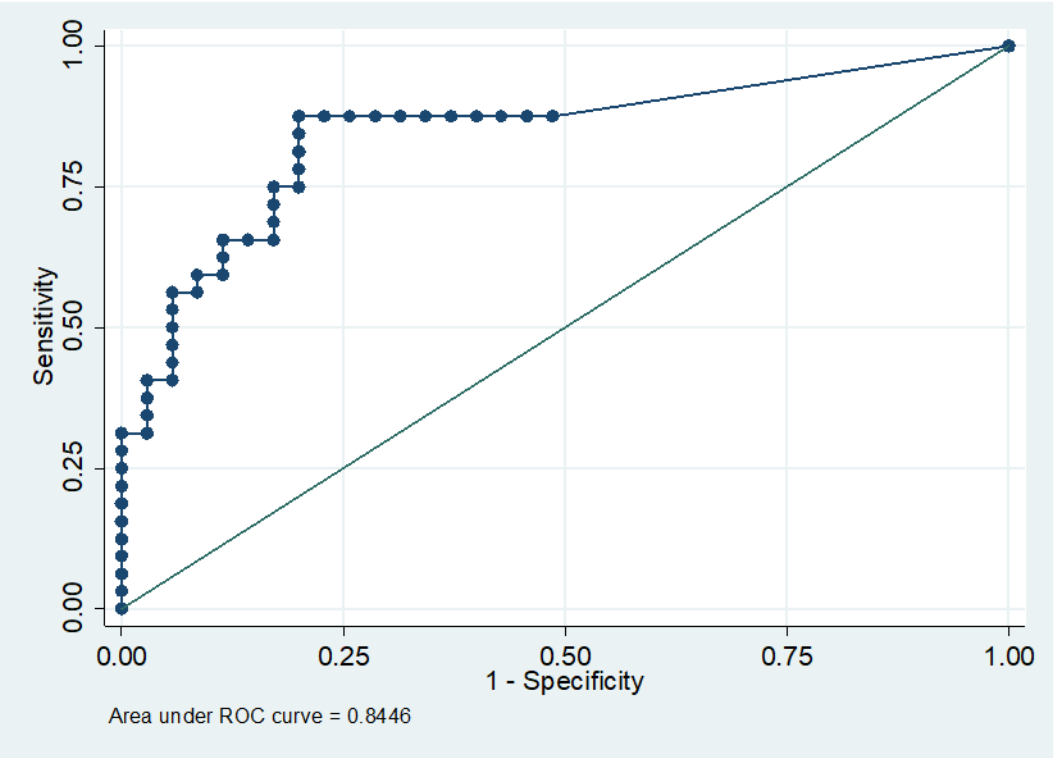


Figure 15: Receiver operator curves of IL21 and IP-10[7]

(A) IP10 and IL21 has a high sensitivity and specificity in discriminating AE from viral infection and normal. ROC curve analysis using a multivariate model with CXCL10/IP10 and IL21 in comparing a pooled high risk for autoimmune encephalitis cohort (compromising of both antibody positive and antibody negative groups) versus a pooled viral control, NI cohorts and OAND controls. AUC = 0.90.

(B) An IP10/IL21 ratio is a reasonably sensitive and specific single result that differentiates the autoimmune encephalitis group from normal and viral controls. This ROC curve analysis uses a univariate logistic model with IL21/IP10 in comparing a pooled high risk for autoimmune encephalitis cohort (compromising of both antibody positive and antibody negative groups) versus a pooled viral control and NI cohorts.

4.3.5 Neopterin and β2 Microglobulin

CSF samples of VI were insufficient for analysis of neopterin and β-2 microglobulin. Only 5/10 normal/ NI samples had sufficient volume (NI patients only) for neopterin analysis, and 4/10 normal/ NI samples had sufficient volume for β-2 microglobulin. For CSF neopterin, a level of greater than 30 ng/mL was considered high by the diagnostic laboratory these samples were processed in. However, at the time of

analysis for the publication of this chapter, the laboratory that routinely processes all CSF pterins performed at my institution is in a children's hospital and their reference range based on a paediatric population.

There was a trend for higher neopterin within both the AbPAE, AbNAE groups and the overall combined AE group vs NI but this difference was not statistically significant. (Figure 16). There was also some overlap in the results for the NI control group with 40% of NI samples having a level of neopterin above 30ng/mL as did 33% of AbPAE patients and 31% of ABNAE.

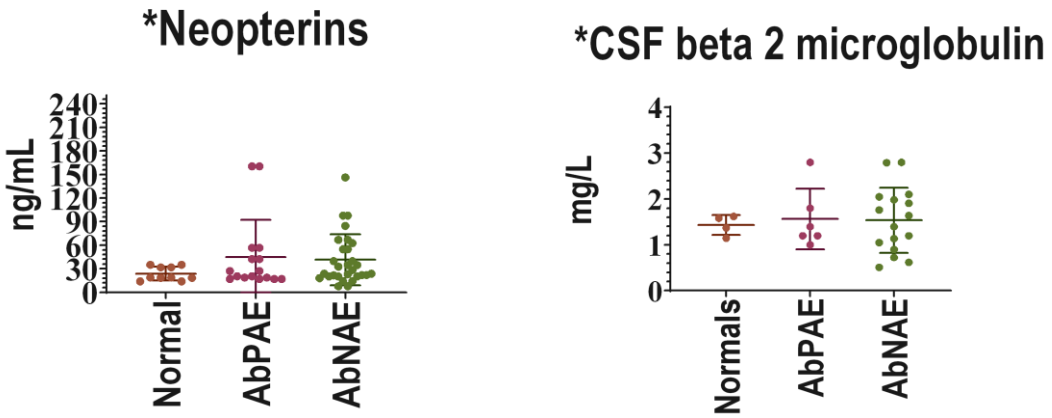


Figure 16: Neopterin and CSF beta-2 microglobulin

Concentrations of CSF neopterin and beta-2 microglobulin in the normal NI cohort and patients clinically at high risk for autoimmune encephalitis.

Abbreviations: AbPAE = patients clinically high risk for autoimmune encephalitis who had identified associated antibodies; AbNAE = patients clinically high risk for autoimmune encephalitis without identified associated antibodies; normal controls = samples from patients either undergoing perioperative anaesthesia or diagnosed with non-inflammatory neurological disease

There was no significant difference between AbPAE and AbNAE compared to NI samples for β-2 microglobulin. Unfortunately, only 53% of samples from the AE group collected had IgG:albumin ratios performed due to preanalytical issues, therefore data from this was not analysed any further.

4.4 Discussion

The diagnosis of antibody-negative AE remains largely one of exclusion[179] and better biomarkers are required to assist with diagnosis to limit the potentially severe sequelae associated with treatment delays. Currently utilised criteria for diagnosis of AE requires supportive investigation findings including congruent MRI abnormalities, CSF pleocytosis, CSF oligoclonal bands, elevated CSF IgG:Albumin index or brain biopsy with exclusion of other disorders[179,452]. However, as demonstrated by this study these biomarkers may not be sensitive or indeed specific for AE, being raised in other causes of neuroinflammation including infection [224,516]. My study has demonstrated that selected CSF cytokines are promising biomarkers of AE, with or without characterised antibodies being present.

4.4.1 Cytokines May Help Differentiate AE from Other Potential Diagnoses

The most promising surrogate marker in AE is IL21 which was raised in both AbPAE and AbNAE groups. While the detected levels of IL-21 in the CSF were in the low range of the assay (0-6pg/ml) they were significantly increased compared to viral and normal controls. Considering the short serum half-life of IL-21 (1-3h)[517], this may represent sustained IL-21 production.

IL21 has many roles in B, CD8 T, and NK cell activation. In B cells, IL-21 acts as both an inhibitor and activator[518,519]. It stimulates apoptosis of B cells that become activated in the absence of T cell help[518,519] but also stimulates B cell proliferation in the setting of T cell help. In combination with IL-4, IL21 has a significant role in switching B cells to IgG1 and IgG3 production[518-520]. IL-21 also stimulates B lymphocyte-induced maturation protein transcription 6 (BLIMP6), which induces differentiation of B cells into

long-lived plasma cells[518,519]. Therefore, IL-21's role in humoral immunity may explain the increased levels in antibody associated AE.

Antibodies associated with AE are continually being described. It is possible that patients diagnosed with antibody-negative AE may have an antibody that is yet to be discovered. Another consideration is that the finding of higher IL21 indicates a role in non-antibody mediated inflammation. IL21 down regulates FOXP3+ regulatory T cells leading to enhanced autoimmunity[518-520]. IL21 plays a synergistic effect on proliferation of CD8+ T cells in combination with IL-7 or Il-15[518-520]. IL-21 stimulates the proliferation of NK and NKT cells as well as enhancing NK cytolytic function[521]. It has distinct effects on CD8+ T cells preventing IL-15 induced downregulation of CD28 and CD62L and potentially preserving their costimulatory function[518,519]. In addition to being a T and NK cell activator, IL21 also critically regulates Th17 cell development, expansion and function. With IL-7 or IL-15, IL-21 further enhances CD8+ T cell proliferation [518-520]. These effects are demonstrated in anti-tumour models[518,519] and may contribute to a predominantly cell-mediated autoinflammatory encephalitis.

The standout cytokine indicative of viral infection in my study was IP-10/CXCL10. IP-10 or CXCL10 is secreted in response to interferon gamma[522], which is produced as part of the Th1 response to viral infection. IP-10/CXCL10 is a chemoattractant for T cells, monocytes, natural killer (NK) cells and dendritic cells and promotes T cell adhesion to endothelial cells via activation of the CXCR3 receptor[223,523]. IP-10/CXCL10 was raised in all viral infections included in my study. Physiologically, this is consistent with IP-10/CXCL10 being a part of the antiviral response promoted by interferon gamma.

The very high levels of IP-10/CXCL10 in the CSF of patients who are PCR positive for a virus suggests that this may be a good marker to help rule out an infective cause in AE. Other reports have associated IP-10/CXCL10 with herpes and flaviviruses.[524] However, this needs to be validated across a greater range of infections as it has been demonstrated that different viral infections have different associated patterns of cytokine expression[65,524-526]. A limitation in my study is that by using deidentified CSF aliquots that were PCR positive for virus it was impossible to correlate these samples to clinical phenotype of patient presentations or previous CSF analysis results, especially in the case of EBV PCR positivity which may not be a clinically significant finding. Additionally, there was insufficient sample to perform other standard tests e.g. oligoclonal bands and cell count and the deidentified nature of the provided PCR positive viral samples mean that previous patient results could not reviewed.

Translating these findings into routine clinical practice, IL-21 and IP-10 may contribute to the diagnostic armamentarium in the investigation of encephalitis, possibly helping to differentiate AE from conditions presenting in a similar fashion where immunosuppression may be harmful. A pragmatic way of comparing these values may be through an IL-21/IP-10 ratio. In my cohort, this ratio had an excellent AUC on ROC curve analysis when AE was compared to NI and VI, but this needs to be further validated.

4.4.2 Comparison with Other Studies

A comprehensive summary of the currently available data examining CSF cytokines in the detection of AE was discussed in the introduction to this chapter. My findings of IL-21 being associated with AE have not been demonstrated in the available

literature where IL17a and CXCL13 were the more consistently reported cytokine raised in AE. This may be because most other studies in the literature have focused on antibody positive AE. Generally, reports of other cytokines associated with AE have also varied, as different investigators have focused on varying panels of cytokines and employed diverse detection methods and commercial kits. Most studies did not include IL-21 in their studied cytokine panel. Even when a Luminex-based bead assay was used, the choice of commercial kits differed between studies. Hence, the studies have reported a range of cytokines associated with AE and many need to be more extensively examined with other methods such as proteomics.

At the time of write up of this study for publication, the only study published that examined CSF cytokine profiles in adult patients was by Micheal et al. [527]. This study used a Bio-Rad platform to examine cytokines in serum and CSF of patients presenting with an acute encephalitis and included 38 patients with an infectious cause 20 who were immune mediated (antibody positive, although which antibodies were present were not specified) and 20 of unknown aetiology. They observed that higher myeloperoxidase (MPO), a marker of neutrophil degranulation, and IL8 was higher in their infectious and their unknown aetiology cohorts. They found that IL-4, IL-10, IL-1RA and IL1-beta was higher in their cohort of patients with immune mediated disease [527]. The kit that they used did not include IL-21 and CXCL10/IP-10 was not statistically significant cross their cohorts. Many of this study's infectious patients had evidence of neutrophils on CSF which may explain the raised MPO and indicate that their cohort was predominantly from bacterial rather than viral pathogens. Michael et al also had a significant number of patients with demyelinating diseases in his autoimmune group which may have influenced the difference in cytokine findings.

Subsequently, most of the studies in the literature have focused on CSF cytokine profiles in antibody-positive AE and mostly in NMDAR-ab associated AE. Even when focused on AE associated with one particular associated antibody positivity, reported cytokines have varied from study to study. Cytokine detection kits used in these studies varied: some used a Luminex based platform, others used an ELISA, and this heterogeneity may explain the variation in results. This may also be one reason why my findings were not well replicated across other studies: I had chosen a commercial kit with very high levels of sensitivity for T cell cytokines. When I had examined lower limits of detection for different cytokine detection assays there was much variation from provide to provider.

In NMDAR-ab associated AE the CSF cytokines IL-17 (associated with Th-17 activity), IL6 (associated with the acute inflammatory response) and CXCL13 (associated with B cells) have been reported to be elevated across more than one study[507-509,512]. However, cytokines may differ with different antibody associations as a study looking at LGI-1 and CASPR-2 encephalitis found a different associated cytokine profile: CSF CXCL-13 and ICAM[513]. CXCL-13 is also known at the B-lymphocyte chemoattractant and has roles in promoting germinal centre development for antibody production[528]. Hence, this may be the reason why it was found to be significant in these studies examining antibody positive AE in contrast to my study which was predominantly antibody negative. Many of these studies also did not directly examine for IL21 in the CSF.

There is a paucity of data on cytokines associated with antibody-negative AE. My study's AE cohort however, had majority antibody negative AE in addition to a mix of

different associated antibodies in the AbPAE cohort. The differences in the AE cohort of my study compared with cohorts in published studies may be another reason why the findings of these studies have differed from those published subsequently in the literature.

My study found that IP-10 was highly associated with CNS viral infections. This finding has been reported in other studies looking at cytokine profiles in CNS infections and has been proposed as a potential biomarker for CNS infection[529-531] including in a metanalysis[532] and proteomic studies using mass spectrometry, subsequently validated by ELISA[533]. However, one study found CSF IP-10 to be associated with AE[508]. This study did not have an infectious encephalitis control arm.

As discussed earlier, differences in assay type, as well as variations in sensitivity and lower limits of detection between kits from different manufacturers, can lead to discrepancies in measured cytokine levels. Moreover, AE remains a relatively rare condition, and many published studies have included small patient cohorts: sometimes fewer than ten per treatment group, leaving them underpowered to detect significant differences in cytokine levels between patient and control groups. Thus, the need to replicate the comparisons of my study (AE, VI and NI) in a larger study is highlighted. IP-10 may still prove to be a worthwhile biomarker of infection even if it is found to high in AE than NI, as long as it is markedly higher in VI than AE.

4.4.3 Other Novel CSF Biomarkers Did Not Differentiate AE from Other Inflammatory Differential Diagnoses

My study did not find other novel markers useful in differentiating AE from NI or VI.

CSF FLC levels have been demonstrated in several other studies to be associated with neuroinflammation. This includes infection [534-536] and multiple sclerosis, where increased CSF kappa, in particular, may have a diagnostic role [536,537]. The association of CSF FLC with infection or inflammation has also been noted in children[538]. Whilst I also found raised CSF FLC in both my infectious and autoimmune cohorts, when compared to NI controls I did not find any significant differences between viral and autoimmune. Therefore, while this is a good marker for CNS inflammation, it is may not be useful in differentiating between different aetiologies.

My study did not find any significant differences between NI controls and the combined AbPAE and AbNAE patients in the examination of CSF neopterin and β-2 microglobulin, which are both associated with acute neuroinflammation[396,411,463,539]. However, interpretation of this data is constrained by the limited number of cases with available measurements.

4.4.4 Current Conventional CSF Diagnostic Markers of AE

Currently utilised conventional markers are neither sensitive[540-542] nor specific [514,515,543,544] enough for the diagnosis of AE[545]. This study also has demonstrated that in all three of these currently used diagnostic markers, there are a proportion of patients even within the AbPAE group who have normal results in CSF protein, cell count, oligoclonal bands and MRI changes.

Whilst there were increased proportions of positive results in CSF pleocytosis and oligoclonal bands in my study in some AE patients, the presence of these markers has been described in infectious and/or neuroinflammatory states such as Guillian Barre syndrome, meningovascular syphilis or multiple sclerosis. [514,515,543,544]. Similarly,

most cases of AE did not have detectable abnormalities on MRI (2). Elevated CSF protein was not a good indicator of neuroinflammation in this study: there was a higher proportion of patients with raised CSF protein in the NI group compared to the AE groups.

My NI group was composed of a mix of IIH and NPH patients and may not be truly representative of a "normal" population absent of any neurological disease and this will be discussed further in study limitations. There have been some reports of increased neurofilament protein pre shunt insertion in NPH and, increased overall protein post ventriculoperitoneal shunt insertion [546]. Two of the NI patients with raised CSF protein had NPH but only one of the had a VP shunt at time of CSF collection. The other patient with raised CSF protein had IIH. CSF protein is not thought to be a marker of IIH and conversely, there have been reports that CSF protein may be inversely correlated to opening pressure in IIH[547], although this is controversial[548].

There was a trend for an increased proportion of patients (35%) with any positive staining in IIF on primate brain in the AbNAE group compared to one patient in the viral and NI groups respectively. However, there was weak immunofluorescence positivity in 1/5 "normal/ NI' controls and 1/13 VI. The viral control that demonstrated positive immunofluorescence findings was an EBV positive sample with a staining pattern suggestive of a cell surface antigen related antibody. Unfortunately, due to the deidentified nature of the VI aliquots, I was not able to correlate these findings with clinical findings. EBV is associated with other autoimmune disease including multiple sclerosis and rheumatoid arthritis[549,550] and its presence is CSF is often of unclear clinical significance. Viral encephalitis such as herpes simplex virus infections are known to precede the development of some cases of autoimmune limbic encephalitis

but a similar relationship with EBV has not yet been reported[541,551,552]. Thus, while non-specific IIF on commercially fixed slides may may be useful, this needs to be examined further.

4.4.5 Limitations of This Study

This was a pilot study in a small group of patients to try to find a biomarker or a group of biomarkers which could be practically useful in real-time to reliably differentiate between clinical syndromes presenting similarly with encephalitis. Whilst my findings in this pilot study are exciting, there were obvious limitations which may be addressed in a larger scale prospective study.

Firstly, there were preanalytical collection issues with the collection of CSF neopterin and CSF IgG:albumin. For CSF neopterin, there was failure to protect from light and difficulty with specific neopterin tube availability which may have underestimated level. However, there were also samples from non-inflammatory controls collected under the same circumstances which had borderline high neopterin. The interferon gamma cytokines which stimulate neopterin production were not significantly elevated in the autoimmune group which suggests that neopterin may have a limited role in the diagnosis, but this needs further examination. Unfortunately, there were not sufficient viral samples for analysis, and it would be of interest to examine if neopterin could be useful in differentiating infectious from non-infectious. Therefore, considering the limitations of this study, more samples from a normal/ NI cohort as well as an AE cohort are needed to confirm if there is truly a difference in neopterin levels between these patient groups.

Similarly, whilst my intention was also to study IgG:albumin ratios in this group of patients, pre-analytical issues with sample sufficiency and sample leakage en-route to laboratory for analysis resulted in only 20% of samples undergoing IgG:albumin ratio analysis. There were too few samples in the normal/ NI control and antibody positive groups to comment on whether this marker is useful in this clinical setting.

There were issues with the number and nature of our comparator cohorts. Our sample size was also small, reflecting the rarity of this disease but also the difficulty in obtaining sufficient NI samples of CSF. My NI cohort was composed predominantly of patients with NPH and IIH with a small subset being taken in the perioperative setting. This was because of the ethical difficulty of subjecting normal patients to an invasive procedure such as lumbar puncture. The conditions NPH and IIH were chosen as NI controls as they are traditionally thought to be non-inflammatory in nature[553,554]. However, since the original design of this study, there is emerging discussion that there may be immune system involvement in the pathogenesis of these conditions [555-559] which may have obscured subtle inflammatory findings in my patient cohorts. Ideally, in future studies, CSF from a truly normal (i.e. without neurological disease) cohort should be sought for comparison.

The size of my disease comparator cohorts was also small. I did not have disease controls but disease controls such as CNS lupus which presents similarly to AE is very rare and therefore samples are difficult to obtain. Multiple sclerosis is another inflammatory CNS condition which may have inflammatory CSF findings, but this condition presents very differently clinically, and is believed to have a very different trajectory, pathophysiology and treatment. Most of the patients recruited into my study at presentation did not have a positive viral PCR and hence I needed to rely on de-

identified PCR-positive samples with limited volume from the microbiology laboratory. A greater number of VI, samples from other neurological diseases and normal controls may help in better defining the sensitivity and specificity of these tests and verify my current findings.

Flow cytometry can detect lymphocytes in the CNS and different patterns may be indicative of neuroinflammation. I wished to explore this in my study but could not, due to logistical issues in sample collection of viable cells. I found that little flow cytometric data was obtained if CSF samples were not collected in Roswell Park Memorial Institute (RMPI) media because of lack of cell viability. Henceforth in patients with psychiatric disease (Chapter 5), up to 1mL of CSF was collected in RMPI media to stabilise cells for flow cytometric analysis.

Our data was further limited because not all results on all variables were available due to limited sample size, especially in the control cohort. ROC analysis in my study indicated that CSF cytokines (IL21 and IP-10) may be better markers than currently available surrogate markers of neuroinflammation for the diagnosis of AE, but this finding needs to be confirmed. In addition, there is conflicting evidence in the literature about the significance of different cytokines in different causes of neuroinflammation and different detection limits across different cytokine kits that need to be investigated.

In summary, data on CSF cytokines in the literature is difficult to compare because of heterogeneity. Most published studies focused on antibody positive AE. Different cytokines, kits and lower limit of detection are used across different studies

and therefore results are predictably variable. I did not correlate serum cytokines with my CSF samples, but this should be examined in further studies.

4.5 Conclusion

This study has examined a range of surrogate markers including those in routine use and those that are novel in their potential utility in the diagnosis of AE. CSF oligoclonal bands, pleocytosis and MRI changes may indicate neuroinflammation, but these markers are not specific.

CSF cytokines particularly CXCL10/IP-10 and IL-21 have emerged from this study as potential differentiators of AE from viral encephalitis. These cytokines may be potentially able to be combined for analysis in a single bead which would decrease the cost of the assay as well as potential turnaround times.

However, these findings will need to be validated in larger studies. A greater number of normal/ NI and disease samples will also help determine a reference range for these cytokines and confirm an optimal cut point level for sensitivity and specificity for these two cytokines. Longitudinal data on the outcomes of patients in my cohort, including the AbNAE group would also assist in potentially identifying cytokines associated with clinical response to immunosuppression and will be the next stage in my analysis.

Chapter 5: CNS Inflammation for Psychiatric Disease

5.0 Preface[2]

This chapter is based on a manuscript that has been accepted for publication:

Jiang JX, Shvetcov A, Fewings N, Gatt P, Dervish S, Garber JY, Silsby M, Fois AF, Duma S, Bandodkar S, Ramanathan S. et al. Markers of Neuroinflammation in the CSF of Patients with Difficult to Treat Psychiatric Disease. Frontiers in Psychiatry.;17:1665447 (in press)

Will be avaliable at:
https://www.frontiersin.org/journals/psychiatry/articles/10.3389/fpsyt.2026.1665447/abstract

I was the primary author, responsible for study design, patient recruitment, data collection, specimen analysis for cytokine assays, data analysis and manuscription drafting, preparation and revision.

Other author contributions are as listed and detailed in Acknowledgements:

Nicole Fewings for running some of the cytokine assays; Suat Dervish for operating the Luminex platform for cytokine analysis; Sushil Bandodkar for expertise in running and interpretation of the CSF neopterin assay; Artur Shekov and Caitlin Finney for assistance with statistical analysis including in construction of the heatmap and principal component analyses (PCA); Anthony Harris, Elizabeth Scott, Ian Hickie, Justin

[2] After the submission of this thesis for examination, the submitted manuscript this chapter based underwent revision including changes in statistical analysis based on feedback from peer review. This paper has now been accepted for publication and is currently in press.

It will be available, with revisions, at:
https://www.frontiersin.org/journals/psychiatry/articles/10.3389/fpsyt.2026.1665447/abstract

Garber, Alessandro Fois, Stephen Dua, Matthew Silsb, Sudarshini Ramanathan, Andrew Bleasel for patient recruitment; Bryne John for recruitment of non-inflammatory controls; Anthony Harris, David Brown and Ming-Wei Lin for assistance with refining the conceptualisation of the study design and supervision of research undertaken.

All co-authors contributed to review and revision of the published manuscript.

5.1 Introduction: Emerging Biomarkers for Psychiatric disease

Difficult to treat psychiatric disease is common and has high morbidity[560,561] and mortality[562]. One of the key challenges in improving these outcomes is that the pathophysiological mechanisms driving psychiatric disorders remain poorly understood and are likely to be diverse. The recent recognition of AE as a cause of psychiatric presentations has prompted interest in exploring inflammation and immune activation as pathogenic pathways in the genesis of primary psychiatric diagnoses [130,563]. This is in addition to the long-standing links between psychiatric disease and the immune system in associations with infection and autoimmunity, as described in Chapter 1 section 1.4.

The autoimmune/inflammatory mechanisms contributing to disease may be in reaction to local and/or systemic inflammatory factors meditating effects upon the brain or by directly targeted immune attack such as found in autoimmune encephalitis [181]. Whilst there are promising reports for novel biomarkers for neuroinflammation, data for these in psychiatric disease is more limited. One study found that CSF neopterin was increased in up to 30% of a cohort of patients with affective and psychotic disorders. This increase in CSF neopterin was not related to BBB dysfunction.[407] Serum

beta-2 microglobulin has been noted to be higher in schizophrenia but this study did not examine CSF levels[564]. CSF cytokines have also been another area of interest here and are further discussed below.

5.1.1 CSF Cytokines in Psychiatric Disease

Disturbances in CSF cytokines have been a subject of interest and studied in the well-defined psychiatric disease groups[310,565 566] and may give insight into the types of immune dysfunction present in psychiatric diseases in general, as well as be a biomarker for an alternative diagnosis. Metanalysis and primary studies have reported disturbances of levels of CSF IL-6 and IL-8 with psychiatric disease (summarized below). However, some reports are conflicting, which can be due to several factors. This includes differences in methods of cytokine detection, numbers of patients in the study and differences in comparator cohorts.

Metanalyses have reported increases in pro-inflammatory cytokines IL6 and IL-8 in CSF associated with psychiatric disease. A 2017, a metanalysis of 28 studies looking at tryptophane catabolites and CSF cytokines, found CSF IL6, IL8 increased in schizophrenia and depression, and CSF 1β and kynurenic acid increased in schizophrenia and bipolar affective disorder compared to healthy controls[566]. In 2018, a report combined a primary study with metanalysis of CSF cytokines in schizophrenia. Their primary study component examined a panel of 17 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-23, IFNγ, TNFα and TNFβ) using an ELISA based method (Q-Plex™ Human Cytokine array, Quansys Biosciences) to find that CSF IL6 was increased in patients with schizophrenia. However, they were only able to reliably detect IL6, IL8 and IL1β using their assay. Their metanalysis (15 studies) also

found IL8 and IL6 increased in CSF of patients with schizophrenia[567]. Increases in CSF IL6 and IL8 was further reported in another 2023 metanalysis of patients with schizophrenia[568] and CSF IL6 in a 2024 meta-analysis (although this only looked at IL-6)[569].

A meta-analysis examining both schizophrenia and affective disorders (depression, dysthymia or bipolar) included 32 studies was published in 2019. They found that IL-8, IL-6 were significantly raised in the patients with schizophrenia compared with healthy controls. There was no significant increase of cytokines examined in the meta-analysis (IL-1, IL1beta, IL-6, IL-8 and TNF-α) in patients with affective disorders compared to controls [310].

In terms of metanalysis of only affective disorders, a metanalysis of studies in major depressive disorder (12 studies examining cytokines in CSF in depression) studies also found increased CSF IL-6 compared with controls and in suicide attempters, regardless of psychiatric diagnosis. CSF TNF-α was also found to be higher in patients with depression[570]. A second systematic review and metanalysis published in 2022 of 97 studies examining patients with unipolar depression examined a range of CSF biomarkers including cytokines. They found increased CSF IL-6 in patients with depression compared to healthy controls but there were no significant differences for IL8 or TNF-α[571].

Primary studies have reported changes in range of cytokine elevations in the CSF of patients with psychiatric disease. As reflective of the metanalyses results, CSF IL-6[572,573] and CSF IL-8 increases have been reported [565] in schizophrenia. Increased CSF IL6 and IL8 was also reported in a cohort of older women with depression[574]. CSF IL-6

correlated with anxiety in a study with a mix of different psychiatric disease (including schizophrenia, bipolar and depression)[575]. However, there are also studies that reported no differences[313,576-579], or decreases[580,581] in CSF IL6 between schizophrenia or depression vs healthy or non-inflammatory controls.

Other increases in CSF cytokines that have been reported in association with psychiatric disease include IL12β in first episode psychosis using a multiplex proximity extension assay technology [582] and IL12p70 using a multiplexed ELIZA based assay[565], TNFα[565,577], interferon-beta (using multiplex immunoassay[583], IL-4[313] and monocyte chemoattractant protein (MCP)-1[581]. CSF IL-1β has been associated with bipolar disorder [584,585]. However, some studies also reported no significant differences between patients with psychiatric disease and controls in the panel of cytokines they examined[579,586]. One of these studies though, did find that within their patients with major depression, CSF IL-1 IL6 and TNFα was associated with suicidality even overall the levels didn't differ from healthy controls[579].

Disturbances in CSF cytokines has also been reported in the paediatric population psychiatric with a skewing towards Th1 cytokines noted in obsessive-compulsive disorder versus a Th2 cytokine profile in patients with schizophrenia[587].

The published literature on CSF cytokines in psychiatric disease is heterogenous with a range of cytokine disturbances being reported, the most common being increased IL-6 and/or IL-8. The differences between the studies may be due to different cytokine detection kits used which may have different sensitivities as already discussed in Chapter 4, as well as application of different cytokine panels. Control groups ranged from health volunteers[575-577,579,580], non-inflammatory controls (patients with NPH and

IIH)[578] and other neurological symptoms[581,586] or diseases (including multiple sclerosis) [578] which may also have impacted on differing reporting of results. Nevertheless, the current results are suggestive that even in well-defined cases of psychiatric disease, there are immune system disturbances.

5.1.2 Need for Biomarkers in Undifferentiated Disease Groups

Most of the published studies of CSF cytokines in psychiatric disease looked at generally well-defined patient groups, usually according to the ICD-10. This may be because research studies usually have a stricter entry criterion, however data is lacking in more ill-defined, atypical cohorts who pose a clinical challenge. However, as outlined in section 1.4.10, the diagnostic and therapeutic difficulty is in patients who may present with atypical disease or who have strong treatment resistance.

There is a lack of validated biomarkers to help identify patients who may benefit from immunomodulation. Being better able to define to further subclassify this subset of patients may allow patients to access treatments other than routinely used psychotropic agents earlier; and these treatments may be more effective. This is of particular importance in clinical scenarios where patients have already failed several agents and live with the morbidity and higher mortality risks associated with poorly controlled disease. Furthermore, it has been demonstrated that in AE that earlier commencement of immunosuppression where there is an immune related cause can lead to better clinical outcomes.

In chapter 4, I studied routinely available and novel markers of neuroinflammation in cerebrospinal fluid in a cohort of patients with antibody negative autoimmune encephalitis. There, the cytokines IL-21 and IP-10/CXCL10 differentiated

autoimmune encephalitis from viral encephalitis [7]. To determine the possibility that some psychiatric patients had a predominantly autoimmune cause of their psychiatric disease, I applied the same panel of both conventional and novel biomarkers used in my previous study evaluating autoimmune encephalitis [7], to a cohort of patients with atypical psychiatric disease.

5.2 Methods

5.2.1 Patient Enrolment

Patients were enrolled as described in Chapter 2 (Methods), section 2.1. Briefly, patients identified by their treating psychiatrist as requiring further evaluation were referred from their various clinics located in Sydney, Australia for immunological assessment at our clinic site, located in Westmead, Australia. Patients who exhibited "red flag" features were recruited sequentially after undergoing lumbar puncture assessment. Inclusion criteria for entry into this study is described in Chapter 2, section 2.1.

These patients were compared against a cohort of AE, viral infection, other autoimmune neurological disease (OAND) and non-inflammation (NI) controls. Definitions for these cohorts are also described in Chapter 2, section 2.1.

5.2.3 Standard Testing

Patients recruited into the study underwent laboratory tests on serum and blood routinely available in the diagnostic laboratory. List of what diagnostic tests were performed is described in Chapter 2, section 2.6.

All psychiatric and AE patients underwent the tests, as described in Chapter 2. The psychiatric cohort underwent additional serological tests compared to AE cohort to look for other potential autoimmune contributors to their disease. Where specimen sample was very limited (the VI cohort and the NI cohort, where the CSF was obtained during spinal anaesthesia), these samples were reserved for novel assays where the reference range for CSF was not established (CSF FLC and cytokines). This is because diagnostic assays available routinely in the diagnostic laboratory have a standard reference range derived from healthy, normal populations.

5.2.2 Cytokine Measurement

Assay selection and cytokine measurement was performed as described in Chapter 2 section 2.6.3. All assays were performed according to manufacturer instructions.

5.2.4 Statistical Analysis

Analysis of the surrogate markers examined in this study was performed using StataMP 1v3 and scatterplot figures of results were prepared using GraphPad Prism v10. Results that were lower than the limit of detection was designated as “0” for statistical analysis. For scatterplots, results were log normalised by adding 1 to all results including transforming log10 of the new value.

For continuous independent variables, univariate analysis using the Wilcoxon rank-sum test or Kruskal-Wallis test as appropriate, were performed to compare the various disease groups. Pearson’s Chi-squared test was performed for univariate analysis of binary outcome variables. Significant cytokine results were then assessed

using a multivariate logistic model to determine markers that were significantly and independently associated with the disease group classification (psychiatric vs AE viral, NI groups). When cytokine levels were compared between patients in the psychiatric patients who did and didn't improve with immunosuppression, the Bonferroni correction for multiple comparisons was applied.

Heatmap plots were generated using scaled variables in R (v 3.6.3) and R Studio (1.2.5033) using the "*pheatmap" library*. Principal component analyses (PCA) were performed in R (v 3.6.3) and R Studio (1.2.5033) using libraries "*factoextra"* and "*factominer"*. Both heat map generation and PCA were generated with the assistance of Dr Artur Shvetcov, and figures were included with his permission.

5.3 Results

5.3.1 Patient Details

A cohort of 35 patients with treatment resistant psychiatric disease were included in this study. Many of these patients had symptom presentation years before this immunology assessment[588]. These patients were compared with an expanded upon historical cohort of 18 NI, 22 VI and 40 AE controls (described in Chapter 4). Table 19 summarises their demographics, and their clinical presentation are summarised in Table 20.

Within the psychiatric cohort, 29 patients were female, 6 were male with a median age of 20, with an age range of 15-60. Twenty-one patients presented primarily with a mood disorder while 14 patients presented primarily with psychotic features. Eighteen patients had a history of autoimmunity whilst 11 patients had a family history

of autoimmunity. Additional physical symptoms were also identified in these patients including fatigue (15/35), cognitive changes (10/35), a previous history of seizures or pseudo-seizures (6/35), symptoms of sleep disturbance (4/35), paraesthesia(3/35), chronic pain (11/35).

In the AE cohort there were 40 patients in total: 13 who were antibody positive and 27 who were antibody negative. There were 21 females and 19 males. The median age was 38.5 with an age range of 15-73. No clinical data was available for the 21 de-identified VI controls[7].

The NI cohort consisted of 14 female and four males with a median age of 45.5 and age range of 17-81. Ten of these patients had a diagnosis of idiopathic intracranial hypertension (IIH), two had a diagnosis of primary headache disorder, two had normal pressure hydrocephalus (NPH) and four patients had CSF samples taken prior to administration of spinal anaesthesia. No clinical data is available for the VI cohort as they were supplied as de-identified aliquots. Because limited quantities of cerebrospinal fluid were available from the NI and VI control cohorts; novel markers, where no established normal ranges were available, such as CSF cytokines analysis were prioritised.

Table 19: Demographics of the included cohorts[6]

	Psychiatric disease	**Autoimmune Encephalitis**	**Non-inflammatory**
N	35	40	18
Age (yrs) median (range)	20 (15-60)	38.5 (15-73)	15 (17-81)
M:F	1:4.8	1:1.1	1:3.5

Table 20: Clinical Features of Included Psychiatric Patients[6]

Characteristic	N (%)
Psychiatric Diagnosis n (%)	
Depression	10 (29%)
Mania/Bipolar disorder	6 (17%)
Chronic non-affective psychosis/schizophrenia	7 (20%)
First episode psychosis	4 (11%)
Multiple psychiatric diagnosis	8 (23%)
Other Symptoms n (%)	
Sleep Disturbance	4 (11)
Seizures (any previous history)	5 (15)
Pseudo seizures	2 (6)
Fatigue	15 (43)
Cognitive Issues	10 (29)
Movement Disorders	1 (2)
Infectious Prodrome	0 (0)
Paraesthesia	3 (9)
Chronic pain	11 (31)
Other Associated Features n (%)	
History of Autoimmunity	18 (51)
Family history of Autoimmunity	11 (31)
Treatment Effect	
Failed at least 1 psychiatric medication	27 (77)
Failed at least 2 psychiatric medications	20 (57)

5.3.2 Serum Markers of Inflammation and Positive Antibodies

Data for serum markers of inflammation and other antibodies (ANA, ENA, dsDNA, anti-VGCC, anti-GAD, ESR, C3, C4, IgG, IgA and IgM and IgG subclasses) were only available for the psychiatric cohort. The most common autoantibody detected in the serum was an anti-nuclear antibody (ANA), which was detected in 21/32 (65%) of patients with psychiatric disease. However, the majority of these (n=18/21) were low

titre (1:80-1:160) without associated ENA antibodies, which have also been reported (albeit at lower prevalence) in otherwise healthy individuals[589]. The predominant pattern in these patients was speckled (17/21), with mitotic spindle apparatus (1/21) and mixed homogenous and speckled pattern reported (2/21).

Two patients had mid-high titre ANA antibodies (speckled, 1:640) again without any associated ENA or dsDNA positivity or clinical features of a connective tissue disease. There was one patient with a high titre 1:2560 speckled ANA with associated anti-SSa/Ro60 antibodies. The other prominent positive serum antibodies were thyroid related with 3/35 patients having raised anti-thyroglobulin antibodies (8%) and seven patients (20%) having raised thyroid peroxidase antibodies. Of these, one patient had elevation of both antibodies. Only two patients with thyroid autoantibodies had evidence of thyroid dysfunction. Both the rates of positive ANA and thyroid autoantibodies were higher than what has been reported in the general population[590].

One out of 35 patients in my psychiatric cohort had positive coeliac serology and 1/35 had a borderline positive myositis associated antibody of which the clinical significance was not clear as this patient did not have any clinical features of myopathy or amyopathic interstitial lung disease. No patients in my cohort had anti-double stranded DNA antibody, liver autoantibodies (by multiblock immunofluorescence) or other diabetes associated antibodies. One of 35 patients had positive VGCC in the serum, without clinical evidence of LEMS (Lambert-Eaton myasthenic syndrome) nor evident neoplastic process. None of the patients in the study had other positive serum onconeural or limbic encephalitis antibodies.

In terms of non-specific markers of immune activation, ESR results were available for 33/35 patients with 3/33 patients (9%) having an ESR above normal limits.

One patient had elevated IgG. On protein electrophoresis, one patient had a polyclonal elevation of gamma globulins (although their serum IgG was within normal limits) and another had evidence of serum oligoclonal bands.

5.3.3 Conventional CSF markers

Table 21: Conventional Serum and CSF results[6]

	Psychiatric Disease (N =35)	**Non-inflammatory Control (N=9)**	**P-value (psychiatric disease vs NI)**	**Autoimmune Encephalitis (N=40)**	**P-value (psychiatric disease vs AE)**
Elevated CSF Protein	5 (14%)	3 (3%)	0.47	24 (60%)	0.02
CSF Mononuclear >5	1 (3%)	1 (11%)	0.46	22 (55%)	0.002
CSF Oligoclonal bands	4 (11%)	0 (0%)	0.3	15 (38%)	0.008

P-values are calculated as AE vs all other control groups (NI, viral and OAND)

CSF markers of neuroinflammation were only available for a subset of the NI cohort (13/18 for CSF protein quantitation and cell count; 8/18 for oligoclonal bands; 9/18 for neopterin). There was no evidence of oligoclonal bands for any of the NI cohort where data was available. The number of patients with CSF red blood cell (RBC) >10 was 18 in the AE cohort, two in the NI cohort and seven in the psychiatric disease cohort. The range of CSF RBC across the three cohorts was 0-620. CSF RBC count was not available for the infectious disease cohort. One NI had CSF pleocytosis (6 mononuclear cells); this patient had a diagnosis of IIH. Three patients had raised CSF protein (>0.45g/L): two with NPH and one with IIH. Five (56%) patients had evidence of raised neopterin (>20nmol/L). Three of these patients had a diagnosis of idiopathic intracranial hypertension and two of these had a diagnosis of NPH. One of the patients with NPH had a shunt at the time of lumbar puncture.

All patients in the psychiatric cohort and AE cohorts had data for conventional CSF markers of neuroinflammation (Table 21). Of the psychiatric cohort, 1/35 (2%) of patients had evidence of CSF pleocytosis (mononuclear cells >5 cells on CSF microscopy), 4/35 (11%) had CSF specific oligoclonal bands, 5/35 (14%) had elevated CSF protein (>0.45g/L) and 12/35 (34%) had raised CSF neopterin (defined by the diagnostic laboratory as < 20nmol/L at the time of analysis of this study). There were no significant differences in these markers between the psychiatric and NI groups.

The AE cohort had significantly higher rates of abnormal conventional markers of neuroinflammation compared to the psychiatric cohort. In the AE cohort 15/40 (38%) patients had raised CSF protein >0.45g/L ($\chi^2 = 5.14$ p=0.02), 12/40 patients (30%) had evidence of pleocytosis ($\chi 2=9.6$ p=0.002), 15/40 (38%) had evidence of oligoclonal

bands (χ^2=7.036 p=0.008) and 29/35 (72%) had evidence of raised neopterin (χ^2=17.01 p< 0.0001; no neopterin data was available for 5 patients). The only significantly raised marker in the AE cohort compared to NI were CSF-restricted oligoclonal bands (χ2=4.52 p=0.03).

Unfortunately, due to preanalytical issues such as sample leakage during transport, data on CSF IgG and albumin levels for assessment for CSF IgG:albumin ratio was only available on a subset of the psychiatric, AE and NI cohorts. Only 1/22 patients in the psychiatric group had evidence of a raised CSF IgG: albumin ratio versus 10/19 in the AE group ($\chi^{2=}$5.4 p=0.02) and 2/5 in the NI group ($\chi^{2=}$9.5 p=0.002). Of the 2 patients in the NI group with raised IgG: albumin ratio, one had IIH and the other NPH, but the CSF was taken prior to insertion of any shunts.

I also determined whether there was local CNS synthesis of IgG versus a disturbed blood brain barrier (BBB) using the Reibergram [591], with serum and CSF IgG and albumin quotients. In my AE cohort, data was available for 16 patients. Two met criteria for disturbed BBB, two had evidence of intrathecal synthesis of IgG and one patient had both abnormalities. In the psychiatric cohort, however, Reibergrams for all 22 patients fell within the normal range.

No patients in the psychiatric cohort had onconeural or limbic encephalitis associated antibodies present in their CSF nor any evidence of CSF indirect immunofluorescence on primate neural substrate in any recognised or atypical pattern.

5.3.4 Novel Markers Available in the Diagnostic Laboratory

5.3.4.1 CSF Beta-2-Microglobulin

CSF beta-2-microglobulin reflects immune activation and lymphoid cell turnover in the CNS[396] and has been a marker used in the investigation of CNS involvement in infectious such as HIV as well as CNS lymphoma[592]. Whilst there was no significant difference in CSF beta-2-microglobulin levels between the AE and NI cohorts (p=0.597), the level of CSF β-2-microglobulin was significantly lower when the psychiatric cohort was compared with NI (z=2.89 p=0.004) and AE (z=3.58 p=0.0003) cohorts.

There was no significant difference in serum beta-2-microgloublin between the psychiatric and NI (p=0.49) or AE cohorts (p=0.3). Whilst there was correlation between serum and CSF beta-2 microglobulin in the AE cohort (p=0.0092, rho=0.59) and NI cohorts (p=0.04, rho=0.81), there was no correlation between serum and CSF β-2microgloublin in the psychiatric cohort (p=0.08, rho=0.3).

5.3.4.2 Flow Cytometry Results

Lymphocyte percentages in CSF was compared with reported lymphocyte populations studied in normal CSF in the literature which have found a CD4 lymphocyte predominance (CD4:8 ratios of 1.1-6.5) [593] and a sparsity of B cell populations (0.5-1%)[594].

CSF flow cytometric data was obtained on 25/35 patients as some CSF was collected without RPMI media and viable cells for analysis could not be obtained. Twelve of these twenty-five were non diagnostic because of very low cell numbers. Eight of these twenty-five patients had a reduced CD4:8 ratio whilst one patient had an

increased CD4:8 ratio. Three patients had B cells detectable in their CSF but none of these patients had more than 1%.

5.3.5 CSF Cytokines

A heatmap (Figure 17) was constructed to better visualise the trends in cytokine values across the cohorts in my study. As reported in my previous publication(Chapter 3; [7]), there was generally high cytokine expression in the VI cohort. Cytokine expression in the AE and psychiatric cohorts was variable, perhaps reflecting the heterogenous characteristics of the patient groups.

On visual inspection of the heatmap, there was a higher IL-7 signal noted in the psychiatric cohort and to a lesser extent the AE cohort when compared to the VI and NI control groups. The cytokine IL-21, a marker I discussed in chapter 4 as associated with AE[7], also appeared to be elevated in the psychiatric cohort when compared with both my control groups (NI and VI).

On univariate analysis of the psychiatric versus NI, the cytokines ITAC (z=-3.145 p=0.0017), GM-CSF (z=-2.479 p=0.013), IFN-γ (z=-3.5 p=0.0005), IL-12p70 (z=-2.365 p=0.018) IL-13 (z=-1.97p=0.049), IL-21 (z=-2.698 p=0.007), IL-23 (z=-2.072 p=0.038), and TNF-α (z=-2.62 p=0.0087) were raised in the psychiatric cohort. However, despite the trend for higher IL7 in the psychiatric cohort observed on the heatmap, there were no statistical differences between the NI and psychiatric disease cohort (z=-1.42, p=0.16)

When the psychiatric cohort was compared with the VI cohort, IL-13 (z=-3.642 p=0.0003), IL2-1 (z=-4.4 p<0.0001), IL-7 (z=-3.789 p=0.0002) and IL-23 (z=-5.104; p<0.0001) were significantly higher in the psychiatric cohort. The cytokines ITAC

(z=5.606, p<0.0001), IFN-γ(z=5.381, P<0.0001), IL-10(z=5.905, P<0.0001), IL-2 (z=2.036, p=0.0418), IL-6 (z=5.901, P<0.0001), IL-8 (z=6.311, p<0.0001), TNF-α (z=4.246, p<0.0001), BCA-1(z=4.409, p<0.0001), CXCL9 (z=4.365, p<0.0001), GCSF (z=3.298, p=0.001) and IP-10 (z=4.205, p<0.0001) were significantly higher in the VI cohorts.

When the psychiatric cohort was compared to the AE cohort, IL-1b (z=2.981 p=0.0029), IL-4 (z=2.142 p=0.03), IL-10 (z=2.69 p=0.0071) and IL-21 (z=2.013 p=0.04) was elevated in the AE cohort. However, ITAC (z=-2.9 p=0.0037) and IFN-γ (z=-2.812 p=0.005) were greater in the psychiatric cohort.

I then modelled differences between cohorts using multivariate logistic regression, examining the CSF cytokines that were significantly different on univariate analysis. These models compared the psychiatric cohort with each of NI, VI and AE cohorts as dependant variables and contributing cytokines as independent variables. After excluding variables for collinearity, I standardised the results of the cytokines using log-normalisation and z-standardisation before analysing results in the model. CSF RBC did not make a significant contribution to the model.

I found the cytokines ITAC, GM-CSF, IL-6, CXCL9 and IL-4 contributed significantly to the model discriminating groups (Table 22; Figure 18). When the psychiatric cohort was compared to the NI cohort, only elevated of levels of ITAC (relative risk ratio (RRR) = 0.14 95% 95% Confidence Interval(CI) 0.03-0.73 p=0.02) and GM-CSF (RRR=0.13 95%CI 0.02-0.6.p=0.008) were independently significantly associated with psychiatric disease. When the psychiatric cohort was compared to the AE cohort, higher levels of ITAC (RR=0.05 95%CI 0.01-0.25 p<0.0001) were associated with psychiatric disease; there was no significant difference in levels of GM-CSF. Within this comparison, the higher levels of cytokines IL-6 (RRR=5.16 95%CI=1.4-19.5

p=0.016), CXCL9 (RRR=6.1 95%CI=1.6-23.2 p=0.007) and IL-4 (RRR=3.4 95%CI=1.6-7.3 p=0.002) were associated with the AE cohort. When the psychiatric cohort was compared to the VI cohort, higher levels of the cytokines IL-6 (RRR=5.6 95%CI=1.1-28.2 p=0.036) and CXCL9 (RRR=11.1 95%CI=1.9 - 64.7 p=0.007) were associated with the viral cohort. There were no significant differences in the other cytokines.

As previously discussed, β-2 microglobulin, a marker of lymphocyte turnover was lower in the psychiatric group compared to the AE and NI groups but without a corresponding difference in IL-7, a major cytokine responsible for lymphocyte survival[595]. This finding suggested there may be differences in immune regulation between my comparison cohorts. Unfortunately, whilst IL-7 was higher in my viral cohort compared to psychiatric, β-2 microglobulin data was not available for my viral cohort.

There was a subgroup of patients in the psychiatric cohort with far higher levels of CSF cytokines than others within this group. They can be visualised as distinctly separated against the rest of the psychiatric cohort on the heatmap (Figure 17). When the values of CSF cytokines were examined in individual patients, six patients (17%) had at least one CSF cytokine greater than four standard deviations above the mean of the NI cohort group. Three of these patients had at least 2 cytokines elevated four standard deviations above the normal mean. The elevated cytokines were a mix of T-cell related, B-cell related and pro inflammatory cytokines ITAC (n=1), IFN-γ (n=1), IL-10 (n=1), IL=-10 (n=1), IL-12p70 (n=1), IL-17a (n=1) IL-1β (n=1), IL-2(n=2), IL-4 (n=2), IL-23 (n=1), IL-5 (n=2), TNF-α (n=1), BCA-1 (n=1), CXCL9 (n=1) and IP-10 (n=1). This was a heterogenous group of patients encompassing a mix of those who presented primarily with depression (n=1), bipolar (n=3) and psychosis (n=2). Five patients were female with one male and

an age range of 16-30. Three of these patients had a personal history of autoimmunity and two of these five had a family history of autoimmunity. Two reported concurrent fatigue and three reported cognitive dysfunction.

Not all patients who had significantly raised CSF cytokines had any evidence of perturbed conventional markers of neuroinflammation in routinely available diagnostic tests (other than neopterin); two patients' CSF would have been considered normal. Of the other patients, there was evidence of CSF pleocytosis (n=1), oligoclonal bands (n=1) and increased CSF protein (n=3). Interestingly, all had a raised CSF neopterin of more than 20ng/mL.

Table 22: Significant cytokines when the psychiatric cohort is compared to a combined cohort of NI, AE and VI in a multivariate logistic regression analysis[6]

Cytokine	Relative Risk ratio	P-value
AE vs Psychiatric		
ITAC/CXCL11	0.54	<0.001
GM-CSF	0.59	0.39
IL6	5.15	0.016
CXCL9	6.14	0.007
IL4	3.4	0.002
Viral Infection vs Psychiatric		
ITAC/CXCL11	1.23	0.83
GM-CSF	0.5	0.33
IL6	5.63	0.036
CXCL9	11.1	0.007
IL4	1.47	0.369
NI vs Psychiatric		
ITAC/CXCL11	0.14	0.026
GM-CSF	0.13	0.03
IL6	1.61	0.27
CXCL9	3.2	0.61
IL4	1.26	0.1

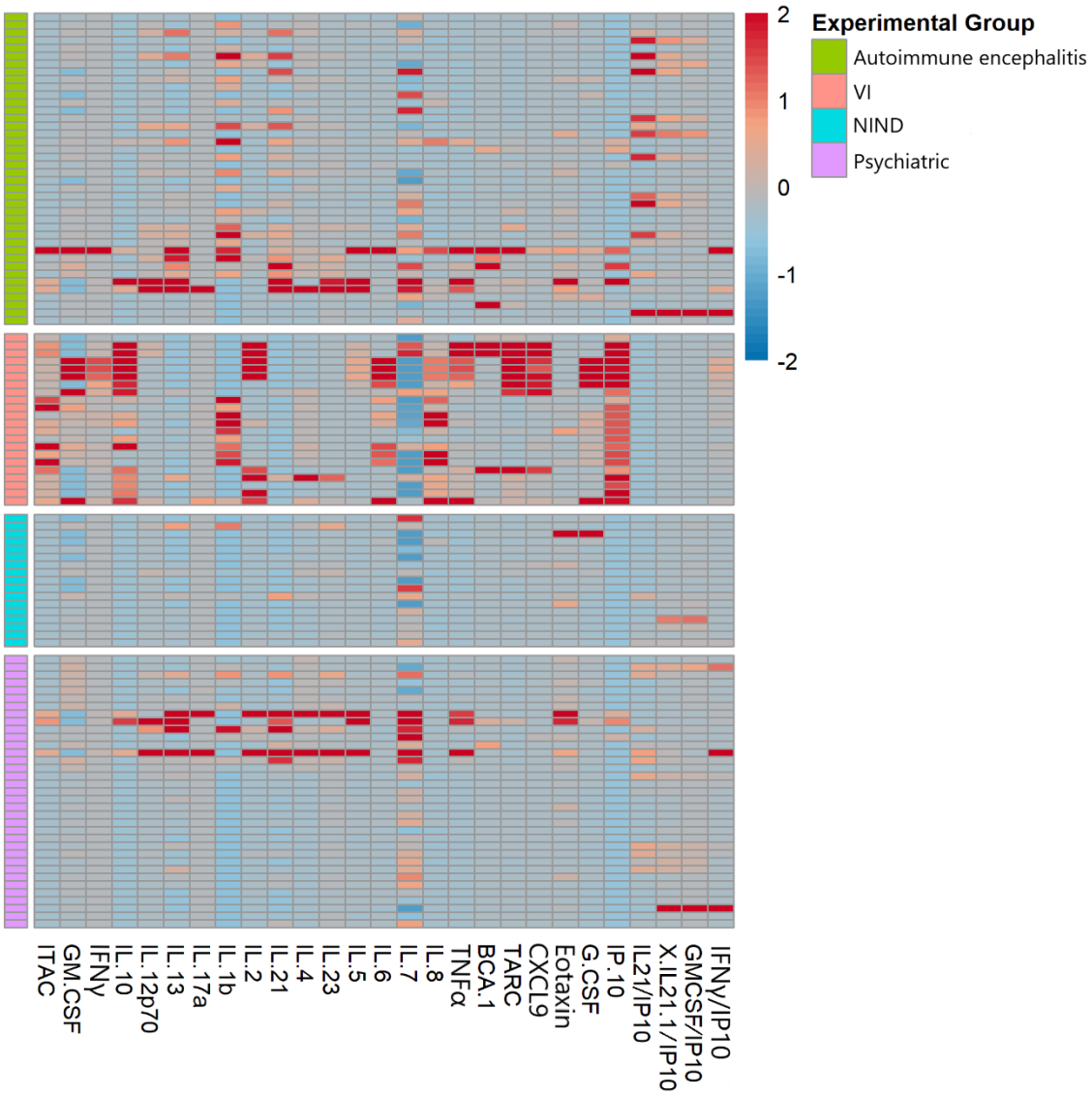


Figure 17: Distribution of CSF cytokines in psychiatric and other cohorts.

Heat map[6] demonstrating distribution of CSF cytokines by cohort group

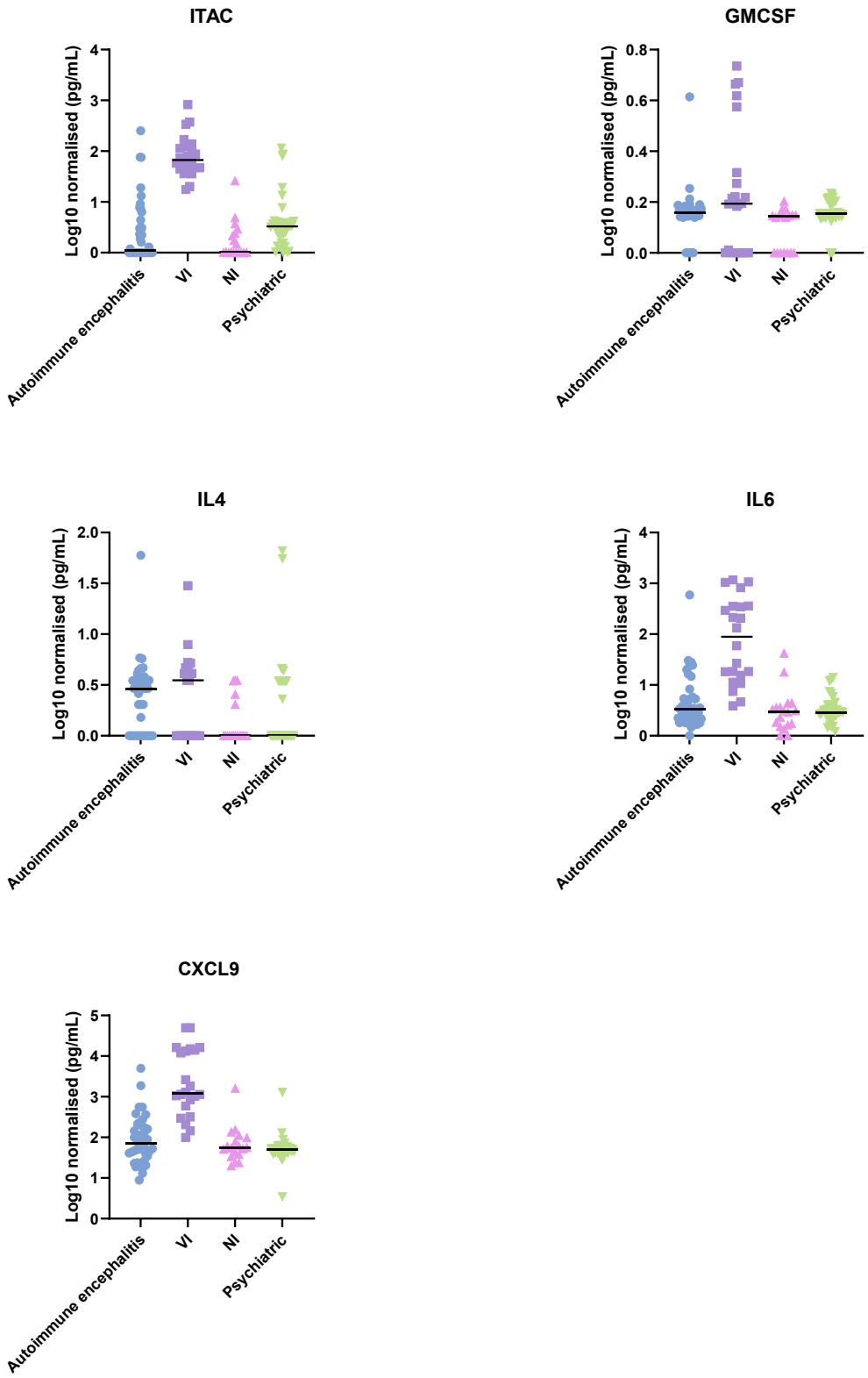


Figure 18: Cytokines in the psychiatric and comparator cohorts

Cytokines significantly different when the psychiatric cohort was compared with a combined cohort of NI, VI and AE controls

5.3.5.1 Effect of Psychotropic Medications On CSF Cytokines

I wished to determine whether prescribed psychotropic medications, particularly antipsychotics, in different patient groups could have influenced their expression of CSF cytokines. As expected, the use of antidepressants was highest in patients with depression (11/13 patients) compared with those who had a history of /bipolar (2/8 patients) and psychosis (5/14 patients). Use of antipsychotics was similar across the groups (depression 9/13 patients), mania/bipolar (5/8 patients) and psychosis (12/14 patients). Antipsychotic treatments are summarised in Table 23.

Use of antiepileptics as mood stabilisers was highest in the mania/bipolar group (4/8 patients) versus depression (1/11 patients) and psychosis (3/14 patients). There were patients prescribed lithium in all three groups of patients: 2/5 patients with mania/bipolar, 2/13 patients with depression and 5/14 patients with psychosis. Only three patients in my cohort were taking clozapine (dose range 200-300mg daily), two of these had a psychotic disorder, and one had a mood disorder with psychotic features.

The only cytokine that significantly differed when antipsychotic usage was examined was IL-13 (mean difference= 3.9pg/mL z=2.191 p=0.028), which was lower in patients taking antipsychotics. However, this may have been driven by three outliers with high IL-13 in the group of patients not taking antipsychotics. There were no significant differences between these two groups when the outliers were removed from the analysis.

When I compared patients who were taking clozapine compared to those who were not taking clozapine (including those taking other antipsychotics), I found that GCSF (mean difference= 17.7 pg/mL z=2.33 p=0.0198) was higher in patients not taking

clozapine. There was no significant difference in IL13 levels between those groups, possibly because of the small number of patients taking clozapine.

Table 23: Antipsychotic use in patient cohort[6]

Antipsychotic	Number of patients	Range of Dose prescribed
Risperidone	2	0.5-3mg
Olanzapine	6	10-300mg
Aripiprazole	4	10-40mg*
Quetiapine	7	25-200mg
Paliperidone	2	3-6mg
Haloperidol	1	3mg (patient recently ceased olanzepine15mg)
Clozapine	3	175-200mg
Amisulpride	1	400mg

* No dose data was available for 4 patients. They were prescribed aripiprazole (2), paliperidone (1) and quetiapine. Six patients were prescribed two concurrent antipsychotics. Of these for 2 patients the second antipsychotic was prescribed on a 'taken when necessary' basis.

5.3.6 Other investigations

Twenty-eight out of 35 patients with psychiatric disease underwent conventional brain MRI. Twenty-two patients had normal studies with six other patients having minor nonspecific white matter changes. Twenty-two patients underwent EEG at the time of entry into the study. Of these, fourteen patients had a normal study. Seven patients were reported to have diffuse cerebral dysfunction, one patient had frontotemporal cerebral dysfunction, and one patient had generalised seizure activity on EEG. This patient did not have elevated CSF cytokines, positive serum autoantibodies and her CSF analysis on routine markers of neuroinflammation was normal. She had already

been taking lamotrigine for her psychiatric disease and gabapentin for chronic pain at the time of EEG.

5.3.7 Principal Component Analysis

Principal component analysis (PCA) of all examined variables in my study across my cohorts indicated that the VI cohort clustered as a separate group based on expressed CSF cytokines. (Figure 19a) However, there was substantial overlap between my NI, psychiatric and AE cohorts. This was consistent with findings in my heatmap and may be consistent with a heterogeneity in pathogenic mechanisms.

As already discussed, there were a small number of individual patients within the psychiatric cohort who had marked elevation of CSF cytokines. These individuals clustered together with individuals from the AE cohort who also appeared to have higher expression of some CSF cytokines.

To further parse the relationship of CSF cytokines and symptoms, I identified the predominant symptoms in my psychiatric cohort: mood or psychotic symptoms reported in these patients. In patients with primarily mood-related symptoms, participants were further divided into whether they experienced unipolar depression or if they had expressed any symptoms of hypomania/mania during the course of their illness. Whilst there was considerable overlap in cytokines expressed in patients with depressed mood and psychotic disease, the patients who had experienced mania tended to be clustered separately, primarily based on expressed CSF cytokines (Figure 19b).

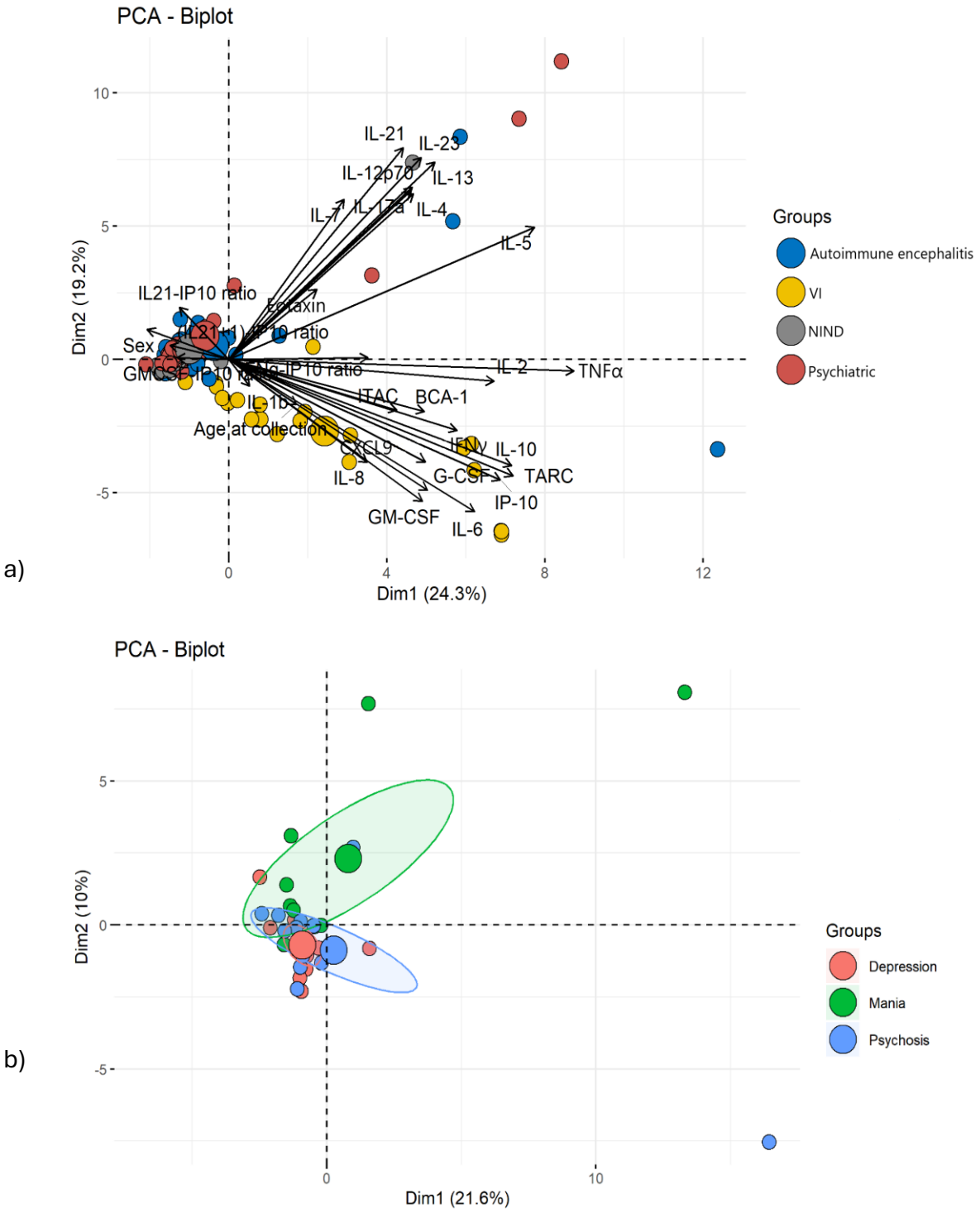


Figure 19: Principal Component Analysis

PCA[6] demonstrating (a) clustering of the infectious cohort by cytokines away from the AE, NI and psychiatric cohorts which are overlapping and (b) separation of patients presenting with mania as part of their psychiatric disease vs patients presenting with depression or psychosis.

5.4 Discussion

Psychiatric disease has a high burden of morbidity and mortality [562], particularly in treatment resistance cases. In Australia, suicide is the leading cause of death in men aged 15-44 [596] and conventional psychiatric medications are not without adverse effects including increasing long-term metabolic risk factors[597,598]. Effective treatment is rendered more difficult in the absence of a clear understanding of causality. Immune dysfunction may contribute to the pathophysiology of a subgroup of patients with atypical psychiatric presentations[265].

This pilot study sought to identify possible immune contribution to disease in a group of patients with atypical or difficult to treat psychiatric disease. These patients were identified by their treating psychiatrists as having atypical features or risk factors for an autoimmune/inflammatory contribution to their disease. This is an area of increasing interest in psychiatry, particularly for patients with treatment resistance to standard treatments (having failed two or more agents) or for whom long term morbidity usually results in long term engagement with psychiatric services.

5.4.1 Analysis of CSF Cytokines Suggest Immune Dysregulation in a Subset of the Psychiatric Cohort

A significant finding of this study was a substantial subset of patients in my psychiatric cohort expressed high CSF cytokines, suggesting a high degree of immune activation amongst these patients. (Figure 17). Six patients had at least one CSF cytokine greater than 4 standard deviations above the normal mean. The pattern of cytokine elevation was heterogenous amongst this group of patients whilst routinely

available CSF markers of neuroinflammation were mostly normal (aside from neopterin). This heterogeneity of response can also be seen in AE, where patients may exhibit elevation in only a limited number, or none, of the many routinely available surrogate markers of neuroinflammation. In the context of completely normal traditional CSF indices, some patients with a suspicious clinical phenotype still respond to a trial of immunosuppression. This finding highlights the need to identify discriminating biomarkers of neuroinflammation in these patients, if the clinical suspicion is high.

Our multivariate analysis identified two candidate cytokines of interest associated elevations in my psychiatric cohort: GM-CSF and ITAC. The interferon gamma cytokine ITAC was elevated in the psychiatric cohort compared with the AE cohort GM-CSF is a pro-inflammatory cytokine of the innate immune system and has a prominent role in the response to infection and in autoimmunity. GM-CSF enhances the survival, adhesion and trafficking of neutrophils and upregulates the antimicrobial functions of neutrophils and macrophage activation[599]. It has been associated with autoimmunity such as disease flares in rheumatoid arthritis and identified as a key mediator in inflammatory models of colitis. [599,600]. GM-CSF is essential to the development of predominantly cellular autoimmune neuroinflammation in experimental autoimmune encephalomyelitis[601].

The increase in GM-CSF in my psychiatric patient group may therefore suggest a pathological pathway veering away from antibody associated Th2 immune responses, typically associated with antibody formation, toward a more adaptive cellular immune response. In autoimmunity, GM-CSF has been associated with the Th17 response [602]. It is produced by Th17 T-cells and their differentiation and survival are facilitated by IL23

from antigen presenting cells in a positive feedback loop promoting TH17 effector function [603]. Whether this feature is related to the lack of anti-CNS antibodies in my psychiatric cohort needs further investigation.

We noted that 30% patients in my psychiatric cohort had some degree of chronic pain interpreted as having a central CNS component along with their psychiatric presentation. GM-CSF also appears to have a role in the development of both inflammation and associated pain through its interaction with CCL17[601]. However, there were no significant difference between my cohorts when levels of CSF GM-CSF in patients with chronic pain were compared with those who did not ($p=0.87$, $\chi^2=0.026$). This could be because of my small sample size.

Many innate and adaptive immune cells are implicated in CNS immune responses[604] including resident CNS microglia and subsets of peripheral immune cells, cytotoxic T cells, NK cells and NKT cells [605-607]. Microglia express the chemokine receptor CXCR3 which bind several chemokines, including ITAC/CXCL11[605], my second cytokine of interest. This was elevated in the psychiatric cohort when compared with both the NI and AE cohorts.

In the context of T-cell activation and polarization, CXCR3 receptor engagement leads to T-cell expression of IFN-γ (Th1 polarization), which was relatively elevated in my psychiatric cohort on univariate analysis. CXCR3 also modulates activation of microglia, the resident macrophages of the CNS [124], in animal models of disease [608]. Microglial activation has been implicated in the CNS immune responses in infection [124,125], autoimmunity [127] and in psychiatric disease [129].

The Th1 axis cytokine ITAC/CXCL11 is secreted by monocytes, endothelial cells, fibroblast and cancer cells in response to IFN-gamma and the proinflammatory cytokine

TNF-α[606]. Increased levels of ITAC/CXCL11 are associated with autoimmune diseases including connective tissue disease associated interstitial lung disease[609,610] and autoimmune thyroid disorders, such as Hashimoto's thyroiditis[611]. Increased levels of ITAC/CXCL11 have also been demonstrated in the CNS of a mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE)[612].

5.5.1.2 Comparison With Other Studies

Studies of CSF cytokines in psychiatric disease has been summarised in the introduction of this chapter (section 5.1.1). The prominent cytokines within my psychiatric cohort differed somewhat from what has been previously reported in CSF in the literature, which are primarily disturbances in levels of the pro-inflammatory cytokines IL-8 and IL-6. Whilst there are conflicting reports in these cytokines between single studies, results of meta-analysis have found increased IL-8 and IL-6 with schizophrenia[310]. [568] [567]and IL-6 with depression[571].

Increases in other CSF cytokines have been reported in primary studies including IL12β, IL12p70, TNFα, IL-4, IL-1β and interferon-beta [313,565,577,581-585]. Interferon-beta and IL12β were not in the panel of cytokines in my study. IL12p70 and TNF α were increased in my study on univariate analysis but not in multivariate analysis. There was no difference in the psychiatric cohort versus NI cohorts in IL6, IL8, IL-4 or IL1b. Indeed, IL-6 was significantly more elevated in the VI and AE cohorts compared to the psychiatric cohort. However, many of the studies in the literature used healthy control comparators. While my comparator cohort had "non-inflammatory" neurological diseases, these samples may not be representative of "normal". This issue was discussed in detail in chapter 4, section 4.4.5.

Comparison between the cytokines reported in the literature with my study and even indeed between other published studies is also difficult because of differences in patient definitions, different comparison cohorts (healthy versus "non-inflammatory" versus other neurological disease). As described in section 5.1.1, published studies in the literature used different cytokine detection assays and each of them would have differing sensitivities and lower limits of detection. Most other the published studies have looked at cohorts with well-defined psychiatric diagnoses. My cohort of patients was defined by having atypical disease and the presence of clinical red flag features that required further assessment to exclude an underlying cause such as autoimmunity. Hence, my patient group may to have different dominant pathophysiological pathways compared to cohorts studied in other reports, and finding of different cytokine profiles here is not entirely unexpected.

5.4.2 Normal Conventional Markers of Neuroinflammation in Disease

Most of my psychiatric disease cohort had normal conventional CSF parameters. MRI was also considered largely normal in all patients who underwent this study. A separate study of CSF in patients with recent onset depression demonstrated increased CSF white cell count when compared with a healthy cohort. However, the mean white cell count of this cohort was 1.49x10^6/L; only one patient of this cohort of 106 had CSF pleocytosis. There were no significant differences in CSF serum:albumin ratios, CSF total protein or IgG index [312]. Increased CSF protein has been described in patients with schizophrenia and affective disorders in a systematic review showing no differences in cell count were seen between these disease groups or healthy controls. This systematic review found conflicting reports of presence of oligoclonal bands with some studies

reported no presence of oligoclonal bands but up to 12.5% positivity was reported in others [310].

Even in my AE cohort, only a minority of patients had positive biomarkers for neuroinflammation in CSF. This known lack of sensitivity to currently available investigations highlight the need for better biomarkers for the detection of potential autoimmune contribution to psychiatric disease.

5.4.3 CSF Beta-2-Microglobulin was Significantly Lower in the Psychiatric Cohort

The finding of an overall decrease in CSF β-2 microglobulin in my psychiatric cohort was an unexpected one and appeared not to be due to serum levels of beta-2-microglobulin. Beta-2 microglobulin stabilises the heavy chains of the class 1 major histocompatibility complex (MHC Class I) and is present on all nucleated cells[613]. During metabolism and degradation, it disassociates and is released into all biological fluids and is hence thought to be a marker of cellular turnover[396,592,614]. Serum β-2 microglobulin has been associated with both haematological and solid organ malignancies[615].

While MHC Class I is expressed on the surface of all nucleated cells it is abundant in lymphocytes and macrophages[396]. Markers of lymphocyte turnover and survival are of particular interest in neuroinflammatory disease given that T-lymphocytes and NK cells produced brain-derived neurotrophic factor (BDNF), which regulates neurogenesis, neuronal survival as well as synaptic plasticity[616,617].

Beta-2-microglobulin has been used in the investigative pathway for CNS lymphoma and infection. Interestingly, when injected into the lateral ventricles of rats, β-2-microglobulin induced behavioural changes suggesting anxiety and depression[618] raising the possibility of a contributory role in these domains. Raised CSF levels of beta-2-microglobulin are associated with CNS infection[396] neuroinflammatory diseases such and neuro-Behcet's disease[619] and multiple sclerosis[620], although some reports in the latter are conflicting[396].

It would be expected that with levels of a marker of lymphocyte turnover such as beta-2-microgluobulin reduced, there would be an associated reduction of IL-7, a cytokine critical to lymphocyte development, maturation and survival[621]. However, this was not the case in my cohort where, in contrast to a decreased beta-2-microglobulin, there was a trend towards elevated CSF IL-7 in my psychiatric cohort, though it was not statistically significant. This may be suggestive of a degree of CNS immune dysregulation within the psychiatric cohort but again, further study is required.

Another consideration regarding beta-2-microglobulin and its essential role in stabilising the heavy chains of HLA Class I (part of the MHC Class I complex), is that HLA Class I molecules also play a role in brain development, particularly in cortical development[622]. It is expressed in neurons and influence processes including neurite outgrowth, synaptic plasticity and synaptic refinement. HLA Class I influences the development and function of cortical connections, activity-dependent refinement in the visual system and homeostatic plasticity[622,623]. Thus, the decrease in beta-2-microglobulin in the CSF of my psychiatric cohort might indicate a significant decrease

in these important neural processes. This could be secondary to a lack of trophic factors, some of which are delivered by immune cells.

It should be noted that a limitation of my study is that my laboratory does not provide a normal reference range for CSF β-2 microglobulin and the number of patients within the NI cohort who had this assay performed was limited. Hence, further studies with a range of patient groups are needed to confirm this finding and explore the role of this potential biomarker.

5.4.4 Evaluation of Immune Parameters in Atypical Psychiatric Disease is Worthwhile

Currently, the diagnostic criteria for AE include an acute or 'subacute onset of symptoms'[540]. However, as the possibility of an autoimmune aetiology for their illness has only been recently realised, patients with treatment resistant psychiatric disease may present years after disease onset during which multiple different psychotropic therapeutic strategies were trialled, and the appreciation of timing and rapidity of initial symptom onset may then be difficult to ascertain. Delays in presentation to medical attention after first symptom onset is common for many psychiatric disorders because of failure to recognise symptoms or because of social stigma. Additionally, the cognitive difficulties often associated with immune contribution to psychiatric disease represent another barrier to determine the characteristics of disease onset.

In my psychiatric disease patient cohort 6 (17%) patients had their diagnosis revised based on clinical, routinely available diagnostic results and response to immunosuppression, thus indicating their pathology was largely the result of a specific autoimmune disorder. These patients are summarised in Table 24. Each of these

patients responded to disease specific therapy and reduced or ceased their psychotropic medications.

One patient had her diagnosis formally revised to include probable seronegative AE. This patient presented with long standing treatment resistant depression and psychosis with visual hallucinations. There was a history of seizures three years ago attributed to a medication side effect and routine EEGs previously were normal. She also had physical symptoms: severe chronic pain, muscle spasms, migraines and unilateral piloerection which is a symptom suggestive of focal autonomic seizures. A prolonged, video recorded EEG then identified generalised seizure activity. Her diagnosis was eventually revised 2 years after she was referred for further neurology and immunological assessment, but after a trial of immunosuppression was commenced.

This patient was subsequently treated with antiepileptics and immunosuppression including prednisolone, mycophenolate, intravenous immunoglobulin (IVIg) as well as Rituximab. She did have initial improvement with immunosuppression, particularly after commencement of IVIg with improvements in concentration, memory and hallucinations. Symptoms worsened prior to when her next IVIg dose was due. Despite this, she has had a fluctuating clinical course and has required ongoing antipsychotics including the introduction of low dose clozapine. This above case illustrates the importance of earlier clinical suspicion and re-evaluation where there are 'red-flag' or atypical features present which may influence overall outcome. It has also been observed that delay in treatment in patients with AE also increases the risk of long-term sequalae[624].

Two patients were diagnosed with Hashimoto's encephalopathy, with one patient having concomitant Sjogren's syndrome. One patient had cryoglobulinemic

vasculitis. Another patient had likely autoimmune contribution to psychiatric disease and improved in functional ability (able to return to work), after a trial of Rituximab and mycophenolate treatment. This patient had positive VGCC in serum, with no evidence of malignancy, features of Lambert-Eaton syndrome or other muscle changes. One patient had coeliac disease and had resolution of symptoms with a gluten free diet and did not initially require immunosuppression. When she represented with the physical symptoms of vertigo and fatigue, further improvement was seen after treatment with mycophenolate which was eventually ceased with maintained health.

Hence, when diagnosis and appropriate disease management was instituted, at least four of these patients with treatment resistant disease had resolution of most of their psychiatric symptoms and three patients longer needed psychotropic medication. This underlines the importance of a thorough clinical workup in patients with psychiatric disease, and clinical reassessment in the context of treatment resistance or clinical features that might suggest an autoimmune disease.

The time between onset of symptoms and initiation of immunosuppression in this treatment group ranged from 2 months to 2 years. When I compared the 'red flag' clinical features between these patients who responded to treatment and other patients in my psychiatric group, there were no clear difference between them ($p=0.69$), although this may be due to small sample size.

Additionally, CSF examination did not necessarily discriminate between these two groups. One patient in the cohort of those who had their diagnosis changed had no abnormalities in routinely available CSF diagnostic results. The other patients had some elevation of markers of neuroinflammation although only two of the six patients had elevation of a marker that is used in the diagnostic criteria for AE (e.g. MRI

abnormalities, CSF pleocytosis, CSF specific oligoclonal bands or elevated CSF IgG index). Both patients had oligoclonal bands present and one of them also had elevated CSF neopterin and CSF protein. Of the remaining three patients with some elevation in CSF markers of neuroinflammation, 3/3 had elevation in CSF neopterin, and 2/3 additionally had elevation in CSF protein. Three of the six patients whose diagnosis was revised had evidence of elevation of at least one of the CSF cytokines I tested at 4 standard deviations above the normal mean.

Change of diagnosis after CSF analysis was also seen in 2 studies reported in a systematic review with between 3.2% and 6% of diagnoses respectively being revised. [310]. In a recently published multi-centre study of 1114 individuals who had taken part in clinical trials for first episode schizophrenia, 3.7% tested positive to anti-NMDAR autoantibodies. These individuals had better functioning and fewer negative symptoms of psychosis but were otherwise very similar to the other patients diagnosed with schizophrenia[625]. In comparison, my cohort had a slightly higher rate of revision of diagnosis, but this was high-risk group selected for possible autoimmune involvement.

Where an alternative diagnosis was found in my cohort, response to immunosuppression was still seen years after first onset of symptoms[588]. Hence, patients who may have had a longer duration of difficult to control disease may still warrant reassessment and treatment[588].

However, not all patients responded to immunosuppression/modulation. A total of 19 other patients were offered immunotherapy: 14 were offered a disease-modifying anti-rheumatic drug (DMARDs) with or without corticosteroids and IVIg, one was offered

glucocorticoids and IVIg, four offered minocycline only and four offered minocycline and another immunomodulatory agent (IVIg, hydroxychloroquine or corticosteroid).

Interestingly in my high cytokine group (at least once cytokine >4 standard deviations above the normal mean), n=3/6 patients responded to therapy whilst n=3/6 patients did not. When CSF cytokines were compared across responders and non-responders, there was no significant difference between the two groups when examined with a Bonferroni adjusted p-value threshold of 0.002.

Of the non-responders in this cohort, one was offered minocycline and then hydroxychloroquine but discontinued the medications within 2-3 months because of side effects and lack of perceived efficacy. One was treated with Plaquenil for 6 months with no clinical benefit seen. Mycophenolate was offered but the patient was lost to follow up. The third patient was treated with methotrexate, sirolimus and mycophenolate, with a duration of immunosuppression for greater than 12 months, with some modest improvements. Interestingly, she represented 2 years after her immunosuppression trial with retinal vasculitis. Further investigations including a repeat lumbar puncture was recommended, but the patient was lost to follow up.

Table 24: Patients where diagnosis changed[6]

Patient Number	Age	Sex	Presenting features	Positive test results	Treatment	Time from symptom onset to treatment response (months)	CSF cytokines increased?
1	16	M	Treatment resistant depression and psychosis	High positive thyroperoxidase antibody Single band seen on CSF oligoclonal bands Increased CSF neopterin	Pulse methylprednisolone, IVIg, rituximab, mycophenolate	24	Y, up to greater than 4 SD above mean
2	30	F	New onset peripartum mania with short term memory loss. No previous psychiatric history	Positive cryoglobulins 1:80 speckled ANA Increased CSF neopterin	Prednisolone and azathioprine with further consolidation of immunotherapy with rituximab post delivery	4	Y, up to greater than 4 SD above mean
3	16	F	New onset mania with prodromal period of short-term memory loss	Non-specific VGKC antibody	mycophenolate	4	Y, up to greater than 4 SD above mean

			and brain fog. History of Hashimoto's thyroiditis and new diagnosis of type 1 diabetes Strong family history of type 1 diabetes.	GAD65 serology (likely indicative of Type 1 diabetes) 1:2560 speckled ANA			
4	20	F	Treatment resistant psychosis with paranoid delusions	Speckled 1:160 ANA Positive coeliac serology Positive CSF oligoclonal bands	Gluten free diet	9	N
5	28	F	Treatment Resistant psychosis	VGCC channel antibodies Positive CSF neopterin	Pulse methylprednisolone, plasmapheresis, mycophenolate, IVIg	12	N
6	17	F	Atypical mood and psychiatric symptoms with episodes of confusion and collapse. Problems with impulsive thoughts and attempts at suicide and self-harm. Unilateral	Nil; MRI brain also normal	Prednisolone, mycophenolate, IVIg, Rituximab	5 years	N

			(right sided) piloerection, chronic pain, muscle spasm and severe headache. Generalised seizure activity was seen on prolonged video EEG. Previous EEG results prior to recruitment were non-specific.				

5.4.5 Diagnostic Results Must Be Considered in Their Clinical Context

Despite the promising results in a subset of my cohort, care still needs to be taken in the interpretation of clinical and investigative findings in re-evaluating patients. Decisions regarding diagnosis should not be made based on a single symptom or test. A large proportion of patients in my psychiatric cohort reported additional physical symptoms including fatigue, cognitive disturbance, seizures or seizure like episodes, sleep disturbance, paraesthesia and chronic pain (Table 20).

Whilst these symptoms may be interpreted as an indication of autoimmune driven disease, they are also reported in psychiatric disease and may also be consequence of medication use[269,626-631]. The new onset of these symptoms in the context of established psychiatric disease or their predominance in the clinical picture should prompt careful re-evaluation but the significance of each symptom must be interpreted in the context of other clinical and investigational findings.

Autoimmune serology must always be interpreted within the clinical context. While the maxim that the presence of a detected autoantibody, without corresponding clinical features, may suggest an autoimmune propensity but does not equate to disease remains broadly true, it is important to recognise that the clinical spectrum of autoantibody-mediated diseases is continually expanding. This evolving understanding may warrant a more nuanced interpretation. Furthermore, this landscape is complicated by the limited knowledge of the epitope specificity of detected autoantibodies, and by the uncertainty of whether the antibody is a primary mediator of disease or a secondary consequence of other processes, such as neurodegeneration. The patient in my cohort with positive VGCC in the serum had no features of associated muscle changes or

evidence of malignancy. The relevance of this in the context of her clinical presentation was unclear, though she improved on immunosuppression Interestingly, association of VGCC and schizophrenia has been reported in the literature[632]. Another of the patients in my cohort had borderline positive myositis associated antibodies but the clinical significance of this was again unclear in the absence of clinical evidence of myositis. However, with the increasing discovery of relevant autoantibodies to CNS disease and the recognition dual antibody conditions[405,633,634], the presence of other yet to be defined autoantibodies may be in play.

The ANA positivity rate in my study exceeded what is expected in a normal population. However, the majority of these had a low titre and were not associated with clinical features of a connective tissue disease or with ENA or dsDNA positivity. One of these patients was eventually trialled on immunosuppression with pulse methylprednisolone because of concurrent features suggestive of autoimmune contribution including treatment resistance and recent history of ovarian cancer but had minimal improvement.

ANA has high sensitivity for autoimmune disease but is less specific and should always be correlated with clinical features and more specific antibody testing. Higher titre ANA antibodies (>1:640) are seen in up to 2.5% of the general population [590] but can also be present years before the development of any autoimmune disease. my patients with mid-level positive ANAs did not exhibit any clinical features of connective tissue disease, nor more specific autoantibody positivity. Similarly, myositis antibodies can have a high false positivity rate [635,636] and if present without the correct clinical features, their significance in psychiatric disease is unclear.

The second highest frequency serum autoantibody in my study was that of thyroid-related antibodies with up to 20% of my cohort with positive antibodies to thyroid peroxidase, which can be found in Hashimoto's encephalopathy. However, thyroid autoantibodies are also present in up to 11% of the general population [372] and cannot in themselves be used to confirm a diagnosis of thyroid disease [637]. Additionally, Hashimoto's encephalopathy is an area of diagnostic controversy. It is thought to be rare, with a wide range of reported clinical presentations and there are no specific biomarkers of the disease. In some large cohorts of suspected Hashimoto's encephalopathy, the majority of patients were subsequently found to have a non-immune mediated diagnosis[540]. Whilst steroid responsiveness has been previously considered to be important for diagnostic confirmation, a recent case series of patients with suspected Hashimoto's encephalopathy found only 31% had a complete response to steroids, requiring multimodal immunosuppression to achieve disease resolution[638]. This is an experience consistent with observations in this centre.

It may be argued that, in patients with severe psychiatric morbidity who have exhausted conventional treatments, a therapeutic trial of immunosuppression merits consideration. However, current evidence and practice support offering such therapy only when there is demonstrable immune-mediated pathology, ideally guided by elevated inflammatory biomarkers, and after a thorough risk–benefit assessment (75). Meanwhile, the knowledge of antibody-mediated disease is rapidly expanding novel autoantibodies are continually identified, cytokine alterations are detected in cerebrospinal fluid despite normal classical indices, and delays in immunotherapy carry a substantial risk of further harm. These developments prompt the question of whether withholding an informed-consent trial might, in certain cases, pose greater harm than proceeding without unequivocal proof of autoimmune involvement.

Immunosuppressive regimens inherently entail risks and side effects. In my own cohort, combining immunotherapy with psychiatric treatment did not significantly increase adverse events compared to psychotropic medication alone, although this small study was not definitive[588]. In light of these uncertainties, and given the absence of conclusive evidence either supporting or refuting the use of immunotherapy in this population, treatment decisions should be made within the context of a multidisciplinary team. Patients, or their legal guardians, should be fully informed of the current evidence base, the potential risks, and the anticipated benefits, particularly given the prospect of lifelong care and social exclusion of the affected person is high. Moreover, I advocate rigorous monitoring and systematic documentation of treatment outcome and banking of appropriate specimens to validated, disease-associated biomarkers to build the evidence necessary for future therapeutic innovation in this challenging patient group.

5.4.6 Study Limitations

Our study is limited by my relatively small and heterogenous cohort in which not all results were available for all patients. This is in the context of there being no clear standard of what investigations are required for this group of individuals, the scope of possible investigations being very wide. There is a referral bias in my study groups, implicit in my recruitment method. Nevertheless, all included patients had at least one 'red flag' feature in their clinical presentation giving indication for a need for further evaluation.

The same limitations with my comparator cohorts as outlined in Chapter 4, section 4.4.5 also apply here, particularly that my NI cohort consisted of patients with NPH and IIH rather than true "normal" participants.

The quantity of CSF collected for lumbar puncture and how it is collected is important. My patients had 10-15mL collected in total to facilitate enough volume for all neurological testing. This volume is greater than that used for "standard" CSF investigations such as confirmation of infection (usually 1-2mL). Unfortunately, not all CSF was collected in RMPI which made flow cytometry results for these patients [639].

We did not examine serum levels of cytokines in my cohorts which means I am not able to see if CSF cytokine levels have been influenced by circulating serum cytokines. This should be examined in future studies.

Like many autoimmune diseases most often associated with autoantibodies, my cohort was female predominant[640]. There is female predominance noted in most (but not all) antibody associated AE [641]. In contrast, antibody-negative AE is reported to have a 1:1 male: female gender divide [642], or in the case of my previously reported cohort, a male predominance [7]. This may indicate a difference in the immune pathogenesis of AE between males and females similar to that seen in other systemic autoimmune diseases. Therefore, it is important to broaden the psychiatric patient groups investigated and compare cytokine signatures in more 'typical' psychiatric populations to establish the sensitivity and specificity of my selection criteria to identify immune contribution to psychiatric disease.

The recently published consensus statement [265], aiming to identify patients with psychiatric disease with an underlying component of immune/inflammatory pathogenesis, included in their case definitions a subacute onset i.e., rapid progression of less than three months. However, there is further concern that patients whose investigation was more distant from disease onset may not be able to be able to be identified for many reasons including cognitive impairment. Due to chronicity of disease,

symptoms in my patients may be due to CNS injury rather than ongoing immune activity, and investigations may be impacted by various psychotropic drugs [643,644]. However, without appropriate investigations, the possibility of immune contribution cannot be excluded in these treatment resistant patients. The question is also raised as to whether newer serum testing that identifies sustained neural damage, such as serum neurofilament light will identify patients with a predominant immune inflammatory contribution and/or be useful in monitoring treatment response[645].

5.5 Conclusion

Our study demonstrates the clinical use of a large and comprehensive panel of immunological investigations in a psychiatric patient cohort selected based on "red flags" or treatment resistance which may identify patients where an alternate diagnosis should be pursued. This study highlights the need for recurrent re-evaluation in patients with difficult to treat or atypical presentations of psychiatric illness to avoid diagnostic anchoring. However, in reevaluating patients, the clinical scenario must be used with investigation results to consider whether an immune-mediated disease process is possible and may be causative rather than simply an association, and whether directed immune modulatory therapy may provide benefit.

Basic questions remain about the general role autoimmunity and inflammation in the pathogenesis of psychiatric illness. Therefore, clinical boundaries of the target group of people with a psychiatric illness who should be referred for this intensive work-up or circumstances when a trial of immunotherapy should be considered remain unclear. However, while not formally investigated, from a health economic view, any cost saving in care requirements from significantly improved function of patients responding to

immunomodulation may be significantly higher than the costs of investigations for the whole group. Further study of these patients may yield a better understanding of both diagnostic algorithms to identify patients with immune contribution to disease and targets for treatment.

Chapter 6: Proteomics and Biomarker Discovery

6.1 Introduction: Mass Spectrometry for Biomarker Discovery

6.1.1 Proteomic Analysis of CSF

So far, the studies in this thesis have focused on biomarkers identified using routinely available biochemistry techniques or, in the case of novel biomarkers, immunoassay techniques. One of the main challenges for CSF biomarker detection in these contexts is the low abundance of protein in the CSF mandating more sensitive assays for detection than those needed for serum. This was already discussed in chapter 4, in the context of differing sensitivities of cytokine detection kits contributing to different findings between studies. Mass spectrometry provides a potential solution for this. It is a powerful tool which able to accurately detect and quantify proteins present at low levels amidst abundant matrix proteins. Thus, it presents a unique advantage in biomarker discovery studies in CSF across a range of neurological diseases where the availability of sample volume may be small[646].

Mass spectrometry has already been utilised for biomarker discovery in other neurological diseases. As an example of the applications of mass spectrometry in other neurodegenerative or neuroinflammatory conditions, I searched the following conditions: Alzheimer's Disease, Parkinson's Disease, amyotrophic lateral sclerosis and multiple sclerosis in PubMed and combined using "AND" the keywords "CSF" and "mass spectrometry", limiting results to the last 10 years. Examples of some potential biomarkers identified using this technique is outlined in Table 25.

Table 25: Examples of mass spectrometry findings in neurodegenerative disorders and multiple sclerosis

Condition	Number of studies identified PubMed search	Examples of some of the potential biomarkers identified
Alzheimer's Disease[647-649]	434	Amyloidβ peptides, Tau and phosphorylated tau, Tau, SOD1, PARK7, YKL-40, NPTX2, SNAP-25, 14-3-3 zeta/delta, β-synuclein, neurogranin
Parkinson's Disease[649,650]	68	OMD, CD44, VGF, PRL, MAN2B1, neuronal pentraxin receptor, syntaxins, LAMP2
Multiple sclerosis[651-653]	67	Secretogranin I and II, Protein 7B2, haptoglobin, alpha-1-anitchymotrypsin, Ig mu chain C region, alpha-2-glycoprotein, Vitamin D binding protein, chtiniase-3-like protein, protein jagged-1, contactin-1, kallikrein-6 and apolipoprotein

6.1.1.1 Proteomics biomarker discovery in Autoimmune Encephalitis

The diagnosis of AE remains difficult, and the pathophysiology of AE likely involves more than the B cell-mediated humoral immune response, with T cell effector mechanisms also likely having a role and with variable contribution of other immune cellular compartments. Hence CSF proteomics using mass spectrometry holds promise as a method to help identify novel biomarkers to aid in diagnosis.

There has only been one study published examining proteomic profiles in AE[654]. This study compared a cohort of 26 patients with antibody positive AE with a cohort of 10 healthy controls and nine patients with relapsing-remitting multiple sclerosis (RRMS). They demonstrated that whilst there was overlap in the proteomic profiles of different antibody subtypes of AE, there was little overlap compared to RRMS, suggesting different

pathological mechanisms at play in these two immune-mediated diseases that respond to b-cell depleting therapies.

This study identified several pathways implicated in AE. This included the complement system, humoral and cellular immune responses, protease function and the contribution of neurodegeneration, as well as synaptic neuronal function, all contributing to the pathological mechanisms resulting in the clinical phenotype[654]. In addition, there were unique proteins expressed in the CSF associated with different antibody associated AE subtypes suggesting the possibility of unique pathways within different AE subtypes. This is not a totally unexpected result given the different clinical phenotypes associated with different antibody associated disease[654].

However, this is a single study with a small cohort of patients and therefore the initial data will need to be interpreted with caution. Given the breadth of data available with proteomics, more studies with replication of results and the concordance of candidate proteins across different studies needs to be achieved for novel biomarkers of disease to be implemented for routine testing.

6.1.1.2 Proteomics biomarker discovery in Psychiatric Disorders

There has been much interest in the application of proteomics to a wide range of body fluids and tissues in psychiatric disease to identify biomarkers and discover new pathological pathways. Earlier studies used microarray chips to detect proteins of interest[655] with later studies employing mass spectrometry. Studies have examined blood, CSF and brain tissue in mostly postmortem studies in defined psychiatric populations to try and find new pathological pathways and biomarkers that can assist with a psychiatric diagnosis.

Proteomic studies of post-mortem brain tissue in patients with schizophrenia have consistently found alterations of energy metabolism with disturbed levels of proteins involved in glycolysis, Krebs cycle, mitochondrial function and oxidative stress[656]. This same review found some six proteomic studies also described alterations of oligodendrocyte-related proteins in schizophrenia brain tissue, consistent with findings of oligodendrocyte dysfunction in suggested by neuroimaging, epigenetic and transcriptomic studies.

In the CSF of patients with schizophrenia, altered levels of proteins involved in cholesterol transport and phospholipid metabolism have been described[656]. Identification of potential disease biomarkers using mass spectrometry in the CSF of patients with first onset psychosis have also found alterations in glucose and lactate as well as increases in VGF derived peptide[657] in one study and a down regulation of collagen alpha-2 and neuron derived neurotrophic factor in patients in another[658]. In a review looking at biomarkers in patients with established schizophrenia however, proteins in cholesterol transport and phospholipid metabolism including apolipoprotein A-1I, A-IV and apolipoprotein E, transthyretin and prostaglandin H2 D-isomerase were altered in patients. Again, VGF peptide was also altered in patients in schizophrenia versus controls[656]. Interestingly, in a study examining patients with symptoms prodromal for psychosis, there was no statistical differences in the between patients and normal controls. This is despite the study confirming that there were different proteomic signatures between patients with first onset schizophrenia and controls[657].

In proteomic studies of patients with affective disorders, there was some overlap in potential biomarkers reported between multiple studies but there was also a variety of differing results. In major depressive disorder, a review reported two studies of CSF using

mass spectrometry and a further seven studies examining for biomarkers using other methodologies (ELISA, gel electrophoresis, protein microarrays and line immunoassay)[659]. A further six studies in this review examined brain tissue. One study (using Surface-enhanced laser desorption ionization mass spectrometry) which largely examined patients with first episode psychosis, also found altered levels of the neurosecretory protein VGF and lower secretogranin II peptides in the CSF of patients within their major depressive disorder comparison cohort[660]. A second study (using 2-dimennsional polyacrylamide gel electrophoresis and time-of-flight mass spectrometry) found thirty-nine proteins either up or down regulated in patients with major depressive disorder, although different to what was reported the previous study. These identified proteins including those involved in the regulation of angiogenesis, acute phase response, adult locomotory behaviour and cell to cell signalling[661]. The two studies differed in their mass spectrometry technique which may have contributed to differing results. When comparing proteins of interest across the studies included in this review, reproducibility emerged as a concern: of the 42 identified biomarkers, only 28 showed consistent findings in at least two studies. Only 7 of these 42 potential biomarkers were measured in CSF, and there were no proteins that showed consistent results between CSF studies.

A further study published in 2020 examined CSF proteomics using mass spectrometry on the CSF of patients with major depressive disorder had comparisons to not only controls, but also patients with schizophrenia and bipolar disorder[662]. Samples were first examined using liquid chromatography mass spectrometry (LC-MS) proteomic analysis, using mass spectrometry then the findings validated using a multiple reaction monitoring (MRM), a targeted MS approach. VGF was shown to be downregulated across all psychiatric disorders when compared to controls. Patients in the major depression

and bipolar cohorts had downregulation of leucine-rich repeat and immunoglobulin-like domain-containing protein 1(LINGO1), glutamate ionotropic receptor AMPA type subunit 4 (GRIA4), contactin associated protein family member 4 (CNTNAP4), and neuroserpin (SERPIN1) when compared to controls with patients with major depression also having downregulation of proprotein convertase subtilisin/kexin type 1 inhibitor (PCSK1N), neuronal pentraxin receptor (NPTXR) and cocaine- and amphetamine-regulated transcript protein (CARTPT). [662].

In bipolar disorder proteomic studies across two cohorts found that eotaxin-1, placenta growth factor, CXCL-6 and CXCL1 had high concentrations in patients compared with controls[663]. Only growth hormone was consistently lower in patients versus controls across the two cohorts. However, this study did not use mass spectrometry for proteomics analysis but rather a multiplex immunoassay. The disadvantage of using this method is that differences in proteins that are not in the immunoassay panel will remain cryptic.

Hence, whilst mass spectrometry and proteomics has been applied across several tissue types in psychiatric disease, there has not yet emerged any biomarkers to use in routine practice. Further study in larger cohorts is required to validate current findings. Furthermore, most of the published studies focus on biomarkers for a well-defined psychiatric diagnosis rather than patients with atypical presentations who may have different pathological pathways underpinning their clinical presentation.

6.1.2 Future Directions of Proteomics

Proteomics and mass spectrometry have a large potential for identification of novel biomarkers beyond what is currently available. The sensitivity of mass spectrometry is allowing for biomarker discovery in studies with fewer cases, which is

useful in conditions with lower incidence like AE where study of large cohort of hundreds of patients may be difficult [664]. Currently published studies in other neurological diseases have already uncovered potential new biomarkers and pathogenic pathways. However, data is lacking in AE and atypical psychiatric disease.

6.2 Methods

6.2.1 Patient Selection

Inclusion of patients for mass spectrometry analysis is described in Chapter 2 (methods), section 2.7.1. Briefly, the aim of this pilot study was to see if there was any indication of differences in proteomic signatures in patients with AE (antibody positive and negative) and psychiatric disease. Patients already included in the cohorts in Chapters 4 and 5 and where there was sufficient (at least 200uL) of available CSF for further analysis were included in this study.

6.2.2 Mass Spectrometry Analysis

Methods used in performance of sample preparation for mass spectrometry analysis is described in Chapter 2, section 2.7.4. The evidence supporting the choice of DIA LC-MS for analysis of samples in this study is described in Chapter 3.

6.2.3 Statistical Analysis

6.2.3.1 Identification of Candidate Proteins

Analysis of mass spectrometric acquisition results and differential abundance analysis (including statistical analysis) to identify candidate proteins was conducted in Spectronaut®. This software package was used for the construction of heatmap, volcano and PCA plots. Statistical testing in Spectronaut is performed using either paired or unpaired t-test with a q-value of less than 0.05 (Spectronaut produces a q-value after

multiple testing correction for p-values are performed using the Storey's false discovery rate (FDR) procedure [665]).

Candidate proteins were defined as differentially abundant proteins with a q value <0.05, a log2 ratio of difference of at least 0.58 and with at least 2 unique peptides identified[666]. For the purpose of discussing the candidate proteins with the greatest difference in the comparisons, a threshold of log2 ratio of 2 was chosen.

6.2.3.2 Principal Component Analysis and Volcano Plots

Spectronaut was used for construction of PCA plots and volcano plots. Volcano plots were also constructed after removal of potential contaminants[667]. To highlight candidate proteins that had the most difference between comparator cohorts, proteins with a log 2 ratio difference of greater or more than 2 are represented in the volcano plots.

6.2.3.3 Reactome Pathways

Further Reactome Pathways and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) interactome analysis were performed with all candidate proteins nominated by Spectronaut (as defined in section 6.2.3.1) after removal of contaminants such as keratin, haemoglobin and saliva[667]. All candidate proteins with a q value <0.05 and log 2 difference of >0.58 (default Spectronaut setting) and with at least 2 unique proteins were subject to pathways and interactome analysis.

Pathways analysis was conducted using Reactome Pathways Knowledgebase [12,668]. For the AE vs NI and psychiatric vs NI comparisons, all differentially abundant proteins (up- and down-regulated) from each comparison were combined into a single list to obtain a global overview of implicated biological processes In contrast, for the AE vs psychiatric and AbPAE vs AbNAE comparisons, pathway analysis was conducted

separately for the proteins increased in each group (e.g., proteins upregulated in AbPAE were analysed independently from those upregulated in AbNAE) to identify pathways specifically enriched within each condition, to enable direct comparison of condition-specific pathway signatures and to avoid cancellation of opposing effects.

For each pathway analysis we report the FDR *q*-value, nominal *p*-value, entities found/total and the entity ratio returned by Reactome. Analyses used the Reactome web interface with default pathway size settings and *Homo sapiens* as the reference species; no evidence type filters were applied.

6.2.3.4 STRING Interactome Analysis

Interactome analysis was performed using STRING[669-671]. All differentially abundant proteins in each comparison (up- and down-regulated combined) were submitted to STRING (Homo sapiens) in the analysis to get a global overview of protein-to-protein interactions between comparisons. Networks used the active evidence sources excluding text mining (i.e. curated databases, experiments, co-expression, neighbourhood, and gene co-occurrence) and a minimum required interaction score of 0.70 (high confidence). STRING's protein-protein interaction (PPI) enrichment *p*-value was used to test whether observed connectivity exceeded random expectation. Functional modules were identified with the Markov Cluster Algorithm (MCL) using an inflation factor of 3, and clusters were annotated via STRING's GO/Reactome enrichment.

6.2.3.5 Further Independent Analysis

Raw data of identified peptides was also extracted from Spectronaut® for data analysis independent of the software package. This was analysed using the standard limma package in R (using linear models and empirical Bayers shrinkage).

6.3 Results From Proteomic Analysis Using Mass Spectrometry

6.3.1 Initial Patient Numbers

Forty-five patients (including NI controls) were analysed. This included a cohort of 19 patients with AE (7 AbPAE and 12 AbNAE), 19 patients with psychiatric disease (11 with primarily mood-based symptoms and 8 with primarily psychotic symptoms) and 7 NI controls. The NI patient with a ventriculoperitoneal shunt inserted prior to lumbar puncture was not included in this cohort.

6.3.2 Protein Identifications

Mass spectrometry overall identified an average of 13 222 peptide profiles with 2201 peptides identified in every sample; 8589 peptides were present in >50% of samples. These peptides represented 565 proteins identified in every sample, and 1346 identified in >50% of samples.

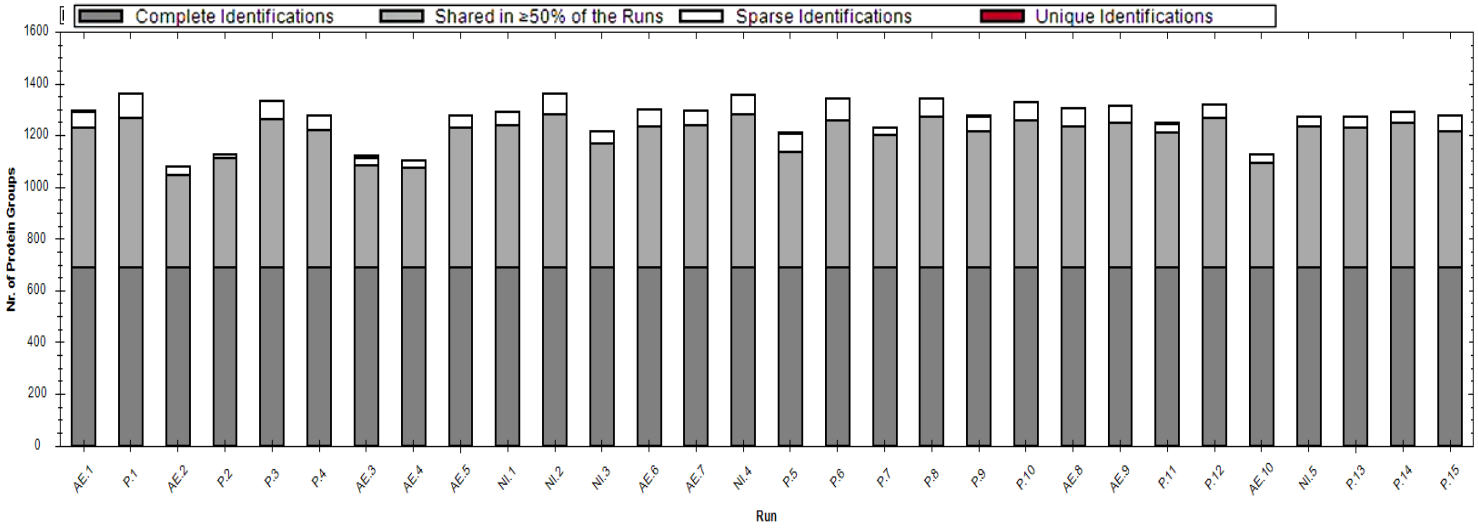


Figure 20: Histogram of run identifications for proteins groups across different samples in the study

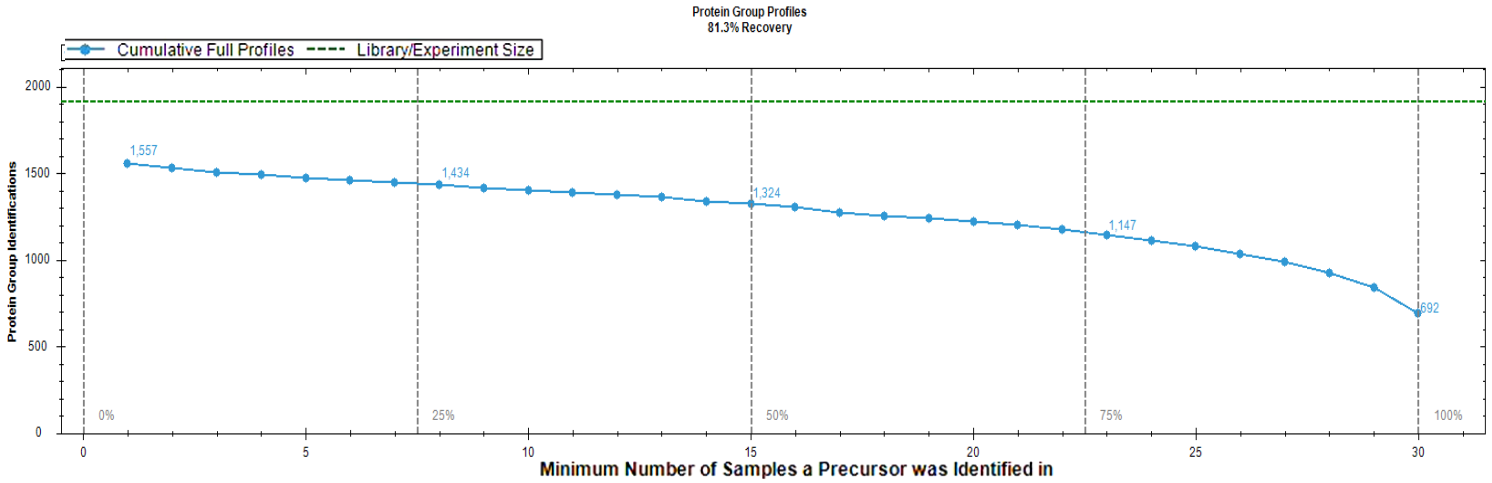


Figure 21: Cumulative Full Profiles

The blue line represents the decline of full protein profiles identification across verses the number of samples the protein group was identified in.

a)

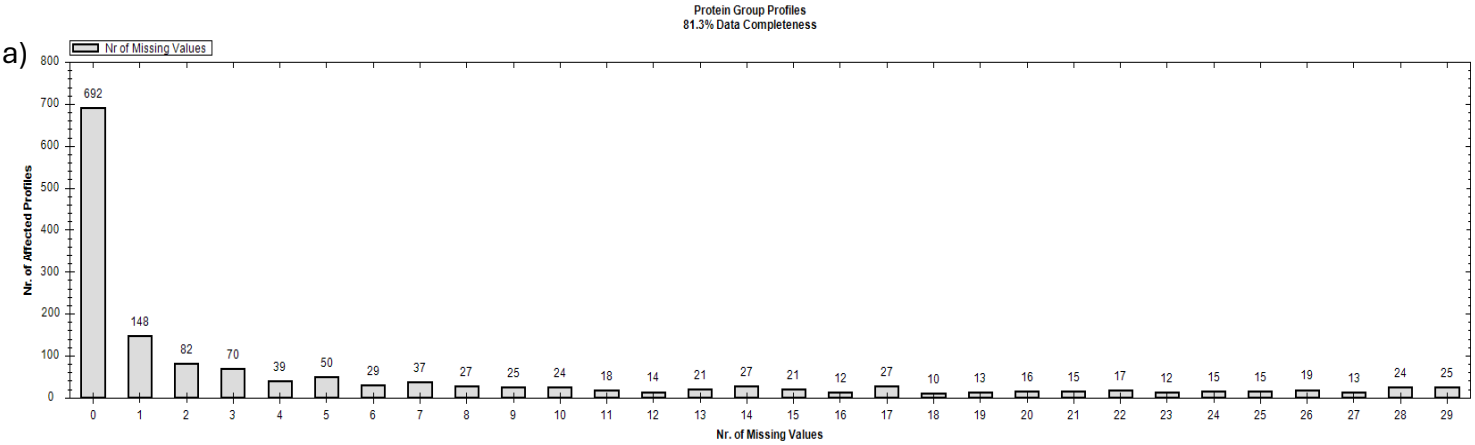


Figure 22: Histogram showing the number of missing values compared to number of affected protein profiles (A)

6.3.3 Removal of Patients from Cohort Due to Blood Contamination

6.3.3.1 Coagulation-Related Proteins as Potential Confounders

During initial data analysis, one of the most prominent differences between the AE (AE) and psychiatric cohorts was the enrichment of proteins related to coagulation pathways (including proteins involved in scavenging of heme from plasma and response to wounding). Given that these proteins are abundant in blood but typically present at very low levels in cerebrospinal fluid (CSF), their elevation raised concerns about potential blood contamination of the CSF samples.

6.3.3.2 Evaluation of Blood Contamination Across Cohorts

To assess the extent of blood contamination, I reviewed CSF red blood cell (RBC) counts across all samples. This revealed a higher prevalence of blood-contaminated CSF (RBC > 10/µL) in the AE cohort compared to NI control groups ($p=0.02$, $\chi^2=5.1$ 1df) There was a trend for increased red blood cell counts in the AE group compared to the psychiatric group although it did not quite meet the threshold for statistical significance

(p=0.06 χ^2=3.8 1df) which may be due to overall low numbers in the corresponding cohorts. There weren't any significant differences in the CSF red cell count between psychiatric and NI cohorts (p=0.2 χ^2=0.2 1df) .

Hence, there appeared to be a disproportionate number of AE patients, including both antibody-positive and antibody-negative cases, that exhibited CSF RBC counts above the threshold. This suggested that traumatic lumbar puncture or blood-brain barrier disruption may have contributed to the observed proteomic differences. In the context of the increased sensitivity of mass spectrometry, this raised concerns that findings in these blood contaminated samples may skew the overall results because detected proteins found in CSF may not be because of the disease state, but because of blood contamination. This concern has also been reflected in published literature with studies reporting effects of blood contamination of CSF and CSF spiked with blood skewing mass spectrometry results[672,673].

Removal of blood contaminated participant samples from the analysis resulted in other candidate proteins being identified. This suggests that inclusion of the blood contaminated samples did change overall proteomic results. It was unclear if this was due to the lower number within the cohorts, or if there was an overrepresentation of proteins from blood rather than CSF in the blood contaminated samples. For this reason, it was decided that I continue the analysis with removal of blood contaminated samples.

CSF blood contamination was not a major consideration in my earlier analysis because the CSF RBC counts noted in my CSF study was not likely to have a major impact on the other conventional markers of neuroinflammation including mononuclear cell/neutrophil count (where normal mononuclear cell/neutrophil count is less than 5 with a correction factor of -1 leukocyte for every 500-1000 red blood cells/uL detected[209]).

However, my analysis suggested that including patients with even minor elevations in CSF RBC count (>10) may cause aberrant results that may be artefactual and attributed to preanalytical factors. An additional concern was raised when there had been a significant increase in red blood cells but in long preanalytical time frames they had lysed releasing their contents.

6.3.3.3 Evidence for exclusion of RBC Contaminated Samples

To minimise the influence of blood-derived proteins on my findings, I implemented a strict exclusion criterion, removing all samples with CSF RBC counts > 10/µL. This led to the exclusion of 9 AE patients (6 antibody-negative, 3 antibody-positive), 2 NI control patients, 4 psychiatric disease patients (2 primarily mood, 2 primarily psychotic).

6.3.4 Final Patient Numbers

The final numbers in my new cohort of patients were therefore 10 patients with AE (4 antibody-positive, 6 antibody-negative), 15 patients with psychiatric disease (9 with primarily mood and 8 with primarily psychotic symptoms) and 5 NI controls. Demographic data are summarised in Table 26. The median ages of patients in the psychiatric disease and NI control cohorts were lower than the AE cohort.

Table 26: Demographic data of included Patients

Group (N)	**Age Range (Median)**	**Sex (M:F)**
AE (10)	17-70 (48)	6:4
Psychiatric Disease (15)	17-63 (22)	2:8
Non-Inflammatory Controls (5)	16-28 (18)	1:4

Candidate proteins (q-value <0.05 and using the default Spectronaut threshold of a log 2 ratio > 0.58 difference) identified on differential abundance analysis before and

after removal of these potentially blood contaminated samples were compared. In the AE vs NI comparison, 214 candidate proteins were identified compared to 309 prior to removal of blood contaminated samples. In the psychiatric vs NI comparison, 215 candidate proteins were identified compared to 224 prior to removal of blood contaminated samples. In the AE vs psychiatric comparison, 185 candidate proteins were identified after removal of potentially blood contaminated samples compared to 339 candidate proteins before potentially blood contaminated samples were removed.

In both groups including samples with blood contamination, and groups without, presence of candidate proteins associated with haemoglobin were detected. That the differentially expressed candidate proteins between comparator groups still included haemoglobin associated proteins, even after blood contaminated samples were removed, raised the hypothesis of red cell lysis of samples in transit also causing contamination. This has also been reported in the literature and will be expanded upon in the discussion[673].

6.3.4.1 Analysis of Minimally Blood Contaminated CSF Cohort

6.3.4.1.1 Principal Component Analysis

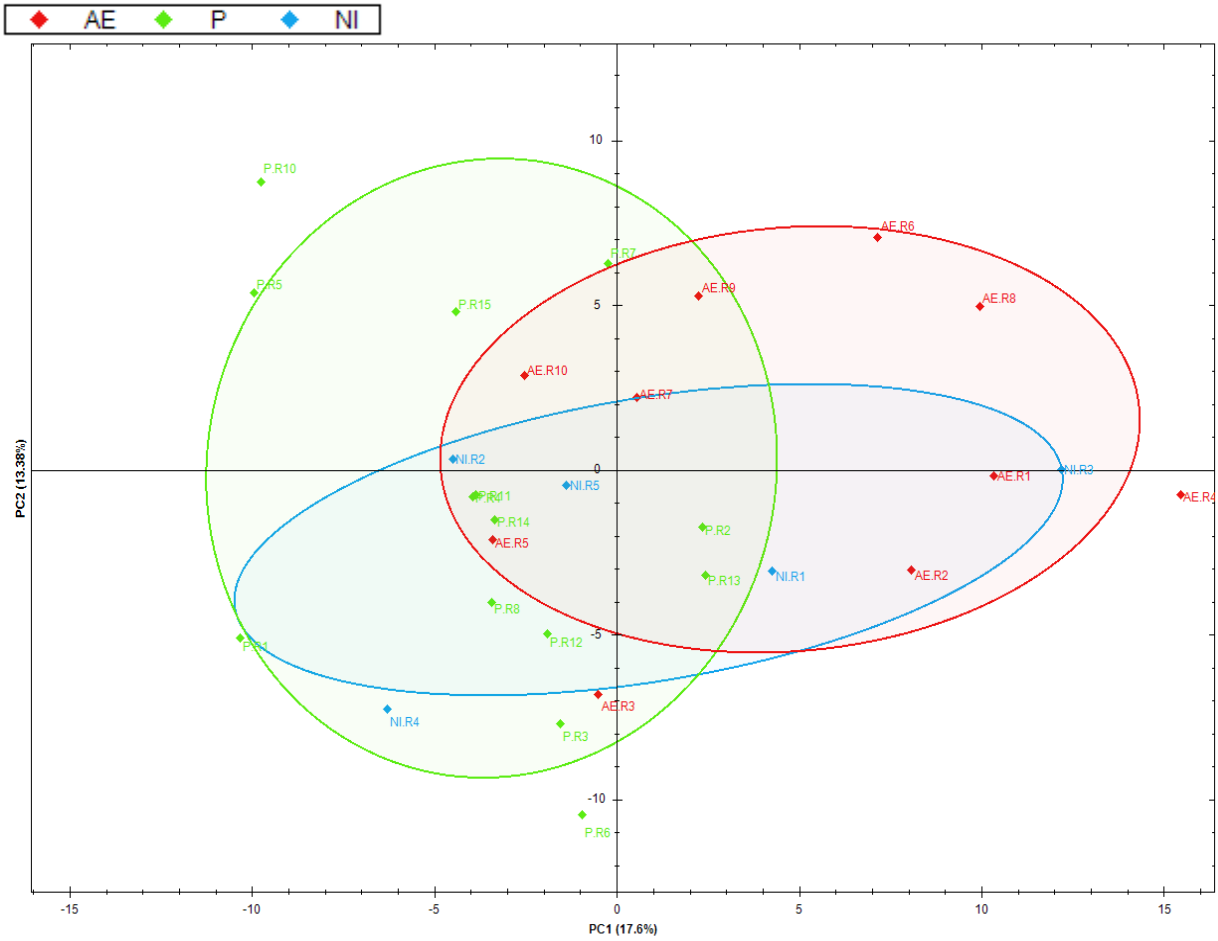
Principal component analysis (PCA) of candidate proteins in my dataset showed large areas of overlap in my AE, NI and psychiatric cohorts with all three cohorts spread widely across PC1 and PC2 (Figure 23a).The variance explained by PC1 was 17.6% and PC2 was 13.4%. This may be due to the relatively small sizes of each cohort, the issues with my NI patients discussed previously and the overall heterogeneity of each cohort.

Each cohort appears homogenously dispersed with a few outliers at the edges of the ellipses. There is some separation between the AE and P cohorts: AE sits mainly on the right (positive PC1), psychiatric sits mainly on the left (negative PC1), although AE

and psychiatric groups do overlap. The NI cohort is spread in between and overlapping both these cohorts.

When the PCA is repeated with the AE cohort subdivided into AbPAE and AbNAE (Figure 23b), the AbNAE cohort sits towards the right of PC1 with some spread along PC2; it overlaps the NI and psychiatric cohorts. The AbPAE cohort sits in the middle-right, overlapping with AbPAE and psychiatric but also with NI. The spread of the AbPAE cohort is smaller. in that it occupies a smaller area, but still has overlap with the NI, psychiatric and AbNAE cohorts.

a)



b)

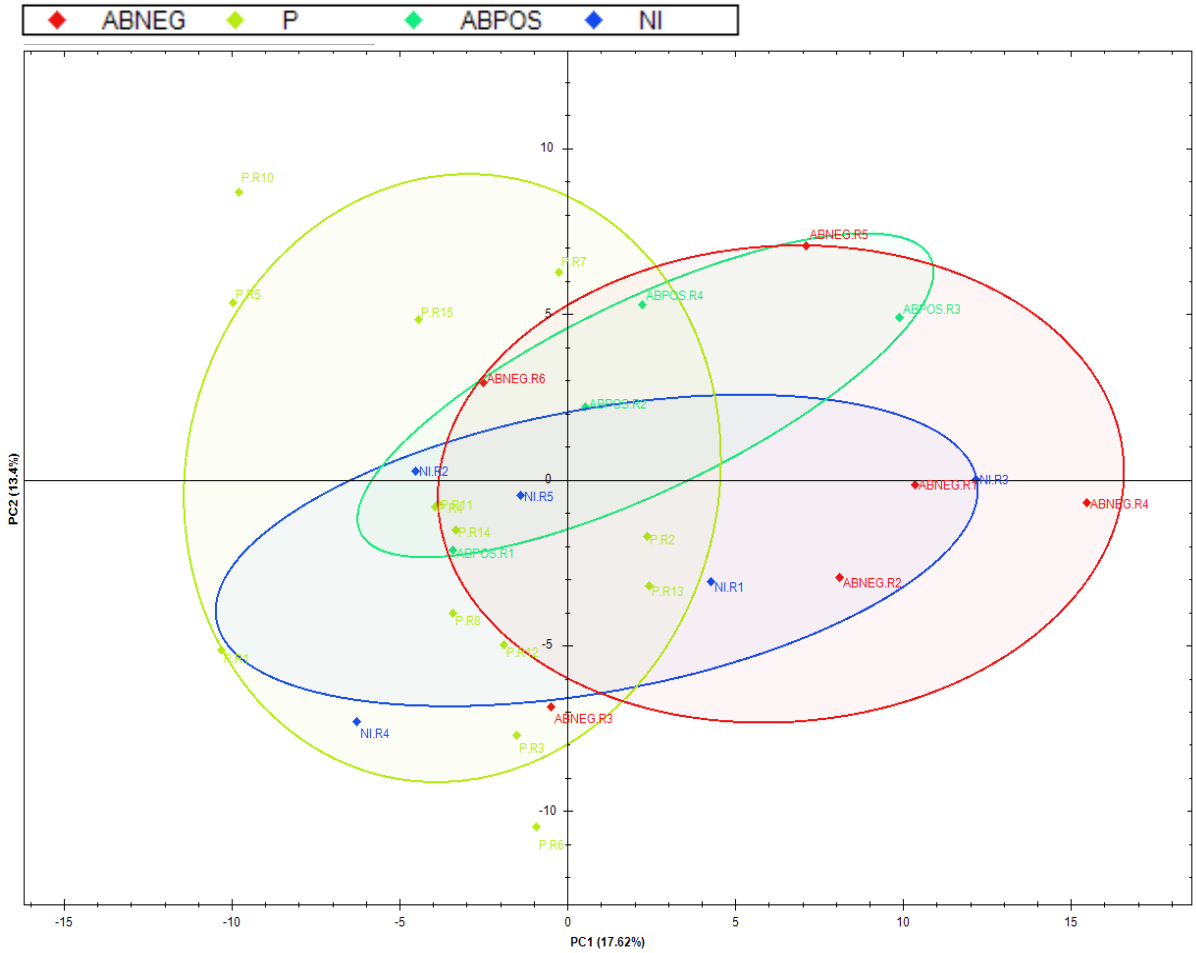


Figure 23: PCA of AE, psychiatric and NI cohorts (A) and all cohorts with AE subdivided into antibody positive and antibody negative (B).

6.3.4.1.2 Reactome Pathway Analysis: Overview

Reactome Pathway analysis of differentially abundant proteins in all comparisons returned pathways with relatively low entity ratios, indicating limited pathway coverage (i.e. the number of constituent proteins in that pathway identified). Despite this, in many of the comparisons there were very small P-values and false discovery rate (FDR). This indicates that, despite limited pathway coverage, the enrichments are statistically robust[12,668]. Detailed results will be discussed in the relevant subsections below.

6.3.5 Autoimmune Encephalitis vs Non-inflammatory Cohort Comparison

6.3.5.1 Candidate Proteins

Differential abundance analysis identified 214 differentially expressed candidate proteins when the AE group was compared to the NI cohort with a log 2 ratio of greater or equal to 0.58. Seventeen of these had an absolute log2 difference of greater or equal to 2 and were all increased in the AE cohort and are summarised in Tabe 27. Of these seventeen proteins, 4 were associated with haemoglobin and most likely contaminants. Five proteins were immune related processes: chitinase-3 like protein 2, prothymosin alpha, parathymosin, purine nucleoside phosphorylase and tumour necrosis factor receptor superfamily member 12A. Two were related to neural function: ubiquitin carboxyl-thermal hydrolase isozyme L1 and cell cycle exit and neuronal differentiation protein 1. The remaining six proteins increased in AE were associated with other process: band 3 anion transport protein, carbonic anhydrase 1 and 3, histones (2 proteins associated) and profilin.

Table 27 : Candidate proteins AE vs NI

Increased in	Absolute AVG Log2 Ratio	Qvalue	Genes	Protein Descriptions	Number of Unique Total Peptides
AE	3.735166	0.030796	SLC4A1	Band 3 anion transport protein	7
AE	2.183829	0.006922	CA1	Carbonic anhydrase 1	20
AE	2.28893	0.00791	CA3	Carbonic anhydrase 3	6
AE	2.556368	0.02619	CEND1	Cell cycle exit and neuronal differentiation protein 1	4
AE	2.282704	0.001059	CHI3L2	Chitinase-3-like protein 2	3
AE	2.471153	0.006273	HBA2	Haemoglobin subunit alpha	25
AE	2.521955	0.002301	HBB	Haemoglobin subunit beta	17

AE	2.442288	0.001929	HBD	Haemoglobin subunit delta	22
AE	2.028969	0.015901	HBG1	Haemoglobin subunit gamma-1	8
AE	2.659047	0.015907	H1-4	Histone H1.4	8
AE	2.399799	0.02449	H2BC12;H2BC11;H2BC17;H2BC3;H2BC12L;H2BC5;H2BC10;H2BC21;H2BC18;H2BC9;H2BC15;H2BC14;H2BC13	Histone	4
AE	2.740098	0.016526	PTMS	Parathymosin	5
AE	2.142625	0.045707	PFN1	Profilin-1	9
AE	2.62791	0.005886	PTMA	Prothymosin alpha	6
AE	2.294709	0.012723	PNP	Purine nucleoside phosphorylase	5
AE	2.247693	0.032429	TNFRSF12A	Tumor necrosis factor receptor superfamily member 12A	4
AE	2.411678	0.019723	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	5

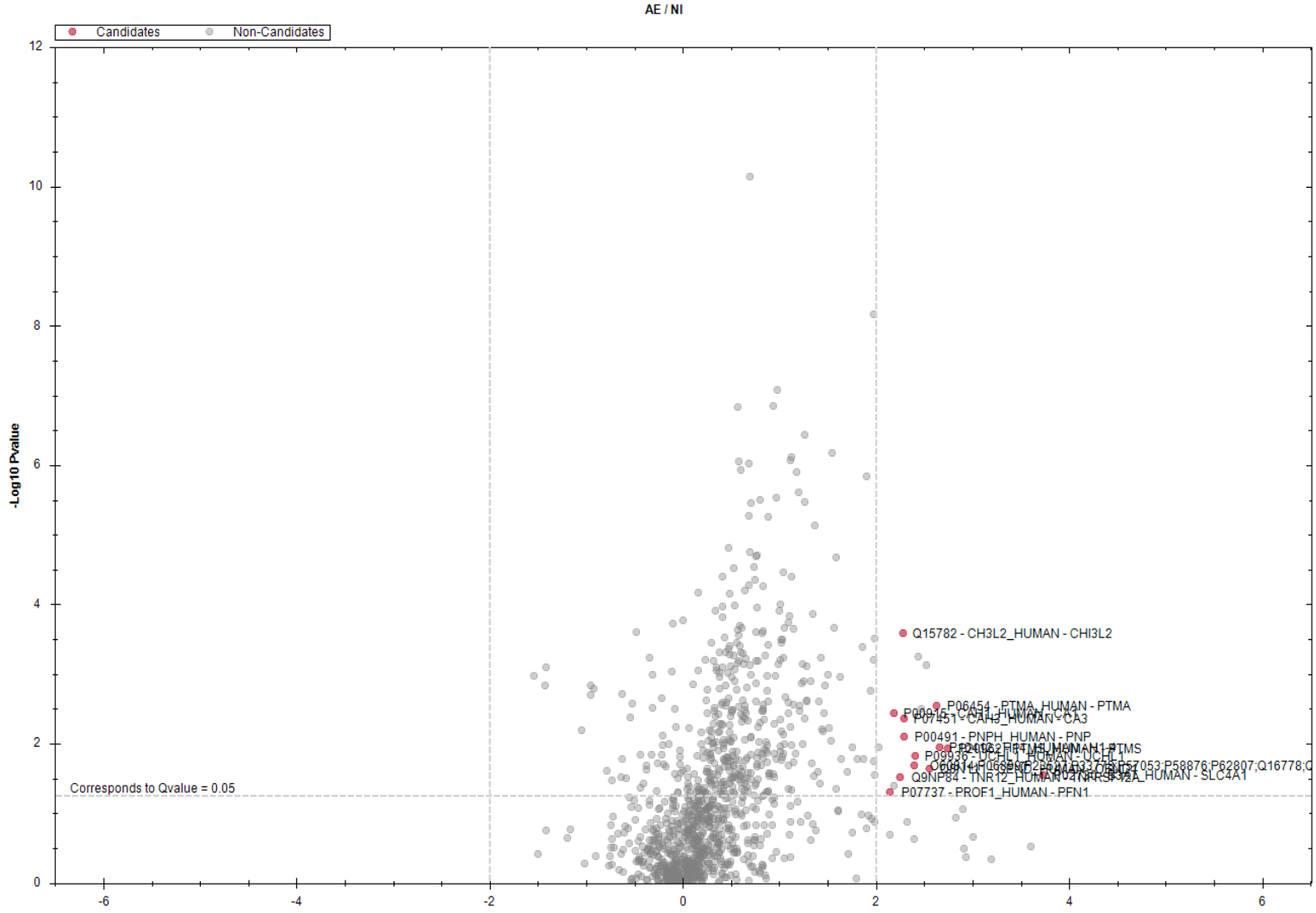


Figure 24: Volcano plot of AE vs NI

6.3.5.2 Reactome Pathways analysis

As expected, in an autoimmune disease, thirteen out of the top 24 pathways featured immune responses and 9 of these pathways were in the top ten. There was a mix of innate and adaptive mechanisms which is not unexpected in an autoimmune process. The most significant pathway was that of neutrophil degranulation[674]. The pathway involving interactions between lymphoid and non-lymphoid cells[675] also had a relatively higher entities ratio compared to other pathways. Other pathways were suggestive of antibody complex mediated autoimmunity. This included activation of complement via

the classical complement pathways[676] and Fc Gamma Receptor (FCGR) activation[677](Table 28).

Table 28: Top 24 most significant Pathways in AE vs NI comparison[668]

Pathway name	Entities found	Entities total	Entities ratio	Entities pValue	Entities FDR	Reactions found	Reactions total	Reactions ratio
Neutrophil degranulation	31	478	0.029763	1.11E-16	1.85E-13	10	10	6.43E-04
Initial triggering of complement	17	120	0.007472	1.12E-12	7.19E-10	20	21	0.00135
Platelet degranulation	20	142	0.008842	1.30E-12	7.19E-10	7	11	7.07E-04
Post-translational protein phosphorylation	14	109	0.006787	6.58E-12	2.74E-09	1	1	6.43E-05
Classical antibody-mediated complement activation	13	97	0.00604	2.25E-11	7.46E-09	2	2	1.29E-04
Response to elevated platelet cytosolic Ca2+	20	149	0.009278	2.18E-10	6.03E-08	8	14	9.00E-04
Creation of C4 and C2 activators	13	111	0.006912	4.35E-10	1.03E-07	7	8	5.14E-04
Regulation of Complement cascade	19	139	0.008655	3.44E-09	7.15E-07	41	42	0.0027
FCGR activation	12	103	0.006413	5.91E-09	1.09E-06	6	6	3.86E-04
Complement cascade	19	156	0.009714	1.28E-08	2.12E-06	68	72	0.004629
CD22 mediated BCR regulation	12	72	0.004483	2.17E-08	3.27E-06	3	4	2.57E-04
Scavenging of haem from plasma	12	106	0.0066	7.29E-08	1.01E-05	3	12	7.72E-04
Binding and Uptake of Ligands by Scavenger Receptors	15	168	0.010461	8.17E-08	1.05E-05	11	33	0.002122
FCGR3A-mediated IL10 synthesis	12	141	0.00878	3.60E-07	4.25E-05	11	20	0.001286

Endosomal/Vacuolar pathway	5	15	9.34E-04	4.60E-07	5.06E-05	4	4	2.57E-04
Role of phospholipids in phagocytosis	12	129	0.008032	5.30E-07	5.51E-05	7	12	7.72E-04
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	14	127	0.007908	7.71E-07	7.48E-05	10	14	9.00E-04
FCERI mediated Ca+2 mobilization	10	129	0.008032	1.89E-06	1.74E-04	5	11	7.07E-04
Role of LAT2/NTAL/LAB on calcium mobilization	10	107	0.006663	2.40E-06	2.09E-04	4	7	4.50E-04
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	21	249	0.015504	3.14E-06	2.61E-04	21	44	0.002829
FCGR3A-mediated phagocytosis	12	157	0.009776	3.82E-06	2.75E-04	14	27	0.001736
Leishmania phagocytosis	12	157	0.009776	3.82E-06	2.75E-04	14	27	0.001736
Parasite infection	12	157	0.009776	3.82E-06	2.75E-04	14	27	0.001736
Antigen activates B Cell Receptor (BCR) leading to generation of second messengers	12	103	0.006413	7.41E-06	5.11E-04	16	26	0.001672

*FDR = false discovery rate

6.3.6 Antibody Positive Autoimmune Encephalitis vs Antibody Negative Autoimmune Encephalitis Cohort Comparison

6.3.6.1 Candidate Proteins

A hundred and twenty-eight differentially expressed proteins were identified in the AbPAE vs AbNAE comparison with a log 2 ratio of greater or equal to 0.58. Nine of these proteins had an absolute log 2 ratio of greater than 2. Of these, there was only one protein increased in the AbPAE group. This was keratin, which has been well recognised as a common contaminant noted in mass spectrometry analysis including CSF[667]. Proteins increased in the AbNAE group included 5 proteins with potential immune association: leukocyte-assisted immunoglobulin like receptor 1, V-set and immunoglobulin domain-containing protein 4, lactotransferrin (also known as lactoferrin), proteoglycan 4 and HLA class I histocompatibility antigen. The other three proteins were methallothionein-2, a-kinase anchor protein 12 and histone H1.5. This is described in Table 29.

Table 29: Proteins increased in the AbNAE group

Over expressed in	Absolute AVG Log2 Ratio	Qvalue	Genes	Protein Descriptions	Number of Unique Total Peptides
AbNAE	2.953609	0.046755	MT2A	Metallothionein-2	4
AbNAE	2.486349	0.033041	AKAP 12	A-kinase anchor protein 12	2
AbNAE	2.41418	0.023662	LAIR1 ;LAIR 2	Leukocyte-associated immunoglobulin-like receptor 1;Leukocyte-associated	2

				immunoglobulin-like receptor 2	
AbNAE	2.358556	0.000676	VSIG4	V-set and immunoglobulin domain-containing protein 4	3
AbNAE	2.290122	0.005385	LTF	Lactotransferrin	3
AbNAE	2.142577	0.035826	H1-5	Histone H1.5	2
AbNAE	2.119654	0.02879	PRG4	Proteoglycan 4	7
AbNAE	2.097156	0.015676	HLA-B	HLA class I histocompatibility antigen, B alpha chain	9

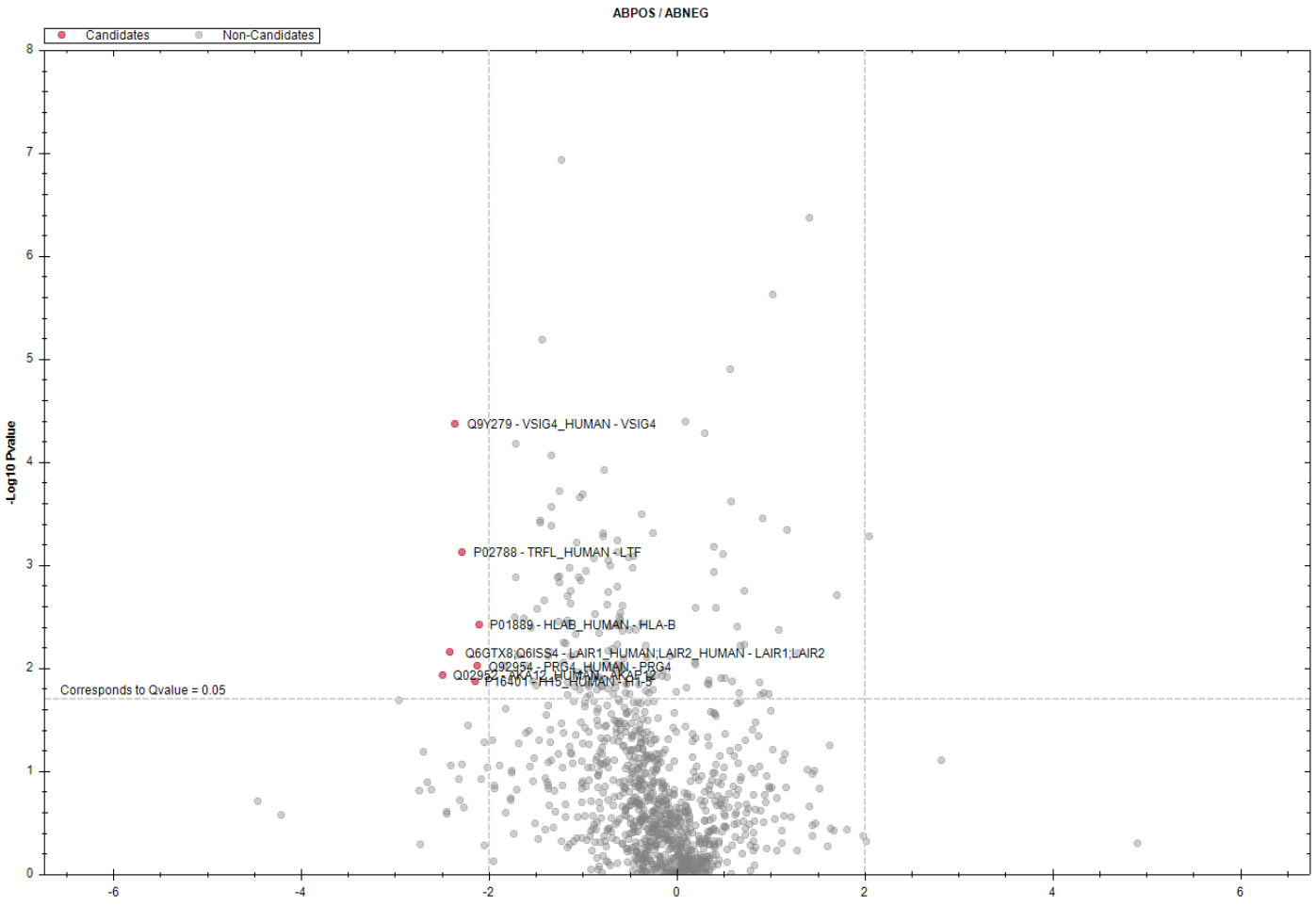


Figure 25: Volcano plots of candidate proteins when AbPAE is compared with AbNAE

6.3.6.2 Reactome Pathways Analysis

Reactome Pathway analysis of proteins increased in AbPAE versus AbNAE revealed only two pathways where the FDR was greater than 0.05 (Table 30). These were neutrophil degranulation[674] and degradation of the glycosaminoglycans chondroitin sulphate/derman sulphate (CS/DS)[678]. Other pathways identified exhibited higher false discovery rates (greater than 0.05). This included pathways involving other processes with glycosaminoglycan. However, when the higher FDRs are considered alongside their very low entity ratios, this indicates both limited pathway coverage and a reduced likelihood that these pathways represent genuine biological enrichment. Interestingly, the glycosaminoglycan pathways were also enriched in the psychiatric pathways compared to AE with more statistically significant FDRs (section 6.3.8.2).

Reactome Pathway analysis of proteins increased in the AbNAE cohort (Table 31) also included those involved in pro-inflammatory responses including neutrophil degranulation[674], innate immune system[679] and platelet degranulation[680]. There were many pathways associated with fibroblast growth factor receptors (FGFR)[681-685]. This is an interesting finding as not only is this pathway implicated in some neuroinflammatory processes, but there are also drug therapies that target this pathway which is implicated in many cancers and will be discussed further below. Interestingly, "regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding proteins (IGFBPs)" was also a highly relevant pathway[686]. These proteins are structured similarly to insulin and are important for growth and metabolism but also has important immune functions.

Whilst these pathways also had low entity ratios suggestive of limited pathway coverage their lower false discovery rates provide greater confidence that the observed enrichment is unlikely to be due to chance and may reflect biologically relevant processes

Table 30: Top 24 relevant pathways increased in AbPAE when compared to AbNAE

Pathway name	**#Entities found**	**#Entities total**	**Entities ratio**	**Entities pValue**	**Entities FDR**	**#Reactions found**	**#Reactions total**	**Reactions ratio**
Neutrophil degranulation	5	478	0.0396	1.01E-04	0.0308	6	10	6.59E-04
CS/DS degradation	2	20	0.00166	1.96E-04	0.0308	2	12	7.91E-04
MPS II - Hunter syndrome (HS-GAG degradation)	1	1	8.29E-05	0.00102	0.0796	1	1	6.59E-05
MPS II - Hunter syndrome (CS/DS degradation)	1	1	8.29E-05	0.00102	0.0796	1	1	6.59E-05
Chondroitin sulphate/dermatan sulphate metabolism	2	37	0.00307	0.0014	0.0867	2	26	0.001714
Defective SLC7A7 causes lysinuric protein intolerance (LPI)	1	2	1.66E-04	0.00204	0.106	1	1	6.59E-05

Metabolism of carbohydrates and carbohydrate derivatives	4	304	0.02519	0.00275	0.1121	8	237	0.015625
Regulation of CDH1 posttranslational processing and trafficking to plasma membrane	0	32	0.00265	0.00287	0.1121	10	14	9.23E-04
Defective HEXA causes GM2G1 (Hyaluronan metabolism)	1	1	8.29E-05	0.00407	0.1385	1	1	6.59E-05
Glycosphingolipid catabolism	1	39	0.00323	0.00611	0.1866	6	32	0.00211
Inhibition of nitric oxide production	0	4	3.31E-04	0.00712	0.1866	1	5	3.30E-04
NFE2L2 regulating antioxidant/detoxification enzymes	0	20	0.00166	0.00718	0.1866	1	28	0.001846
Sphingolipid metabolism	1	107	0.00886	0.00809	0.1943	8	78	0.005142
Basigin interactions	1	25	0.00207	0.00941	0.2071	10	10	6.59E-04
Lysosomal oligosaccharide catabolism	1	4	3.31E-04	0.01117	0.2121	1	4	2.64E-04
Mucopolysaccharidoses	1	11	9.11E-04	0.01117	0.2121	2	22	0.00145

Metal sequestration by antimicrobial proteins	1	6	4.97E-04	0.01419	0.2388	2	5	3.30E-04
Keratan sulphate degradation	1	15	0.00124	0.0152	0.2388	1	7	4.61E-04
Maturation of spike protein	0	6	4.97E-04	0.01673	0.2388	1	8	5.27E-04
Glycosphingolipid metabolism	1	58	0.00481	0.01706	0.2388	6	47	0.003099
Tryptophan catabolism	1	14	0.00116	0.01721	0.2409	1	13	8.57E-04
Hyaluronan degradation	1	16	0.00133	0.01921	0.2497	1	10	6.59E-04
IRAK4 deficiency (TLR2/4)	1	20	0.00166	0.02021	0.2623	1	1	6.59E-05

Table 31: Top 24 relevant pathways associated in AbNAE compared with AbPNAE

Pathway name	**#Entities found**	**#Entities total**	**Entities ratio**	**Entities pValue**	**Entities FDR**	**#Reactions found**	**#Reactions total**	**Reactions ratio**
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	9	124	0.010273405	2.41E-08	2.30E-05	12	14	9.23E-04
Post-translational protein phosphorylation	9	107	0.008864954	3.25E-08	2.30E-05	1	1	6.59E-05

Neutrophil degranulation	16	478	0.03960232	6.37E-08	3.01E-05	10	10	6.59E-04
FGFR2 mutant receptor activation	7	38	0.003148302	2.24E-07	7.92E-05	17	18	0.001187
Signaling by FGFR2 IIIa TM	6	23	0.001905551	1.02E-06	2.87E-04	2	2	1.32E-04
Developmental Cell Lineages	5	139	0.011516156	1.44E-06	3.39E-04	15	17	0.001121
Signaling by FGFR2 in disease	7	49	0.004059652	1.96E-06	3.65E-04	27	28	0.001846
HDL remodelling	2	11	9.11E-04	2.06E-06	3.65E-04	2	13	8.57E-04
Phospholipase C-mediated cascade; FGFR2	4	21	0.001739851	2.57E-06	4.04E-04	3	3	1.98E-04
Platelet degranulation	7	128	0.010604805	3.13E-06	4.41E-04	5	11	7.25E-04
Developmental Cell Lineages of the Exocrine Pancreas	3	84	0.006959403	5.23E-06	6.69E-04	8	10	6.59E-04
Chylomicron remodelling	2	10	8.29E-04	1.50E-05	0.0016748	3	3	1.98E-04
FGFR2 ligand binding and activation	4	23	0.001905551	1.54E-05	0.0016748	4	5	3.30E-04
FRS-mediated FGFR2 signaling	4	29	0.002402651	2.04E-05	0.0020632	9	9	5.93E-04
Response to elevated platelet cytosolic Ca2+	7	133	0.011019056	2.90E-05	0.002726	5	14	9.23E-04
SHC-mediated cascade:FGFR2	4	28	0.002319801	3.29E-05	0.0028989	4	4	2.64E-04
EPH-ephrin mediated repulsion of cells	6	51	0.004225352	5.01E-05	0.0040962	9	9	5.93E-04

Plasma lipoprotein remodelling	2	35	0.002899751	5.25E-05	0.0040962	7	31	0.002044
Signaling by FGFR in disease	7	73	0.006048053	6.62E-05	0.0048958	35	99	0.006527
Innate Immune System	22	1187	0.098342999	7.38E-05	0.0051634	142	709	0.046743
Signaling by FGFR2 amplification mutants	2	2	1.66E-04	7.73E-05	0.0051818	4	4	2.64E-04
Chylomicron assembly	2	10	8.29E-04	9.43E-05	0.0060368	4	5	3.30E-04
Binding and Uptake of Ligands by Scavenger Receptors	6	129	0.010687655	1.05E-04	0.0064348	13	33	0.002176
Extracellular matrix organization	10	321	0.026594863	1.13E-04	0.0065837	118	330	0.021756

6.3.7 Psychiatric vs Non-inflammatory Cohort Comparison

6.3.7.1 Candidate Proteins

When the psychiatric cohort was compared to NI controls, 215 candidate proteins were identified. Of these, eight had absolute log2 ratio greater than or equal to two difference. There were four which were all likely contaminants[667]: two haemoglobin associated proteins, 1 keratin associated protein, and one saliva associated protein. The four remaining proteins were all increased in the psychiatric cohort (Table 32). Two of these were associated with immune function: fibroleukin and acid sphingomyelinase-like phosphodiesterase 3a. One was associated with neural function: Ephrin-A5; the other was corticosteroid-binding globulin.

Table 32: Proteins increased in the psychiatric cohort group

Increased in	AVG Log2 Ratio	Qvalue	Genes	Protein Descriptions	Number of Unique Total Peptides
psychiatric	2.163309	6.20E-11	FGL2	Fibroleukin	13
psychiatric	2.219985	0.000731	SMPDL3A	Acid sphingomyelinase-like phosphodiesterase 3a	4
psychiatric	2.227034	0.000795	SERPINA6	Corticosteroid-binding globulin	17
psychiatric	2.701773	0.031064	EFNA5	Ephrin-A5	6

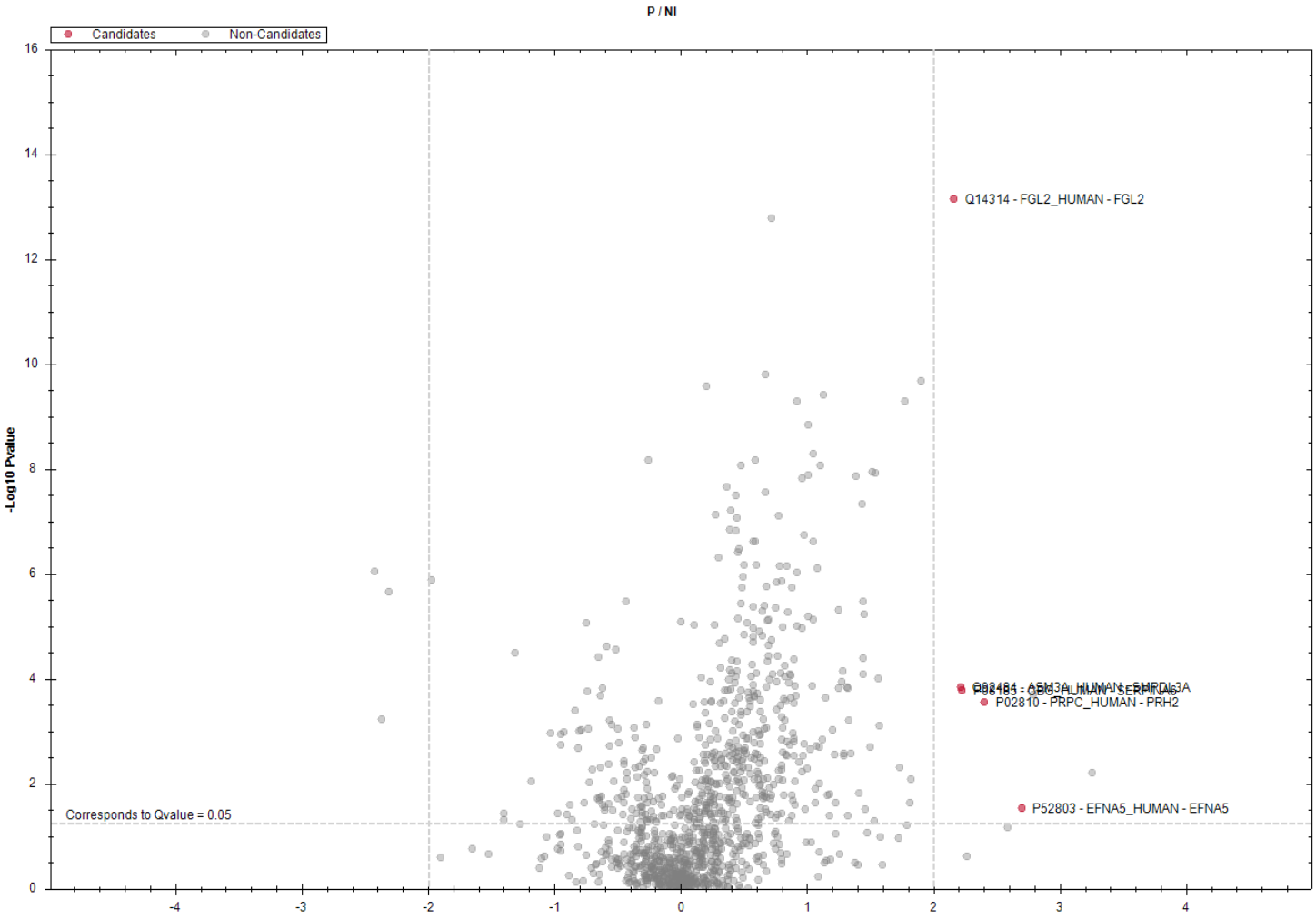


Figure 26: Volcano plots of candidate proteins when P is compared with NI

6.3.7.2 Reactome Pathways Analysis

Reactome Pathways analysis in the psychiatric cohort compared to the NI cohort (Table 33) mapped very similarly to the pathways the increased proteins in AE when compared to NI mapped to albeit in slightly differing order of significance. The top 24 pathways were also dominated by those related to innate and adaptive immune function including neutrophil degranulation[674], platelet degranulation[680], phagocytosis[687] and immune responses to parasites[688] as well as pathways involving complement[676] and FCGR activation[677] that are associated with immune complexes.

Table 33 : Top 24 relevant pathways proteins increased in psychiatric vs NI comparison[668]

Pathway name	#Entities found	#Entities total	Entities ratio	Entities pValue	Entities FDR	#Reactions found	#Reactions total	Reactions ratio
Neutrophil degranulation	37	478	0.0297634	1.11E-16	1.87E-13	10	10	6.43E-04
Classical antibody-mediated complement activation	15	97	0.0060399	1.52E-13	1.28E-10	2	2	1.29E-04
Scavenging of haem from plasma	17	106	0.0066002	1.21E-12	6.79E-10	7	12	7.72E-04
Creation of C4 and C2 activators	15	111	0.0069116	4.77E-12	2.00E-09	7	8	5.14E-04

FCGR activation	15	103	0.0064134	5.96E-12	2.00E-09	6	6	3.86E-04
Post-translational protein phosphorylation	14	109	0.006787	1.14E-11	3.19E-09	1	1	6.43E-05
Initial triggering of complement	16	120	0.007472	2.35E-11	5.65E-09	18	21	0.001350135
Binding and Uptake of Ligands by Scavenger Receptors	19	168	0.0104608	6.11E-11	1.28E-08	14	33	0.002121641
FCGR3A-mediated IL10 synthesis	15	141	0.0087796	1.12E-09	2.08E-07	11	20	0.001285843
Platelet degranulation	17	142	0.0088418	1.41E-09	2.37E-07	4	11	7.07E-04
Role of phospholipids in phagocytosis	15	129	0.0080324	1.84E-09	2.79E-07	7	12	7.72E-04
Parasite infection	15	157	0.0097758	2.33E-08	2.79E-06	14	27	0.001735888
Leishmania phagocytosis [687]	15	157	0.0097758	2.33E-08	2.79E-06	14	27	0.001735888
FCGR3A-mediated phagocytosis	15	157	0.0097758	2.33E-08	2.79E-06	14	27	0.001735888

CD22 mediated BCR regulation	12	72	0.0044832	3.40E-08	3.81E-06	3	4	2.57E-04
FCERI mediated Ca+2 mobilisation	12	129	0.0080324	3.95E-08	4.15E-06	5	11	7.07E-04
Role of LAT2/NTAL/LAB on calcium mobilisation	12	107	0.0066625	5.30E-08	5.19E-06	4	7	4.50E-04
FCERI mediated NF-kB activation	15	162	0.0100872	7.41E-08	6.89E-06	3	19	0.001221551
Response to elevated platelet cytosolic Ca2+	17	149	0.0092777	9.50E-08	8.36E-06	5	14	9.00E-04
Complement cascade	18	156	0.0097136	1.29E-07	1.08E-05	50	72	0.004629034
Regulation of Complement cascade	17	139	0.008655	2.03E-07	1.62E-05	29	42	0.00270027
Regulation of actin dynamics for phagocytic cup formation	16	158	0.0098381	4.49E-07	3.23E-05	14	24	0.001543011

Anti-inflammatory response favouring Leishmania parasite infection	15	192	0.0119552	4.62E-07	3.23E-05	12	40	0.002571686
Leishmania parasite growth and survival	15	192	0.0119552	4.62E-07	3.23E-05	12	40	0.002571686

6.3.8 Autoimmune Encephalitis vs Psychiatric Cohort Comparison

6.3.8.1 Candidate Proteins

In comparing the AE cohort versus the psychiatric cohort, there were 185 candidate proteins and 26 had a difference of a log 2 ratio greater or equal to two. Of these, only two proteins over expressed in the psychiatric cohort but both were keratin associated proteins and likely to be contaminants.

Twenty-four proteins were increased in AE with a difference of log 2 ratio of greater than or equal to 2. Of these, six were potential contaminants: 4 were related to haemoglobin. Myoglobin was also increased AE. Whilst this isn't a well reported contaminant of CSF, it is theoretically possible to introduce it into the sample during the lumbar puncture procedure. It may also reflect increased serum myoglobin levels in the setting of rhabdomyolysis such as after a prolonged seizure activity. Similarly, beta-enolase is a potential contaminant, possibly related to myoglobin contamination as it is a muscle-specific enzyme with critical roles in energy metabolism[689]. Whilst it has not been described as a common CSF contaminant in mass spectrometry, it has also never

been described as detectable in CSF. In CSF, the proteomically different neurone-specific enolase is present and a marker for neuronal injury.

Of the other eighteen increased proteins, six were related to immune function: Immunoglobulin lambda variable, prothymosin alpha, CD27 antigen, macrophage inhibitory factor, complement C2 and complement C4-B. Five were associated with neural processes or neurones: microtubule-associated protein 2, ubiquitin carboxyl-terminal hydrolase isozyme L1, fatty acid-binding protein, heart and band 4.1-like protein 3. Whilst fatty-acid binding protein, heart or fatty acid binding protein 3 (FABP3) gene is expressed in high levels in the heart, there is also significant expression in neural tissues particularly in the CNS[690]. The remaining seven were associated with other processes: small ubiquitin-related modifier, histone, carbonic anhydrase 1, profilin 1, acidic leucine-rich nuclear phosphoprotein 32 family member A, purine nucleoside phosphorylase and phosphoglycerate mutase 1. Of these, phosphoglycerate mutase 1 also has roles in regulation of immune cell function and is also expressed in neural tissues.

Table 34: Proteins increased in the Psychiatric vs AE comparison

Increased in	Absolute AVG Log2 Ratio	Qvalue	Genes	Protein Descriptions	Number of Unique Total Peptides
AE	4.942214	2.50E-05	HBB	haemoglobin subunit beta	17
AE	4.7845	5.13E-05	HBA2	hemoglobins subunit alpha	25
AE	4.413797	2.50E-05	HBD	haemoglobin subunit delta	22
AE	3.58589	0.006365	SUMO1P1;SUMO1	Small ubiquitin-related modifier	2

				5;Small ubiquitin-related modifier 1	
AE	3.38119	0.01913	H1-4	Histone H1.4	8
AE	3.363309	0.0036	CA1	Carbonic anhydrase 1	20
AE	3.08863	0.000355	PFN1	Profilin-1	9
AE	3.072336	0.038097	ENO3	Beta-enolase	8
AE	2.916328	0.027617	SPTB	Spectrin beta chain, erythrocytic	7
AE	2.760253	0.008832	HBG1	haemoglobin subunit gamma-1	8
AE	2.723646	0.001649	MAP2	Microtubule-associated protein 2	15
AE	2.653221	0.031531	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	5
AE	2.498585	0.047593	IGLV3-19	Immunoglobulin lambda variable 3-19	4
AE	2.363985	0.018006	PTMA	Prothymosin alpha	6
AE	2.344196	0.019905	FABP3	Fatty acid-binding protein, heart	10
AE	2.308696	0.014125	ANP32A	Acidic leucine-rich nuclear phosphoprotein 32 family member A	5
AE	2.243658	0.012682	CD27	CD27 antigen	3
AE	2.183063	0.000622	PNP	Purine nucleoside phosphorylase	5
AE	2.180152	0.023423	EPB41L3	Band 4.1-like protein 3	3
AE	2.179096	0.005758	MIF	Macrophage migration inhibitory factor	2
AE	2.101527	0.005732	C2	Complement C2	29
AE	2.093667	0.025019	MB	Myoglobin	13
AE	2.040408	0.019905	PGAM1	Phosphoglycerate mutase 1	17

AE	2.033633	0.001373	C4B_2	Complement C4-B	2
psychiatric	2.015243	0.000492	KRT14	Keratin, type I cytoskeletal 14	61
psychiatric	2.180922	0.000452	KRT10	Keratin, type I cytoskeletal 10	58

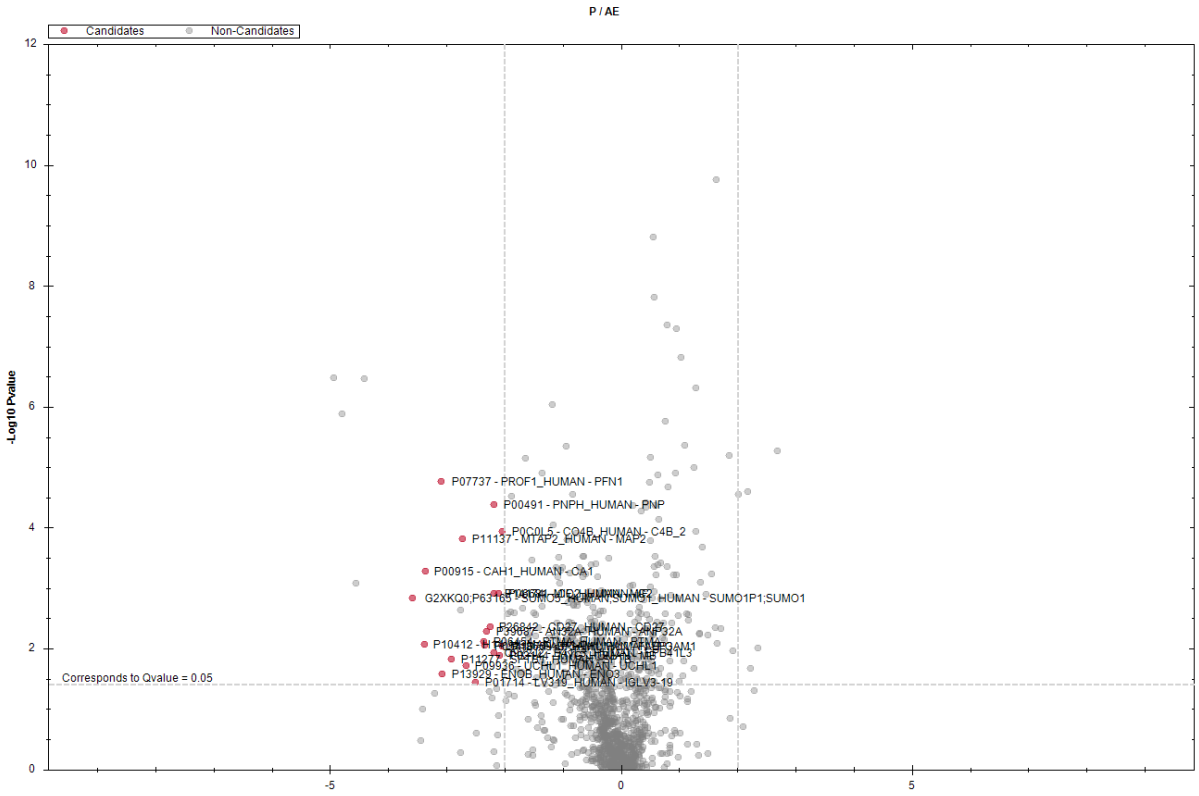


Figure 27: Volcano plot of Candidate proteins in the psychiatric vs AE comparison

6.3.8.2 Reactome Pathways Analysis

When Reactome Pathways analysis was conducted using candidate proteins of greater or equal to a log 2 ratio of 0.58 difference (see section 6.2.3.2), two of the 25 most significant pathways mapped from proteins increased in the psychiatric cohort (Table 35) compared to AE mapped to innate immune pathways: neutrophil degranulation[674], and innate immune system[679]. Glycosphingolipid metabolism and catabolism,

was also represented[691,692]. Interestingly there were similarities in pathways here with some of the pathways seen in the AbPAE when compared with AbNAE. This includes CS/DS degradation[678], as well as glycosaminoglycan related pathways[691,693,694] which had more statistically significant FDRs here than that those listed in the AbPAE vs AbNAE comparison.

The top eight pathways mapped by proteins overrepresented in the AE cohort versus psychiatric cohort had very low FDRs despite low entities ratios. These also involved innate and adaptive pathways including neutrophil degranulation[674] and immunoregulatory interactions between a lymphoid and non-lymphoid cells[675]. There were also pathways associated with post-translational phosphorylation[695], DNA checkpoint pathways (Chk1/Chk2)[696] and metabolic processes such as regulation of insulin-like growth factor transport and uptake[686]. Outside of these top eight pathways, FDR increased above 0.05, and when considered alongside the lower entity ratios, suggest that these pathways may be less biologically relevant (Table 36).

Table 35: Pathways increased in psychiatric cohort when compared to AE[668]

Pathway name	#Entities found	#Entities total	Entities ratio	Entities pValue	Entities FDR	#Reactions found	#Reactions total	Reactions ratio
Neutrophil degranulation	15	478	0.039602	7.935208046205844E-12	5.9752116587930004E-9	5	10	6.592827004219409E-4
CS/DS degradation	4	20	0.001657	4.453856061736871 7E-7	1.6746498792130637E-4	6	12	7.911392405063291E-4
Glycosphingolipid catabolism	3	39	0.003231	1.299456156123035 6E-6	3.2616349518688819E-4	9	32	0.0021097
Glycosphingolipid metabolism	3	58	0.004805	2.044114297050647 6E-6	3.8429348784552175E-4	10	47	0.00309863
The activation of arylsulfatases	1	13	0.001077	3.207591781961838 3E-6	4.8113876729427574E-4	2	2	1.3185654008438817E-4
Mucopolysaccharidoses	3	11	9.113504556752278E-4	5.370251594638553E-6	6.7128144932981 91E-4	5	22	0.00145042
Differentiation of Keratinocytes in Interfollicular Epidermis in Mammalian Skin	4	39	0.003231	7.508880394135886E-6	8.0345020217225398E-4	3	4	2.6371308016877635E-4
Keratan sulphate degradation	3	15	0.001243	1.350278019685014E-5	0.001269	3	7	4.6149789029535865E-4

Chondroitin sulphate/dermatan sulphate metabolism	4	37	0.003065	2.195513643976632E-5	0.001822	20	26	0.00171414
Defective HEXA causes GM2G1 (Hyaluronan metabolism)	1	1	8.285004142502072E-5	6.861254952605922E-5	0.005146	1	1	6.592827004219409E-5
Breakdown of the nuclear lamina	1	3	2.4855012427506213E-4	7.678004793387 76E-5	0.005221	2	3	1.9778481012658228E-4
Developmental Cell Lineages of the Integumentary System	4	59	0.004888	9.064119628887646E-5	0.005617	3	7	4.6149789029535865E-4
Glycosaminoglycan metabolism	6	136	0.011267605633802818	9.854794629071684E-5	0.005617	34	96	0.00632911
Formation of the cornified envelope	4	129	0.010687655343827672	1.4376473834243342E-4	0.00762	20	27	0.00178006
Innate Immune System	18	1187	0.098343	1.701406299535746E-4	0.008507	83	709	0.04674314
Sphingolipid metabolism	3	107	0.008865	2.1503911086828698E-4	0.009927	11	78	0.00514241
Keratan sulphate/keratin metabolism	3	37	0.003065	2.2561729450087942E-4	0.009927	3	16	0.00105485

Diseases associated with glycosaminoglycan metabolism	3	41	0.003397	3.206020709795032E-4	0.013144684910159632	9	23	0.00151635
Keratinisation	4	217	0.017978458989229496	6.216287353899563E-4	0.024243520680208297	21	34	0.00224156
Diseases of carbohydrate metabolism	3	34	0.002817	7.489764433774582E-4	0.026628939527119644	5	44	0.00290084
Gamma carboxylation, hypusinylation, hydroxylation, and arylsulfatase activation	3	61	0.005054	7.608268436319898E-4	0.026628939527119644	9	50	0.00329641
SEMA3A-Plexin repulsion signaling by inhibiting Integrin adhesion	0	14	0.00116	8.242845183236147E-4	0.028026	5	8	5.274261603375527E-4
Depolymerisation of the Nuclear Lamina	2	16	0.001326	0.001073	0.034335	2	6	3.9556962025316455E-4

Diseases of metabolism	6	265	0.021955260977630488	0.001294	0.040113	14	201	0.013251582278481012
Hyaluronan degradation	2	16	0.001326	0.001504	0.045133	2	10	6.592827004219409E-4

Table 36: Most relevant pathways in AE when compared to psychiatric[668]

Pathway name	#Entities found	#Entities total	Entities ratio	Entities pValue	Entities FDR	#Reactions found	#Reactions total	Reactions ratio
Neutrophil degranulation	25	478	0.02976	1.11E-16	1.66E-13	10	10	6.43E-04
Endosomal/Vacuolar pathway	4	15	9.34E-04	2.19E-06	0.00159	3	4	2.57E-04
Activation of BAD and translocation to mitochondria	5	19	0.00118	3.49E-06	0.00159	5	5	3.21E-04
Post-translational protein phosphorylation	7	109	0.00679	4.25E-06	0.00159	1	1	6.43E-05
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	14	249	0.0155	4.34E-05	0.01106	20	44	0.002829
Scavenging of heme from plasma	7	106	0.0066	4.69E-05	0.01106	8	12	7.72E-04
Chk1/Chk2(Cds1) mediated inactivation of Cyclin B:Cdk1 complex	5	17	0.00106	5.19E-05	0.01106	5	5	3.21E-04

Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	8	127	0.00791	2.52E-04	0.04706	11	14	9.00E-04
Activation of BH3-only proteins	5	36	0.00224	3.94E-04	0.06537	5	19	0.001222
Diseases of glycosylation	7	203	0.01264	5.02E-04	0.07477	21	72	0.004629
G2/M DNA damage checkpoint	5	82	0.00511	0.00107	0.13326	7	12	7.72E-04
Binding and Uptake of Ligands by Scavenger Receptors	7	168	0.01046	0.00107	0.13326	8	33	0.002122
SARS-CoV-2 targets host intracellular signalling and regulatory pathways	5	19	0.00118	0.0021	0.24182	2	5	3.21E-04
Regulation of localization of FOXO transcription factors	3	14	8.72E-04	0.0026	0.27516	3	5	3.21E-04
Activation of C3 and C5	3	7	4.36E-04	0.00292	0.27762	3	4	2.57E-04

Initial triggering of complement	5	120	0.00747	0.00299	0.27762	8	21	0.00135
Diseases associated with glycosaminoglycan metabolism	3	53	0.0033	0.00449	0.38014	18	23	0.001479
Reversible hydration of carbon dioxide	2	17	0.00106	0.00458	0.38014	2	8	5.14E-04
Regulation of Complement cascade	7	139	0.00866	0.00531	0.41395	28	42	0.0027
Defective B3GALT6 causes EDSP2 and SEMDJL1	2	21	0.00131	0.00688	0.46807	1	1	6.43E-05
Defective B4GALT7 causes EDS, progeroid type	2	21	0.00131	0.00688	0.46807	1	1	6.43E-05
Erythrocytes take up oxygen and release carbon dioxide	2	16	9.96E-04	0.00688	0.46807	1	6	3.86E-04
Defective B3GAT3 causes JDSSDHD	2	22	0.00137	0.00753	0.48918	1	1	6.43E-05
Complement cascade	7	156	0.00971	0.00819	0.49695	43	72	0.004629
G2/M Checkpoints	5	142	0.00884	0.00842	0.49695	9	24	0.001543

6.3.9 Interactome Analysis

I analysed candidate proteins in the psychiatric versus AE comparisons using STRING[669,671] of known and predicted protein-protein interactions (based on currently available evidence) to determine if there were interactions amongst these candidate proteins. Each node represents a protein, and nodes are colour coded for their cluster. Edges between the nodes represent protein-protein associations with each line representing a separate association between proteins.

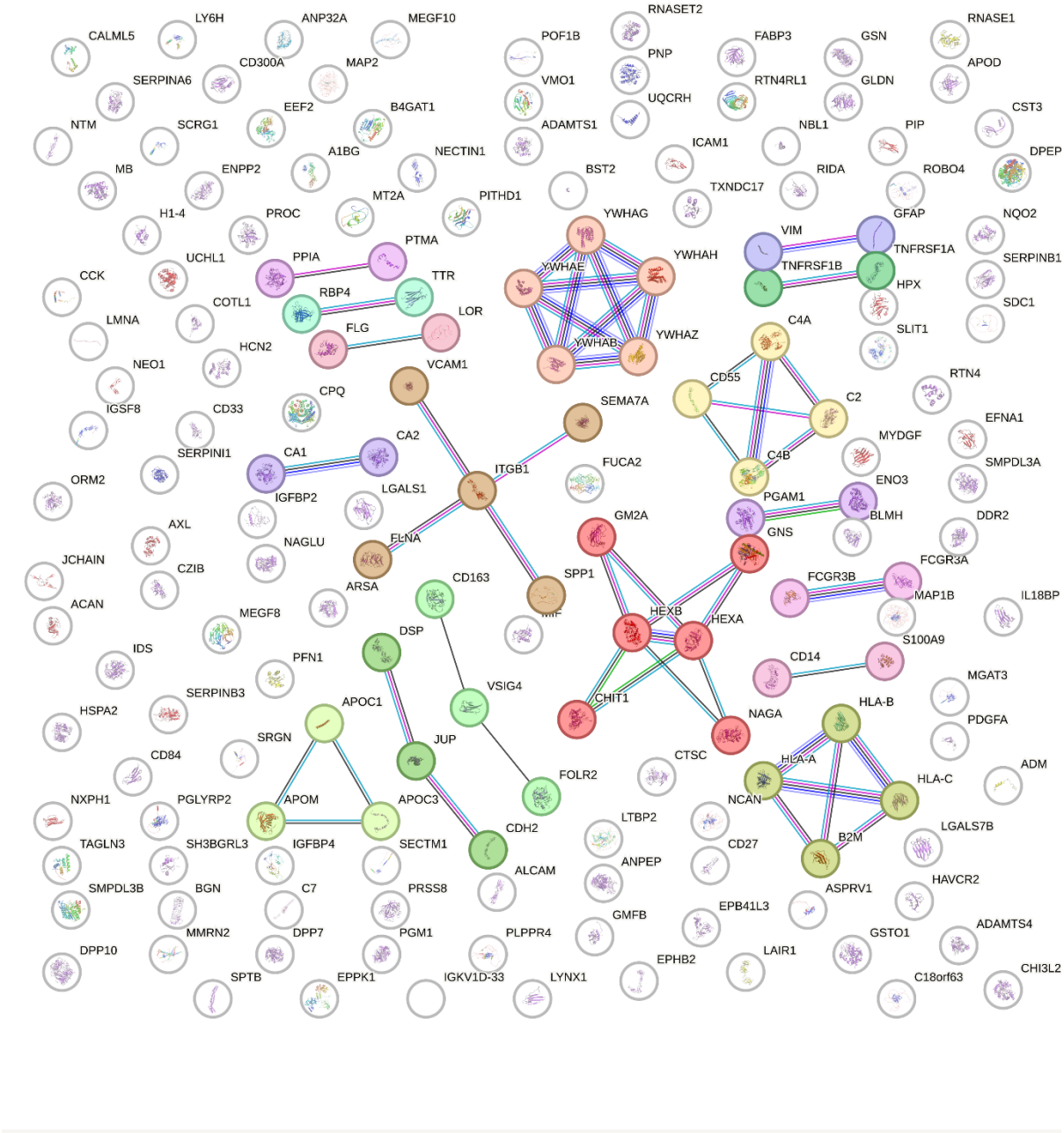
6.3.9.1 Psychiatric vs AE

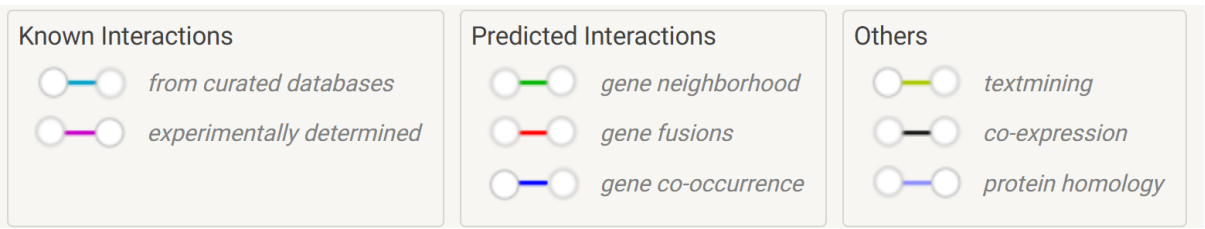
There were 171 protein nodes present in the analysis with 51 edges or interactions identified (Figure 28). This was significantly more than the expected number of edges (7) and the PPI enrichment p value was < 1.0e-16. The average node degree was 0.596 and average local node clustering coefficient was 0.274. Hence, as demonstrated by figure 28, whilst most of the proteins were sparsely interconnected, there were pockets of proteins with high connectivity between them. These can be seen in the protein clusters.

The top 3 protein clusters identified were glycosphingolipid catabolism, SARS COV-2 targets host intracellular signalling and regulatory pathways and cell-extracellular matrix interactions.

The three top STRING Gene Ontology enrichments in this protein network for biological processes were immune or neural related: regulation of immune effector

process, negative immune response and negative regulation of neurone projection of development (Figure 29)





color	cluster Id	gene count	description
	Cluster 1	6	+ Glycosphingolipid catabolism
	Cluster 2	5	+ SARS-CoV-2 targets host intracellular signalling and regulatory pathways
	Cluster 3	5	+ Cell-extracellular matrix interactions
	Cluster 4	4	+ Complement activation, classical pathway
	Cluster 5	4	+ Antigen processing and presentation of endogenous peptide antigen via MHC cl...
	Cluster 6	3	High-density lipoprotein particle remodeling
	Cluster 7	3	Fascia adherens
	Cluster 8	3	CD163, FOLR2, VSIG4
	Cluster 9	2	+ Negative regulation of extracellular matrix constituent secretion
	Cluster 10	2	Retinoid cycle disease events
	Cluster 11	2	+ Bergmann glial cell differentiation
	Cluster 12	2	+ Nitrogen metabolism
	Cluster 13	2	+ Glycolysis
	Cluster 14	2	PPIA, PTMA
	Cluster 15	2	+ IgG-binding protein
	Cluster 16	2	MyD88 deficiency (TLR2/4)
	Cluster 17	2	Ichthyosis vulgaris, and Epidermolytic acanthoma

Figure 28: STRING Interactome analysis of AE vs Psychiatric cohort comparison

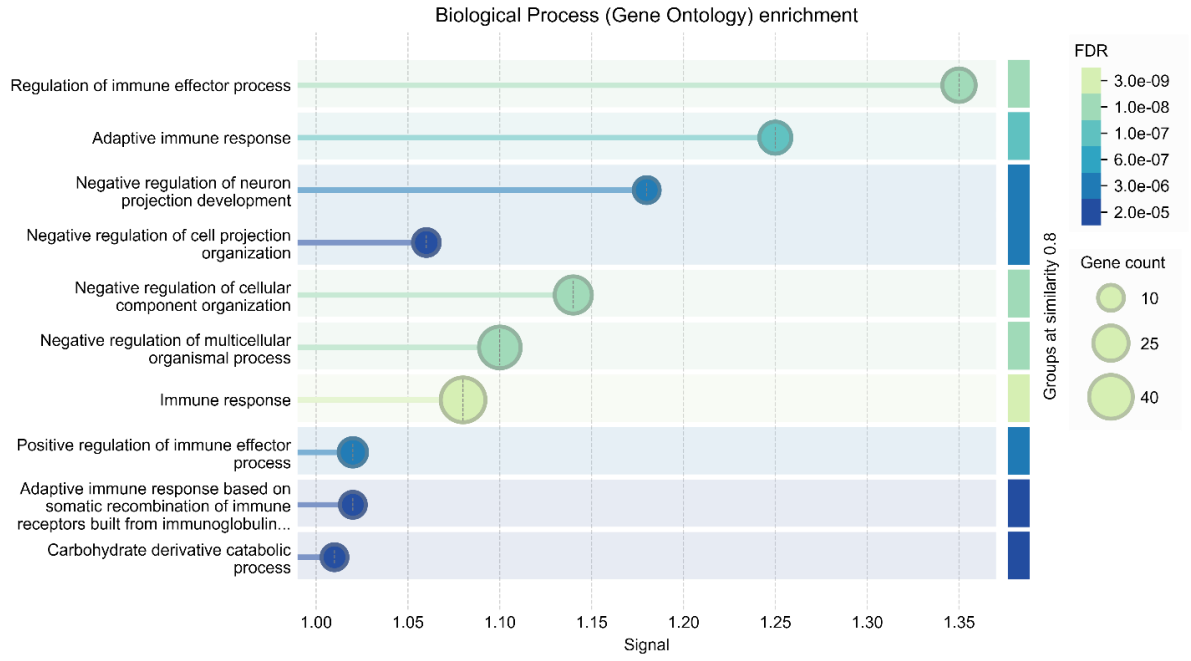


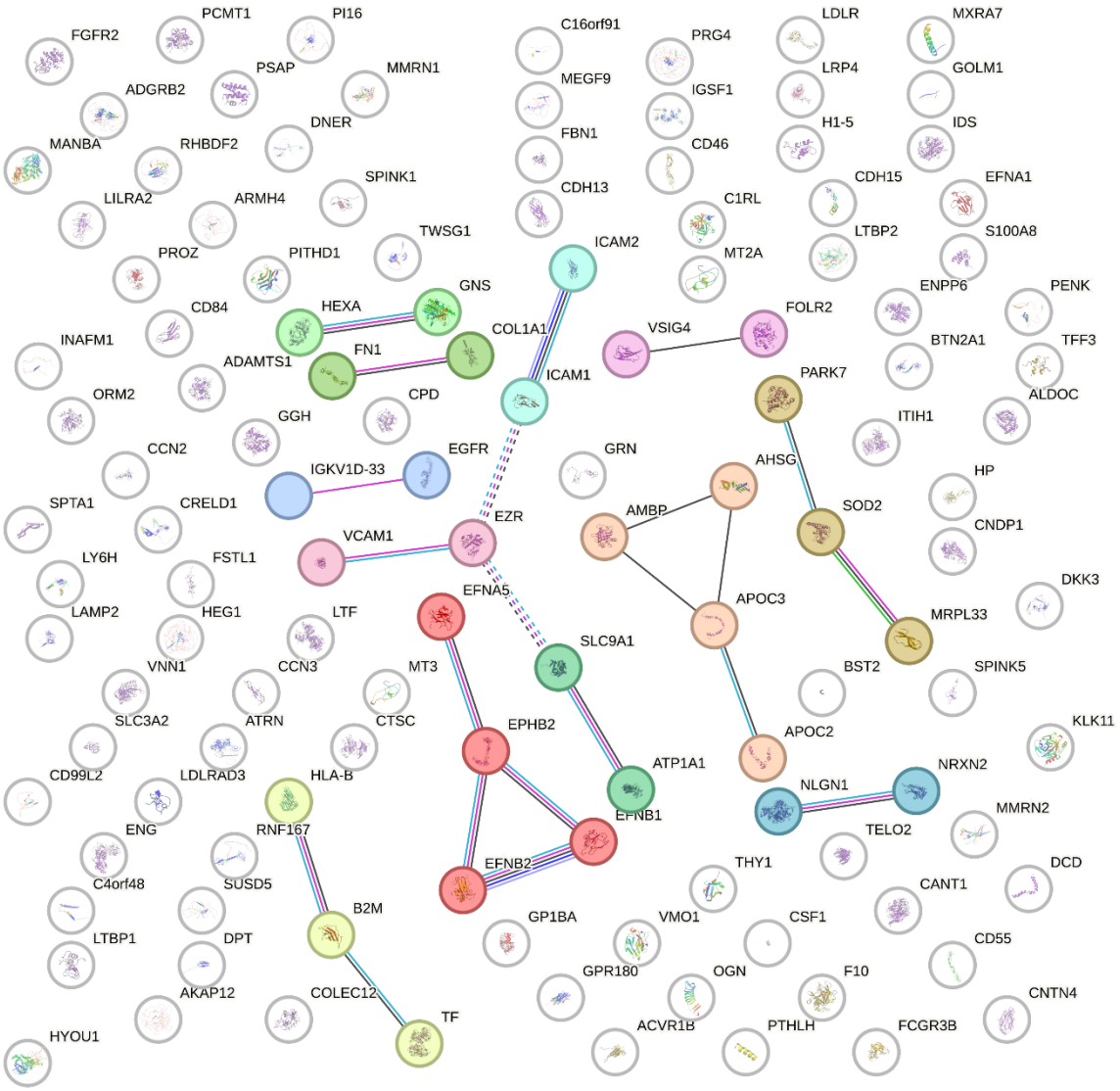
Figure 29: AE vs psychiatric comparison: STRING functional enrichment for biological processes (Gene Otology)

6.3.9. 2 AbPAE vs AbNAE

There were 124 protein nodes present in the analysis with 22 edges/interactions identified. This was significantly more interactions than expected (Figure 30) with a PPI enrichment p-value of 1.51e-12. The average node degree was low at 0.355 and average local clustering coefficient was 0.191. This indicates a sparsity of interconnectedness throughout the total number of proteins but there were pockets of nodes with high interconnectivity.

The most prominent clusters were the Ephrin signalling pathway, negative regulation of very low-density lipoprotein clearance, positive regulation of reactive oxygen species biosynthesis and MHC I.

The three top STRING Gene Ontology enrichments in this protein network for biological processes were cell adhesion (related to proteins including integrin, vitronectin, protein CBR-IGM3 and activated leukocyte adhesion molecule), regulation of immune system process and negative regulation of multicellular organismal process (Figure 31).



Known Interactions

from curated databases

experimentally determined

Predicted Interactions

gene neighborhood

gene fusions

gene co-occurrence

Others

textmining

co-expression

protein homology

color	cluster Id	gene count	description
	Cluster 1	4	+ Ephrin receptor signaling pathway
	Cluster 2	4	+ Negative regulation of very-low-density lipoprotein particle clearance
	Cluster 3	3	Positive regulation of reactive oxygen species biosynthesis
	Cluster 4	3	+ MHC I
	Cluster 5	2	Syndecan interactions
	Cluster 6	2	Glycosaminoglycan degradation
	Cluster 7	2	+ Sodium ion export across plasma membrane
	Cluster 8	2	ICAM1, ICAM2
	Cluster 9	2	+ Postsynaptic density protein 95 clustering
	Cluster 10	2	EGFR, IGKV1D-33
	Cluster 11	2	FOLR2, VSIG4
	Cluster 12	2	Membrane to membrane docking

Figure 30: STRING Interactome analysis of AbPAE vs AbNAE cohort comparison

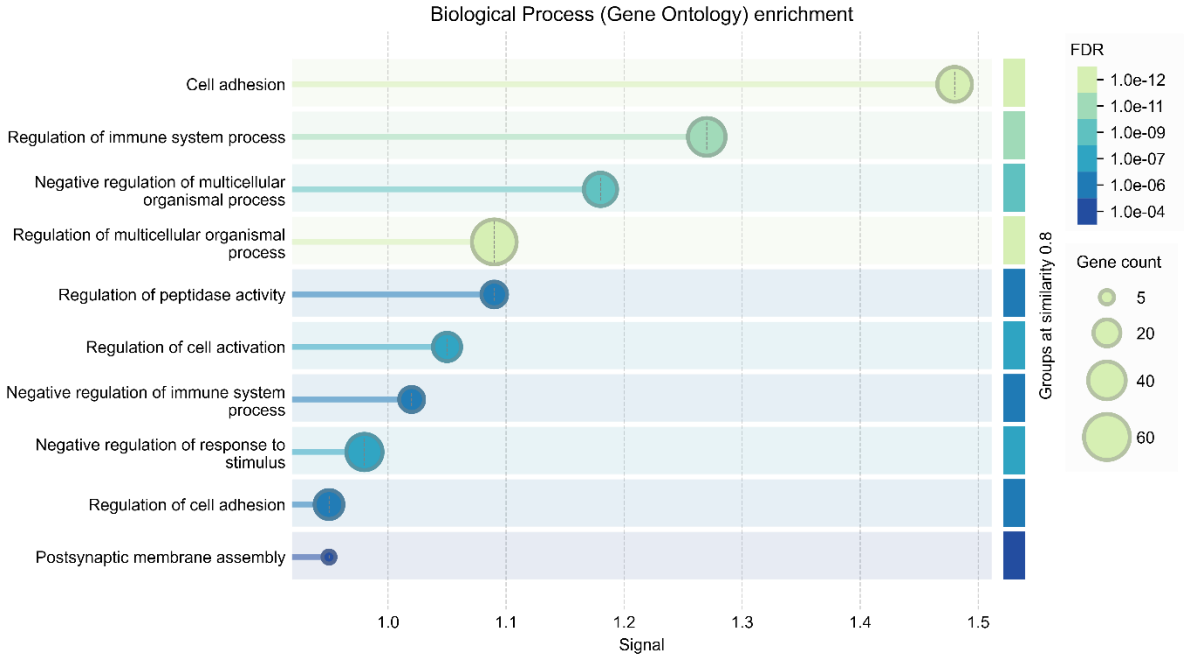


Figure 31: AbPAE vs AbNAE: STRING functional enrichment for biological processes (Gene Otology)

6.3.10 Inter-Assay Correlation

In chapter 3, the key results of my study of surrogate markers of neuroinflammation in the CSF of patients with AE found increased CSF mononuclear cells and oligoclonal band present in a proportion of patients with AbPAE and AbNAE versus none in the NI cohort. CSF lambda but not kappa FLC were increased in the AE cohort compared to NI controls. Compared to normal/ NI groups, GM-CSF, IL12p70, IL13, IL17a, IL1b, IL21, IL4, IL5, BCA-1/CXCL13 and GCSF were associated with AE. When a multivariate logistic regression model was used to compare the AE cohort with a combined group of NI, VI and OAND controls. There were no differences in cytokines between the AbPAE and AbNAE cohorts.

In chapter 4, I also found evidence of oligoclonal bands in patients with psychiatric disease and none with NI controls. On univariate cytokine analysis, I found ITAC, GM-CSF, IFN-γ, IL-13, IL-21 and TNF-α increased in the psychiatric cohort with GM-CSF and ITAC increased compared to NI in a multivariate logistic regression analysis.

Proteomic analysis with mass spectrometry did find similarities with my previous study results. In examining all candidate proteins identified by Spectronaut with the default absolute log 2 ratio of greater than 0.58, 27 proteins identified as belonging to immunoglobulin (both heavy and light chains) was increased in AE. Oligoclonal bands represent intrathecal production of immunoglobulins and so this result could represent the finding of oligoclonal band positivity in AE described in chapter 3. Whilst both kappa and lambda FLC were increased in the AE cohorts, there were proteins identified as part of lambda FLC (10 proteins) vs kappa FLC (7 proteins).

In Chapter 4, I found CSF beta-2 microglobulin was significantly lower when the psychiatric cohort was compared to NI and AE cohorts. On proteomic analysis, CSF beta-2 microglobulin was again lower in the psychiatric cohort, when compared to AE (q-value 0.01, average log 2 ratio -0.79). However, this was not seen when compared to the NI cohort. Again, no cytokines that were significantly different in chapter 3 was identified here as candidate proteins. This may be due to overall lower cohort numbers in the proteomic analysis as the cohorts in this chapter were only a subset of those in Chapter 4.

6.3.11 Package Independent Data Analysis

Data was exported from Spectronaut with the aim of further analysis, independent of the Spectronaut software package. The data was reanalysed independently using the limma package in R (using linear models and empirical Bayes shrinkage) by Artur Shvetov (biostatistician). No significant differences between AE, psychiatric and NI cohorts were found. This discrepancy in analysis results raised concerns especially given the number of candidate proteins. Any proteomic profile where there was insufficient data for identification or quantification were explicitly set to “0” to allow for statistical analysis. However, no significant differences between AE, psychiatric and NI cohorts were found.

6.4 Discussion

6.4.1 Summary of Results

Analysis with Spectronaut found several candidate proteins that were overrepresented between the AE and NI cohorts, the AE and psychiatric cohorts and AE and psychiatric cohorts. In the AE vs NI cohort, proteins increased in AE included those associated with immune activation and neuronal damage. Similarly, the P vs NI cohort also included candidate proteins associated with immune processes, neural processes and interestingly also corticosteroid binding globulin (corticosteroids are used in the treatment of autoimmune disease). On pathways analysis, there was much similarity in the AE vs NI and psychiatric vs NI comparisons, particularly in those associated with immune functions.

When AE was compared to the psychiatric cohort there was, as expected proteins associated with immune functions increased in AE. However, pathways analysis still identified overexpression of processes related to immune function as well as glycosaminoglycan metabolism in the psychiatric cohort. When antibody negative AE was compared to antibody positive AE, proteins associated with innate immunity and lymphocyte (including T-lymphocyte) regulation was increased in antibody negative AE.

Interactome analysis of psychiatric vs AE and AbPAE vs AbNAE found sparse interactions between all candidate proteins. However, there were pockets of nodes with high interconnectivity.

Unfortunately, candidate proteins identified in Spectronaut were not able to be verified on independent analysis.

6.4.2 Spectronaut Results

6.4.2.1 Principal Component Analysis

PCA indicated a large spread of each cohort with a considerable degree of overlap. PC1 explained 17.6% of variance and PC2 explained 13.4% of variance. AE was slightly more PC1 positive and psychiatric was slightly more PC1 negative (although still overlapped), but the NI cohort was spread in between these two. Of the cohorts in my study, the AbPAE group were grouped closer together but still overlapped with psychiatric, AbNAE and to a smaller degree, the NI cohort.

There are several reasons for this degree of overlap. This likely represents my small sample size and the heterogeneity of patients in each cohort: particularly in the psychiatric and AbNAE groups. However, this may also reflect that there are common biological processes shared between each cohort, particularly as our psychiatric cohort was selected for a higher likelihood of a secondary cause. The overlap with the NI cohort could be due to the issues with the nature of my NI cohort as already raised in Chapter 4. The NI cohort had lumbar punctures performed for what is traditional considered “non-inflammatory” neurological disease: IIH and NPH but their CSF proteome may not reflect a true normal.

6.4.2.2 Autoimmune Encephalitis

All proteins with a log 2 ratio of greater than 2 were increased the AE cohort (Table 27). As expected, many of these proteins were immune-related. Interestingly, the immune-related proteins showing the largest differences between groups were not confined to antibody-mediated immune mechanisms, which have traditionally received the most research attention, but instead reflected broader inflammatory activity

involving both innate and adaptive immune processes. These proteins included purine nucleoside phosphorylase (PNP), tumour necrosis factor receptor superfamily 12A, CH3L2 and both prothymosin and parathymosin. Two other proteins increased in AE: Ubiquitin carboxyl-terminal hydrolase (UCH-L1), cell cycle exit and neuronal differentiation protein 1 (CEND1) and histones may also have a role in neuroinflammation. These will be discussed below.

PNP is an enzyme in the purine salvage pathway and is essential for lymphocyte survival, particularly T cells[697]. It has roles for breakdown and recycling of purine nucleosides making it critical for cell turnover. It prevents the toxic buildup of intracellular deoxyguanosine triphosphate which, in excess, can cause apoptosis of rapidly dividing cells such as those in the immune system[697]. Mutations in the PNP gene result in a severe combined immunodeficiency with severe reduction in T cells and variable NK and B cell function[697,698]. Hence its elevated levels here may be a marker of increased T cell turnover and activity in the setting of this autoimmune disease [699]. This is in contrast with beta 2 microglobulin which was not identified as a candidate protein in this analysis, nor was a significant difference found between AE and NI beta-2-microglobulin in my results in Chapter 4 (albeit with limitations).

Likewise, tumour necrosis factor receptor superfamily member 12 binds the TNF-like weaker inducer of apoptosis (TWEAK) to trigger a proinflammatory signalling cascade[700]. TWEAK has been upregulated in several autoimmune conditions such as rheumatoid arthritis and multiple sclerosis[701,702]. Hence, the presence of this protein may also reflect increased inflammatory processes.

Chitinase-3-like protein 2 (CHI3L2) is a member of the chitinase-like protein family. It is from the glycoside hydrolase family of chitinase-like proteins as chitiniase-3-like protein 1 (CHI3L1) and shares sequence homology with CHI3L1, which has roles in inflammation and tissue repair. CHI3L1, but not CHI3L2 has been reported to be elevated in the CSF of antibody positive AE including LGI-1[439] and NDMAR encephalitis[440] as well as multiple sclerosis [438]. CHI3L1 has been proposed as a biomarker for neuroinflammation and neurodegeneration[703].

Unlike CHI3L1, CHI3L2 has not yet been reported to be elevated in the CSF of patients with AE. CSF CHI3L2 has been associated with multiple sclerosis: particularly in relapsing-remitting multiple sclerosis and also increased disability in progressive multiple sclerosis[703,704]. One published study observed this association where both CSF CHI3L1 and CHI3L2 were reported as associated with multiple sclerosis after proteomic analysis with mass spectrometry[705]. This was then validated using an ELISA based technique. Hence, this is a potentially new biomarker for neuroinflammation in AE that has been seen in other neuroinflammatory disease, and worth further investigation to confirm my findings.

Prothymosin alpha has roles lymphocyte, particularly cytotoxic CD8 T cell activation as well as NK cell activation. It also stimulates the innate immune system include inducing oxidative killing by neutrophils and activation of dendritic cells and macrophages[706]. Interestingly, in the context of neuroinflammation, it has neuroprotective effects and inhibits necrosis of cortical neurones and hence may play a role in prevention of neuronal damage in this setting.

Another protein increased in AE which is structurally related to prothymosin is parathymosin, which modulates prothymosin-α activity by interacting with overlapping pathways and molecular targets [707]. This interaction may serve as a regulatory mechanism to temper immune responses and protect against excessive inflammation [708,709]. The concurrent overexpression of both immune-activating and immunomodulatory proteins in the setting of inappropriate inflammation suggests a state of immune dysregulation, consistent with mechanisms underlying many autoimmune conditions.

In terms of overexpression of neural related proteins in the AE cohort, UCH-L1 may be a biomarker of interest. UCH-L1 is a neural protein essential for maintenance of axonal integrity and health. It is elevated in neuroinflammatory and neurodegenerative disorders including traumatic brain injury[710], Guillian-Barre syndrome[711] and also in neuropsychiatric lupus[712]: another autoimmune neuroinflammatory condition that can present with florid psychiatric symptoms. Hence, while there is not yet reported literature on the presence of UCHL-1 in AE, this is another potential biomarker worth further exploration.

CEND1 is a membrane-associated protein crucial for neural development and a marker of neurogenesis[713]. Low expression of CEND1 has been associated with increased NF-κB pathways activation, which is a central driver of inflammation[714]. Hence CEND1 may modulate neuroinflammatory responses.

Finally, histones are increased in the AE cohort compared to the NI and psychiatric cohorts as well as increased in the antibody negative cohort AE compared to antibody positive AE cohort. Histones, as part of extracellular nucleosomes in the CSF

may have a role in driving neuroinflammation through the activation of microglia via the danger-associated molecular pattern (DAMP) signalling of the innate immune system as demonstrated in a mouse model. This study demonstrated that injection of recombinant nucleosomes (but not histones themselves) induced microglial oxidative stress and pro-inflammatory cytokine activation[715]. This may be another area that warrants further study.

Pathways analysis likewise reflected the increased immune activation as expected in AE. Again, involvement of the innate and adaptive immune mechanism systems is seen (Table 28). The finding of increased representation of pathways associated with complement activation[716] and FCGR receptor activation[677] is consistent with there being a role for humoral immunity of immune complexes. Whilst my AE cohort is predominantly AbNAE, with subset of AbPAE it has been discussed that even in AbNAE, there may be pathogenic antibodies driving disease that are yet to be characterised.

6.4.2.2.1 AbPAE vs AbNAE, beyond antibody mediated pathogenesis

Antibody-negative AE is a subset of AE where no specific neural autoantibodies are detected but shares clinical and radiological similarities to antibody-positive AE[179]. However, novel anti-neuronal antibodies are still being identified even in neurological conditions not traditionally thought to be immune driven[717]. Some patients classified as having antibody-negative AE may have antibody detected when their serum and CSF samples are examined in different assays, many of which are in the research phase, or available in only a few centres globally[718]. Hence, it is possible that a subset of patients with antibody-negative AE have an antibody that has not yet been described[719].

Beyond pathogenic antibodies, both T cell lymphocyte activation and the innate immune system play important roles in the pathophysiology of AE. Activation of CD4+ lymphocytes and the action of cytotoxic CD8+ T cells on neurones has been demonstrated in antibody positive autoimmune encephalitis[720-722].

All the candidate proteins with a difference greater than a log2 ratio of 2 in the AbPAE vs AbNAE comparison were increased in the AbNAE cohort (Table 29). Five of these proteins were immune related and these were all indicative of immune processes outside that of antibody mediated pathogenesis and their downstream engagement of complement and other associated proteins. These were: leukocyte-assisted immunoglobulin like receptor 1 (LAIR-1), V-set and immunoglobulin domain-containing protein 4, lactotransferrin, proteoglycan 4 and HLA class I histocompatibility antigen.

HLA class I histocompatibility antigen is part of the MHC class I complex which presents antigen to cytotoxic T lymphocytes. If the peptide presented is foreign (e.g. in the setting of a virally infected cell), then the CD8 T lymphocyte will initiate apoptosis of the target cell though the delivery of cytotoxic proteins including granzymes and perforin[4,41]. IFN-γ upregulates expression of MHC I[723], so increased abundance may indicate a Th1 response in the AbNAE group of patients. However, no overt differences in cytokines were noted on proteomic analysis here or were seen between the AbPAE and AbNAE comparisons in Chapter 4 and confirmation needs to be made in a larger cohort.

Lactoferrin is an iron-binding protein found in human milk but is also part of innate immunity and is released from the granules of neutrophils with direct antimicrobial effects[724,725]. Lactoferrin has other immune functions including activation

and recruitment of antigen presenting cells[725] and bridging innate and adaptive responses[726]. Hence, its presence may represent pro-inflammatory activation of the innate immune system. Interestingly though, Lactoferrin has been recently demonstrated to have neuroprotective effects[727], anti-inflammatory effects[728] and immunomodulatory actions on and reactive astrocytes[729]. This raises questions about its role in this setting: as a promoter of neuroinflammation or as an immunomodulator/neuroprotective agent in the setting of a dysregulated immune response.

Other immune related proteins increased in AbNAE were also immunomodulatory. V-set and immunoglobulin domain-containing protein 4 is a checkpoint inhibitor which suppresses T cell proliferation and cytokine production[730]. LAIR-1 is another immunoregulatory protein[731]. It is an inhibitory receptor in immune cells including B and T lymphocytes, NK cells, monocytes and macrophages as well as microglia[732]. It inhibits secretion of proinflammatory cytokines and attenuates proinflammatory polarization of macrophages[733]. It is also essential for resolving neuroinflammation and microglial activation.[734].

Proteoglycan4 is another immunomodulatory protein. It is a large glycoprotein responsible for lubricating joints and protecting cartilage surfaces[735], which raises the possibility of it being a contaminant. However, interestingly, it has been described to cross the blood brain barrier (BBB) after traumatic brain injury where it has been observed to inhibit neuroinflammatory responses.[736]. Indeed, proteoglycan4 has a multifaceted role in the immune system, particularly in attenuating inflammatory states and promoting wound healing[737-739].

It is thus, interesting that these immunomodulatory proteins including lactoferrin are increased in the setting of inappropriate immune activation, and their roles in these pathologies could be further defined. Both AbPAE and AbNAE are states of immune dysregulation although the specific processes in each may differ. AbPAE, with its antibody associations necessitates involvement of the adaptive immune system. AbNAE may very well be a heterogenous mix of entities with dysfunctional upregulation of different aspects of the immune system between different patients. The presence of immunoregulatory proteins, particularly those that inhibit the adaptive immune system in the setting of inappropriate inflammation in AbNAE may indicate a shift towards an innate immune response. However, the actions of the immune system are complex and often interrelated and more study here with a larger cohorts may need to confirm this hypothesis.

Pathway analysis showed enrichment of innate immune processes in both AbNAE and AbPAE, with neutrophil degranulation[674] emerging as a shared upregulated pathway. This confirms that the immune response in AbPAE is beyond simply the adaptive humoral response mediated by antibodies.

Many pathways reportedly mapped by proteins increased in AbPAE were associated with glycosaminoglycans, although only one (CS/DS degradation)[678] had a FDR <0.05. However, enrichment of this pathway: associated with the degradation of chondroitin sulphate and dermatan sulphate is still of interest as glycosaminoglycans can have pro-inflammatory roles.

Glycosaminoglycans are long, linear polysaccharides with roles in structural support, signalling regulation, maintaining normal tissue architecture, hydration and

tissue homeostasis and barrier properties[740,741]. Glycosaminoglycans like chondroitin, hyaluronan and heparan sulphate have been identified the CSF. They can be major players in neuroinflammation of the CNS and are critical to BBB integrity[741-743], the compromise of which may facilitate autoantibody access to the CNS. Heparan sulphate and chondroitin can bind to and localise cytokines, interact with growth factors and activate innate immunity[741 742]. Heparan sulphate fragments can, through engagement of toll-like receptor 4, activate microglia and promote pro-inflammatory cytokine signalling[744]. It is possible the increased metabolism of chondroitin sulphate/dermatan sulphate could reflect a shift to adaptive immunity.

However, whilst chondroitin sulphate can be pro-inflammatory within the CNS[742], chondroitin sulphate has been shown to inhibit NF-κB induced inflammation in the joint space[745]. Chondroitin sulphate also participates in glial scar formation[741]. Thus, the up-regulated chondroitin metabolism here may indicate broader state of immune dysregulation, but targeted studies will be required to define the specific mechanisms.

Pathways mapped by proteins overexpressed in AbNAE also reveals engagement of innate immunity[674,679,680], rather than humoral responses. Interestingly, the pathway that emerged as the most relevant was regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding proteins[686].

Insulin-like growth factors are a family of polypeptide hormones: structurally similar to insulin and mediate many of the effects of growth hormone[746]. However, they also have important role in the immune system. IGF-1 supports the growth, maturation and survival of lymphocytes[747-749] as well as the innate immune system[750]. It also stimulates the proliferation of T-regulatory cells and depending on the context can

promote pro and anti-inflammatory responses[747,751]. In circulation, IGFs are usually bound in complexes with IgGF binding proteins and become active when they are released. However, some IGFs still have activity whilst still bound[752]. It remains unclear whether the overexpression of pathways regulating this protein reflects an increase or a decrease in immune activation. However, it may serve as another marker of immune dysregulation, warranting confirmation and further investigation.

Several pathways increased in AbNAE were related to fibroblast growth factor receptor (FGFR)2[681,683,684,753]. FGFR2 is a critical cell signalling cascade that regulates many important processes including cell proliferation, differentiation and survival It has an interesting role in CNS regulation and neuroinflammation as, depending on the context, it can influence both neuroprotective and neuroinflammatory processes. One of the main pathways triggered by FGFR2 activation is the phosphoinositide-3-kinase (PI3K) pathway[754]. Not only is the PI3K pathway essential for immune cell survival activation and trafficking, but it is also crucial for the proliferation and regulation of apoptosis of neurones[755-757].

In a mouse model of Parkison's disease, FGF7/FGFR2 autocrine signalling increased astrocyte release of pro-inflammatory cytokines. Attenuation of this signalling pathway in the mouse model, including inhibition of FGFR2 in astrocytes, inactivated NF-κB signalling and improved outcomes[758]. In CNS infection with borrelia burgdorferi (Lyme neuroborreliosis) which can have psychiatric and cognitive dysfunction presentations, FGFR1 ,2 and 3 were all upregulated in microglia as part of the immune response[759]. Conversely, FGF10 signalling through the FGFR2 pathway is involved in downregulation of inflammation and recovery after spinal cord injury via the

PI3K/AKT pathways[760]. Interestingly, FGFR2 appears to reverse depressive behaviours in a rat neuroinflammatory model of depression but inhibiting the FGFR receptor evoked microglial activation and depressive-like behaviours[761].

Another reason why FGFR2 warrants further investigation is that it can be a potential therapeutic target. Not only is FGFR2 implicated in neuroinflammatory processes, dysregulation of the FGFR2 pathway also is implicated in conditions including abnormal bone development and cancer. Here, drugs that target this pathway have been developed[762]. These include monoclonal antibodies (bemarituzimab), tyrosine kinase inhibitors and fibroblast growth factor (FGF) ligand traps. Hence, it may be a potential therapeutic target in AbNAE, utilising drugs that are already available. Thus, the overrepresentation of the FGFR2 pathway here with the evidence for its role in neuroinflammation is worth future study. However, at this stage it is difficult to know if FGFR2's role in the CNS is neuroinflammatory or neuroprotective. A neuroprotective mechanism may help regulate neuroinflammation, and a neuroinflammatory state, this may also activate neuroregulatory mechanisms. A deeper understanding of this pathway is required to determine how it might be most effectively manipulated for therapeutic benefit.

6.4.2.3 Immune Activation in the CSF of Patients with Atypical Psychiatric Disease

The finding that the top 25 significant pathways in the psychiatric cohort versus NI comparison overlapped substantially with those in the AE versus NI comparison: including pathways related to neutrophil degranulation[674], complement activation[676], and Fc gamma receptor regulation[677], suggests that the immune system plays a role in both conditions. Indeed, the degree of similarity in immune-related pathways between

the psychiatric and AE cohorts may be suggestive of shared pathogenic mechanisms. Benefit of immune suppression in the patients within this cohort has been explored in another published paper where it has been demonstrated that most patients tolerate immune suppression[588], with a trend for improvement of function, requiring further study to confirm therapy related improvements.

The overlap of pathways in the psychiatric cohort versus NI and AE versus NI are not due to cytokines. Unlike the cytokine studies (chapters 4 and 5), cytokines were not identified as candidate proteins by proteomics. Additionally, only 2 patients with high cytokines expressed in the CSF were present in the atypical psychiatric cohort (Chapter 5). Hence, the similarities of pathways here more likely represent broader immune activation across the AE and atypical psychiatric cohorts.

In terms of the candidate protein in the comparison between psychiatric and NI cohorts, there were two increased proteins in the psychiatric cohort related to immune function with a log 2 ratio difference of greater or equal 2. Both have potential immunoregulatory roles: fibroleukin and acid sphingomyelinase-like phosphodiesterase 3a (Table 32).

Fibroleukin is also known as fibrinogen-like protein 2 and has prothrombinase activity, particularly in the setting of inflammation and sepsis[763-765] but also has broader immune actions. It has two forms: membrane bound on macrophages and endothelial cells and a soluble form secreted primarily by T-regulatory cells[764]. Soluble fibroleukin reduces proinflammatory cytokines while increasing anti-inflammatory cytokine IL10 in a mouse model[766]. In contrast, membrane-bound fibroleukin has pro-inflammatory effects through its actions as an immune associated coagulator and also potentially by

interaction with toll-like receptor 4 of the innate immune system[765]. Fibroleukin detected in the CSF is most likely the soluble, secreted form[767,768] although theoretically, membrane bound proteins can be detected in CSF in extracellular vesicles[769]. Given the association of infection with psychiatric disease and the possibility of a subset of psychiatric disease being immune dysfunction related in origin, this is an interesting protein for further study.

The other over expressed immune related protein was both immunoregulatory. Acid sphingomyelinase-like phosphodiesterase 2a (SMPDL3A) functions as a nucleotide phosphodiesterase secreted by cholesterol-loaded macrophage foam cells and may decrease pro-inflammatory nucleotides[770]. It is also emerging as a regulator of inflammation through its role in innate immune signalling pathway particularly in relation to restricting the cyclic guanosine monophosphate-adenosine monophosphate synthase(cGAS)- cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) Stimulator of Interferon Genes (STING) pathway[771,772]. cGAS is a pattern recognition receptor for double stranded DNA and activation leads to synthesis of cyclic GMP-AMP (cGAMP) which then activates STING and induces the production of type 1 interferon and other proinflammatory pathways via Interferon Regulatory Factor 3 (IRF3) and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFk-B)[771,773]. Sphingomyelin Phosphodiesterase Acid-like 3A (SMPDL3A) selectively hydrolyses 2'3'-cGAMP, attenuating cGAMP mediated STING activation, limiting the inflammatory responses[771]. The cGAS-STING pathway has emerged as a possible therapeutic target in neuroinflammation: it seems to be implicated in several conditions including ischemic brain injury, traumatic brain injury and also neurodegenerative diseases such as Parkinson's and amyotrophic lateral sclerosis[774].

In contrast to the increase of mostly immune-regulatory proteins discussed above, Ephrin A5 (EphA5) is a neurogenesis related protein which may have neuroinflammatory actions, also increased in the psychiatric cohort. EphA5 is a receptor tyrosine kinase belonging the larger family of Eph receptors which mediate and regulate cell to cell signalling through regulation of receptor trafficking[775]. EphA5 is expressed almost exclusively in the nervous system and is widely distributed in the CNS. In adults, it has a role in supporting synaptic plasticity[776]. EphA5 is induced in activated astrocytes following CNS injury, although its role there seems to be an inhibitor of axonal sprouting and neural circuit reorganisation rather than actively participating in the neuroinflammatory state[777]. Decreased EphA5 has been associated with decreases in central monoamines in animal models[778]. Disruption in this group of neurotransmitters has been strongly associated with psychiatric disease and hence the unexpected relative overexpression of EphA5in this atypical psychiatric cohort may be worth further study.

There is also emerging evidence that the ephrin signalling family have roles in immune activation. Whilst the role of EphA5 in immunity is less well-defined, it has been noted that other receptors within this signalling family are present on human B and T cells and may contribute to activation, proliferation and T cell differentiation as well as cell trafficking[779]. Therefore, it is unclear if EphA5 serves a dual role in the CNS in both neuroregulation and inflammation.

Cortisol-binding globulin was also increased in the psychiatric cohort. This is the main protein for transport of cortisol in the blood, binding about 80-90% of circulating cortisol. In its bound state, cortisol is inactive[780]. Cortisol is an important stress

hormone and belongs to the glucocorticoid class of hormones. It has important roles in metabolism regulation, managing the body's fight or flight response, regulates the sleep-wake cycle and has anti-inflammatory effects[781,782]. Corticosteroids such as prednisolone which are used at high doses to quickly supress the inflammatory response belongs to the same class of hormones[783].

In critically ill patients, decreases in cortisol-binding globulin are associated with increases in free cortisol[784]. Cortisol-binding globulin also may regulate free cortisol levels, although how it does this is complex and influenced by temperature and glycosylation[785]. The relative overexpression of cortisol binding globulin may influence the psychiatric patient group's CSF cortisol levels and their stress responses. Interestingly, higher cortisol levels have been associated with cognitive dysfunction and may be neurotoxic[786]. Increased cortisol binding globulin in this context may therefore be an attempt to limit cortisol-induced neuronal damaged at the cost of dysregulated immunity. Changes to cortisol binding globulin function have been associated with chronic fatigue syndromes[787] and to chronic pain[788]. Hence this is also an interesting finding as a significant proportion of my psychiatric patients develop or have pain and/or fatigue. Whilst these symptoms may be related to their underlying psychiatric diagnosis, this finding may give some further insight into why these patients develop such symptoms.

6.4.2.4 Immune Protein Expression in Psychiatric Disease Compared to AE

Unsurprisingly, the AE cohort had a relative increase in several proteins related to immune function when compared to the psychiatric disease cohort. While the two cohorts had similar pathways inferred when they were compared with NI, the

psychiatric disease cohort had distinct differences in pathway related protein expression. Additionally, in the AE cohort, there was relative abundancy of four proteins which are related predominantly to neural function FABP3, MAP2, Band 4.1 protein 3 and UCH-L1.

As already mentioned in results, FABP3 or heart type fatty acid binding protein is primary expressed in heart and skeletal muscle[690,789]. Given the presence of myoglobin and beta enolase also increased in AE, it being a contaminant cannot be completely ruled out. However, in other studies, elevated CSF FABP3 has is thought to be reflective of neuronal membrane disruption and lipid dyshomeostasis in Alzheimer's disease[790,791]. It is elevated in several neurodegenerative disorders: Lewy body dementia[792], Parkinson's disease[690], and may also be a predictor of dementia in clinically healthy adults[793]. Its presence in this AE cohort may therefore reflect underlying neuronal injury. Clearly carefully controlled studies are required to ensure that these later results are not due to CSF contamination, related to the preanalytical factors that have been discussed above. This may include the difficulties in the lumbar puncture procedure in patients that are older and have more disturbed CNS function.

Microtubule associated protein 2 (MAP2) stabilises and regulates microtubules, supporting neuronal structural growth and synaptic plasticity. It is closely related to microtubule associated protein Tau, which has been well described in the pathogenesis of numerous neurodegenerative diseases[794]. MAP2 disruption may affect the structural and functional plasticity of synapses and lead to dysregulation of protein synthesis.[794]. Reduction in MAP immunoreactivity has been also described in neuropsychiatric disorders such as schizophrenia[795] and major depressive disorders.[794], indicating

abnormal chemical modification of this protein may contribute to the pathogenesis of psychiatric disease. That MAP2 is increased in AE when compared to the psychiatric cohort may indicate increased neuronal damage: increased CSF levels in amyotrophic lateral sclerosis correlated with disease progression[796]. Thus, the increase of both FABP3 and MAP2 which are both linked to neurodegenerative brain injury may more neuronal damage or neuronal perturbation in the AE cohort compared to the psychiatric cohort.

Band 4.1 protein 3 is a membrane skeletal protein with roles in apoptosis and inhibition of cell proliferation[797]. It is expressed in brain tissue and is involved in synaptic function[798]. UCH-L1 is increased again in AE in this comparison, as it was when AE was compared with NI (discussed in section 6.4.2.2).

Interestingly, when mapping proteins to pathways, increased proteins in the psychiatric cohorts also mapped to pathways related to innate immune activation including neutrophil degranulation[674]. Additionally, pathways related to innate and adaptive immune function were increased in both the psychiatric and AE cohorts compared to NI, which may reflect immune pathways being active in the pathogenesis of both cohorts.

There were also several pathways increased in psychiatric vs AE related to glycosaminoglycans metabolism and degradation including that of chondroitin[693], hyaluronan sulphate[694] and keratan sulphate[799,800]. Keratan sulphate has diverse roles in the CNS including those in regulatory, repair and neuroprotection[801-803]. Hence the implications of enrichment for pathways for its metabolism in this setting may imply increased neuronal injury and this needs to be explored further. The proinflammatory

nature of other glycosaminoglycans and the anti-inflammatory actions of chondroitin have been discussed in section 6.4.2.2.1. Their role in activating the innate immune system [744,804] and the enrichment for pathways for their breakdown, in the setting where other immune activation has been described may indicate a shift towards more adaptive immune system engagement or broader immune dysregulation. Further study of immune processes in these patients, however, is needed to confirm this.

These glycosaminoglycan pathways were also present in the list of pathways identified AbPAE vs AbNAE comparison, but the FDRs did not reach statistical significance which could indicate that these results do not represent genuine biological enrichment. Alternatively, because the Reactome Pathways database is based on the current literature, it may be because knowledge about proteins represented in these pathways is more limited. If the latter is the case, then then this again, raises the possibility of an overlap between the pathological processes in AE and these atypical psychiatric patients.

6.4.4 STRING Interactome Analysis

Interactome analysis with STRING found pockets of high proteins with high connectivity between them in the AE vs psychiatric comparison and AbPAE vs AbNAE comparison. The STRING database "compiles, scores and integrates protein–protein association information" from evidence currently available in the literature[669]. Amongst their sources is the Reactome Pathways database. Hence some of the clusters identified including "glycosphingolipid catabolism" and "SARS CoV-2 targets intracellular signalling and regulatory pathways" in AE vs psychiatric were also seen in the Reactome Pathway analysis. In the AE vs psychiatric comparison, clusters of protein

interactions did feature complement pathway and its activation which may interact with antibody-antigen or antibody-antibody immune complexes.

Similarly, in the AbPAE vs AbNAE comparison, the ephrin receptor signalling pathway, which has previously discussed as immunoregulatory and effects on neuronal function, was one of the prominent clusters (section 6.4.2.3). The top STRING Gene Ontology enrichment in this comparison was cell adhesion involving proteins such as integrins, vitronectin and activated leukocyte adhesion molecule[669,671] all which have important roles in blood brain barrier integrity and movement of immune cells across the blood brain barrier[805-807]. Given the organisation of the blood brain barrier and avenues of immune entry into the CNS described in Chapter 1, it is not surprising that these processes are involved in AE. Hence the finding in the STRING analysis[669,671] supports the results already discussed, with the caveat that this analysis uses the interactome data that is published as part of its approach.

6.4.3 Comparison with Other Studies

Findings in other studies examining CSF proteomics in AE and psychiatric disease have already been outlined in the introduction to this chapter but will be revisited here in reference to the current results.

6.4.3.1 Autoimmune encephalitis

There has only been one study published examining proteomic analysis by mass spectrometry of CSF in patients with AE[654]. This study investigated patients diagnosed with antibody-positive AE, most of whom had anti-NMDAR encephalitis using a different methodological workflow. The AE patients were compared to a cohort with relapsing-remitting multiple sclerosis and a cohort of patients with somatic symptom disorder as

"non-inflammatory controls". Enrichment analysis of differentially abundant proteins found biological processes involved in innate and humoral immune responses such as complement activation and immunoglobulin mediated immune response in NMDAR-Ab AE. Biological processes associated with immune function was also associated with LG1-1 Ab AE, and GAD Ab AE. In all these AE subtypes, proteins involved in synaptic transmission, neurotransmitter receptor and axonal development was also observed. [654].

Interestingly, while immune-related pathways were predominant across all autoimmune encephalitis (AE) patients, the above study identified distinct pathway signatures within the different pathogenic antibody subtypes. This suggests that even among antibody-positive encephalitis cases, there may be divergent underlying pathological processes. This finding is also not unexpected: disease processes involving antibodies directed at different antigenic targets are inherently likely to result in distinct pathophysiological mechanisms[654].

While the exact proteins reported were not seen in the most differentially abundant proteins in my analysis, the differentially abundant proteins in my AE cohort also mapped to pathways involving innate (e.g. neutrophil degranulation) and humoral(e.g. classical antibody-mediated complement activation). Neural protein essential for axonal maintenance (e.g. UCH-L1) was also seen in my cohort.

In contrast to the above study's well-defined inclusion criteria of antibody positive AE patients, one of the challenges in my study is the heterogeneity of comparison groups. The antibody positive group in my study was combined because of the overall small cohort size. If I had greater numbers of AE patients, the AbPAE group

could potentially be more easily subdivided into their antibody specificities. My study also included AbNAE, which is also likely more a heterogenous mix of differing conditions, some potentially having uncharacterised antibodies and some with predominantly innate immune pathogenesis. However, in combining different antibody positive AE subtypes with AbNAE, there may be the advantage of being able to identify common pathophysiological pathways and therefore potential future diagnostic biomarkers and therapeutic targets for this group of diseases. Recruitment of larger cohorts, which may need a multi-centre approach given this is a rare condition, may assist analysis and perhaps identify clinical phenotypes that might allow diagnostic categorisation and guide therapy.

Finally, this study used different methodology to mine. Their CSF samples were depleted using a ProteoMiner Small Capacity Protein Enrichment Kit (Bio-Rad)[808] which uses a diverse bead-based library of combinational peptide ligands to remove high abundance proteins whilst concentrating low abundance proteins. This study did perform DIA analysis of the CSF samples, but removal of more high abundance proteins may have better enhanced discovery of lower abundance proteins because of their more targeted approach of reducing highly abundant proteins. However, as with all methods, there may have been non-specific protein depletion compromising the identification of a different subset of low abundance CSF proteins [809]. Their data was analysed using Progeneisis IQ for Proteomics (Waters) which also may have a different imputation or other analysis strategies, when compared to Spectronaut may also impact their reporting of disease associated protein expression changes.

6.4.3.2 Psychiatric Disease

Numerous studies have applied proteomic methods to investigate potential biomarkers in psychiatric disease in a range of biological tissues: blood, CSF and postmortem brain tissue. This has already been discussed in the introduction to his chapter (section 6.1.1.2).

The most consistent biomarker reported in proteomic studies of CSF was disturbances of VGF, which has been seen in both psychotic and affective disorders[656,657]. Disturbances of proteins associated with cholesterol transport and phospholipid was also reported in studies of psychosis[656] as well as downregulation of transthyretin [660,810] . Proteins associated with angiogenesis and acute phase response were reported in affective disorder [661] with another study reporting down regulations in neuronal/synaptic protein, [662] and a study on bipolar disorder reporting raised eotaxin-1, CXCL6, CXCL-1 and placenta growth factor[663].

Among the candidate proteins I identified as most differentially expressed between psychiatric and neuroinflammatory (NI) cohorts (with a threshold of a log 2 ratio greater or equal to 2), none overlapped with the proteins reported in previous studies. Rather, in my psychiatric cohort, the most pronounced differences were observed for increased fibroleukin, acid-sphingomyelinase-like phosphodiesterase 3a, Ephrin A5 and corticosteroid binding protein. However, when I examined the complete list of all 215 candidate proteins nominated by Spectronaut, (with a threshold difference of log2 ratio of greater or equal to 0.58), I found that transthyretin and apolipoprotein A I and II were upregulated in my psychiatric patients, consistent with previous reports in first episode psychosis[660,810]. Hemopexin, which was reported to be differentially

expressed in depression[661], was also reduced in my psychiatric cohort. Differences between the most differentially expressed proteins in my study and what is reported in the literature could be due to several factors. As discussed in the introduction to this chapter, the mass spectrometry acquisition techniques also differed from study to study reported in the literature. This could change the sensitivity of detection of low abundance proteins and be another reason for differences. Issues with co-depletion of non-albumin-based proteins and Spectronaut analysis could also be contributory, this will be discussed further in Limitations (section 6.4.5).

Most published reports have cohorts of patients with well-defined diagnoses of psychiatric disease. In contrast, recruitment of my psychiatric cohort was based on atypia, which would have excluded them from the diagnostically well-defined cohorts examined in previous studies. By prioritising "red flag" features suggestive of immune involvement, the selection process likely biased toward detecting evidence of immune activation, which may explain why the candidate proteins and biological pathways identified here differ from those reported in the literature. At the same time, the similarities observed between my atypically defined cohort and more narrowly defined patient groups suggest that shared, and perhaps more fundamental, pathways of pathogenesis may be involved.

Given the relatively small size of my cohort, larger and more resource-intensive studies in patients with similar clinical phenotypes will be important for confirming these findings. Direct comparisons between "atypical" and "typical" cohorts could be particularly informative in determining whether distinct biological pathways underlie

these different clinical presentations, and whether they represent divergent trajectories emerging from initially shared pathogenic mechanisms.

6.4.5 Limitations

Although some of my proteomic findings were consistent with results from earlier chapters that used different assay methods, I did not replicate the previously observed cytokine correlations within the cohorts of interest. One reason for this is because the cohorts used in this mass spectrometric analysis was a subset of my previous comparator cohort (Chapter 5), limited by availability of CSF samples available. With the small numbers of participants across my studies, changes in included numbers may lead to changes in overall results.

The approach used for my albumin depletion strategy could have significantly affected some protein detection. Although most cytokines are not generally albumin-bound, the reduction in overall total protein yield before and after depletion, as demonstrated by gel electrophoresis, suggests the possibility that some cytokines may have been lost during this step. This will be further discussed in section 6.4.5.1 below. Additionally, DIA was performed with 1D LC–MS technique and has its own limitations. While DIA often provides greater depth than 1D DDA LC-MS, 2D LC–MS can better handle interferences by adding a second, orthogonal separation that helps resolve co-eluting species. Nevertheless, my pre-analysis evaluation indicated that DIA with 1D LC-MS was the most suitable option for this study, even compared with 2D LC–MS.

6.4.5.1 Protein Losses During Albumin Depletion

Depletion of high abundance proteins can cause co-depletion of non-target proteins, reducing the overall proteome coverage or biasing the sample[809]. This is an

ongoing issue in proteomic analysis. Whilst my albumin depletion strategy showed similar results between kits from two independent manufacturers, they both used Cibacron Blue dye-agarose resin which may cross react with other proteins leading to non-albumin proteins being removed [811]. However, other protein depletion strategies can still result in unintentional protein loss: a study comparing a multiple affinity protein removal system with a relative protein enrichment strategy also showed retention of proteins of potential interest discarded in the "waste" fractions of both methods[499].

This problem with current approaches was demonstrated in my samples with the post depletion protein concentration being less than would be expected based on pre depletion measurement of albumin. This loss in non-albumin proteins was also confirmed on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Figure 13). As discussed in Chapter 2, CSF is a challenging biological fluid to analyse using mass spectrometry because of the low total protein concentration and large dynamic range of individual proteins. Depletion of high abundance proteins to improve detection low abundance proteins is widely employed in mass spectrometry analysis[504]. The benefit of albumin depletion is demonstrated in Chapter 3, where the number of proteins groups identified using mass spectrometry was much higher after albumin depletion, despite evidence of some post-depletion losses on gel electrophoresis.

Unfortunately, in this study, the evidence of other protein losses on gel electrophoresis was in my study was only noted after all participant samples had been analysed. While it is clear that there were non-specific losses of proteins other than albumin, it is not clear whether this was a proportional loss of all protein classes, which may not have had a large impact on protein detection, or whether some proteins were disproportionately affected. However, overall, with the increased numbers of protein

peptides detected, while this depletion step does introduce uncertainty, it would seem appropriate for our aim of discovering protein differences. While beyond the scope of this thesis, these changes can be validated with a targeted proteomic approach to measure the protein of interest's differential concentration in samples

6.4.5.3 Issues with Independent Analysis

Findings from my Spectronaut analysis could not be replicated in independent statistical analysis. An in-depth discussion about the potential reasons for this discrepancy requires detailed, expert statistical knowledge and is beyond the scope of this thesis. However, this discrepancy in analysis results raises concerns about the validity of results, especially given the number of candidate proteins raised by Spectronaut.

Nevertheless, Spectronaut is a well-established software package widely used in DIA mass spectrometry and is highly regarded compared to other software packages available[812]. While the results of this study should be interpreted with caution, it will be important to determine whether they can be replicated in a larger cohort or with alternative detection methods. Such validation would confirm whether the identified biomarkers and pathogenic pathways are truly of interest and warrant further study.

6.4.5.2 Pre-Analytical Issues

6.4.5.2.1 Potential Blood Contamination

One major issue in my study is that of potential CSF contamination: by blood, fluids and other body tissues that may affect the outcome of results. Mass spectrometry is a highly sensitive analytical method and contaminants that may not interfere with standard diagnostic assays may interfere or produce significant results.

For example, even after removal of samples that would likely have been affected by blood contamination, these blood-contamination associated proteins remained elevated when stratified by differing collection methods (radiology assisted verses unassisted lumbar puncture). This may also be due to differing transport times associated with different collection methods that may have led to red cell lysis and failure to detect significant contamination. This stratification also indicated the presence of protein related to hair, muscle and even saliva which should not be normally present in CSF.

Hence, as discussed above, contamination risks are likely related to pre-analytical variability due to differences in clinical settings and transport logistics, as well as contamination during sample processing. Moving forward, in addition to meticulous adherence to precautions in sample preparation, approaches to sample collection and processing should be standardise as much as possible. This is further discussed in the sections below.

6.4.5.2.2 Operator Factors and Potential for Contamination

In the Australian public hospital system, lumbar punctures are usually performed by medical staff at the bedside in the emergency department, on the ward or (if an outpatient) in the day surgery unit under aseptic technique but unguided by any imaging modalities. Where lumbar puncture is anticipated to be difficult because of anatomical variation (such as severe scoliosis or very large body habitus), or when multiple bedside attempts at lumbar puncture have failed then patients are referred to radiology for fluoroscopy guided lumbar puncture. Patients who have spinal anaesthesia likewise have the procedure at the bedside, usually not guided by imaging.

In the outpatient setting, patients can be directly referred to private radiology for lumbar puncture. The advantage of this is that with radiological guidance it is easier to insert the needle into the correct anatomical space, without multiple attempts for successful lumbar puncture. This is not available routinely in the public hospital setting however, because of limitations on resources or dictated by the urgency of the procedure (such as emergency presentation with an encephalitis like phenotype),

A “blind” bedside lumbar puncture, with variable operator experience has increased risk of introducing contaminants as CSF is collected. This is because without being able to visualise the path the needle is tracking, the tip may pass through surrounding structures including small blood vessels, muscle and perhaps even peripheral nerve tissue. This risk is increased the more failed attempts occur before the lumbar puncture is successful: it is not unusual for some practitioners to take several passes with the same needle before successful sample collection.

Most of my AE cohort had their CSF collected at the beside because of the urgency of diagnostic need. Hence, when it appeared that samples in the AE cohort were disproportionately affected by proteins related to blood and coagulation, my hypothesis was that this was due to the method of collection. In contrast, many of my psychiatric cohort had their CSF collected at a private imaging facility, under Computed Tomography(CT) guidance, because of patient anxiety about the procedure and their preference for the procedure to be successful the first time. While the likelihood for blood contamination is low in this setting, the transport time to the laboratory may have increased red cell lysis, reducing the detection of blood contamination and perhaps introducing a different profile of proteins related to released red cell content.

6.4.5.1.3 Needle Gauge size

The gauge or needle bore size may also increase risk or decrease risk of contamination as a larger bore may have increased risk of passing through and retaining components of surrounding tissue structures and introducing contaminants into the subarachnoid space or collected CSF. In both the public hospital ward, emergency department and day surgery setting as well as in the private imaging setting, the standard lumbar puncture needle size is 22-gauge. However, in the operating suite for spinal anaesthesia the needle size used is 28 gauge. Hence, there may be less risk of contamination for a subset of my NI controls (whose samples were collected whilst undergoing spinal anaesthesia with a small gauge needle) compared to the rest of the study cohort. This may reflect why keratin was increased in the psychiatric cohort (where the 22-gauge needle was used) compared to NI (where part of the cohort was collected in the operating theatre where the 28 gauge needle was used).

6.4.5.1.4 Transport Factors and Specimen Processing

Examination of CSF red cell count did reveal that a disproportionate number of patients in the AE cohort had potential blood contamination. However, after removal of these patients, haemoglobin-related protein still appeared as candidate proteins in the analysis suggesting a degree of blood contamination that is not easily detected by mere cell count and may be related to red cell lysis in the time between transport and processing for analysis.

Haemoglobin associated proteins were increased the NI cohort (who underwent lumbar puncture as a “blind” bedside procedure) when compared to the psychiatric cohort (who had their lumbar punctures performed under imaging guidance). However,

collection at an external private imaging setting also poses potential challenges for blood contamination to go undetected. This is because there is possibility for any cells collected lysing due to increased time to sample processing.

In the public hospital setting the laboratory is on site and can immediately process the samples after collection. Samples collected in a private imaging facility, however, need to be transported back to the hospital laboratory for processing where they are subsequently centrifuged and separated. This may involve samples waiting several hours for routine transport which may occur a few times a day. During this period, even samples containing very low numbers of red blood cells may undergo haemolysis As discussed, it has been demonstrated that red cell lysis can increase the haemoglobin content of a CSF sample[673]. This is a more difficult situation to correct, for as red cell count for the sample may fall below the detection threshold due to haemolysis, despite the presence of haemoglobin and other blood associated proteins in the sample.

Finally, in significant transport delays, there may be decline in white cell counts in the CSF as indicated by our flow cytometry experience, reducing the CSF white cell count (WCC), a key diagnostic criterion for the diagnosis of AE and other inflammatory CNS conditions.

Outside of blood contamination, there were other possible contaminants identified in our results including skin and saliva related proteins[502]. The possibility of introduction of these contaminants into the sample due to a “blind” lumbar puncture procedure and needle gauge size has been discussed in the immediate previous 2 sections. In this study, we used usual diagnostic practices including standard specimen

processing practices in the diagnostic laboratory. Sample processing is another area where contaminants could have been introduced including in producing sample aliquots for use in different diagnostic tests or for storage. Here proteins from the operator (e.g. hair, skin or saliva) or laboratory environment could potentially contaminate the sample and this has been reported in other CSF mass spectrometric analysis[502,813]. This is especially a problem in discovery proteomics such as in this study because the whole proteome is sampled for potential biomarkers.

To minimise risk of contamination, the samples arriving at the laboratory should be marked as requiring specialised processing steps. Precautionary practices such as processing samples in a dedicated clean workspace, use of a laboratory coat, nitrile gloves, hair nets and face masks by the operator, processing samples in laminar flow hoods and making sure pipette tips used for processing are stored in boxes kept closed when not in use, as well as use of low-binding plastics and glassware and frequent equipment cleaning will help reduced the risk of contamination by the operator or laboratory environment. Discussion with the receiving laboratory for CSF samples to have these practices in place before samples are collected may help prevent sample contamination in future studies.[813,814].

6.4.5.1.6 Considerations for Future Studies

In planning for future qualitative/quantitative mass spectrometry studies, controls for the above factors (as much as possible) should be made. This can be challenging for patients who are presenting in an emergency setting, where diagnostic lumbar puncture is to be prioritised for patient care but consideration of needle bore size and experience of the proceduralist (a junior doctor may have less experience or be

less likely to be successful on first attempt than a senior doctor who is familiar with the procedure) needs to be made. Red blood cell count data should also be entered at time of collection to help with decisions about whether the sample collected is able to be entered into the study or too potentially contaminated by blood.

A decision could be made that all patients either have (as much as possible) have radiological guidance for the lumbar punctures (if the radiology department agrees to be involved), or all patients undergo lumbar punctures at the bedside to better reduce skewing of the data because of contamination. Protocols could be put into place regarding these so collectors are aware of the correct procedure and standardised needle gauges before specimen collection takes place. Transport for a sample could also be made uniform to prevent issues with red cell lysis. The receiving laboratory should have a set protocol in place to minimise any potential sample contamination from the operator.

However, this would still need to depend on the clinical urgency of the procedure, available radiological resources and technical difficulties that may prevent a successful “blind” lumbar puncture. For instance, image guided lumbar puncture appointments may be difficult to access in a busy public hospital system with competing patient demands for resources.

If a particular biomarker has been identified as important, quantitative mass spectrometry can be used as a diagnostic assay to ascertain if this is present or not. Whilst in this approach there is less chance of recognising irrelevant contaminant proteins as possibly significant, levels of the biomarker may still be affected by blood contamination if it is highly available in blood. Hence, keeping contamination risk in

mind, protocols should be established if designing such a diagnostic assay to control for risk of procedural failure and size of needle used. The analysis of matching serum may also assist in some of the above issues in CSF proteomics.

6.4.5.3 Other Study Limitations

This study examines a heterogenous cohorts with small numbers of patients in each study group including different age ranges across each studied cohort. It would have been important to examine the impact of age on proteomic expression using multiple regression or other advanced statistical methods, but this cannot be performed using only Spectronaut. Thus, there is a risk that data may be arbitrary rather than representative of the general populations these cohorts arise from. However, the inclusion of broadly diverse clinical phenotypes into a single group may identify common pathogenic themes. Additionally, this is a single centre study and inherently, prone to bias in recruitment methods. Further study in an expanded population using a multicentre approach will help validate these findings.

6.5 Conclusion

These findings highlight the importance of pre-analytical considerations in biomarker discovery and potential assay development as well as scrutinising processing steps in mass spectrometry, particularly when using closed source software. However, despite the limitations of this study, there are some proteins and pathways highlighted that do warrant further study. Whilst the results have not identified one key biomarker that can be used for diagnosis, it does reinforce the hypothesis of immune system activity playing a role in difficult to control psychiatric disease. Further study in a larger cohort with data analysed independent of Spectronaut can further

validate these findings. Future studies could also compare patients with atypical psychiatric disease with those with more typical presentations to determine similarities and differences in biological pathways between these two groups of patients.

Chapter 7: Overview and Final Conclusions

Increasingly in medicine, with advancing diagnostics, it is recognised that the spectrum of clinical presentation of autoantibody disease and indeed other disease entities are expanding. As these presentations evolve it can be difficult to correlate clinical findings with investigation results. Therefore, ascribing autoantibody mediated disease, when the clinical presentation is atypical or when investigational results lack sensitivity or specificity is particularly challenging. This challenge extends to highly sensitive and specific tests if the clinical setting is not considered congruent with the result, particularly in rarer diseases, where disease prevalence leads to highly sensitive and specific test results having high negative predictive values but reduced positive predictive values

Against this background, I examined two recently defined conditions: autoimmune encephalitis (AE) and atypical psychiatric disease. In AE, I compared available biomarkers of neuroinflammation in antibody-positive and -negative patients, focusing on CSF analysis. In this context it is notable that even in antibody positive patients, currently available routine diagnostic testing can be negative[8,15]. This is more so in antibody negative patients with AE. Currently, patients in the context of both antibody positive and negative AE are diagnosed using clinical criteria, which often leads to significant diagnostic uncertainty if criteria are not fulfilled. To attempt to address this issue I examined CSF cytokines as a potential biomarker to assist with diagnosis with the finding that IP-10 and IL-21 may be able to differentiate infection from AE. However, this result needs to be replicated in a larger cohort.

One of the challenges in the study of CSF cytokine signatures is the range of discrepant results being reported in the literature. This may be due analytical characteristics of varied cytokine assays commercial kits which have differing sensitivities and lower limits of detection, with some cytokines having low CSF concentrations. Another challenge is the replication of results in larger cohorts as the incidence of AE is low, especially when confined to single centre studies. Further, the ethical concerns around collection of normal CSF significantly impairs the development and validation of CSF biomarkers of disease.

With the recognition of NMDAR autoantibody mediated encephalitis in 2008 and the subsequent recognition of antibody negative encephalitis, it was recognised that these conditions could present with predominantly psychiatric symptomatology. However, initial classifications required clear evidence of neurological involvement. Therefore, I extended my study to patients with atypical and difficult to treat psychiatric disease.

In my cohort, extensive investigation led to patients having their diagnosis revised and responded well to immune suppression when they previously were treatment resistant. This finding emphasises the importance of re-evaluation of patients with atypical or treatment resistant psychiatric disease when the routine treatment algorithm is not effective. Whilst it is most likely that the symptoms of various psychiatric diagnoses are contributed to by different pathological mechanisms, my findings identify a subset of patients where the dominant contributor to disease may be immune driven. This was characterised by positivity in both current markers of neuroinflammation and elevated CSF cytokines. However, the range of elevated

cytokines in the CSF was mixed, likely indicating diverse inflammatory pathways and by implication, differing cellular participants as being activated in these atypical psychiatric symptom complexes. Nevertheless, these findings point to potential value in applying the same investigations to a larger cohort of patients, with a trial of immune suppression in patients who have CSF findings indicating high immune activity to ascertain which biomarkers are the best value in identifying those who will respond to immunomodulation and whether treatment may be tailored to various patterns of immune activation based on CSF biomarker testing.

Proteomic analysis of AE and psychiatric cohorts identified inflammatory pathways for further exploration. A major challenge, however, was the discrepancy between results from Spectronaut, a proprietary tool, and independent statistical analysis. Despite multiple enquiries, the reasons for this discrepancy could not be obtained. In addition, preanalytical factors in CSF collection and processing were found to strongly influence results, underscoring the need for standardisation if such workflows are to be translated into routine diagnostics.

In conclusion, my studies identify potential biomarkers that may aid AE diagnosis, though validation in larger cohorts is required given conflicting reports in the literature. My studies have also shown that further exploration of the immune system involvement in atypical psychiatric disease is worthwhile: to assist with suggesting alternative diagnoses, and for consideration of other therapeutic options, particularly where standard treatments have failed. Finally, whilst proteomics offers promise for biomarker discovery and pathway mapping, the handling of biometric data resulting

from analysis remains a challenge. Furthermore, strict control of preanalytical factors is critical for generating reproducible and clinically meaningful results.

Chapter 8: References

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