Improving the diagnosis of encephalitis through analysis of the host transcriptome, proteome and metabolome.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Mark Alexander Ellul

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Table of Contents

Abstract

Background
Encephalitis (inflammation of the brain) is a devastating neurological condition. It is most often caused by either acute viral infection or an autoimmune process. These can be difficult to distinguish clinically and require very different treatments. Encephalitis can also be mimicked by other neurological or systemic disease states. Improved biomarkers to distinguish encephalitis from its mimics, and autoimmune from viral encephalitis, could improve diagnosis and expedite effective treatment. Previous studies have identified signatures based on small numbers of proteins but none have investigated proteome, transcriptome or metabolome approaches to diagnosis using unbiased methodology.

Aims
1. To establish whether multiomic methods can identify biomarkers to distinguish encephalitis from mimicking conditions, and autoimmune from viral encephalitis.
2. To investigate whether host responses in cerebrospinal fluid (CSF) and blood correlate with clinical outcome.
3. To explore how the pathogenesis of autoimmune and viral encephalitis differ from other mimicking conditions by pathway and network analysis.

Methods
1. Using microarray, I investigated gene expression patterns in blood of patients with encephalitis and mimicking conditions.
2. Using liquid chromatography/mass spectrometry, I explored the CSF proteome of patients with encephalitis and mimics, validated by two separate cohorts.
3. Using ^{1}H nuclear magnetic resonance (NMR) spectroscopy, I analysed metabolic profiles in CSF in patients with encephalitis and mimics.

Results
All three techniques revealed potential biomarkers to distinguish aetiological groups. Protein candidate biomarkers were the most effective and included adenosine deaminase 2 (ADA2) which distinguished encephalitis from mimics with a high degree of accuracy, including in patients without CSF pleocytosis. Encephalitis could also be distinguished from mimics by gene expression or metabolite profiles. Distinguishing viral from autoimmune encephalitis was more challenging, but could be achieved by combining small panels of genes or metabolites. Several markers correlated with clinical outcome in autoimmune and viral encephalitis, including proteins involved in leucocyte adhesion and cytokine signalling. Pathway analysis identified mRNA transcripts and protein networks which were enriched in encephalitis when compared with mimicking conditions, especially concerned with the innate and adaptive immune response and the complement and coagulation cascades.

Conclusions
Through analysis of transcriptome, proteome and metabolome, it was possible to identify candidate biomarkers to improve the aetiological diagnosis of encephalitis, to predict clinical outcome and to elucidate disease mechanisms. These findings now need to be validated through prospective clinical studies and should direct future mechanistic studies and the development of potential therapies.

Chapter 1. Introduction

Introduction to encephalitis

Definitions and general clinical features

Encephalitis means inflammation of the brain, deriving from the Greek *ἐγκέφαλος* meaning "within the head". The strictest pathological definition requires the presence of an infiltrate of inflammatory cells within the brain parenchyma, which can only be proven by examination of tissue from either a brain biopsy specimen or at post-mortem examination. However, in clinical practice this is usually not feasible, and brain biopsies are now seldom performed (although they may occasionally be necessary in diagnostically challenging cases) (Wong et al. 2010).

In practice, therefore, clinicians rely on other indicators for the presence of brain inflammation. Chiefly, there must be evidence of the clinical effect of brain inflammation in the form of a change in consciousness or behaviour, together with evidence from investigations (either from cerebrospinal fluid (CSF) testing, brain imaging or neurophysiology) that inflammation is the cause. Occasionally encephalitis can be diagnosed on less strict clinical evidence if a pathogen or immune process known to cause encephalitis is identified.

Encephalopathy is a term used to denote a change in conscious level or behaviour attributable to brain dysfunction. This may be caused by brain infection or inflammation,

but also by systemic disorders which have a secondary effect on the brain, particularly non-neurological infections, metabolic or toxic disorders (Erkkinen et al. 2019).

Meningoencephalitis is a pathological term which refers to parenchymal inflammation of the brain which is associated with meningeal inflammation. This pattern of inflammation is particularly associated with certain organisms, particularly bacteria and certain parasites, and can produce a clinical picture with features typically associated with both encephalitis and meningitis. Some pathological processes may also affect the spinal cord (encephalomyelitis).

Epidemiology

The incidence of encephalitis in Western countries is estimated to be between 0.7-13.8/100,000, although estimates vary widely owing to differences in case definitions, diagnostic procedures and population of study (Jmor et al. 2008; Granerod et al. 2013). Viral causes constitute the most common aetiologies in all settings, but the causative viruses vary widely between geographic distributions.

Epidemic viruses are generally more frequent in resource poor settings, in particular flaviviruses such as Japanese encephalitis virus, dengue and West Nile virus (Turtle et al. 2012). Epidemic causes may also affect the more developed world, for instance West Nile virus has been increasingly detected in the USA and Southern Europe. Over the last three years, I have been part of a team showing that Zika virus is associated with encephalitis in

South America and SARS-CoV-2 appears to be associated with encephalitis and encephalopathy globally (Turtle et al. 2012; Soares et al. 2016; Ellul, Benjamin, et al. 2020). Influenza has also been associated with cases of encephalitis and encephalopathy, particularly in children, and novel strains such as H1N1 were associated with cases of encephalitis (Goenka et al. 2014).

Sporadic causes of encephalitis are ubiquitous throughout the world, with herpes simplex viruses (HSV) and varicella zoster virus (VZV) the most common causative agents. HSV accounts for about 20% of identified causes of encephalitis in several studies in the UK and USA (Granerod et al. 2013; Steiner et al. 2007).

Determining the incidence of autoimmune encephalitis is challenging owing to significant differences in diagnostic approach, the rapid discovery of novel antibodies and the probability of substantial numbers of undiagnosed cases. However, autoimmune encephalitis is thought to account for at least 20% of cases of encephalitis according to prospective studies, therefore the UK overall might expect approximately 1,000 cases/year (Granerod, Ambrose, et al. 2010; Granerod et al. 2013).

Pathophysiology

Viral encephalitis

Viruses can invade the human host by a number of routes including via the respiratory or gastrointestinal tracts or the skin. From their point of entry, several of the important viral

causes of encephalitis initially infect cells of the peripheral nervous system, including HSV which invades sensory nerve endings (Cunningham et al. 2006). Many viruses spread via the blood, including human immunodeficiency virus (HIV). Most viruses then infect specific cells within the central nervous system (CNS), which may include neurons, glial cells, immune cells or epithelia. HSV can infect several cell types including neurons, glia and meninges (Esiri 1982). The tropism of viruses for different cell types relies on the characteristics of cell surface receptors, which are utilised by the virus to allow entry into cells.

The gross pathology of viral encephalitis normally shows oedema of the affected parts of the brain which progresses over the clinical course to tissue destruction, sometimes with haemorrhage (Hatanpaa et al. 2014). The cerebral ventricles may be compressed and cysts may form in affected tissue. Histopathologically, there is frequently a lymphocytic infiltrate (with varying involvement of neutrophils, which may be more prevalent early in the disease course). Lymphocytes access the parenchyma through the walls of parenchymal blood vessels, classically via postcapillary venules (Engelhardt 2006). Normally the distribution of inflammatory cells is predominantly around blood vessels (perivascular cuffing) and there may be evidence of white or grey matter necrosis. Microglial nodules (localised collections of microglia) may sometimes be seen. In some causes of encephalitis, including VZV, a necrotising or granulomatous vasculitis may occur.

The immune response to viral infection of the CNS is a key determinant of clinical outcome, not only in terms of the clearance of viral infection, but also because immune processes can result in neuronal destruction in their own right (Michael et al. 2015). In the setting of viral

infection, initial innate immune responses lead to increases in blood brain barrier permeability and permit the trafficking of leucocytes. This process is co-ordinated by the interplay of numerous cytokines and chemokines released by immune cells and cells of the brain parenchyma, as well as glycoproteins such as vascular cell adhesion molecule (VCAM) and intercellular adhesion molecules (ICAM) (Al-Obaidi et al. 2018). Innate immune responses, coordinated by neutrophils, macrophages, dendritic cells and natural killer cells, are key to the initial response to viral infection, since antigen-specific adaptive immune responses take hours to days to develop. An overzealous innate immune response appears to be related to poor outcome; in particular the abundance of interleukin (IL)-1α and IL-1β in CSF, the abundance of myeloperoxidase, a marker of neutrophil activity, and changes in the C-X-C motif ligand (CXCL)1- CXCR2 axis, have been linked to outcome in human samples and animal models in HSV encephalitis (Michael et al. 2020; Michael et al. 2015).

The generation of an adaptive immune response requires presentation of antigen to T cell receptors via peptide presented in the antigen binding groove of a major histocompatibility complex (MHC) molecule in the presence of appropriate co-stimulation. Both cell-mediated and humoral arms of the adaptive immune system then play a role in the clearance of viral CNS infection, and may contribute to tissue destruction (Mancini et al. 2018).

Autoimmune encephalitis

The immune system evolved to defend against invading pathogens, and complex checks and balances exist to ensure that tolerance is exhibited towards self antigens. Important mechanisms of T cell tolerance include the clonal deletion of T cells in the thymus and periphery, T cell anergy in response to encountering antigen in the periphery in the absence of appropriate co-stimulation, and the sequestering of antigen in immunologically privileged sites (Zheng et al. 1994; Kraig et al. 1996; Johnson et al. 1994). B cell self-tolerance is regulated by maturational arrest of autoreactive B cells in the bone marrow (Schuurman et al. 1992), receptor editing, and apoptosis of autoreactive B cells within germinal centres (Cyster et al. 1994).

The CNS parenchyma has previously been considered to be immunologically privileged, however it is now understood that there is a complex dynamic of trafficking and surveillance by leucocytes across the blood brain barrier in health (Gibson et al. 2004) and that the CNS has a functioning lymphatic system that can drain antigen to cervical lymph nodes (Engelhardt et al. 2016). The development of autoimmunity against CNS antigens is incompletely understood, but in the case of most autoimmune encephalitis syndromes there appears to be failure of B cell tolerance, leading to antibody secreted cells (ASCs) producing antibodies directed against a single neuronal target, with pathogenic potential. Indeed, circulating B cells with specificity for a certain neuronal antigen have been detected in autoimmune encephalitis (Ramberger et al. 2020). The mechanism by which peripheral immune cells gain access to CNS antigen is unclear, although two scenarios relating to N-methyl-D-aspartate receptor (NMDAR) antibody encephalitis have the potential to

illuminate pathophysiology. Firstly, in the case of young women with ovarian teratoma who develop NMDAR antibody encephalitis, the NR1 subunit of the NMDA receptor is expressed on tumour cells, and tertiary lymphoid structures have been identified in association with teratoma cells (Tüzün et al. 2009). Cultured B cells from teratomas in patients with NMDAR antibody encephalitis are capable of secreting NR1 specific IgG *in vitro* (Makuch et al. 2018). Secondly, we and others have described cases in which HSV encephalitis has been followed, after several weeks, by a secondary NMDAR antibody encephalitis, implying that the inflammatory environment and exposure of self antigen in the setting of viral infection is capable of inducing breakage of immune tolerance (Armangue et al. 2018; Ellul et al. 2016).

Causes of encephalitis

Viral encephalitis

The likelihood of infection with individual viruses associated with encephalitis depends on host factors including age and the presence of immunocompromise, as well as social or environmental influences, including the local prevalence of disease, recent foreign travel, seasonality and behavioural risk factors (Solomon et al. 2011). Worldwide, the most common causes of encephalitis are arboviruses, in particular flaviviruses such as Japanese encephalitis virus (Griffiths et al. 2014). However, in this chapter I will focus on the most

frequently encountered viral causes of encephalitis in the UK which make up the predominant cases in the clinical cohort of this thesis.

HSV encephalitis

HSV types 1 and 2 are large deoxyribonucleic acid (DNA) viruses which occur worldwide (Whitley et al. 1998). HSV type 1 is associated with orofacial lesions (cold sores) and genital herpes, while HSV type 2 causes genital herpes and aseptic meningitis. Both viruses can cause encephalitis, although HSV type 1 is responsible for about 90% of cases (Aurelius et al. 1993). HSV type 2 encephalitis is more common in immunosuppressed patients. HSV type 1 encephalitis is thought to result from primary infections in around one third of patients, while the remainder represent reactivation of latent virus (Nahmias et al. 1982).

Around one third of the global population is thought to have clinically active HSV infection (Whitley 2009), however only a very small proportion develop CNS complications. The age distribution of HSV encephalitis is bimodal, with highest incidence in the (predominantly neonates and infants) and the elderly (Tyler 2004).

The main route of entry of HSV to the nervous system is via retrograde axonal transport along sensory fibres (Roizman et al. 2007). The virus can remain latent in sensory ganglia which act as a reservoir, resulting in a lifelong risk of reactivation. The route of access of the virus to the CNS is less clear, although it is postulated that invasion of the cranial nerves, either olfactory or trigeminal, may provide a route of infection (Tyler 2004).

HSV encephalitis characteristically causes inflammation and necrosis in the temporal and frontal lobes, usually bilaterally although often asymmetrically. Occasionally brainstem encephalitis can occur. Clinically, patients present with an acute syndrome (usually over a few days to a week) of headache, fever and change in conscious level, often with seizures and focal neurological signs, reflecting the area of inflamed brain (Aurelius et al. 1993).

If untreated, mortality from HSV encephalitis is around 70%. Treatment with intravenous aciclovir has reduced mortality to around 20% (Skoldenberg et al. 1984; Whitley et al. 1977). However, neurocognitive deficits are very common amongst survivors, particularly in memory and executive function (Harris et al. 2020).

VZV encephalitis

Primary infection with VZV causes chickenpox and infection occurs in around 99% of the population (Kilgore et al. 2003). During primary infection the virus establishes latency in neurons, and can reactivate in older age or with immunosuppression to cause shingles, characterised by dermatomal rash and pain. Encephalitis, meningitis or cerebellitis may occur with either primary zoster infection or reactivation. Infection of the CNS is associated with haemorrhagic inflammation, and demyelination may also occur. VZV may also infect the cerebral arteries causing a vasculopathy, and this is reflected in an increased risk of stroke following VZV infection (Kang et al. 2009).

Other viral causes of encephalitis

Human enteroviruses are ribonucleic acid (RNA) viruses of the picornavirus family. Enteroviruses are predominantly spread by the faeco-oral route and the virus replicates in the gastrointestinal tract. Enteroviruses occur in seasonal epidemics, although sporadic cases may also occur. Young children primarily harbour and transmit the viruses, although older adults are vulnerable to more severe clinical manifestations (Moore 1982). Encephalitis associated with enteroviruses is predominantly caused by coxsackieviruses, echoviruses and enterovirus (EV)-71 (Horstmann et al. 1968).

Adenoviruses generally cause infection of the upper respiratory tract or gastrointestinal tract but may rarely involve the CNS, particularly in immunocompromised patients (Rumboldt 2008).

HIV has a wide spectrum of associations with nervous system disease. It may cause an encephalitis or meningoencephalitis at the time of primary infection (Valcour et al. 2012), but with chronic infection can result in HIV-associated neurocognitive impairment (HAND), of which the most severe clinical manifestation is HIV-associated dementia (HAD). However, of greater importance worldwide is the propensity to opportunistic infection resulting from immunosuppression with HIV infection.

Autoimmune encephalitis

In recent years, autoimmunity has been increasingly recognised as an important cause of encephalitis. Many different antibodies are now known to target antigens on the neuronal surface, and some of these are associated with characteristic clinical presentations, although seronegative cases are also recognised. Autoimmune encephalitis may mimic other conditions, including primary psychiatric disorders, particularly early in the disease. Because early immune treatment of autoimmune encephalitis improves patient outcomes, and indeed many make a good recovery, it is important to recognise these syndromes promptly.

Many patients with autoimmune encephalitis have the capacity to make a good recovery if treated appropriately, despite a stormy disease course. Therefore, it is important to have a high index of suspicion to recognise cases early.

<u>Clinical syndromes in autoimmune encephalitis</u>

The spectrum of clinical presentations associated with autoimmune encephalitis is wide and partly reflects the antibody involved (Table 1). The most well characterised syndromes are associated with antibodies directed against neuronal surface antigen, and these antibodies are generally considered to be directly pathogenic. Antibodies associated with intracellular antigens are often paraneoplastic and immune therapy responses may be limited.

Table 1. Key features of syndromes associated with neuronal surface antibodies (modified from Ellul et al. 2020 (Ellul, Wood, et al. 2020))

Antibody	Demographics		Key distinguishing features	Tumour associations
	Age predominance	F:M ratio		
NMDAR	Children Adults <40 years	4:1	Psychiatric features followed by movement disorder (classically orofacial dyskinesia), seizures, encephalopathy and autonomic dysfunction	About 50% of female patients have ovarian teratoma. Other tumour associations have been described .
LGI1	Older adults	1:2	Faciobrachial dystonic seizures, amnesia, hyponatraemia	<10% Breast, lymphoma, thymoma, thyroid
CASPR2	Older adults	1:9	Sleep disorders (insomnia, sleep-wake cycle disturbance), peripheral nerve hyperexcitability, ataxia	<5%, various tumours
$GABA_AR$	Children or adults	1:1	Seizures	Thymoma in 30%
$GABA_BR$	Older adults	1:1.5	Seizures, amnesia	SCLC in 50%
AMPAR	Older adults	2:1	Amnesia	65% Breast, SCLC, thymoma
DPPX	Adults	1:2	Severe diarrhoea and weight loss, myoclonus, hyperekplexia	<10% Lymphoma

NMDAR = N-methyl-D-aspartate receptor; LGI1 = leucine rich glioma inactived-1; CASPR2 = contactin-associated protein-like 2; $GABA_AR$ = γ-aminobutyric acid type A receptor; $GABA_BR$ = γ-aminobutyric acid type B receptor; AMPAR = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; DPPX = dipeptidyl-peptidase-like protein 6; SCLC = small cell lung cancer.

The most recognisable clinical syndromes are limbic encephalitis and NMDAR antibody encephalitis. Limbic encephalitis, reflecting inflammation of the medial temporal lobe, may present with changes in behaviour, seizures or memory difficulties. Two of the most common and well-characterised antibodies associated with limbic encephalitis are those directed against leucine-rich glioma-inactivated 1 (LGI1) and contactin-associated protein-

like 2 (CASPR2). Autoimmune encephalitis associated with LGI1 antibodies generally affects older patients and is unusual in those less than 40 years old. LGI1 antibodies may be associated with faciobrachial dystonic seizures: brief jerking movements affecting ipsilateral arm and face, which are highly specific to LGI1 and may precede the onset of encephalitis, but may not be present in many (Irani et al. 2011). Hyponatraemia is also a common finding (O'Sullivan et al. 2016). CASPR2 antibody encephalitis can present with a crossover of central and peripheral nervous system features including memory difficulties, sleep disturbances and peripheral nerve hyperexcitability syndromes. Testing for voltage gated potassium channel (VGKC)-complex antibodies should now be abandoned in favour of testing for LGI1 and CASPR2 antibodies, which target proteins associated with the ion channel. VGKC positivity in the absence of LGI1 or CASPR2 antibodies has been reported in a heterogenous group of clinical syndromes, but is not thought to be a true marker of disease (van Sonderen et al. 2016).

NMDAR antibody encephalitis most often affects children and younger adults, has a 4:1 female predominance and is associated with ovarian teratoma in around half of females. The disorder begins with abnormal behaviour, often with psychotic or affective features, developing by 1 month into a characteristic clinical picture. This may include seizures, movement disorder (classically orofacial dyskinesia, but limb dyskinesia/chorea is often predominant), reduced consciousness level and autonomic dysfunction (particularly bradycardia/tachycardia, hyperthermia and fluctuating blood pressure) (Titulaer et al. 2013). As described above, NMDAR antibody encephalitis can occasionally be triggered by HSV encephalitis: in a recent prospective study of 51 patients with HSV encephalitis, 14 developed secondary autoimmune encephalitis associated with neuronal antibodies,

although 3 also developed NMDAR antibodies in the absence of clinical evidence of encephalitis (Armangue et al. 2018). Other, more recently described syndromes are associated with antibodies against γ-aminobutyric acid receptors (GABAR) type A or B, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and dipeptidyl-peptidase-like protein 6 (DPPX) (Table 1).

Acute disseminated encephalomyelitis (ADEM) is characterised by the presence of widely scattered foci of inflammation and demyelination and is most frequently associated with antecedent infection (Pohl et al. 2016). ADEM occurs most frequently in children and is generally considered to be monophasic, although recurrent variants are also described. Varicella, rubella, mumps, influenza and mycoplasma are all frequently associated with the syndrome, and a minority of cases follow vaccination. Recently a proportion of cases have been associated with myelin oligodendrocyte glycoprotein (MOG) antibodies, and these patients have a significant risk of relapse (Santoro et al. 2019; Jurynczyk, Messina, et al. 2017). Clinical features of ADEM are protean, but often onset is rapid and associated with fever. Seizures are common and focal signs depend on the distribution of inflammation and may include hemiparesis, sensory loss or brainstem features.

Hashimoto's encephalopathy is a controversial entity in which patients, predominantly female, develop encephalopathy sometimes in association with seizures or other clinical signs, in association with anti-thyroid antibodies (Schiess et al. 2008). Although the pathophysiology of this condition is unclear, and authorities differ on whether it represents a true autoimmune encephalitis, in patients with no other identified cause steroids are often administered, which have been associated with favourable outcome. The antibodies

themselves, usually directed against thyroid peroxidase (anti-TPO) or thyroglobulin, are generally considered to represent an epiphenomenon rather than being truly pathogenic (Graus et al. 2016).

Syndromes associated with antibodies against intracellular antigens are more commonly paraneoplastic. Glutamic acid decarboxylase (GAD) antibodies are associated predominantly with stiff-person syndrome and cerebellar ataxia but are also reported in association with limbic encephalitis, in which around 25% of patients have an underlying malignancy, most commonly thymoma or small cell lung carcinoma (Malter et al. 2010). Other paraneoplastic antibodies include anti-Hu (ANNA1) and anti-Ma2, both of which cause a limbic encephalitis syndrome which is usually poorly responsive to immunotherapy, and in which an underlying cancer is almost universal (usually small cell lung cancer and testicular seminoma respectively) (Alamowitch et al. 1997; Dalmau et al. 2004).

Formes frustes within these classical syndromes may also occur, for example some patients with incipient NMDAR antibody encephalitis may present with first episode psychosis or prominent movement disorder.

Diagnosis of encephalitis in current clinical practice

Confusion is a common presentation to the acute medical unit and has a wide differential diagnosis. The initial clinical challenge is distinguishing causes of encephalopathy, including septic, metabolic, toxic and others, from patients who have encephalitis and therefore need

specific treatments. Initial history should identify clues as to possible causes, including a full collateral history if available, in order to ascertain the true duration of the problem. Evidence should be sought of a change in personality or behaviour, or periods of drowsiness or seizures (which may be subtle). A travel history should be obtained, including any contact with animals, fresh water, mosquito or tick bites, or exposure to illnesses in the community. Known immunocompromise or risk factors for HIV infection should be established. Examination should establish conscious level, any focal neurological deficit, seizure activity or movement disorder.

Patients presenting with symptoms suggestive of encephalitis should be admitted urgently and undergo lumbar puncture and neuroimaging. The main concern at the outset is to exclude an infectious cause such as HSV, which can cause rapidly progressive brain oedema, necrosis and death. Aciclovir treatment in HSV encephalitis is a life-saving intervention.

The key to establishing evidence of CNS inflammation is the analysis of CSF. In viral encephalitis there is typically a CSF pleocytosis (greater than 5 white cells x 10^9/L), comprising predominantly lymphocytes. However, early in the illness neutrophils can predominate, or occasionally the white cell count (WCC) may be normal (Saraya et al. 2016). Protein is normal to moderately raised and glucose is normal. Polymerase chain reaction (PCR) for the most frequent viral causes should yield results within 24–48 hours from most laboratories. CSF PCR for HSV has a sensitivity and specificity of over 95% for HSV encephalitis in immunocompetent adults (Cinque et al. 1996). In some cases where CSF is obtained very early in the disease course, PCR for HSV can be falsely negative. Therefore, if

clinical suspicion of HSV remains, lumbar puncture should be repeated and will often be positive, despite aciclovir treatment (Weil et al. 2002).

Magnetic resonance imaging (MRI) is the gold standard technique for brain imaging in encephalitis, (Bertrand et al. 2017) and is abnormal in 90% of cases of HSV encephalitis (Granerod, Ambrose, et al. 2010). Electroencephalography (EEG) is useful in identifying and monitoring seizure activity, but is non-specific and can be abnormal in a number of other causes of encephalopathy.

All patients with suspected brain infection should have an HIV test. Meningoencephalitis can occur at the time of HIV seroconversion (del Saz et al. 2008) and immunocompromise vastly alters the differential diagnosis. Since HIV serology may be negative at seroconversion, if there is clinical suspicion HIV RNA testing should be requested (Palfreeman et al. 2009).

The role of brain biopsy in the diagnosis of encephalitis has declined since the advent of PCR testing in CSF and it does not form part of the initial assessment. However, it still has a place for patients in whom diagnosis has not been obtained following extensive investigation, particularly if there are focal abnormalities on imaging (Wong et al. 2010).

If tests for an infective cause are negative, or if patients present with a recognisable phenotype of autoimmune encephalitis at the outset, then autoantibody testing should be considered (Graus et al. 2016).

Although autoimmune encephalitis is now usually a positive diagnosis, other mimics of AE should be excluded, including primary CNS lymphoma, neurosarcoidosis, CNS vasculitis, tumours, genetic epilepsy syndromes, mitochondrial encephalomyopathies, and prion disease.

CSF findings are often abnormal in autoimmune encephalitis, with a mild lymphocytic pleocytosis and/or moderately raised CSF protein. However, a completely normal CSF occurs in up to a third of patients and does not exclude the diagnosis (Dubey et al. 2018).

Neuroimaging with MRI in autoimmune encephalitis is essential, although this may also be normal in up to a third of cases (Baumgartner et al. 2013). In general, MRI findings are nonspecific although medial temporal lobe signal change frequently occurs in limbic encephalitis.

EEG in autoimmune encephalitis often reveals encephalopathic changes which can support the diagnosis but are nonspecific. Caution is required as psychiatric and antiepileptic drugs can cause similar features (Venkatesan et al. 2019). EEG is helpful in diagnosing non-convulsive status epilepticus, or in distinguishing seizures (e.g. epilepsia partialis continua (EPC)) from a movement disorder. NMDAR antibody encephalitis may be associated with characteristic extreme delta brush pattern.

Several antibody-associated encephalitides, particularly NMDAR antibody encephalitis, can present similarly to primary psychiatric illness. “Red flag” early features for autoimmune

encephalitis include an infectious prodrome, rapid progression, movement disorder, focal neurological signs, seizures or unexplained hyponatraemia (Pollak et al. 2020).

Antibody testing for encephalitis is a rapidly evolving area of neuroimmunology. Certain autoimmune encephalitides, particularly those associated with NMDAR antibody and LGI1 antibodies, present with recognisable clinical syndromes and diagnostic suspicion may already be high so that antibody testing can be targeted. Panels of immunofluorescence tests have been developed commercially and their role is still evolving, considering the balance between increased chance of detecting a pathogenic antibody versus the potential for false positives. Antibodies have hitherto mostly been tested in serum. However, intrathecal antibody synthesis occurs in most forms of autoimmune encephalitis and, particularly for NMDAR antibody antibodies, CSF testing is more sensitive and specific than serum and should be undertaken where clinical suspicion is high and in challenging cases.

Consensus criteria also allow for a diagnosis of seronegative autoimmune encephalitis, based on clinical features in the absence of antibodies which may respond to immune therapy (Graus et al. 2016; Graus et al. 2018).

Because of the paraneoplastic associations of several antibodies, investigation for malignancy is crucial. Computed tomography of the chest, abdomen, and pelvis is indicated in all patients with suspected autoimmune encephalitis. For those with particular associations, such as $GABA_BR$, positron emission tomography scanning is advised and, if negative, should be repeated at 3-6 months (Titulaer et al. 2011). Additionally, in NMDAR

antibody encephalitis, because of the association with teratoma, ultrasound of the ovaries or testes should be performed.

Management of Encephalitis

Management of viral encephalitis

Intravenous aciclovir is a life-saving treatment in HSV encephalitis and has reduced mortality from above 70% to around 10– 20% (Skoldenberg et al. 1984; Whitley et al. 1977). Aciclovir is also used in VZV encephalitis although evidence of efficacy is less well founded. It is relatively safe, although there is a small risk of renal impairment owing to a crystal nephropathy (Yarlagadda et al. 2008). Renal function should be monitored; in patients with known renal impairment the dose should be reduced.

Ideally, lumbar puncture should be performed immediately in patients with suspected brain infection, and empirical treatment started immediately thereafter. However, if lumbar puncture is delayed for more than 6 hours empirical aciclovir may be needed before lumbar puncture (Solomon et al. 2011). Patients with HSV encephalitis are likely to remain PCR positive in CSF for at least the first few days after commencing treatment, so lumbar puncture should still be performed as soon as possible in patients who have commenced aciclovir. This will help to establish the diagnosis and therefore dictate the duration of treatment. The UK guidelines recommend that aciclovir should be continued for at least 2 weeks, at which point the lumbar puncture should be repeated. If the HSV PCR is still positive, aciclovir should be continued with repeat lumbar puncture every week until the

PCR is negative (Solomon et al. 2011). If the patient is completely well, some would suggest that repeat CSF is not necessary (Stahl et al. 2017).

Corticosteroids have occasionally been used in HSV encephalitis, particularly where cerebral oedema is profound or clinical response to aciclovir treatment is not forthcoming. An open-label clinical trial of dexamethasone adjunctive therapy for HSV encephalitis is ongoing (NCT03084783). In VZV encephalitis short courses of corticosteroids are occasionally used, particularly when vasculopathy is suspected.

Currently specific treatments do not exist for other viral causes of encephalitis. Management is supportive, with pragmatic management of elevated intracranial pressure and intensive care support where indicated. Of patients with encephalitis, 50–60% have seizures in the acute phase, which may be clinically subtle (Glaser et al. 2003; Misra et al. 2008) and seizure control is a key aspect of management.

Management of autoimmune encephalitis

Evidence for optimal management of autoimmune encephalitis is largely based on retrospective studies, together with principles adapted from other antibody mediated diseases, such as myasthenia gravis. Patients with encephalitis should be managed at a centre with appropriate facilities and specialist expertise, ideally a regional neuroscience centre.

First line therapy, aimed at reducing antibody levels rapidly, normally comprises intravenous corticosteroids often in combination with intravenous immunoglobulin or plasma exchange. I am part of a team leading an upcoming trial of intravenous immunoglobulin or placebo (in combination with corticosteroids) for autoimmune encephalitis, due to begin recruiting soon, which should provide much-needed evidence for optimal first-line therapy. In those who fail to respond to first-line agents, more aggressive immune therapy is usually started, with concomitant higher risk of adverse effects, including cyclophosphamide or rituximab (Nosadini et al. 2015). In NMDAR antibody encephalitis other agents such as bortezomib have been used in a small number of refractory cases. Relapses may occur when immune therapy is reduced, or may reflect tumour recurrence or persistence of missed tumour (Dalmau et al. 2018).

Starting immune therapy early has been associated with improved outcome in NMDAR antibody encephalitis in retrospective series. Also, in those who fail to respond to first-line therapy, moving to more aggressive second line treatments is associated with better outcome and reduced rate of relapse (Titulaer et al. 2013). In patients with ovarian teratoma, tumour resection is associated with faster rate of recovery and reduced relapse rate.

LGI1 antibody encephalitis generally responds well to first-line treatment, particularly corticosteroids, and outcomes are generally good, however, in the long-term cognitive problems are common (Irani et al. 2014; Arino et al. 2016).

Patients with autoimmune encephalitis often require extensive supportive care, including prolonged intensive care unit stays. Issues may include managing seizures, autonomic instability, infective complications, and agitation. Antipsychotic medications may be required, but should be used with caution due to the risk of extrapyramidal side effects or neuroleptic malignant syndrome, which may be higher in NMDAR antibody encephalitis (Lejuste et al. 2016).

With appropriate treatment, outcome overall compares favourably with infectious encephalitis, with >80% of patients with NMDAR antibody encephalitis having no more than slight disability (modified Rankin Scale 2) at 2 years (Titulaer et al. 2013). However, evidence is emerging that subtle neurocognitive/psychosocial issues may be more prevalent than previously appreciated (Yeshokumar et al. 2017).

The care of patients with encephalitis is challenging for nursing staff. Patients often have physical, neuropsychological and communication difficulties which make interaction with their environment and their relatives demanding. Most patients with encephalitis are left with some degree of neuropsychological impairment (Raschilas et al. 2002) and the prevalence of attentional, behavioural and emotional disorders in survivors remains high up to 3 years after diagnosis (Mailles et al. 2012).

Encephalitis of unknown cause

Despite optimal investigation, the aetiology of encephalitis remains unknown in 20-30% of cases of encephalitis (Granerod, Ambrose, et al. 2010). In some of these cases, delayed investigation combined with the effect of empirical treatment may have led to an infectious cause being missed (Michael et al. 2010). Alternatively, the cause may have been omitted from testing, particularly in the case of unusual or imported organisms, or uncommon autoantibodies. Novel causes of encephalitis are continuously discovered, for example through metagenomic approaches (Wilson et al. 2019), or through the identification of antibodies against novel neuronal targets. It is therefore likely that a proportion of the unknown cause group may be amenable to diagnosis in the future as novel causes are elucidated. Alternatively, some of these cases may represent misdiagnosis, or encephalitis mimics. Even when a cause is identified, the time taken for investigations to be performed can lead to a delay in appropriate treatment, particularly if specialist tests for rarer antibodies or infectious agents are required.

The management of cases of encephalitis without an identified cause is challenging. In some cases clinical features may provide clues as to whether a viral or autoimmune cause is more likely, for example a more subacute onset without the presence of marked changes on neuroimaging may point to an autoimmune cause, particularly in the presence of movement disorder or psychiatric features. However, in many cases the clinical picture is not sufficiently clear to provide guidance.

Since the advent of aciclovir treatment, empirical administration of the drug has become commonplace in most cases of suspected neurological infection (Solomon et al. 2011), since adverse effects are relatively uncommon. However, for the last ten to fifteen years, awareness of autoimmune causes of encephalitis has led to the conundrum of whether immunosuppressive treatment should be administered in cases where the cause is unclear. Consensus guidelines for the diagnosis of autoimmune encephalitis support the treatment of seronegative cases (Graus et al. 2016). But the consequences of pursuing an immunosuppressive treatment strategy in the presence of an unidentified infection may theoretically be severe, especially if suppression of host immune defences lead to uncontrolled viral replication.

There is therefore a need for more effective biomarkers to differentiate encephalitis from mimicking conditions, and to differentiate viral from autoimmune encephalitis, in order to ensure that the correct treatment strategy is chosen and to improve outcome.

Encephalitis from the perspective of a patient and relative

To understand an illness fully it is essential for clinicians and scientists to look at it from the perspective of the patient and their loved ones. I give such an account here kindly provided by a patient supported by the Encephalitis Society, to which I have connections.

“E” and her husband “M” kindly agreed to reflect on their experiences and memories of surviving encephalitis, including the difficulties of living with a disease for which a cause was never found. They gave consent for the use of their story in this thesis.

Introduction

“E” developed change in conscious level and seizures following an infectious illness in the winter. She had a week of treatment on the ward for a presumed systemic infection before the clinical team discovered that she had developed right sided weakness, along with further seizures, and began to suspect that she may have encephalitis or meningitis. She had a lumbar puncture, which showed elevated protein and WCC. Her MRI brain scan showed multiple areas of abnormality consistent with inflammation in both cerebral hemispheres and the brainstem. By this time her conscious level had continued to fall and she required admission to intensive care and mechanical ventilation.

Tests for infectious causes, including CSF microscopy and culture and PCR for the common viral causes of encephalitis, were all negative, as were tests for autoantibodies associated with encephalitis. She was treated empirically with intravenous corticosteroids then a long oral taper. Slowly she began to improve and gradually regained mobility and speech. After a prolonged stay in hospital of several months she was discharged, although she continued to have occasional seizures at home for several months. In the longer term she has been left with double vision, ongoing neurocognitive difficulties and emotional lability.

E's story

"Last autumn, I felt like I was getting ill. I can remember telling my husband that I maybe needed some bed rest. That's the last thing I do remember about my health, until coming to many weeks later in hospital when my husband said, simply, that my crappy body had just been crappy again! I felt as though I woke up in hospital after some weeks, but my husband said I'd been awake on and off, but confused. Certainly I remember nothing before Christmas and into New Year. Then I remember thinking I'd been kidnapped in Lebanon. I speak no Arabic, but the former colonial language is still spoken a lot in Beirut so I tried French on the hospital ward! Of course, no-one understood why, nor was I able to communicate my fears. Once I tried to persuade my husband to pull out all the plugs, plunging the ward into darkness. I'd planned this all day, believing that this would bring some big-wigs to the ward who would realise the kidnappers now held sway. My husband refused to cooperate so I was very upset with him.

"I also remember thinking back to when, as a probation officer, I'd visited prisons regularly and learned a lot of bad behaviour and terrible language. I think this was in evidence as well, although no one has told me details. My proper wake-up was at the end of January. I then had to apologise to ward staff for treating them so badly and then start to cope with seeing double, half a memory and very bad balance. One day my husband gave me photos of the grandchildren and other important little ones. He talked me through their names but I was so upset that I didn't know this stuff, and desperate for my family not to realise. This still upsets me greatly in hindsight.

“One real difficulty was knowing who people were that came to my bedside when not in uniform. Doctor? Student? Orthoptist? Physio? Sometimes they were single, sometimes in a group: it’s frightening to be trying to talk sense when you have no memory of what’s being discussed, nor who the people are. One guy that I remember with real warmth was the man responsible for giving drinks, twice a day. I don’t know what I said to him to start with, but explained clearly that he had nothing to do with my medicines or my body, his job was to offer me drinks and clear up after. What a relief! He was no kidnapper; he said hello each time he passed and reminded me who he was if I needed it. He was so reliably steady, I have really good memories of him.

“Everyone else coming to my bedside seemed to know me but I remembered none of them, nor anything that they’d told me before. From more recent conversations, I think the neurologist thought I’d been resistant to hearing about auto-immune encephalitis at this stage. I simply have no memory but doubt I was resistant – any diagnosis through my lifetime of illnesses has been something to be welcomed, as it means you can learn about what’s wrong and what may help improve things.

“Moving on, it’s now a year since I came home from hospital. My double vision and poor balance remain a pain, plus my unreliable memory. My husband’s phrase about my crap body has simply behaved in a crap way again, has been the best explanation I’ve had. Its not a bad description of auto-immune illness! I can’t care for the grandchildren as we did

before, but fortunately my husband is now retired so he cares for the little ones and for me, very well indeed. Sometimes my brain and stamina are good, so I risk boredom - but the next day I can be tired out, and still have to cope with endless medicines plus their possible side-effects upon me."

M's story

"The first response from the 999 operator and then the paramedics was superb, and very reassuring. As was the initial assessment at the hospital. They didn't know what was going on, but they were clearly making every effort to find out. At this stage, E was conscious (though sleepy) and could still recognise and name me and the grandchildren. An attempted MRI scan on the Friday afternoon failed to produce any decent images because E couldn't keep still.

"Over that weekend nothing much happened other than continuous monitoring by nursing staff. Certainly and visibly E slipped further into unconsciousness. They kept popping in to do what turned out to be GCS tests. It turned out that having been scoring about 13 on the Friday, by Sunday afternoon, she was down to about 7. The upshot was that by Monday morning they had decided that they really needed an MRI scan, that they couldn't do one at the local hospital with a patient on a ventilator, so they were going to arrange a transfer to the specialist unit. The process took all day, but at least it was in hand.

“Everyone in the ICU was extremely supportive. The staff nurse phoned me at home first thing on Tuesday morning, and everyone I dealt with was great. There was still, of course, the question of exactly what was going on with E, but I felt that everyone was trying their best – and making sure they communicated with me. I felt confident they were doing all they could. They kept me informed about their thinking; and when the MRI scan showed definite areas of the brain affected, it began to pin things down. The consultants and their staff were very approachable and willing to share their thinking, but they were clearly unsure for some considerable time.

“Once we got into the New Year, she clearly began to make progress. She had the idea that she had been kidnapped in Lebanon, and one day asked me to unplug all the electrics so we could escape. When I refused, she looked at me and said, in a venomous tone: you are useless; you are pathetic; and you always have been. That was the point where I realised the real E was definitely still there!

“I don’t think they ever did clearly communicate a diagnosis. The word encephalitis had been used. They had ruled out viral or bacterial causes, but I think the first time I saw the official diagnosis of “auto-immune encephalitis” was on the discharge note. I don’t think we really understand why E’s system decides to go crazy from time to time. But it’s happened over the 40+ years we’ve been married, and I think we just accept that this is how it is.

“E is still not strong, and gets tired very easily. Her mobility is limited - not helped by the fact that she has been seeing double (when not wearing her glasses with the corrective prism). Mentally she is slowly developing more of her old intellectual curiosity and starting to read more than whodunits and thrillers. Her emotions – or expressions of emotion - like tears and laughter are a bit uncontrolled, also. Looking back 12 months she has made enormous progress, but she is still a woman with significant disability.”

Comments

E’s story highlighted several key problems in the diagnosis of encephalitis. Initially, she was treated for an infectious encephalopathy, and it was several days before CNS infection or inflammation were suspected and ruled out. Distinguishing between encephalopathy related to systemic disease and encephalitis can be challenging. Secondly, both her and M were left with no clear answer of what caused her illness. This uncertainty also contributed to a delay in the administration of appropriate treatment. Her condition would meet diagnostic criteria for possible autoimmune encephalitis, but without the clarity of a positive antibody in CSF or serum.

For me, E’s case illustrated the importance of improving diagnostics in encephalitis, both to distinguish encephalitis from mimicking conditions such as infectious encephalopathy, but also to distinguish autoimmune from viral encephalitis in order to expedite appropriate treatment and improve outcome.

Host immune responses in the diagnosis of encephalitis: existing literature

In order to improve the diagnosis of encephalitis, and to look for associations with outcome and clinical features, several small studies have investigated cytokines and chemokines as biomarkers.

Using host responses to distinguish viral from autoimmune encephalitis

Several authors have specifically examined host immune responses as a diagnostic tool to distinguish causes of encephalitis. Kimura et al. investigated levels of BAFF and APRIL in CSF in patients with autoimmune encephalitis, HSV encephalitis and bacterial meningoencephalitis (Kimura et al. 2015). BAFF and APRIL were significantly higher in HSV encephalitis than in autoimmune encephalitis.

Ygberg et al. investigated a panel of mediators in children with infective encephalitis (the majority of whom had tick-borne encephalitis virus), NMDA receptor antibody encephalitis and controls (Ygberg et al. 2016). They found significantly increased IL-6, IL-7, and IL-13 levels in infective encephalitis compared with both controls and autoimmune encephalitis patients.

Ichiyama et al. (Ichiyama et al. 2008) compared mediator profiles in patients with non-herpetic autoimmune limbic encephalitis with HSV encephalitis. The CSF concentrations of IFN-gamma and sTNFR1 levels of patients with HSE were significantly higher than those with autoimmune encephalitis.

Michael et al., using a cytometric bead array platform, found that serum and CSF myeloperoxidase (MPO) was able to identify correctly 91% of patients with infectious encephalitis as opposed to autoimmune encephalitis (Michael et al. 2016).

Host responses associated with outcome in encephalitis

Michael et al., using the same panel of mediators as above, investigated CSF and serum in patients with autoimmune, infectious and unknown cause encephalitis (Michael et al. 2015). They found that proinflammatory interleukin signalling was associated with an increased severity of illness, and specifically that a higher ratio of CSF IL-1 β to IL-1RA was associated with a worse outcome.

Kamei et al. (Kamei et al. 2009) assessed 6 inflammatory mediators in 56 serial CSFs taken from 20 adult HSV encephalitis patients. They found that IL-6 and IFN-gamma levels were elevated, and were associated with poor outcome.

Winter et al. investigated immune responses of 118 patients with Japanese encephalitis, finding that levels of interferon-alpha , IL-6 and IL-8 were higher in CSF of nonsurvivors than the survivors (Winter et al. 2004). In enterovirus-71 encephalitis, Griffiths et al. found that several markers including IL-1β, IL-1Ra, and G-CSF were raised significantly in patients who developed cardio-respiratory compromise, a serious complication of the condition (Griffiths et al. 2012). In addition, IL-1Ra and G-CSF were elevated in fatal cases. The G-CSF:IL-5 ratio was the most accurate prognostic marker for death.

Leypoldt et al. examined CXCL13 specifically in NMDAR antibody encephalitis (Leypoldt et al. 2014), since this chemokine had previously been investigated as a possible biomarker of multiple sclerosis. They found that CXCL13 was elevated in the majority of patients with NMDAR antibody encephalitis, and that marked or prolonged elevation correlated with poor response to treatment.

The literature so far on host responses in encephalitis consists of studies examining small numbers of pre-specified markers. Some themes emerge, such as the elevation of pro-inflammatory mediators in infective encephalitis. However, the above studies were limited in several ways. Firstly, they only examined a very small number of patients; secondly, they could only examine proteins by enzyme-linked immunosorbent assay (ELISA) which have been specified in advance.

To overcome these limitations, it is necessary to use high-throughput techniques to analyse the host response in an unbiased way. These techniques have become known as "-omics", referring to the suffix implying an all-encompassing analysis of a certain aspect of a biological sample, whether nucleic acid, protein or small molecule metabolite.

Clinical Transcriptomics

The transcriptome is the entire repertoire of transcribed genes within a certain cell or set of cells at a certain point in time. It is comprised of RNA molecules, coding and non-coding. Coding RNA (mRNA) may be translated into protein, while non-coding RNA, such as

microRNAs (miRNA), small interfering RNA (siRNA), small nucleolar RNAs (snRNA) and long non-coding RNAs (lncRNAs) are not. Non-coding RNA is increasingly understood to perform important functions, for example siRNAs are capable of causing degradation of mRNA after transcription, affecting translation of protein (Crooke et al. 2018).

Clinical transcriptomics is the study of the transcriptome of a biological sample in order to identify clinical biomarkers or understand mechanisms of disease. DNA microarray and RNA sequencing are both commonly used techniques for transcriptomic analysis. Microarray analysis requires some *a priori* knowledge of genes, but has cost advantages, while RNA sequencing is more expensive but is independent of knowledge of genetic sequences.

DNA microarray

Microarrays are capable of analysing the expression of thousands of genes within a single chip. They rely on hybridisation to a probe, with the hypothesis that the abundance of transcripts can be deduced by the amount of hybridisation to each probe.

Initially, RNA is extracted from the biological sample, and the purity of the extracted RNA is measured. RNA is then reverse transcribed, amplified and labelled with fluorescent dye, before being exposed to an array of probes spotted onto a stable surface. Binding is then measured using a scanner which measures fluorescence associated with each probe, allowing calculation of relative expression. Either single channel (one colour) or two channel (two colour) microarray may be used: two channel compares the hybridisation of two

distinct dyes to the same probes and therefore is able to compare two different disease states, or disease with control.

Existing literature

I searched PubMed with the search terms encephalitis OR meningitis (as MeSH Major Topics) AND transcriptom*[Title/Abstract] OR gene expression [Title/Abstract]. Within these search results, original research articles were identified which included 5 or more human subjects and in which transcriptomic techniques (microarray or RNA sequencing) were used to investigate encephalitis or meningitis. Further articles were identified from the reference lists of identified articles.

Table 2. Literature review of transcriptomics in CNS infection

First author and year	Disease studied	Number of patients	Method	Principal findings
(Bartholomeus et al. 2019)	Enterovirus meningitis	56	RNA-Seq in whole blood	Upregulated type 1 interferon responses in enterviral meningitis
(Erwin-Cohen et al. 2017)	Venezuelan equine encephalitis vaccination	10	Microarray in whole blood	Upregulated interferon response in vaccinated subjects.
(Lill et al. 2013)	Bacterial meningitis	21	Microarray in whole blood	10 significantly different genes including interleukins
(Pan et al. 2019)	TB and viral meningitis	12	Microarray in PBMCs, miRNA analysis	11 differentially expressed miRNAs distinguish groups
(Rohlwink et al. 2019)	TB meningitis	20 children	RNA-Seq in whole blood, ventricular and lumbar CSF	Increase in inflammasome activation and decrease in T cell activation in blood. Neuronal excitotoxicity and cerebral damage in ventricular CSF. Differences in protein translation and cytokine signalling in lumbar CSF.

RNA-Seq = ribonucleic acid sequencing; TB = tuberculosis; PBMC = peripheral blood mononuclear cell; miRNA = micro ribonucleic acid; CSF= cerebrospinal fluid.

Five studies were identified which investigated encephalitis or meningitis by transcriptomic methods. Studies were identified using both microarray techniques and RNA sequencing. All 5 studies identified genes associated with the host immune response which differed between groups, predominantly reflecting a pro-inflammatory state in the setting of neurological infection. The type 1 interferon response and interleukin signalling were commonly identified as differentially expressed.

Clinical Proteomics

The proteome can be defined as the whole array of expressed proteins of an organism, region or cell, consisting of whole proteins, with or without modifications, and peptides. The proteome is location-specific and dynamic, varying with homeostasis of the organism and any disease state. By necessity the proteomic study of a single clinical sample is a single snapshot of the proteome of that physiological system. It is also extremely susceptible to the conditions of sample acquisition and storage, which can bind or denature proteins and reduce capacity for protein identification, or may introduce systematic bias.

The search for protein biomarkers of disease is a rapidly growing and evolving area of clinical science. As laboratory techniques have developed, and the potential for protein identification has increased, emphasis has shifted from the analysis of small panels of

proteins of interest (for example through multiplex ELISA techniques) towards the unbiased acquisition of large datasets covering as much of the proteome as possible. In the last few decades, the number of proteins typically analysed in an experiment of this kind has moved from tens to hundreds to thousands. This transition from a reductionist to a more global approach has increased the potential for identifying novel proteins and interactions, but has also brought challenges in the interpretation of results, since capacity for false conclusions is high, particularly when sample sizes are relatively small.

Liquid chromatography-mass spectrometry

The most common approach to the proteomic analysis of biological fluids is the separation of samples by a form of chromatography, followed by analysis by mass spectrometry. This allows the optimal coverage of the constituent proteins within the mixture. Experimental approaches to proteomic analysis are typically divided into “top-down” and “bottom-up” techniques (Brown et al. 2020). Top-down proteomics involves the analysis of intact proteins. Bottom-up proteomics involves the prior digestion of proteins into peptides. Because of difficulties in analysing large intact proteins, bottom-up proteomics is most commonly used for complex mixtures (also known as “shotgun” proteomics).

In a “bottom-up” approach, initially the proteins in the sample are enzymatically digested into constituent peptides (Hamzeiy et al. 2017). The resulting peptide mixture is separated into fractions, usually using a chromatographic column, before being subjected to tandem mass spectrometry (MS/MS). The spectrometer ionises the samples producing a spray of ions which are separated by mass to charge ratio (m/z). This produces a spectrum which can

be matched to a database to identify the proteins to which the identified peptides belong. Depending on the number of peptides identified from each protein and the coverage the complete protein sequence, proteins may be identified with varying degrees of certainty. Certain peptide sequences may also be common to a number of similar proteins resulting in several possible protein identities.

The identification of proteins within a sample is limited by the abundance of proteins, and by the complexity of the sample. The latter refers largely to the dynamic range of proteins in the sample, i.e. the presence of proteins in very different concentrations. Quantification of peptides, and therefore proteins, may use a labelled or unlabelled approach. Labelled approaches rely on the introduction of stable isotopes which bind to peptides. Unlabelled quantification relies on statistical analysis of the spectra, for example by peak intensity or spectral counting, and can deliver either a relative abundance (comparing analysed samples) or an absolute abundance through statistical methods.

CSF proteomics

Proteomic techniques are chosen carefully according to the biological fluid to be analysed. CSF is often used for the investigation of disorders of the CNS, as its physiological proximity to the parenchyma of the brain and spinal cord allows it to reveal changes in disease states that would not be identifiable in blood or other more distal samples.

However, CSF presents challenges as a medium for proteomic analysis. Although blood samples are relatively easy to obtain, CSF is usually acquired by lumbar puncture, although

in certain circumstances it can be obtained directly from the ventricles by external ventricular drains or if the patient has a CSF shunt in situ. Although the risks of lumbar puncture are low, the procedure can be uncomfortable and it is only performed when clinically required. It is therefore not normally performed on healthy individuals, reducing the availability of true healthy control CSF. In patients who undergo lumbar puncture for clinical reasons, the procedure is often performed as an emergency and acquiring CSF for research purposes can be challenging, as the procedure cannot be repeated for research reasons alone. If the procedure is difficult or the patient uncooperative, clinicians may be unwilling to prolong it in order to collect extra samples for research reasons.

The concentration of protein in CSF is relatively low compared to serum or plasma, with protein abundances approximately one hundred-fold lower (Maurer 2010). This means that if the CSF becomes contaminated with serum or whole blood, as may happen during lumbar puncture, the effects on the measured proteome can be profound.

The dynamic range of CSF is extremely wide, with the proteome dominated by highly abundant proteins such as albumin (Maurer 2010). Some experimental techniques include a depletion of these highly abundant protein using a binding agent in a column or resin (Lee et al. 2019). This reduces the dynamic range and may improve the identification of proteins of lower abundance. However, this risks the possible depletion of other proteins of lower abundance, as the specificity of the binding agent may vary. It also introduces another potential source of variation into the workflow which is already very susceptible to an accumulation of experimental bias.

Existing literature

I searched PubMed with the search terms encephalitis OR meningitis (as MeSH Major Topics) AND CSF[Title/Abstract] AND proteom*[Title/Abstract]. Within these search results, original research articles were identified which included 5 or more human subjects and in which proteomics of CSF was used to investigate encephalitis or meningitis. Further articles were identified from the reference lists of identified articles.

Table 3. Literature review of proteomics in CNS infection

First author and year	Disease studied	Number of patients	Method	Principal findings
(Cordeiro et al. 2015)	pneumococcal vs. enterovirus vs. meningococcal meningitis	24	2D-PAGE and MALDI-ToF-ToF	Unique signatures found in each of the three conditions. Kallikrein-kinin system upregulated in bacterial meningitis.
(Mu et al. 2015)	TB meningitis vs healthy control	24 discovery, 53 validation	LC-MS/MS	4 differentially abundant proteins, all involved in lipid metabolism, including ApoB
(Yang et al. 2015)	TBM patients vs. healthy controls (n=12)	24	LC-MS/MS	Over-representation in inflammation-associated processes, complement and coagulation cascades and cell adhesion molecules in TBM
(Angel et al. 2012)	early disseminated Lyme disease vs. CNS inflammation	45	LC-MS/MS	108 proteins differ significantly in abundance in Lyme disease from controls. Differences in proteins associated with cell death.
(Asano et al. 2011)	Paediatric acute encephalopathy vs febrile seizures	36 discovery, 19 validation	SELDI-TOF MS	Neurosecretory protein VGF precursor (VGF4.8) identified as biomarker for encephalopathy
(Bonnet et al. 2019)	T.b. gambiense early stage vs. late stage vs. controls (CSF < 5 WCCs/µl and no trypanosomes)	10 discovery, 70 confirmation	LC-MS/MS	69 biomarkers identified, including neuroserpine and moesin.

(Fraisier et al. 2014)	West Nile virus vs Non-West Nile virus infection, headache, idiopathic intracranial hypertension and healthy controls.	87	LC-MS/MS	47 differentially abundant proteins including Defensin-1 alpha in West Nile virus.
(Gomez-Baena et al. 2017)	Pneumococcal meningitis vs controls (normal CSF)	12 discovery, 16 validation	LC-MS/MS	Over 200 proteins differentially expressed, proteins involved in the immune response and exosome signalling were significantly enriched in the infected samples
(Njunge et al. 2017)	Acute Bacterial Meningitis vs. Cerebral Malaria	59	LC-MS/MS	Myeloperoxidase and lactotransferrin distinguish bacterial meningitis from cerebral malaria
(Ou 2013)	Tuberculous meningitis, Cryptococcal meningitis vs. Healthy controls	60	iTRAQ LC-MS/MS	9 differentially expressed proteins, involved in calcium ion binding, lipoprotein metabolism, immune response, and signal conduction
(Sengupta et al. 2015)	Japanese encephalitis virus (JEV) vs. Non-JEV Acute Encephalitis Syndrome	20	2D PAGE, MALDI-ToF	Vitamin D binding protein, complement proteins C3 and C4b and fibrinogen beta and gamma chain were increased in expression in JE patients.
(Tiberti et al. 2015)	T. bruceii gambiense, T. rhodesiense vs. controls (CSF < 5 WCCs/ µl and no trypanosomes)	6 discovery, 185 validation	LC-MS/MS	C-reactive protein (CRP) and orosomucoid 1 (ORM1) significantly increased in rhodesiense HAT patients.

2D-PAGE = 2-dimensional polyacrylamide gel electrophoresis; MALDI-ToF = Matrix-assisted laser desorption/ionization- time of flight; TB = tuberculosis; LC-MS = liquid chromatography-mass spectrometry; TBM= tuberculous meningitis; ApoB = apolipoprotein B; CNS = central nervous system; SELDI-ToF = Surface-enhanced laser desorption/ionization- time of flight; T.b.gambiense = Trypanosoma brucei gambiense; CSF = cerebrospinal fluid; WCC = white cell count; iTRAQ = Isobaric tag for relative and absolute quantitation; HAT = human African trypanosomiasis.

Clinical Metabolomics

Metabolomics is the profiling of small molecule components of metabolic pathways within a biological system. The two major platforms used for metabolomic analysis are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. NMR has relatively lower sensitivity when compared to mass spectrometry, but has the advantage of being non-destructive and producing highly reproducible results (Keun et al. 2011).

^{1}H NMR

NMR spectroscopy is based on the principle that magnetic moments of the atomic nucleus can provide information about the structure of molecules (Giraudeau 2020). ^{1}H NMR is based on the spin of single protons which, in the presence of a constant magnetic field, can exist in one of two energy states (-1/2 or +1/2). Protons can be excited into a higher energy state by application of radiofrequency energy, and will subsequently relax emitting a detectable signal. Since nuclei exist within differing molecules, shielding of each nucleus from the magnetic field is different, producing 'chemical shift'. Excitation of the sample using radio waves can therefore detect the resonance frequency of each ^{1}H environment, producing a separate peak for each environment within the sample (Reo 2002).

Existing literature

A literature review was performed to locate articles investigating encephalitis or meningitis in humans using CSF metabolomic approaches. I searched PubMed with the search terms encephalitis OR meningitis (as MeSH Major Topics) AND CSF[Title/Abstract] AND metabolom*[Title/Abstract]. The search was limited to articles published in English, and where the full text was available. Within these search results, original research articles were identified which included 5 or more human subjects and in which metabolomics (NMR or mass spectrometry) of CSF was used to investigate encephalitis or meningitis. Further articles were identified from the reference lists of identified articles.

Table 4. Literature review of metabolomics in CNS infection

First author and year	Disease studied	Number of patients	Method	Principal findings
(O'Sullivan et al. 2013)	Rabies	44 CSF samples from 11 patients, 25 controls	^{1}H NMR	Patients separated well from controls. 56 metabolites identified which cluster into three stages of disease.
(Kawashima et al. 2006)	Influenza-associated encephalopathy	6 patients, 20 controls	FT-MS	752 metabolites identified. 3 unknown metabolites potential biomarkers
(Chatterji et al. 2017)	Meningitis	26 negative controls, 30 positive controls, 49 meningitis	^{1}H NMR	Meningitis differentiated from negative controls well. No clear differentiation of meningitis from positive controls.
(Dai et al. 2017)	TB meningitis	50 TBM patients, 17 viral meningitis, 17 bacterial meningitis,	UHPLC-QTOF-MS	Many metabolites differentially expressed.

		16 cryptococcal meningitis.		
(Mason et al. 2017)	TB meningitis	33 paediatric TBM, 34 suspected meningitis but negative controls	GC-MS	Alanine, lysine, branched-chain amino acids distinguish TBM
(Lamour et al. 2017)	Trypanosoma brucei rhodiense	46 patients	^{1}H NMR	27 metabolites identified. Cannot determine early from late stage. Subset of metabolites associated with presence of neurological signs.
(Ratuszny et al. 2019)	Enterovirus meningitis	10 patients, 19 controls	LC-MS/MS	Phosphatidylcholines were upregulated in enterovirus,
(Kuhn et al. 2018)	Varicella zoster reactivation with CNS involvement	14 shingles, 16 facial nerve zoster, 15 VZV meningitis/encephalitis, 10 enteroviral meningitis, 11 idiopathic Bell's palsy, 15 NPH	LC-MS/MS	4 analytes identified for meningoencephalitis. Did not correlate directly with CSF leucocyte count.
(Li et al. 2017)	Tb and viral meningitis	18 TB, 20 viral meningitis patients	^{1}H NMR	25 metabolites contributed to discriminating groups.
(van Laarhoven et al. 2018)	TB meningitis	33 pts, 22 controls. Validation cohort of 101 patients.	LC-MS/MS	CSF tryptophan concentration predicted mortality and correlated with 11 gene loci associated with tryptophan conc.
(Zhang, Zhang, et al. 2019)	TB meningitis	TBM 25, VM 27, BM 20, NC 28	^{1}H NMR	Large panels of metabolites distinguished between groups.
(Mason et al. 2015)	TB meningitis	TBM 33, south African controls, Dutch controls	^{1}H NMR	16 metabolites differentiated TBM from controls.

CSF = cerebrospinal fluid; NMR = nuclear magnetic resonance; FT-MS = Fourier transform mass spectrometry; TB = tuberculosis; TBM = tuberculous meningitis; UHPLC-QTOF-MS = ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry; LC = liquid chromatography; CNS = central nervous system; VZV = varicella zoster virus; NPH = normal pressure hydrocephalus; BM = bacterial meningitis; NC = normal conrol.

Both NMR and mass spectrometry have been used with success to elucidate differences in metabolomic profiles between disease groups and controls. A striking number of studies have investigated metabolites in tuberculous (TB) meningitis, all finding differences in metabolite profiles with control, most commonly in amino acid metabolic pathways. One study combined mass spectrometry metabolomics with genomic analysis to investigate for polymorphisms associated with differences in tryptophan metabolism. Few studies were found investigating metabolic profiles in viral or autoimmune encephalitis, with the exception of one study in rabies and one in VZV which included a number of patients with encephalitis.

Scope and aims of this thesis

Encephalitis is a devastating condition with substantial mortality and very high morbidity. Diagnosis is often problematic and delayed contributing to worse outcomes, causing distress for patients and relatives and presenting a dilemma for treating clinicians. Discriminating between encephalitis and mimicking conditions, such as encephalopathy owing to peripheral infection, metabolic or toxic encephalopathy, seizure disorders, cerebrovascular disease and psychiatric disorders can be challenging. Once encephalitis is diagnosed, distinguishing viral from autoimmune encephalitis can also be difficult, but these conditions require very different treatment, and proceeding with immunosuppression in the presence of infection may be disastrous. There are few effective predictors of clinical outcome at

presentation. The disease mechanisms of both viral and autoimmune encephalitis are incompletely understood.

Previous studies have attempted to address these questions through analysis of small panels of pre-specified proteins involved in the host immune response. However, none of these studies have included autoimmune and viral encephalitis compared directly to mimicking conditions. They could only detect differences in the proteins specified in advance, which relies on our limited prior knowledge about differences in pathological processes. Only looking at proteins may miss earlier signals in the expression of genes, or differences in the compounds involved in metabolic pathways. Additionally, since the panel of pre-specified mediators varies between each study, it is not clear which results are most relevant to a larger population of encephalitis patients.

Other studies have successfully used powerful laboratory techniques to examine the host transcriptome, proteome and metabolome in other related neurological conditions, including TB meningitis, bacterial meningitis and small numbers of cases of encephalitis of single aetiologies. However no study has used high-throughput techniques to explore differences between autoimmune encephalitis, viral encephalitis and mimicking conditions.

This thesis aims to address these issues, utilising CSF and blood samples collected prospectively from 30 centres, using transcriptomics to analyse gene expression in whole blood, and pairing this with proteomic and metabolic analysis in CSF.

Aims

- To characterise a cohort of patients with encephalitis and mimicking conditions recruited from the UK and establish whether a model based on clinical features can distinguish cases of autoimmune encephalitis or viral encephalitis from other groups.

- To look for new candidate biomarkers to distinguish between autoimmune encephalitis, viral encephalitis and mimicking conditions, and to predict clinical outcome, by:

    - Gene expression profiling by microarray analysis of whole blood
    - Proteomic analysis by liquid chromatography/mass spectrometry in CSF
    - Metabolomic analysis by ^{1}H NMR spectroscopy in CSF.

- To integrate findings from transcriptomic, proteomic and metabolic analyses to look for differences in pathways and networks between autoimmune and viral encephalitis and mimicking conditions.

Chapter 2. Selection and characterisation of the clinical cohort

Introduction

Encephalitis as a whole has an incidence of 3.45-8.66 per 100,000 (Granerod et al. 2013), and in order to study the condition and draw conclusions regarding its diagnosis and management it is necessary to recruit a substantial cohort of patients affected by encephalitis. Ideally such cohorts are recruited prospectively, as this improves the collection of clinical data and allows for the systematic collection of clinical samples, although useful retrospective cohorts have also been described (Titulaer et al. 2013).

For this reason, over the last several decades large multicentre prospective studies have been conducted in the UK recruiting patients with encephalitis. In this study I examined samples from two major multicentre studies of encephalitis in order to ascertain how to improve diagnosis by discriminating between autoimmune encephalitis, viral encephalitis and mimics. Clinical studies of encephalitis often find that patients who meet the clinical case definitions for encephalitis are not in fact infected with a virus, but with a bacterium better known as a cause of bacterial meningitis. I therefore included this important additional group of patients in my study.

Before proceeding to detailed analysis for the identification of novel biomarkers in CSF and blood, in this chapter I will discuss recruitment of the patient cohort, and describe their demographic and clinical characteristics, investigations and outcome. I will then explore how well clinical features and basic investigations discriminate between aetiological groups.

Methods

Clinical material

For this study I used CSF and blood samples recruited over a period of 10 years in prospective cohort studies from 30 sites around the UK.

The **ENCEPH UK study** was co-ordinated by the University of Liverpool and was a 3 year prospective study recruiting adult patients from 30 centres the UK (Cooper et al. 2016). Among the principal aims of the ENCEPH UK study was "to determine the early clinical features predictive of HSV encephalitis to support the development of a predictive tool for HSV encephalitis based on these early clinical features, and to determine clinical predictors of other causes of encephalitis".

The **Health Protection Agency (HPA) Encephalitis study** (Granerod, Ambrose, et al. 2010) which recruited patients of all ages from 24 hospitals in London, the South West and the North West of England (Granerod, Ambrose, et al. 2010).

The **Walton Centre CSF Biobank** was set up to provide a library of CSF for a range of neurological studies. I recruited patients with suspected encephalitis to the biobank when I saw them clinically on the wards.

Case recruitment, consent and sample acquisition

Potential cases in both the ENCEPH UK and HPA studies were ascertained by research nurses, clinicians, microbiologists, virologists or radiologists, by visits to the wards in participating centres and communication with the diagnostic laboratories, in addition to raising awareness through talks and training sessions with hospital staff. From 2013 onwards, I raised awareness amongst clinical colleagues and screened patients personally for inclusion before recruiting them if appropriate.

Patients were eligible for inclusion in the studies when they presented to a participating hospital with a case of suspected encephalitis. Written consent was taken from the patient, if possible, or if the patient lacked capacity then proxy consent was taken from a family member or representative. If the patient subsequently regained capacity then consent was retaken from the patient at an appropriate time.

Samples were taken of CSF and RNA-stabilised blood (PaxGene tubes) as soon as possible after admission. Consent was also taken from patients to allow the use of leftover samples taken for clinical purposes, principally CSF sent to microbiology or biochemistry laboratories.

Diagnostic testing

In all cases, where possible, basic blood and CSF investigations were performed (see table 1), alongside testing for the commonest UK viruses causing encephalitis (HSV types 1 and 2, VZV, enteroviruses and parechoviruses), and further specific tests were performed where clinically indicated and where sample volume allowed. This followed National Guidelines for diagnosis and management of encephalitis (Solomon et al. 2011).

Table 1. Baseline diagnostic testing performed for all patients.

Sample	Investigation
Blood	Full blood count, clotting
	Urea and electrolytes, liver function tests, C-reactive protein, glucose
	HIV serology
	NMDA receptor antibody Voltage gated potassium channel antibody
CSF	Protein and glucose Microscopy, cell count and culture
	CSF PCR panel herpes simplex virus 1 and 2 Varicella zoster virus enteroviruses parechoviruses
Other samples to be considered	Throat swab and stool/rectal samples for enterovirus PCR
	Oral/skin/vesicle fluid swab for HSV/VZV PCR
	Genital swabs for HSV PCR

HIV = human immunodeficiency virus; NMDA = N-methyl-D-aspartate receptor; CSF = cerebrospinal fluid; PCR = polymerase chain reaction; VZV = varicella zoster virus.

In the case of some patients, one or more of these baseline tests had not been performed or the results were unavailable because of difficulties in obtaining adequate sample, patient transfer from one centre to another or incomplete record keeping. If sufficient sample was available I attempted to perform the missing tests retrospectively, but if sample volume was too limited and the patient's diagnosis was already clearly established, I gave priority to retaining CSF or blood for inclusion in the study, rather than completing basic investigations which were unlikely to alter to diagnosis.

Antibody testing was performed for NMDAR antibody by live cell-based assay and VGKC antibody by radioimmunoprecipitation assay, which were the only encephalitis-associated antibodies routinely tested at the time of the studies (O'Sullivan et al. 2016). Since these patients were recruited, several other antibodies have become part of the standard testing panel for autoimmune encephalitis. In particular, VGKC is no longer routinely tested by most neurologists, and it is understood that pathogenic antibodies target subunits associated with the voltage gated potassium channel, most often LGI1 or CASPR2. Where CSF or serum was available for testing, in cases of seronegative autoimmune encephalitis or VGKC antibody positive encephalitis, I tested patient samples using Euroimmun antibody mosaic BIOCHIP (Product no. 112d-6) which incorporates fixed cell assays for LGI-1, CASPR2, glutamate receptors (type AMPA1/2), dipeptidyl aminopeptidase-like protein 6 (DPPX) and Gamma aminobutyric acid (GABA) B receptor.

Neuroimaging was performed at the discretion of the clinician, but all patients had at least computed tomography (CT) brain scanning performed. EEG was performed only when clinically required.

Case selection and categorisation of cases

Patients were eligible for recruitment to the original studies (and hence to my biomarker studies) if the treating clinician suspected a diagnosis of encephalitis. I next established which patients had sufficient sample available for proteomic, metabolomic and transcriptomic analyses. In many cases patients had been recruited to the study but owing to constraints of clinical time, the logistics of patient transfers or difficulty with sample tubes, the requisite samples were not collected. Clinical features were collected according to a standardised case report form for each study at the time of recruitment. For the ENCEPH UK study, further case report forms were completed, where possible, at 3, 6 and 12 months.

Having excluded patients without samples or sufficient sample volume available, I classified the cohort according to widely accepted strict inclusion criteria. Initially, I established whether patients met the case definition for encephalitis (Table 2). Patients who went on to have the diagnosis of encephalitis excluded, and where other diagnoses were made, were eligible for inclusion in the mimics group. In addition, some patients had a final diagnosis of bacterial meningitis, and these were included as a separate group.

For autoimmune encephalitis, patients were classified according to the consensus criteria (Graus et al. 2016). These criteria are subdivided into several parts, and to be defined as autoimmune encephalitis patients had to meet at least the criteria for possible autoimmune encephalitis (table 3), and then diagnosis was assigned by whether they met criteria for a specific antibody or disease. Specifically, a definite diagnosis could be made using criteria for definite autoimmune limbic encephalitis, definite ADEM, or anti-NMDA receptor encephalitis (appendix).

For viral encephalitis, patients were classified according to criteria proposed by Granerod et al. (Granerod, Cunningham, et al. 2010), which set out the diagnostic tests required to make a diagnosis of diagnosis caused by a pathogen. For example, for a definite diagnosis of HSV encephalitis, HSV DNA or antigen must be detected in any CSF/brain specimens, or there must be an HSV-specific intrathecal antibody response, or autopsy neuropathology demonstrating panencephalitis HSV DNA/antigen present in eosinophilic neuronal inclusions. Full criteria for the diagnosis of the viral causes of encephalitis included in this cohort are included in the appendix.

Power calculations

For transcriptomic approaches I performed a power calculation using a model by MDAnderson Cancer Centre, University of Texas (Hu et al. 2005). This model assumes that on a log scale the expression of each transcript is normally distributed and that the measurement of each transcript is independent of the next. The sample size needed to identify a twofold change in transcript abundance between clinical groups (using an array

containing 60,000 transcripts), accepting an average standard deviation in gene-expression of 0.68 (based on data from meningitis patients), a 5% false detection rate (i.e. a per-gene significance level alpha of 0.05) and 80% power, will be 8 in each group. Increasing to 25 per group will allow for detection of transcripts with lower fold-changes in abundance between groups (down to 1.5) or higher average standard deviation (1.25). This is a conservative estimate, as previous microarray studies with fewer patients (n<20) have demonstrated clinically applicable results (Griffiths et al. 2005).

For mass spectrometry and metabolomic studies, using IFNγ in CSF as an example marker, published data suggest a mean concentration of 9 IU/ml (6-75) in non-viral encephalitis (Ichiyama et al. 2008) and 25 IU/ml (0-53) in viral encephalitis (Kamei et al. 2009). To have 90% power to detect this difference with 5% chance of type 1 error will require approximately 29 patients in each group. This is comparable to other studies which have detected significant differences in abundance of small molecules and proteins in CSF (Comabella et al. 2010).

Table 2. Criteria for encephalitis (adapted from Granerod et al. 2010)
Acute or sub-acute (<4 weeks) alteration in consciousness, cognition, personality or behaviour persisting for more than 24 hours
Plus ANY two of:
a. Fever (≥ 38ºC) / Prodromal illness – acute or sub-acute
b. Seizures: New onset
c. Focal Neurological Signs – Acute or Sub-acute onset. Including • Focal weakness • Oromotor dysfunction • Movement disorders including Parkinsonism • Amnesia
d. Pleocytosis: CSF white cell count >4 cells/ul
e. Neuroimaging: Compatible with encephalitis
f. Electroencephalogram (EEG): compatible with encephalitis

Table 3. Criteria for possible autoimmune encephalitis (Graus et al. 2016)
All three of the following criteria must be met:
1. Subacute onset (rapid progression of less than 3 months) of working memory deficits (short-term memory loss), altered mental status, or psychiatric symptoms
2. At least one of the following:
· New focal CNS findings
· Seizures not explained by a previously known seizure disorder
· CSF pleocytosis (white blood cell count of more than five cells per mm^3)
· MRI features suggestive of encephalitis*
3. Reasonable exclusion of alternative causes
*Brain MRI hyperintense signal on T2-weighted fluid-attenuated inversion recovery sequences highly restricted to one or both medial temporal lobes (limbic encephalitis), or in multifocal areas involving grey matter, white matter, or both compatible with demyelination or inflammation.

Statistical analysis

I investigated the differences in basic clinical features and demographics using Fisher's exact test, and laboratory results and other continuous variables using Kruskal-Wallis tests. I used logistic regression analysis with backwards elimination to ascertain models for predicting diagnostic category, with a p value of <0.05 being classed as significant.

Ethics

All patients or an accompanying relative provided written informed consent including for use of leftover CSF for the investigation of disease biomarkers. The Enceph UK study was approved by the NRES Committee East Midlands–Nottingham 1 REC [11/EM/0442]. Additional samples from the HPA study were approved by the North and East Devon Multicentre Research Ethics Committee [05/Q2102/22], and from the Walton Centre Research Biobank, approved by the Wales REC4 National Research Ethics Service [16/WA/0291]. Approval for use of samples was granted by the study steering committees and by the Walton Centre Research Biobank Managing Committee.

Results

In total 566 patients were recruited to all the studies combined (Fig 1). Of these, 399 patients were excluded because no samples were available. Of the remaining 167, 11 had bacterial meningitis, and were included as a separate group for comparison. 121 met the definition of encephalitis. The 31 patients who did not meet this definition were included as the mimic group (i.e. they had suspected encephalitis on admission, but in fact had other diagnoses made). 69 met the definitions for either autoimmune or viral encephalitis. Table 4 shows the diagnoses within each category. The remainder were classed as having unknown cause encephalitis and were excluded.

Of the 111 total patients included in the study, 79 were from the ENCEPH UK study, 20 were from the HPA cohort and 12 were from the Walton Centre CSF biobank. From among these patients 71 had CSF for proteomics and metabolomics, 21 had blood for transcriptomics only, and 19 had CSF and blood (Figure 2).

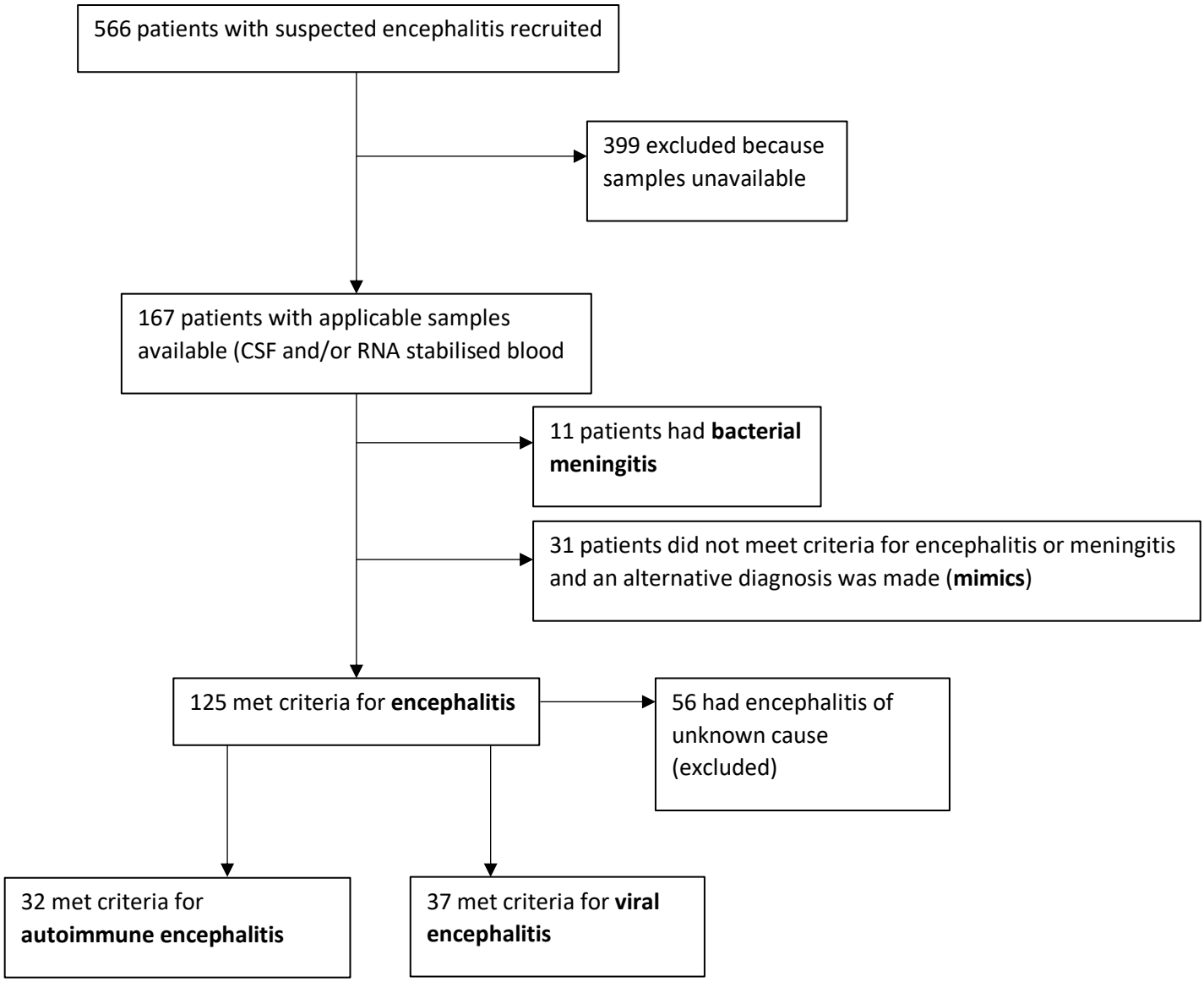


Figure 1: Flow chart illustrating recruitment and initial division into cohorts

Table 4. Profile of diagnoses in each diagnostic category.

Autoimmune cases n=32		**Viral encephalitis n=37**		**Bacterial meningitis n=11**		**Mimics n=31**	
NMDAR antibody	12	Herpes simplex virus type 1	25	*Mycobacterium tuberculosis* (MTB)	4	Headache disorders including migraine	13
ADEM	6	Varicella zoster virus	7	*S.pneumoniae*	4	Seizures or epileptic disorders	5
VGKC antibody	4	Enterovirus	4	Purulent meningitis,no organism identified	3	Cerebrovascular disorders	3
GAD antibody	1	Adenovirus	1			Psychiatric disorder or delirium	5
Rasmussen's encephalitis	1					Toxic encephalopathy/ Korsakoff's syndrome	3
IGLON5	1					Metabolic	1
Hashimoto's encephalopathy	1					Concussion	1
Seronegative autoimmune	6						

Table 5. Demographics and presenting clinical features for 111 patients with encephalitis or mimics.

Characteristics	Autoimmune encephalitis n=32	Viral encephalitis n=37	Bacterial meningitis n=11	All encephalitis n=80	Mimics n=31	p value
Age at admission Median (range)	29 (6-75)	50 (1-91	66 (31-89)	50 (1-91)	48 (18-71)	**<0.01**
Sex M:F	15:17	19:18	7:4	41:39	9:22	0.14
Personality or behavioural change n (%)	21 (65)	24 (65)	7 (64)	52 (65)	15 (48)	0.48
Seizures n (%)	14 (43)	12 (32)	0 (0)	26 (65)	9 (29)	0.44
Fever n (%)	16 (50)	29 (78)	10 (91)	55 (69)	14 (45)	**0.03**
Headache n (%)	17 (53)	22 (59)	8 (73)	47 (59)	21 (68)	0.33
Lethargy n (%)	12 (38)	19 (51)	2 (18)	33 (41)	9 (29)	0.14
Irritability n (%)	14 (43)	8 (22)	4 (36)	26 (33)	10 (32)	0.26
Amnesia n (%)	15 (47)	8 (22)	3 (27)	26 (33)	10 (32)	**<0.01**
Psychosis n (%)	4 (13)	0 (0)	0 (0)	4 (5)	3 (10)	**<0.01**
Movement disorder n (%)	11 (34)	4 (11)	0 (0)	15 (19)	4 (13)	**<0.01**

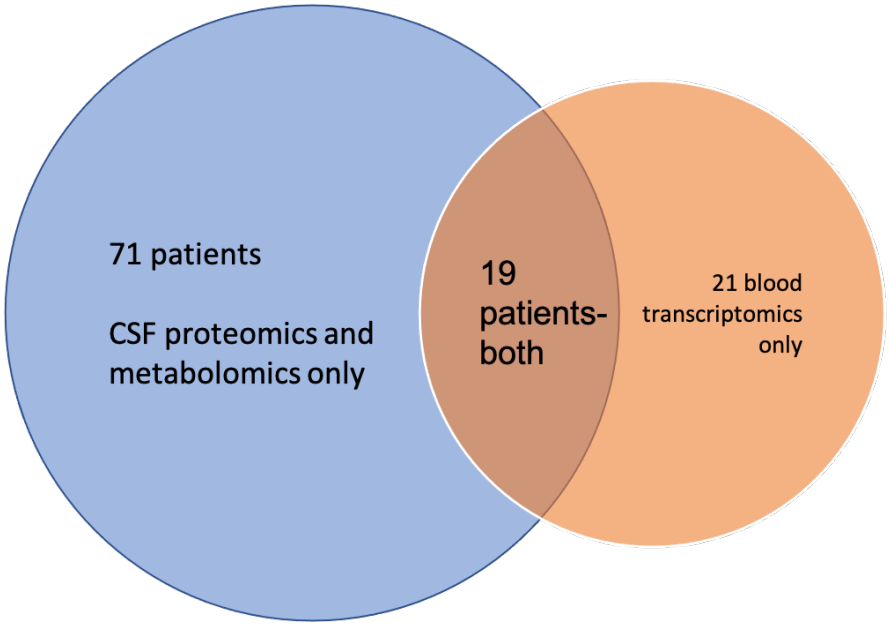


Figure 2: Pie chart illustrating number of patients in each part of the study, according to samples available.

Table 5 shows basic demographics for the patients in the study. The sex distribution was relatively even in all groups apart from the mimic group, which was predominantly female, although the difference was not significant. The median age (range) of the encephalitis patients was 50 (1-91). The age in the autoimmune group was significantly lower than the other groups (29 (6-75) $p<0.01$).

Significant differences in presenting clinical features were seen in the case of fever ($p=0.03$), amnesia ($p=<0.01$), psychosis ($p=<0.01$) and movement disorder ($p=0.01$). Fever was more often seen in infectious cases, although it also occurred in 50% of autoimmune cases. Amnesia occurred at presentation in a significantly higher number of the autoimmune cases. Psychosis and movement disorder were significantly more common in autoimmune cases.

Table 6. Abnormal investigation findings. (Abnormal protein is >0.5g/l, CSF pleocytosis is cells >5/mm^3)						
Investigation	**Autoimmune encephalitis n=32**	**Viral encephalitis n=37**	**Bacterial meningitis n=11**	**All encephalitis n=80**	**Mimics n=31**	**p value**
CSF pleocytosis	21/30 (70)	29/36 (80)	10/10 (100)	60/76 (78)	0/31 (0)	**<0.01**
CSF protein	15/31 (48)	25/32 (78)	8/10 (80)	48/73 (66)	5/25 (20)	**<0.01**
CT or MRI brain	21 (66)	29/37 (78)	8/11 (73)	58/69 (84)	5/26 (19)	**<0.01**
EEG	21/29 (72)	15/19 (79)	2/4 (50)	38/52 (73)	5/6 (83)	**<0.01**

Table 7. Laboratory values for CSF [median (IQR)]						
Parameter	**Autoimmune encephalitis**	**Viral encephalitis**	**Bacterial meningitis**	**All encephalitis**	**Mimics**	**p value**
CSF WCC (cells/mm^3)	7 (1-29)	28 (7-80)	2752 (957-2981)	23 (4-80)	1 (1-1)	**<0.01**
CSF lymphocyte %	90 (86-99)	95 (90-99)	15 (10-73)	90 (76-99)	ND	0.09
CSF protein (g/L)	0.5 (0.3-0.7)	0.8 (0.5-1)	2.3 (1-5.8)	0.7 (0.4-1)	0.4 (0.3-0.5)	**<0.01**
CSF glucose (mmol/L)	3.5 (3.1-4.3)	3.6 (3.2-4.6)	2.5 (2.3-2.9)	3.4 (3-4.3)	3.8 (3.4-4.4)	na

Table 6 shows the proportions of patients in each group who had abnormal investigations. CSF pleocytosis was common in all the encephalitis categories. Abnormal (raised) CSF protein concentration was significantly more common amongst infectious patients. Abnormal neuroimaging and EEG (where performed) were common in all encephalitis groups. All of these parameters were significantly different to the mimic group.

Table 7 shows the absolute values for CSF laboratory tests. CSF WCC was significantly different between groups, with autoimmune showing a mild or moderately raised WCC, with bacterial being extremely high in all cases. The lymphocyte percentage, where calculated, did not reach significance, perhaps due to small sample size, although the bacterial cases mostly showed a neutrophilic pattern. CSF protein was significant different between groups. CSF glucose was not analysed further, as glucose ratios were not available in many cases, and glucose CSF values alone are often not informative.

Table 8: Outcome of patients [no.(%)] measured by Glasgow outcome score at last follow-up by diagnosis.					
Glasgow outcome score	**Autoimmune encephalitis** **n=31**	**Viral encephalitis** **n=37**	**Bacterial meningitis** **n=11**	**All encephalitis** **n=79**	**Mimics** **n=29**
1: death	2 (6)	3 (8)	0 (0)	5 (6)	1 (3)
2: vegetative state	1 (3)	0 (0)	0 (0)	1 (1)	0 (0)
3: severe disability	9 (29)	5 (14)	0 (0)	14 (20)	2 (7)
4: moderate disability	5 (16)	13 (35)	4 (36)	22 (27)	7 (24)
5: good recovery	14 (46)	16 (43)	7 (64)	37 (46)	19 (66)

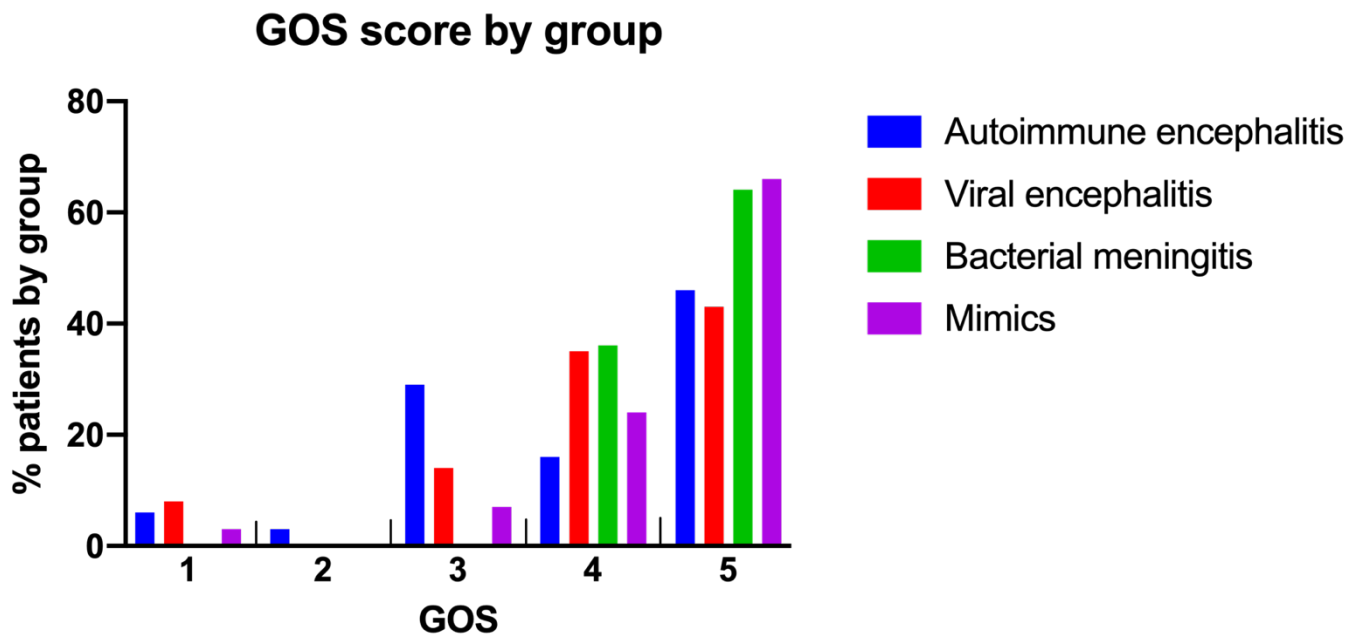


Figure 3: Outcomes by Glasgow outcome scale score by aetiological group.

Table 8 shows outcome measured by Glasgow outcome (GOS) scale score, at last follow-up. There were no significant differences overall between aetiological groups. GOS reflects functional outcome, and ability to live an independent life following brain injury (McMillan et al. 2016), and is therefore highly clinically significant.

Mortality was highest in the viral group (8%), and lowest in the bacterial group (0%). The proportion of patients who had a good recovery was similar between groups, although those with autoimmune encephalitis were more likely than those with viral encephalitis to have severe disability.

Multiple logistic regression analyses including viral, autoimmune, bacterial and mimic patients were performed to determine which clinical features might be predictive of specific diagnoses. They showed that the simplest effective model to predict diagnosis of viral encephalitis versus all other groups comprised CSF protein, abnormal neuroimaging and fever. Abnormal neuroimaging was the greatest contributor to the model (coefficient 12.13)

The simplest effective model to predict a diagnosis of autoimmune encephalitis versus all others comprised younger age, absence of headache, presence of memory problems and presence of movement disorder. Presence of movement disorder was the greatest contributor to the model (coefficient 17.1).

Figure 4 shows receiver-operating characteristic (ROC) curves and probabilities of correct predictions for the performance of these models. Based on the model to predict viral encephalitis versus others, assuming the most likely category, 6 cases (13%) would be misclassified as viral and 11 cases of viral encephalitis (44%) would be missed. Using the model to predict autoimmune encephalitis versus others, 5 cases (10%) would be misclassified as autoimmune encephalitis and 4 cases of autoimmune encephalitis (21%) would be missed.

The cases most likely to be misclassified as viral encephalitis based on the model included patients with autoimmune encephalitis, bacterial meningitis and mimics. One of the cases of

NMDAR antibody encephalitis had a prior HSV encephalitis preceding the development of NMDA antibodies by 6 weeks, and by this model was misclassified as viral encephalitis. Those patients with autoimmune encephalitis who were missed by this model included 3 patients with seronegative autoimmune encephalitis, one with VGKC antibody and one with IgLON5 encephalitis. None of the NMDAR antibody encephalitis patients were missed.

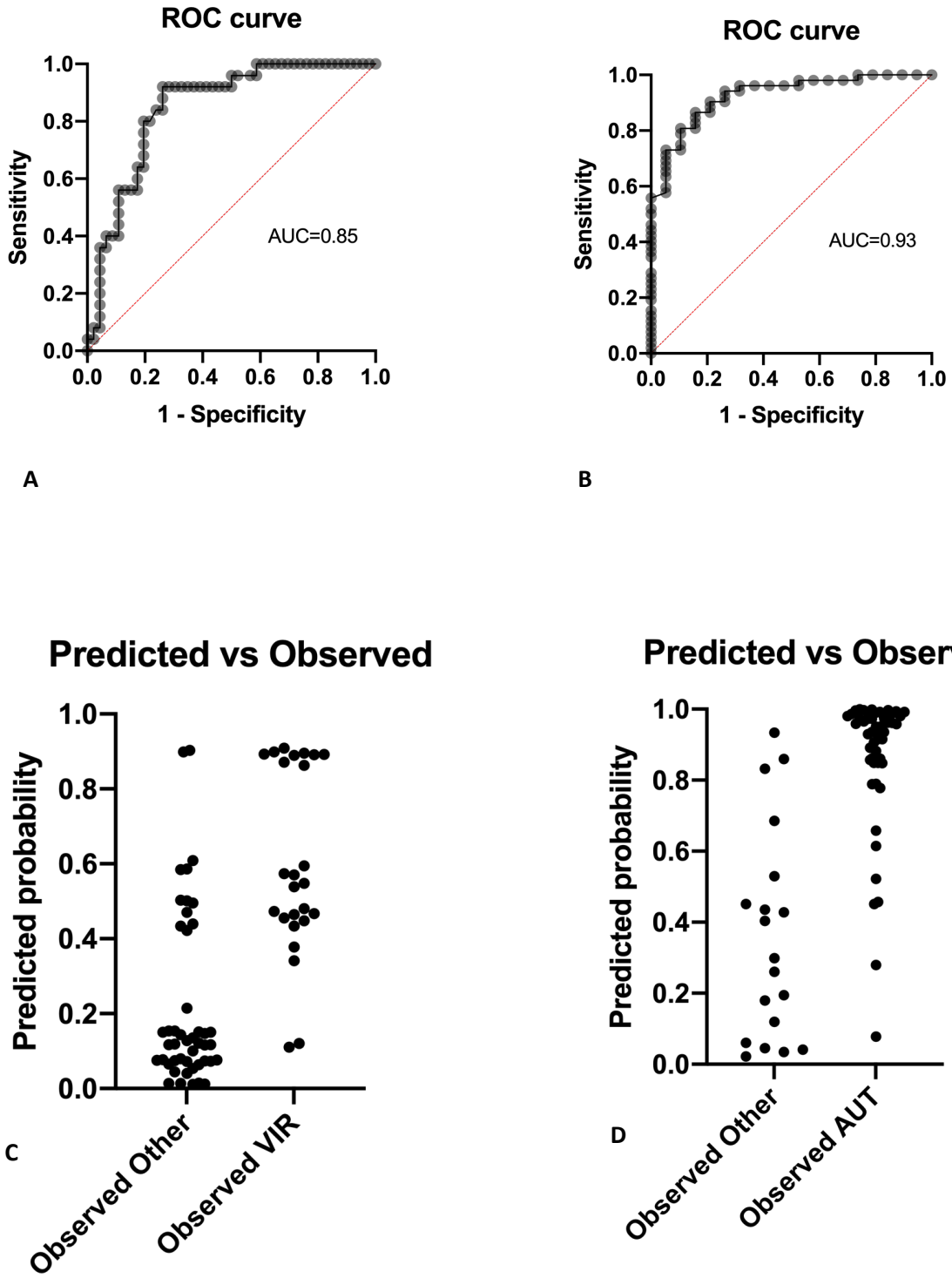


Figure 4. Receiver operating curve (ROC) analysis of models to identify viral and autoimmune encephalitis based on clinical features. A: ROC curve for model to identify viral encephalitis. B: ROC curve for model to identify autoimmune encephalitis. C: probability of correct classification of cases of viral encephalitis based on model (predicted probability 1=viral encephalitis, 0=other). D: probability of correct classification of cases of autoimmune encephalitis based on model (predicted probability 1=autoimmune encephalitis, 0=other).

Discussion

This characterisation of the clinical cohort for the study shows that in many respects it is representative of the population of acute encephalitis patients in the UK (Granerod, Ambrose, et al. 2010). The profile of the diagnoses in each group is in keeping with published UK epidemiological studies of encephalitis, and it appears to be representative of the sampled population. HSV encephalitis is the most common identified cause of encephalitis in the UK, and that is reflected in the number of cases in this cohort. More recently NMDAR antibody encephalitis has been increasingly identified, and is the most frequently identified autoimmune cause, so this also demonstrates that the cohort reflects epidemiology well. There are several rarer autoimmune causes, including a case of Rasmussen's encephalitis in a young adult female, and a case of IgLON5 encephalitis. The mimics represent a reasonable spread of differential diagnoses of encephalitis presenting acutely to a UK hospital (Solomon et al. 2011).

The basic demographics are balanced other than a predominance of females in the mimic group, although this did not reach statistical significance. The age of patients in the autoimmune group is significantly lower than in the other groups, and this is reflective of the pathophysiology of these diseases, especially given the preponderance of cases of NMDAR antibody encephalitis and ADEM, both of which predominantly affect younger people (Titulaer et al. 2013; Steiner et al. 2015).

The presenting clinical features of patients revealed several significant differences. Fever was more common in the infectious group, which might be expected. Although fever does also occur in some cases of autoimmune encephalitis, it is less frequent. Amnesia or memory difficulties were significantly more frequent in the autoimmune group. This may reflect the fact that, as a presenting feature, memory issues are more likely to feature strongly in autoimmune cases, which in infectious encephalitis they may become evident later or be shrouded by more salient features at presentation. Psychosis and movement disorder were significantly more common in autoimmune cases, which is unsurprising given the number of cases of NMDAR antibody encephalitis, of which these are core clinical features.

CSF parameters were significantly different between encephalitis of all causes and mimics, especially CSF pleocytosis, although there were still cases of viral and autoimmune encephalitis with normal cell count. As expected, CSF protein showed a stepwise increase from autoimmune encephalitis, where it was mildly raised, to a moderate increase in viral encephalitis and markedly elevated protein in most cases of bacterial disease.

Regarding outcome, mortality was relatively low compared to previous reports (Whitley et al. 1977; Granerod, Ambrose, et al. 2010). No patients died in the bacterial group, but this may be owing to the small number of patients in that group. The outcome was relatively similar between autoimmune and viral encephalitis, although those with autoimmune encephalitis were more likely to be classed as having severe than moderate disability.

There are several potential sources of bias in selection of this study cohort. Firstly, some patients with encephalitis may be more likely to be screened and recruited than others, for example patients with difficult behaviour or who are acutely unwell may be systematically under-represented. In selecting patients with samples available for study (although this was essential) there may be systematic bias introduced as some patients may be less likely to have leftover clinical samples than others (i.e. those with more straightforward lumbar punctures) which again may bias against those who are more seriously unwell or who are exhibiting challenging behaviour. However, the study cohort is similar to and reflective of large epidemiological studies of encephalitis in the UK which is reassuring that sources of bias have not dramatically altered the makeup of the eventual cohort.

Clearly diagnosis is a complex process involving pattern recognition by the treating doctor, taking into account all of the history, examination and investigations. Through deriving models based on clinical features to identify cases of autoimmune and viral encephalitis I have attempted to quantify this clinical decision making process, and identify the clinical features which contribute most to distinguishing aetiological groups. The optimal model to predict diagnosis of viral encephalitis versus all other groups included elevated CSF protein, abnormal neuroimaging and the presence of fever. Conversely, the model to identify autoimmune encephalitis incorporated younger age, absence of headache, presence of memory problems and presence of movement disorder. The model for autoimmune encephalitis was more accurate overall than the model for viral encephalitis. The cases of

autoimmune encephalitis in this cohort included a large number of cases of NMDAR antibody encephalitis, which have several salient features including the presence of movement disorder, which was the feature contributing most to the model. Conversely, patients with seronegative autoimmune encephalitis were least likely to be classified correctly.

Conclusion

This chapter has described the clinical features of the cohort of patients I studied. It was reassuring that the cohort was similar to the large epidemiological studies performed previously, and thus is likely to be reflective of the incidence of encephalitis in the UK as a whole. Interestingly, although I found some clinical parameters could distinguish between the diagnostic groups, none were sufficiently robust to be relied upon, hence the importance of investigating for potential biomarkers which can do this. In the next chapter I investigate whether RNA transcripts in the blood could be used in this way. I also begin to examine what such transcripts tell us about disease mechanisms in different types of encephalitis.

Chapter 3. Transcriptomic analysis in whole blood

Introduction

Analysis of clinical features from the current study cohort and others (Granerod, Ambrose, et al. 2010) shows that discriminating between autoimmune encephalitis, viral encephalitis, and other disease processes that present in a similar way is challenging. Testing for autoantibodies and infectious agents can take days or weeks, potentially delaying time-critical treatment. In many cases no cause is found and treatment is therefore delivered based on insufficient diagnostic information, often resulting in poorer outcome (Schmidt et al. 2011).

The diagnosis of encephalitis is heavily reliant on the analysis of CSF obtained by lumbar puncture, which can present challenges and delays. Although inflammation and tissue damage in encephalitis occurs within the CNS, varying immune responses in both autoimmune and viral encephalitis are detectable in the peripheral blood, and this has been demonstrated through differing cytokine and chemokine profiles, including from my group in Liverpool (Michael et al. 2016). Modulation of gene expression in peripheral leucocytes is likely to underly these changes in protein abundance. In addition, in the setting of CNS inflammatory disorders, circulating lymphocytes and monocytes/macrophages cross the blood brain barrier, and the signalling pathways allowing this migration require gene expression changes in the peripheral blood (Engelhardt 2006).

Previous studies have investigated gene expression profiles in related conditions involving inflammation in the nervous system, including bacterial meningitis (Lill et al. 2013; Kulohoma et al. 2017), where activation of cellular and humoral immune responses discriminated patients from healthy controls, and enterovirus meningitis (Bartholomeus et al. 2019), in which an innate immune signature based on IL-1 signalling was identified.

Therefore, I hypothesised that RNA transcripts in peripheral blood would differ between viral and autoimmune encephalitis, bacterial meningitis and mimicking disease states, and that these changes could provide a model to discriminate between these conditions. In addition, I hypothesised that gene enrichment analysis may help to understand the pathophysiology underlying these differences between aetiological groups. To test this hypothesis I analysed RNA extracted from whole blood from patients with these conditions by DNA microarray.

Methods

Patient groups and blood collection

The patients which make up the cohort for this chapter are a subset of the whole study cohort described in chapter 2. Adult patients with suspected encephalitis were recruited from participating hospitals in the UK. Following informed consent from patients or representatives, blood was collected using the Vacutainer system into PaxGene tubes (Qiagen), kept at room temperature for 2 hours and then frozen at -80°c until needed.

Once sufficient clinical diagnostic information was obtained, patients were divided into groups according to case definitions (see Chapter 2)- viral encephalitis, autoimmune encephalitis, bacterial meningitis and mimics.

RNA extraction, quality control, amplification and labelling

Samples were thawed and RNA extracted using commercially available PaxGene Blood RNA extraction kits. Briefly, samples were centrifuged, and the pellet washed and incubated with proteinase K to digest the proteins. The lysate was filtered through a shredder spin column and the flow through collected. Ethanol was then added to the lysate and loaded onto an RNA spin column to selectively bind total RNA. The column was washed twice with 30µl of RNAse free water to collect two fractions of total RNA.

To validate RNA quality, wash samples were assessed by UV spectrophotometry. Samples were included in the array cohort if the RNA yield was ≥ 100ng/µl, the 260:280nm absorbance ratio was 1.8-2.2 and the 260:230nm absorbance ratio was ≥1.8.

Labelling was performed using the Agilent Low Input Quick Amp Labelling kit to generate fluorescent complementary RNA (cRNA). For this 2-colour gene expression microarray, study samples were amplified and transcribed to produce cRNA labelled with Cy5, while Universal Human Reference RNA (Agilent p/n 740000) was labelled with Cy3.

Briefly, RNA from samples and reference RNA were mixed with RNA spike-in controls (Agilent spike A mix for reference RNA, spike B mix for study samples), Agilent AffinityScript Reverse Transcriptase and Oligo dT-Promoter primer. Incubation at 40°C for 2 hours and 70°C for 15 minutes resulted in the synthesis of cDNA. Next, t7 RNA polymerase, Cy3 or Cy5 dye and nucleoside triphosphates (NTPs) were added and samples were incubated at 40°c for 2 hours resulting in the synthesis of labelled sample and reference cRNA.

The cRNA was purified using the RNeasy mini kit (Qiagen) and quantified using UV spectrophotometry. Samples were required to meet criteria of ≥825 ng and specific activity of ≥8.0 pmol Cy3/5 per µg cRNA to proceed to hybridisation.

Hybridisation

Labelled cRNA was hybridised using Agilent SurePrint G3 Human Gene Expression 8x60K v2 Microarray chips (Design ID 039494). This design features long non-coding RNA (lncRNA) probes based on the catalogue of lncRNA from the LNCipedia 2.1 database.

Labelled cRNA (Cy3 for reference samples, Cy5 for study samples) was combined with Agilent gene expression blocking agent, nuclease free water and RNA fragmentation buffer to a total volume of 25µl and incubated at 60°C for 30 minutes to fragment RNA before cooling on ice for 1 minute.

Gasket slides were loaded into Agilent SureHyb hybridisation chambers and 40µl of hybridisation sample was dispensed into each gasket well, before application of the Agilent slide. The chambers were incubated at 65°C for 17 hours.

Slides were washed for 1 minute at room temperature and 1 minute at 37°C. Slides were inserted into slide holders and scanned using an Agilent SureScan Microarray Scanner, Agilent Scan Control software v7.0.03, and Agilent Feature Extraction v12.0.2.2.

Expression data filtering and scaling

Data were $\log_2$ transformed. Features with a signal:background ratio of <2.5 were excluded. Within-array normalisation was performed by Loess regression; between-array normalisation was performed by quantile method. Replicates of the same gene were collapsed to the gene with the highest median expression across all samples. Genes that did not show expression in at least one array were removed. This yielded a final dataset of 3156 genes.

Statistical analysis

Clinical features were analysed using chi-squared tests for categorical variables and Kruskal-Wallis test for continuous variables. Correlation between genes and clinical features were analysed using Pearson correlation coefficient. Univariate analysis for differential gene expression was performed using the R *limma* package (Ritchie et al. 2015). *limma* creates a linear model for each gene using an empirical Bayes correction of standard errors to assess whether expression of any genes is associated with the response variable (in this case, aetiological group). This ensures that results are as reliable as possible, even when the sample size is small in comparison to the number of variables. Correction for multiple comparisons was performed using the Benjamini-Hochberg method. Hierarchical clustering used Euclidean distance and Ward's linkage method. Multivariate analysis used principal component analysis and sparse partial least squares discriminant analysis (sPLSDA) using the R package mixOmics (Rohart et al. 2017). For selection of panels from genes identified by

sPLSDA I used multiple logistic regression with backwards elimination using chi-squared tests to assess for difference between models.

Results

Figure 1 is a flow chart illustrating selection of the samples for analysis. Of the entire cohort, 53 patients met the inclusion criteria and had appropriate samples for transcriptomic analysis. All of these samples were processed, and 40 met the RNA quality control standards. Those that failed had either low RNA yields or unacceptable 260:280nm absorbance ratio, indicating RNA impurity or contamination. Figure 2 shows the clinical characteristics of the aetiological groups.

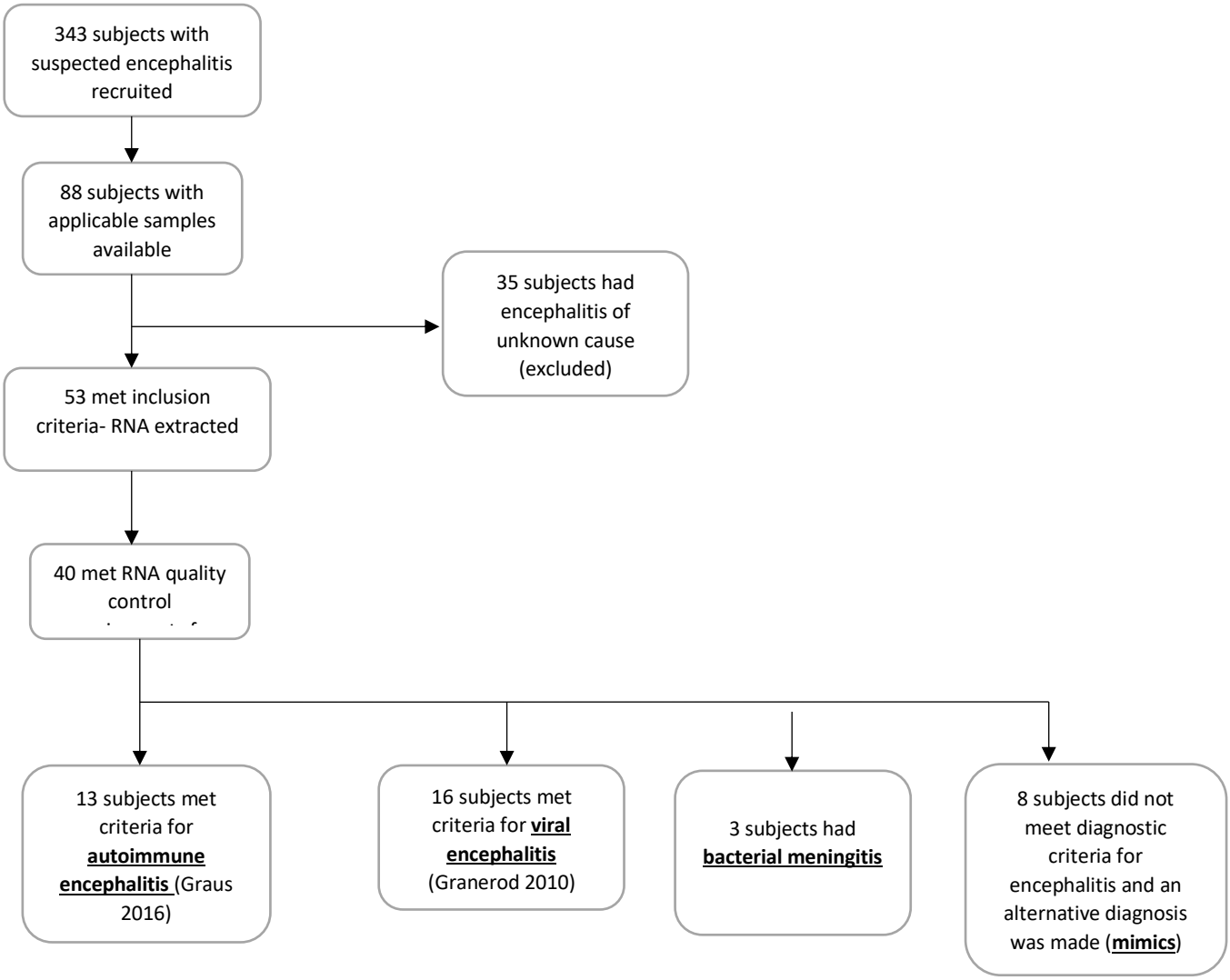


Figure 1. Flow chart illustrating selection of the cohort for transcriptomic analysis

Table 1. Clinical characteristics of the transcriptomics cohort					
Characteristics	**Autoimmune n=13**	**Viral n=16**	**Bacterial n=3**	**Mimics n=8**	**p value**
Age, median (range)	55 (18-75)	48 (30-91)	82 (71-89)	49 (24-80)	0.070
Female, n (%)	6 (46)	7 (44)	1 (33)	6 (75)	0.45
Fever n (%)	3 (23)	13 (81)	2 (67)	5 (63)	**0.017**
Seizures n (%)	7 (54)	4 (25)	0 (0)	2 (25)	0.19
Amnesia n (%)	9 (69)	3 (19)	0 (0)	4 (50)	**0.019**
Headache n (%)	4 (31)	12 (75)	1 (33)	5 (63)	0.093
Psychosis n (%)	2 (15)	0 (0)	0 (0)	2 (25)	0.21
Movement disorder n (%)	7 (54)	2 (13)	0 (0)	1 (13)	**0.033**
Immunosuppression at time of blood sampling, n (%)	3 (23)	0 (0)	0 (0)	0 (0)	0.080
Aciclovir at time of CSF sampling, n (%)	5 (38)	14 (88)	2 (67)	5 (63)	0.055
Brain imaging abnormality	9 (69)	15 (94)	3 (100)	3 (38)	0.017
EEG abnormality	7/11 (64)	6/6 (100)	0/0 (0)	2/3 (67)	0.24
CSF pleocytosis	6/12 (50)	14 (88)	2/2 (100)	0/6 (0)	**0.0024**
Outcome (GOS score) at last follow-up- median (range)	4 (1-5)	4 (3-5)	4 (4-5)	4 (3-5)	0.63
Diagnoses	4 NMDA, 3 VGKC, 1 Rasmussen's encephalitis, 1 Hashimoto's encephalopathy, 1 GAD, 3 seronegative	11 definite HSV1, 2 probable HSV1, 1 VZV, 2 enterovirus	3 S. pneumoniae	1 alcohol withdrawal, 2 headache disorders, 2 metabolic disorders, 1 cerebrovascular disease, 1 first seizure, 1 primary psychiatric disorder	

Univariate analysis

Initially, I used *limma* to look for single gene differences between groups in a pairwise fashion. Bacterial samples were well distinguished from other groups by a number of single genes, and analysis of bacterial cases versus all other groups revealed 285 genes with significant differences of which the most pronounced was *CSGALNACT2* (coding for chondroitin sulfate N-acetylgalactosaminyltransferase 2). The most significant genes are represented in Figure 2A. Figure 2B shows a volcano plot for the analysis of bacterial meningitis samples vs the rest of the cohort, which represents significance on *limma* analysis (y axis) against fold change (x axis). The significant gene with the greatest fold change between bacterial meningitis and other groups was *ARG1* (coding for Arginase-1) However, on pairwise analysis of autoimmune encephalitis, viral encephalitis and mimics no genes were significant on univariate analysis, after correction for multiple comparisons.

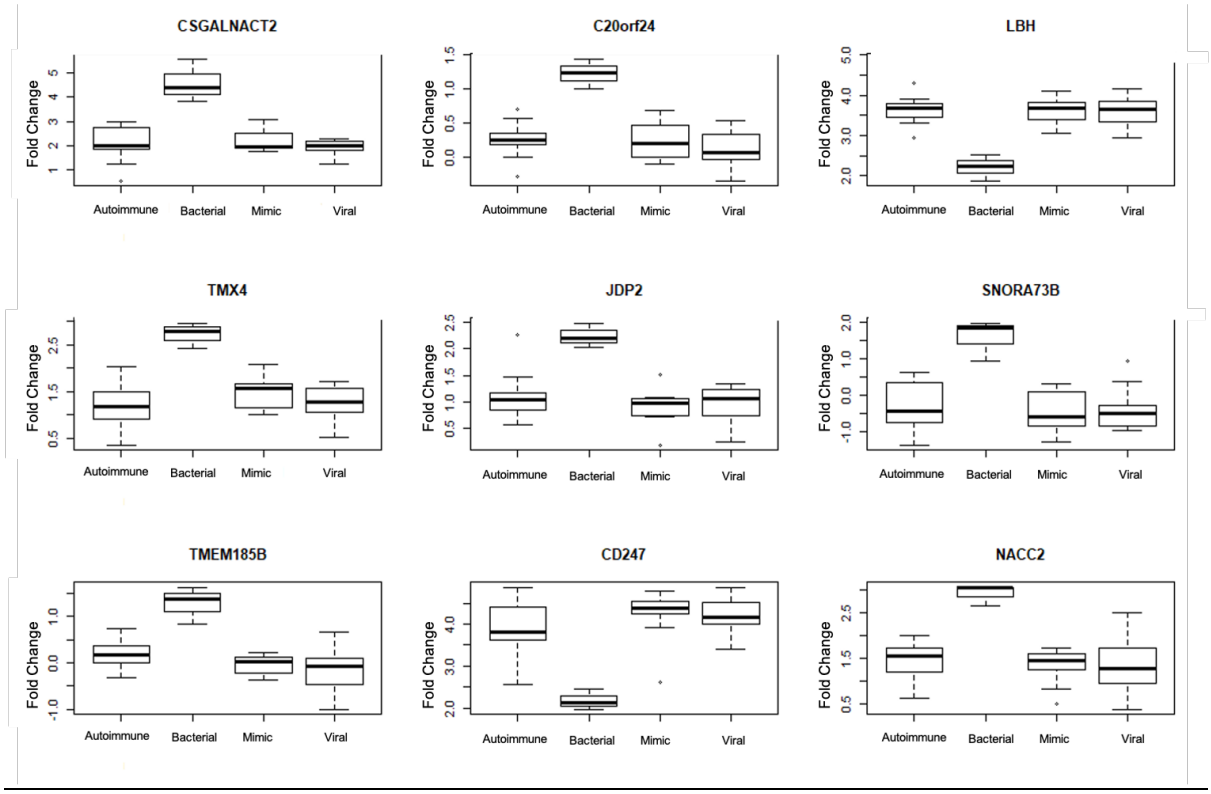
Bacterial samples were removed from further analyses as this group was clearly distinct on univariate analysis, and to see whether subsequent analysis without this group could allow different types of encephalitis to be distinguished.

Cluster and multivariate analysis

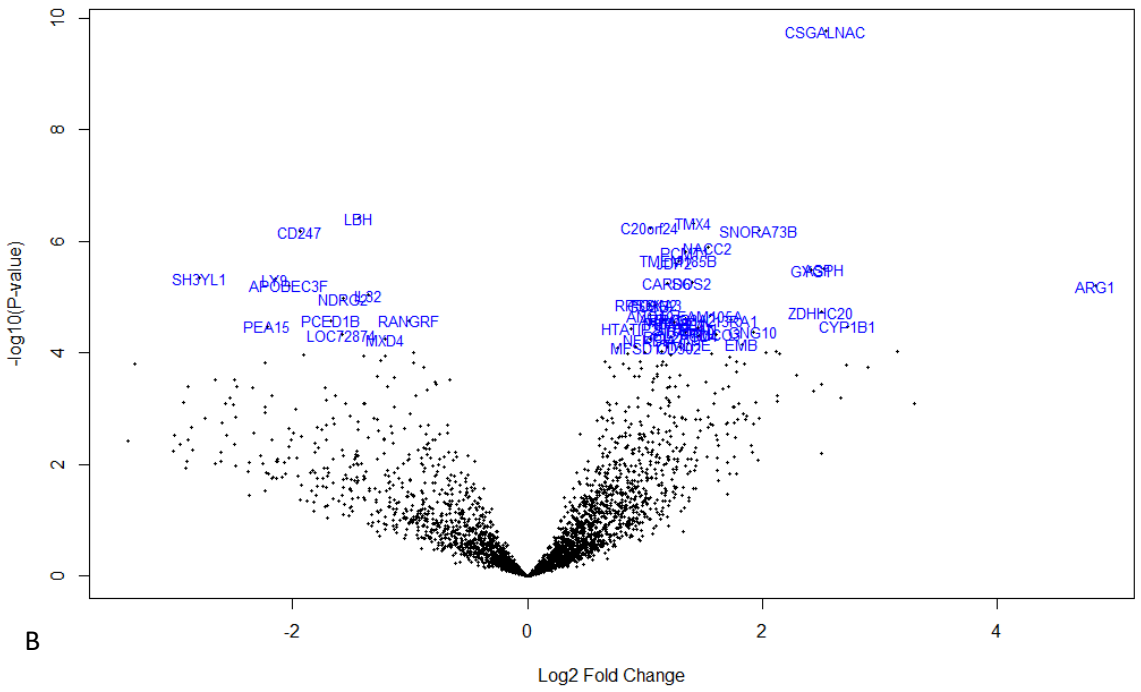
I next performed cluster analysis to explore whether unsupervised analysis of gene expression patterns could stratify patients by aetiological group: viral encephalitis, autoimmune encephalitis and mimics.

Hierarchical cluster analysis by both genes and arrays did not show clustering by aetiological groups (Fig. 3A), indicating a wide range of overlapping variability within and between groups.

In order to identify clusters of interest amongst genes which differed between groups, I performed a more supervised cluster analysis of genes only, restricted to the lowest 25 corrected p values by one-way analysis of variance (ANOVA) (figure 3b). Although this showed 2 broad clusters, the first mostly upregulated in autoimmune encephalitis and mimics, the second mostly in viral encephalitis, clustering showed no clear pattern in the data and no clear functional clustering of genes.

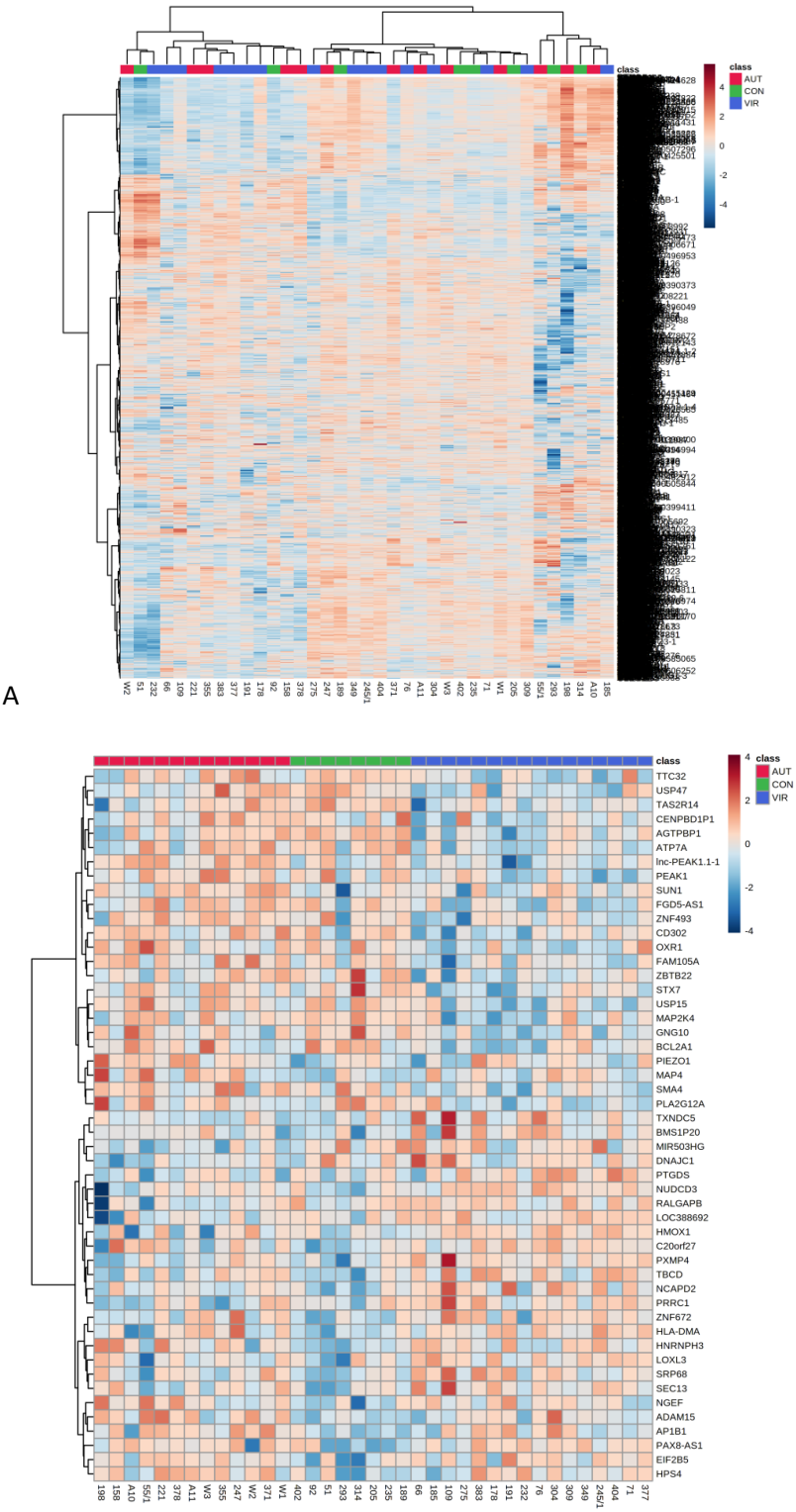


A



B

Figure 2. Significant genes between bacterial meningitis and the rest of the cohort. A: boxplots for the most significant genes by *limma* analysis. B: volcano plot illustrating $\log_2$ fold change and $-\log_{10}$ p value, significant genes by *limma* analysis indicated in blue.



A

B

Figure 3. Heatmaps illustrating cluster analysis. Coloured bar represents aetiological group: red=autoimmune, green=mimics, blue=viral. Each column represents one patient, each row represents one gene. A: entire dataset (filtered) clustered by genes and arrays. B: 25 most significant genes by ANOVA clustered by genes only.

Next, I investigated whether patterns existed within the data between aetiological groups by an alternative unsupervised multivariate approach through principal component analysis.

Figure 4 shows scores plots for principal component analysis for autoimmune encephalitis, viral encephalitis and mimics. Separate plots are shown for pairwise interactions between principal components 1-3, along with the cumulative variance accounted for by principal components. Overall, this analysis did not show substantial patterns in the data for components 1-3, with 9 components required to account for more than 70% of the variance within the dataset, reflecting the complexity and overlapping nature of the transcriptome.

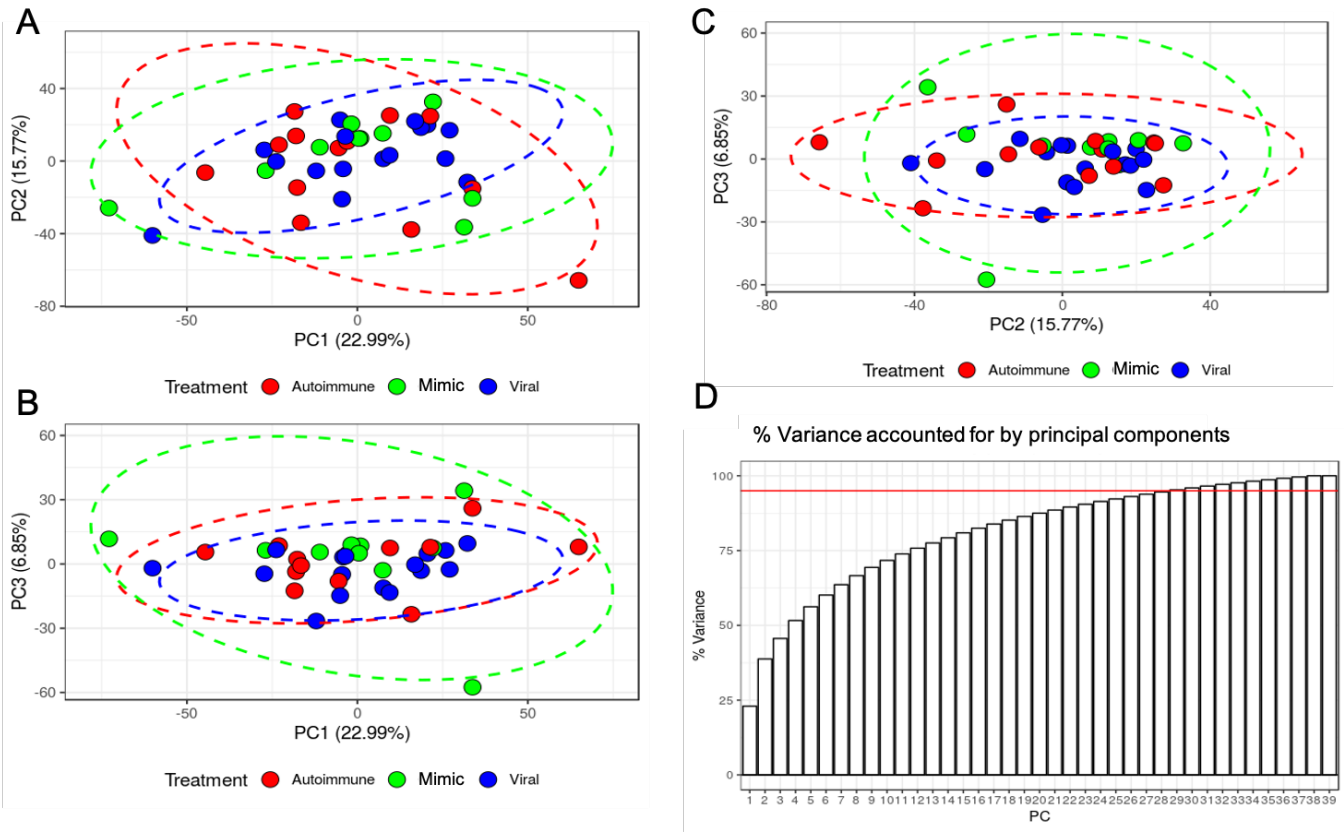


Figure 4. Principal component analysis including autoimmune (red), viral (blue) and mimic (green) groups. A-C: scores plots for components 1-3. D: Cumulative percentage variance accounted for by each principle component.

Since unsupervised techniques did not yield variables to distinguish between aetiological groups, I next used a supervised multivariate technique: sPLSDA, chosen because it is particularly effective at reducing the number of variables in complex datasets (Lê Cao et al. 2011). I looked for variables which could distinguish between aetiological groups in a

pairwise fashion, with a 5-fold cross validation step performed 100 times to select the most appropriate number of components and features for the model (figure 5A-C).

I used the variables with the greatest contribution to the sPLSDA model (figure 5D-F) to construct gene panels to distinguish each group. Variables with coefficient >0.1 were included in multiple logistic regression with backwards elimination in order to select the simplest model without significant loss of prediction of the dependent variable (aetiological group). Table 2 shows the selected gene panels for each pairwise comparison with area under the ROC curve.

Table 2. Gene panels selected for pairwise discrimination between aetiological groups

Comparison	**Genes included in panel**	**Area under ROC curve**
Autoimmune vs. mimics	*ASS1*/*MCFD2*/*HLA-DQA2*/*MSN*/*PRDM1*	0.85
Viral vs. mimics	*HLA-DMA*/*lnc.DTYMK-3*/*PRR36*	0.95
Viral vs. autoimmune	*TMEM40*/*ATP7A*	0.85

ROC = receiver-operating characteristic; lnc = long non-coding.

The model discriminating autoimmune from mimics was the most complex with 5 genes, while separating viral encephalitis from mimics required only 3 genes and achieved more effective discrimination.

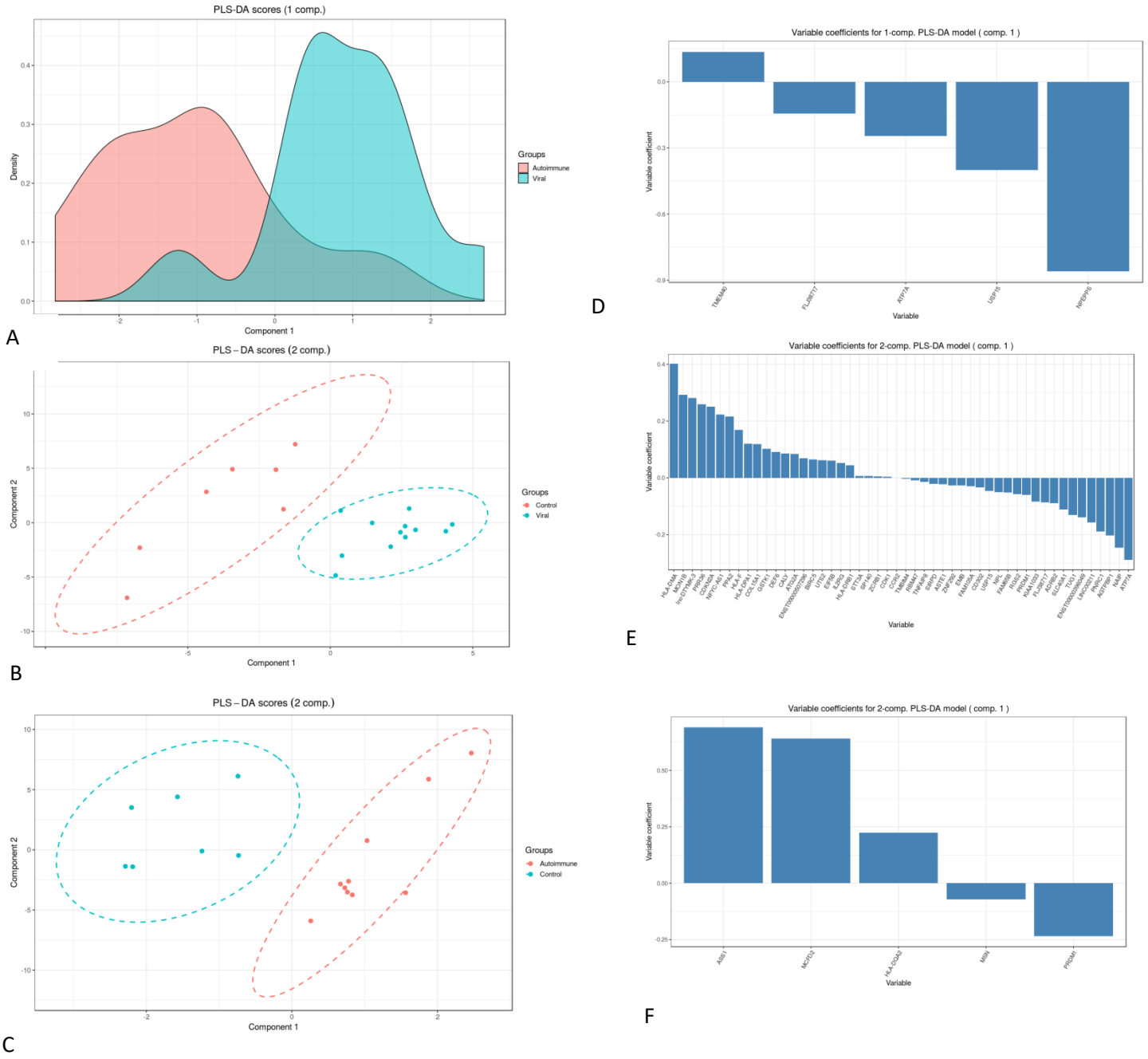


Figure 5. Sparse partial least squares discriminant analysis (sPLSDA) for discrimination between autoimmune encephalitis, viral encephalitis and mimics. A: 1 component model for autoimmune encephalitis vs viral encephalitis. B: 2 component model for viral encephalitis vs mimics. C: 2 component model for autoimmune encephalitis vs. mimics. D-F Contribution of variables to the sPLSDA models, D: autoimmune vs viral, E: viral vs mimics, f: autoimmune vs mimics.

Enrichment analysis

Gene set enrichment analysis uses a list of significant genes to identify biological pathways which are over-represented, thereby building a functional profile of the genes of interest. In this dataset I decided to look for pathways enriched in bacterial meningitis compared to other groups, as this analysis yielded the most significant results and therefore was most amenable to meaningful gene set analysis. I used the gene identifiers with significant differences in expression in bacterial meningitis to analyse genes associated with biological processes, cellular components and molecular function according to the gene ontology (GO) knowledgebase, Reactome database and the Kyoto Encyclopaedia of Genes and Genomes (KEGG). Table 3 shows the top ten enriched pathways, the number of genes associated with each pathway and the significance level. Genes associated with the innate immune system were the most significantly enriched, along with neutrophil degranulation and the VEGFA-VEGFR2 signalling pathway. Genes associated with adaptive immunity were also enriched, although to a lesser extent.

Table 3: Gene set enrichment analysis: bacterial meningitis versus other groups				
Gene Set Name	**Description**	**Number of genes**	**p value**	**FDR q-value**
Reactome Innate Immune System [1114]	Innate Immune System	43	$4.14\ e^{-20}$	$1.19\ e^{-16}$
Reactome Neutrophil Degranulation [479]	Neutrophil degranulation	27	$6.37\ e^{-17}$	$9.13\ e^{-14}$
Vegfa-vegfr2 Signaling Pathway [437]	VEGFA-VEGFR2 Signaling Pathway	17	$1.16\ e^{-8}$	$1.11\ e^{-5}$
Reactome Disease [1580]	Disease	31	$1.65\ e^{-7}$	$1.18\ e^{-4}$
Reactome Eph Ephrin Signaling [92]	EPH-Ephrin signaling	8	$2.45\ e^{-7}$	$1.4\ e^{-4}$
Reactome Nervous System Development [580]	Nervous system development	17	$6.49\ e^{-7}$	$2.8\ e^{-4}$
Reactome Signaling By Rho Gtpases [454]	Signaling by Rho GTPases	15	$6.84\ e^{-7}$	$2.8\ e^{-4}$
Kegg T Cell Receptor Signaling Pathway [108]	T cell receptor signaling pathway	8	$8.42\ e^{-7}$	$2.91\ e^{-4}$
Biocarta T cytotoxic Pathway [12]	T Cytotoxic Cell Surface Molecules	4	$1.01\ e^{-6}$	$2.91\ e^{-4}$
Biocarta T helper Pathway [12]	T Helper Cell Surface Molecules	4	$1.01\ e^{-6}$	$2.91\ e^{-4}$
FDR = false detection rate				

Discussion

I analysed blood from 40 patients with encephalitis and mimicking conditions by 2-colour human gene expression DNA microarray. Univariate analysis of significantly upregulated or downregulated genes revealed that bacterial meningitis differed from encephalitis and mimics by expression of 285 genes. On univariate analysis, no genes showed significant differences between autoimmune encephalitis, viral encephalitis and mimics. Cluster and principal component analysis did not show clear patterns in the data. Multivariate analysis using sPLSDA provided models which were able to discriminate between autoimmune encephalitis, viral encephalitis and mimics, and I built simple gene panels that could distinguish viral encephalitis from mimics with good accuracy (ROC AUC 0.95), and autoimmune encephalitis from viral encephalitis and mimics with lower accuracy (AUC 0.85).

The transcriptomic profile of bacterial meningitis reflected activation of the innate and adaptive immune system, as found by previous studies (Lill et al. 2013). The most significant gene upregulated in bacterial meningitis, *CSGALNACT2*, is involved in chondroitin sulphate synthesis and is not clearly functionally connected to infection and immunity, although it has been identified in transcriptional analysis in severe sepsis, and in neurovascular disease (Le et al. 2019; Aung et al. 2014). The significant gene with the greatest fold change in bacterial meningitis was *ARG1*, whose protein product Arginase-1 is a key regulator of neutrophil function and has previously been found to be upregulated in sepsis (Ahmad et al. 2019). Pathways enriched in bacterial meningitis mostly reflected immune function, with

significant enrichment of gene sets associated with innate immunity. Both VEGF and Ephrin signalling, involved in angiogenesis, are known to be upregulated in sepsis and bacterial infection (Almasy et al. 2020; Lee et al. 2017). However, there were only three bacterial meningitis cases in this cohort, so interpretation of these results must be cautious.

Although no single genes were identified to distinguish between aetiologies of encephalitis and mimics, sPLSDA modelling identified several genes which in concert were able to discriminate groups within this cohort. The genes identified included several of the human leucocyte antigen (HLA) group. In viral encephalitis, four genes of this group were upregulated compared to mimics, mostly of the MHC class II locus (*HLA-DMA*, *HLA-DPA1* and *HLA-DRB1*), concerned with presentation of extracellular antigen to CD4+ helper T cells. *HLA-F* was also upregulated in viral encephalitis compared to mimics, and codes for an MHC class 1 protein concerned with presentation of intracellular antigen to CD8+ cytotoxic T cells, as in the case of viral infection. In autoimmune encephalitis, another MHC gene *HLA-DQA2* was upregulated compared to mimics.

Although bacterial meningitis was well distinguished from other groups, gene expression signatures were not as effective as anticipated at differentiating between autoimmune encephalitis, viral encephalitis and mimics. The panels developed from multivariate analysis were effective within this cohort of 40 samples but would require validation in an independent cohort to exclude overfitting. There are several possible reasons for the lack of significant differences in blood transcriptome. The numbers of patients were small in relation to the number of variables, increasing false detection rate and potentially leading to

suppression of signals which could be detected in a larger cohort. The mimic group included hospital patients with suspected encephalitis, some of whom had peripheral infection or other acute illnesses which may have reduced the possibility of detecting upregulation of genes associated with host immune responses in encephalitis. A healthy control group could have provided increased sensitivity for these changes, but may not have yielded biomarkers that were useful in real clinical practice, because healthy controls do not usually come to hospital with suspected encephalitis.

Conclusion

My results from this chapter suggest that gene expression profiles from blood may have the potential to yield biomarker combinations to improve the diagnosis of encephalitis, particularly in cases where CSF is unavailable or delayed. However, in order to obtain stronger signals to distinguish these groups, I decided in the next chapter to study the CSF, which is a closer proxy to the environment of the brain parenchyma, the site of inflammation in encephalitis.

Chapter 4. Proteomic analysis in cerebrospinal fluid

Introduction

Diagnosis of inflammation or infection of the CNS in clinical practice relies heavily on the analysis of CSF. Having analysed gene expression profiles in patients with encephalitis and meningitis in Chapter 3, it is clear that transcripts in the blood can distinguish bacterial meningitis from encephalitis and mimicking conditions. However, distinguishing viral from autoimmune encephalitis and mimics requires analysis of a biofluid providing a closer proxy to brain tissue. From previous studies and analysis of this study cohort (Chapter 2), it is evident that routine laboratory analysis of CSF is not always sufficient to distinguish autoimmune from viral encephalitis. In both cases, the CSF WCC may be normal, and CSF total protein and glucose changes are non-specific. Therefore, I decided to analyse the CSF through the unbiased and high-resolution approach of mass spectrometry-based proteomics.

Several previous studies have measured panels of proteins in either CSF or serum in order to identify diagnostic or prognostic biomarkers, many focusing on immune proteins such as chemokines and cytokines. Previous studies from my team In Liverpool identified MPO, which could distinguish infectious from autoimmune encephalitis, and identified that in HSV encephalitis a higher IL-1:IL-1RA ratio correlated with worse outcome (Michael et al. 2016;

Michael et al. 2015). In other studies, interferon-gamma (IFNγ), IL-6, IL-10, and sTNFR1 were found to be elevated in HSV encephalitis, and IL-6 and IL-6 and IFNγ were associated with worse outcome (Ichiyama et al. 2008; Kamei et al. 2009). IL-6, IL-7, and IL-13 have been shown to be elevated in infective encephalitis (predominantly associated with tick-borne encephalitis virus) compared with autoimmune encephalitis or controls (Ygberg et al. 2016). In NMDAR antibody encephalitis, CXCL13 has been found to be elevated and correlated with worse outcome (Leypoldt et al. 2014).

The proteome of an organism or tissue is the entire array of expressed proteins and is dynamic, varying over time and with disease states. Clinical proteomics is an attempt to analyse this complex mixture using approaches to measure the abundance of thousands of proteins in parallel, albeit from the snapshot of a clinical sample. Although proteomic analysis of CSF has been used to investigate meningitis, CNS vasculitis, Parkinson's disease and neurodegenerative conditions amongst others (Cordeiro et al. 2015; Mandel-Brehm et al. 2019; Bereczki et al. 2018), the method has not previously been applied to samples from patients with encephalitis of different aetiologies.

Given the clinical challenge of diagnosing and managing patients with encephalitis, the aim of this chapter was to analyse the proteomic profiles of patients with viral encephalitis, autoimmune encephalitis and mimicking conditions in order to identify protein candidates for diagnostic use or to provide prognostic information for clinicians.

Methods

Patients and diagnostic subclassification

Patients with suspected encephalitis were recruited through participating hospitals as discussed in chapter 2. Division of patients into aetiological groups is illustrated in Figure 1. These patients represent a subset of the total cohort described in chapter 2.

Patients were further divided into approximately equal 'discovery' and 'validation' cohorts, cohorts 1 and 2 respectively. Cohort 1 (discovery cohort) included only patients with definite diagnoses of either autoimmune encephalitis (Definite autoimmune limbic encephalitis, NMDAR antibody encephalitis or definite acute disseminated encephalomyelitis (ADEM) (Graus et al. 2016) or viral encephalitis ("confirmed" criteria for each organism according to Granerod 2010 (Granerod, Cunningham, et al. 2010) (see appendix)). Cohort 2 (validation cohort) also included patients with possible diagnosis. In cohort 1 I included patients with mimics who proved to definitely not have systemic infection – i.e. those with non-inflammatory final diagnoses including headache disorders, without fever and with normal brain imaging and EEG. In cohort 2 I included mimics who presented with inflammatory or infectious disorders, or in whom the final diagnosis was uncertain. The reason I divided the mimics like this was because in cohort 1, the discovery cohort, I wanted to identify potential biomarkers using cleanly divided groups, which could then be tested in cohort 2, the validation cohort, with a mimic group representing more closely the situation in real clinical practice.

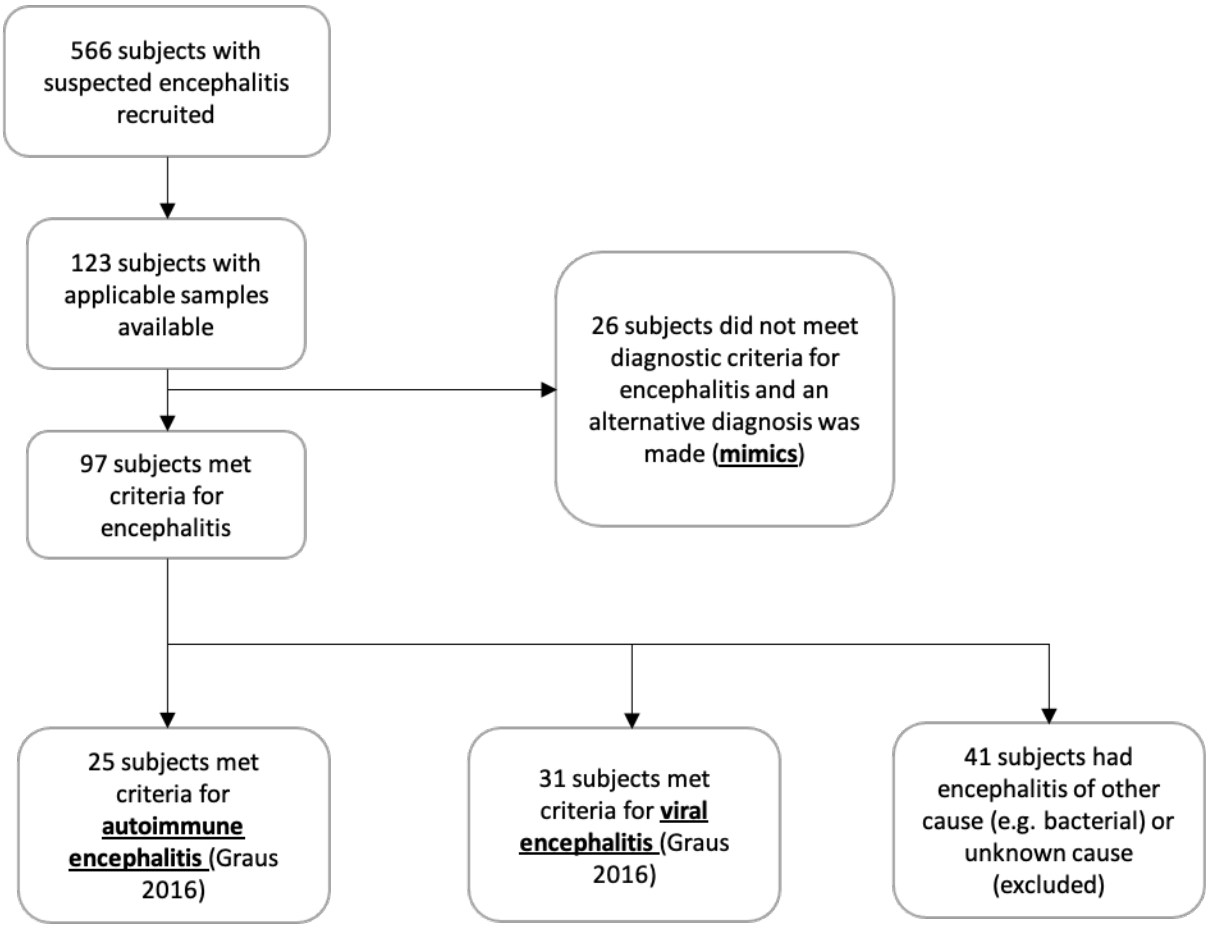


Fig 1. Flow chart illustrating cohort selection.

Sample collection and LC-MS/MS analysis

Samples were collected by lumbar puncture, aliquoted and frozen within 2 hours at -80°C. Freeze thaw cycles were minimised. 83 samples eligible for inclusion were then prepared using Thermo Scientific SMART Digest Trypsin kit with SOLAμ Solid-Phase Extraction (SPE) (product code 60109-103) Briefly, 50μl of CSF was added to 150μl SMART Digest buffer on ice and vortexed. The resulting mixture was added to the SMART Digest tubes containing immobilised trypsin. The samples were placed on a heat shaker for 1 hour at 70 °C and 1400 rpm. Following digestion samples were acidified with 1% formic acid. Samples were desalted using 96-well SOLAμ SPE plates in a vacuum manifold. Peptides were eluted with 50μl (70% acetonitrile/30% water) and dried and re-suspended in 2 % acetonitrile and 0.1% formic acid ready for analysis by LC-MS/MS.

LC-MS/MS analysis was performed using a Dionex Ultimate 3000 nano-ultra high-pressure reverse phase chromatography column coupled on-line to a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific). Samples were separated on an EASY-Spray PepMap Rapid Separation Liquid Chromatography (RSLC) C18 column (500 mm x 75 μm, 2μm particle size, Thermo Scientific) over a 60 minute gradient of 2-35 % acetonitrile in 5 % DMSO and 0.1% formic acid at a flow rate of 250 nl/min. Full MS (MS1) scans were acquired at a mass resolution of 70,000 full width at half maximum (FWHM) at m/z 200 and the top 15 most abundant precursor ions were selected for higher-energy collisional dissociation (HCD) fragmentation.

MRI analysis

For four patients with HSV encephalitis, data were available from a previous study which analysed MRI scans by stereology, with methods as described previously (Defres et al. 2017). Briefly, stereological analysis was performed using Easymeasure software. The total volume of signal abnormality on T2-weighted fluid-attenuated inversion recovery (FLAIR) images, loosely defined as cerebral oedema, was measured for each hemisphere of the brain. Separation between test points on the square grid used for point counting was 10 pixels, and slice interval was between 4 and 5 mm (every MR section). The region of interest was based on the area of FLAIR image hyperintensity. All hyperintense FLAIR image voxels were classified as being oedema and as previously observed for the pattern of HSV encephalitis, were in the temporal and limbic regions. All images for FLAIR assessment were reviewed with a neuroradiologist blinded to clinical information.

Data processing and statistical analysis

Protein quantitation was performed using the MaxQuant software suite (Cox et al. 2014). Three samples where <1500 peptides were identified were removed from analysis (2 samples from the viral and 1 from the autoimmune group) and 79 complete datasets were included in the final analysis. Candidate proteins with missing values in more than 30% of each group were excluded. Protein distributions were normalised to the median in order to allow comparison between samples with differing overall protein concentrations and to account for variability in sample storage conditions and processing.

Univariate analyses were performed using the R package *limma* (Ritchie et al. 2015). Multivariate analysis was performed by Random Forest using the R packages RandomForest and varSelRF, and panels were assessed using logistic regression. Candidate biomarkers were selected by these methods from cohort 1 (discovery cohort), and validation in cohort 2 (validation cohort) was performed using ROC analysis. P-values obtained from hypothesis tests were corrected for multiple comparisons throughout using the Benjamini-Hochberg method. Gene set enrichment analysis was performed using the full set of measured proteins as the background, using the DAVID platform and Reactome pathway analysis through the clusterProfiler package in R.

Results

Demographics and clinical characteristics

The clinical and demographic characteristics of the patients included in the final analysis are shown in table 1. Significant differences existed between the aetiological categories in age (the patients with autoimmune encephalitis were generally younger), while fever was more common in viral encephalitis and movement disorder more common in autoimmune encephalitis. Some patients with autoimmune encephalitis had already received immune therapy at the time of CSF sampling, predominantly corticosteroids. There was a trend towards earlier administration of aciclovir in patients with a final diagnosis of viral encephalitis. Abnormality on brain imaging and CSF pleocytosis were significantly more frequent in encephalitis of any cause than in mimics. At the time of recruitment VGKC antibody was commonly used diagnostically, these samples were tested retrospectively where possible for LGI-1 and CASPR2 antibodies in CSF and were found to be negative; serum was not available for testing. These patients were therefore classified as if they were antibody negative according to clinical criteria (Graus et al. 2016).

Table 1: Clinical characteristics and diagnoses in proteomic cohort.							
	Autoimmune encephalitis n= 24		**Viral encephalitis n=29**		**Mimics n=26**		**p value**
Age, median (range)	29 (6-73)		50 (1-91)		46 (18-80)		**0.03**
Female, n (%)	12 (50)		14 (48)		18 (69)		0.24
Fever	14 (58)		21(72)		11 (42)		0.07
Seizures	10 (42)		10 (34)		8 (31)		0.72
Amnesia	10 (42)		6 (21)		7 (27)		0.24
Headache	13 (54)		15 (52)		18 (69)		0.38
Psychosis	3 (13)		0 (0)		2 (8)		0.17
Movement disorder	7 (29)		3 (10)		4 (15)		0.19
Immunosuppression at time of CSF sampling, n (%)	10 (42)		0 (0)		0 (0)		**<0.01**
Aciclovir at time of CSF sampling, n (%)	10 (42)		17 (59)		10 (38)		0.27
Brain imaging abnormality	16 (67)		20 (69)		3/21 (12)		**<0.01**
EEG abnormality	14/21 (67)		13/16 (81)		3/4 (12)		0.61
CSF pleocytosis	16 (73)		24 (83)		1 (4)		**<0.01**
Outcome (GOS score) at last follow-up- median (range)	5 (1-5)		4 (1-5)		5 (1-5)		0.11
Diagnoses	**Cohort 1 n=15** 9 NMDA 1 VGKC (LGI-1 and CASPR2 negative in CSF) 4 ADEM 1 IgLON5	**Cohort 2 n=9 of which definite diagnoses = 3** 1 NMDAR 3 seronegative 2 ADEM 2 VGKC (LGI-1 and CASPR2 negative in CSF) 1 GAD	**Cohort 1 n= 13** 10 HSV 2 VZV 1 enterovirus	**Cohort 2 n=16 of which definite diagnoses = 4** 8 HSV 5 VZV 2 enterovirus 1 adenovirus	**Cohort 1 n=10** 9 headache disorders 1 epilepsy	**Cohort 2 n=16** 4 seizures/epilepsy 3 headache disorders 2 confusion of unknown cause 1 concussion 3 toxic/metabolic encephalopathy 1 PRES 1 MoyaMoya 1 primary psychiatric disorder	

Identification of candidate biomarkers

Univariate analysis was initially performed in a pairwise fashion in Cohort 1 to identify single proteins with significant differences between groups. Corrected p values of <0.05 were considered significant. This revealed 219 proteins with significant differences between viral encephalitis and mimics (Fig. 2). Twenty-nine proteins distinguished autoimmune encephalitis from mimics and only one protein (neurocan core protein (NCAN)) distinguished autoimmune from viral encephalitis. 26 of the proteins distinguishing autoimmune encephalitis from mimics were found to be common with those distinguishing viral encephalitis from mimics, so a further analysis was performed to look for proteins distinguishing encephalitis (of any cause) from mimics, identifying 35 proteins with significant differences.

Multivariate analysis was performed using the iterative random forest algorithm, with 2 iterations. In the first iteration 20 models were fitted on different subsets of data and protein importance for each model was assessed by the "reduction in Gini index" criterion. The proteins that appeared among the top 10 most important in at least one model were deemed important. This was repeated for each group comparison.

To obtain small scale diagnostic panels of proteins the second iteration of multivariate analysis was performed using the varSelRF method for each group comparison. Briefly, random forest models were iteratively fitted to the data each time reducing the number of

proteins used by removing the least contributing protein, using out-of-bag error as the exclusion criterion. This resulted in diagnostic protein panels of minimal size while preserving performance. The performance of the protein panels was assessed by ROC analysis using logistic regression for better model transparency. Logistic regression was repeated 10 times on different subsets of data in cohort 1 (discovery cohort) in order to assess the robustness of the panel. Subsequently a model was fitted to the whole dataset in cohort 1 and tested on cohort 2 (validation cohort).

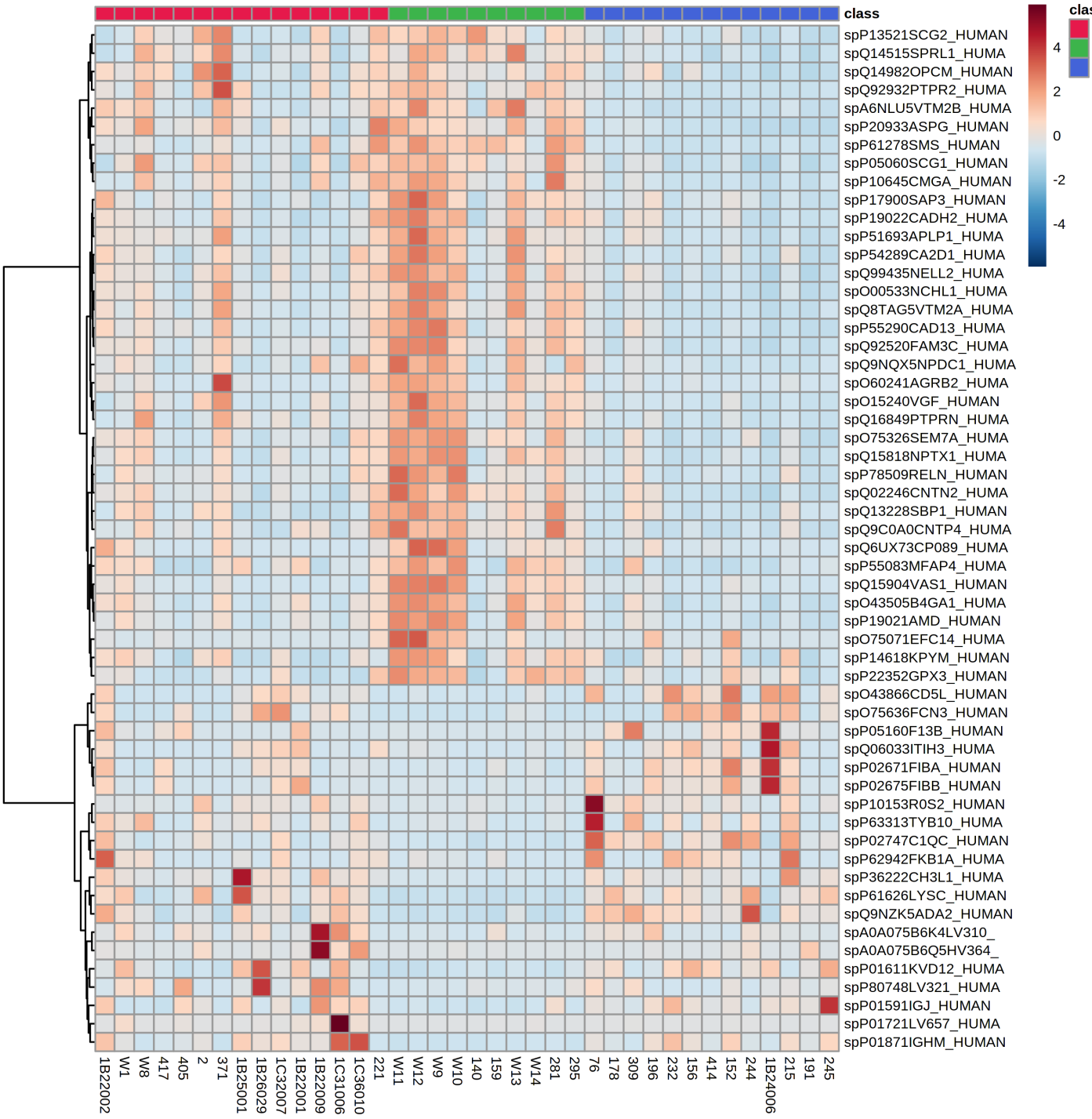


Figure 2. Heatmap illustrating the relative abundance of the proteins with the most significant difference between aetiological groups on univariate analysis. Columns represent individual patients; the coloured bar shows group, red= autoimmune, green=mimics, blue=viral.

Validation of candidates

Proteins identified as significantly different between groups by univariate analysis, in addition to proteins most often selected by random forest construction and panels built from random forest models, were tested in the validation cohort using ROC analysis. The five proteins with the largest area under the ROC curve (AUC) are shown in table 2 and Fig. 3.

Adenosine deaminase 2 (ADA2) was selected in pairwise comparisons of autoimmune vs mimics and viral vs mimics in cohort 1, and also distinguished encephalitis of any cause from mimics. This finding was validated in cohort 2, where ADA2 was able to discriminate viral encephalitis from mimics without error, and distinguished autoimmune encephalitis from mimics with AUC 0.84. It did not significantly distinguish viral encephalitis from autoimmune encephalitis in cohort 1, although in cohort 2 it did distinguish significantly, with AUC 0.94.

Neurocan core protein (NCAN) was the most effective single protein in distinguishing autoimmune from viral encephalitis in cohort 1, and AUC in cohort 2 was 0.72. Somatostatin was less abundant in autoimmune encephalitis than in mimics, and less abundant still in viral encephalitis. Several immunoglobulin subunits were identified on univariate analysis. IgM heavy chain was significantly higher in abundance in both viral and autoimmune encephalitis compared to mimics, with immunoglobulin kappa and lambda variable regions significantly more abundant in viral encephalitis than mimics, consistent across both cohorts 1 (discovery) and 2 (validation). V-set and transmembrane domain-containing protein 2A

(VTM2A and B) and apolipoprotein B100 were significantly less abundant in viral and autoimmune encephalitis than in mimics.

Table 2. Most promising candidate biomarkers. Proteins identified by significant differences in abundances from cohort 1 which had the largest area under the receiver-operating characteristic (ROC) curve in cohort 2 for each pairwise analysis.

Comparison	Biomarker candidates	FDR	logFoldChange	Number unique peptides	AUC in cohort 2
Viral encephalitis vs. mimics	Adenosine deaminase 2 (ADA2)	2.84E-05	3.02	17	1
	Immunoglobulin heavy constant mu (IGHM)	5.45E-05	3.16	18	0.89
	V-set and transmembrane domain-containing protein 2A (VTM2A)	5.45E-05	-2.45	6	0.92
	V-set and transmembrane domain-containing protein 2B (VTM2B)	7.81E-05	-3.00	3	0.7
	Somatostatin (SMS)	0.00015	-3.24	4	0.78
Autoimmune encephalitis vs. mimics	Adenosine deaminase 2 (ADA2)	0.0062	2.42	17	0.84
	Immunoglobulin heavy constant mu (IGHM)	0.0069	3.10	18	0.74
	Chitinase-3-like protein (CH3L1)	0.0069	2.16	20	0.61
	Glutathione peroxidase 3 (GPX3)	0.0080	-2.04	6	0.61
	Immunoglobulin kappa variable 3D-15 (KVD15)	0.012	1.94	3	0.6
Viral encephalitis vs. autoimmune encephalitis	Neurocan core protein (NCAN)	0.026	-1.84	14	0.72
	V-set and transmembrane domain-containing protein 2B (VTM2B)	0.072	-1.83	3	0.68
	Trans-Golgi network integral membrane protein 2 (TGON2)	0.072	-1.30	6	0.71
	G-protein coupled receptor 37-like 1 (G37L1)	0.072	-1.56	3	0.67
	Apolipoprotein B-100 (APOB)	0.072	1.99	71	0.72

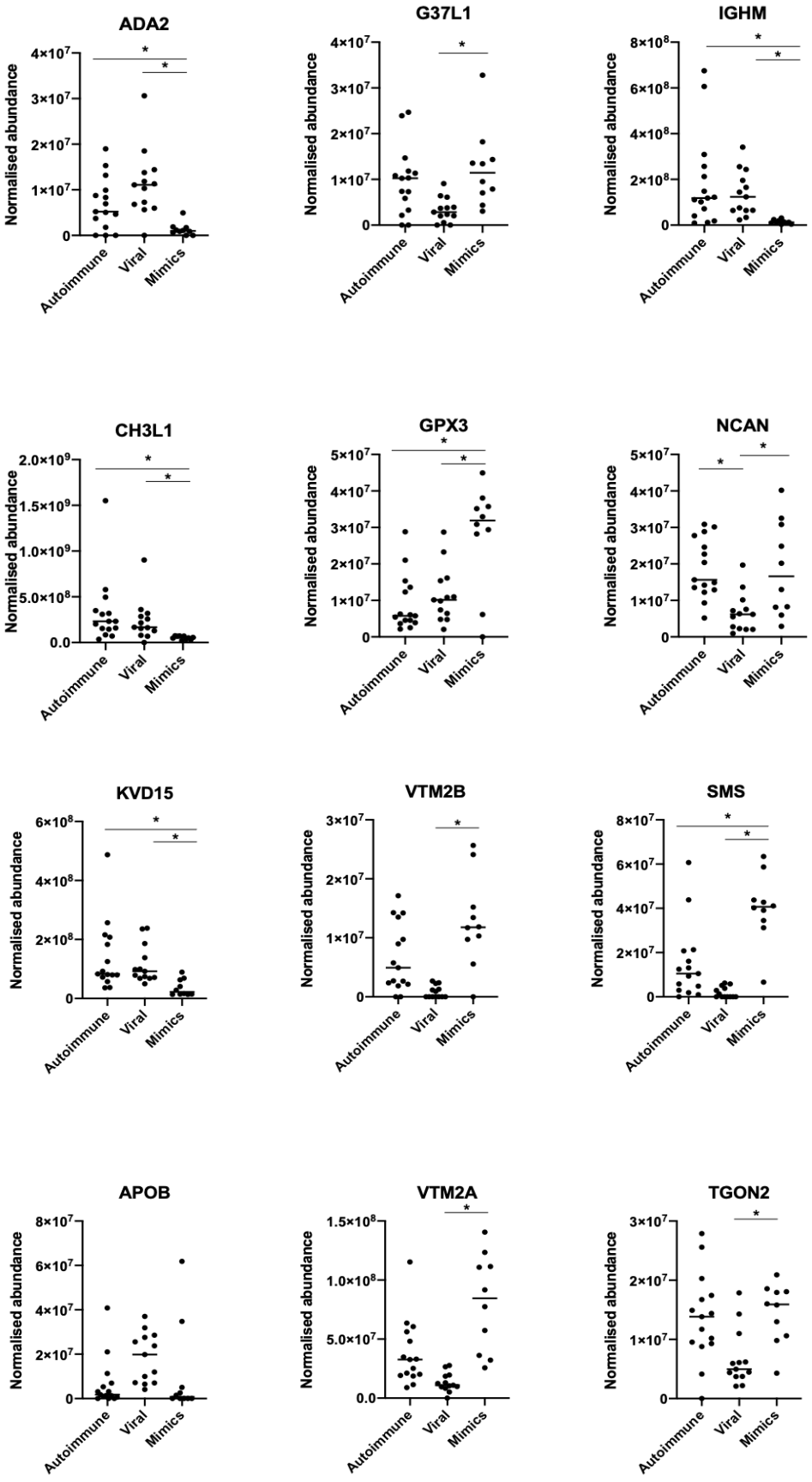


Figure 3. Boxplots showing normalised abundances in cohort 1 of the best performing candidate biomarkers. Having been selected in the discovery cohort for statistical significance on univariate analysis, these proteins showed the highest area under the receiver-operating characteristic (ROC) curve in the validation cohort. Horizontal lines

None of the top candidates identified showed significant correlation with CSF WCC on analysis of the whole cohort (Fig 4). In particular, ADA2 was able to distinguish viral encephalitis from mimics in those with normal WCC (<5/mm3) with AUC of 0.98.

Although panels derived from random forest analysis performed well on cross-validation in cohort 1 (in which they were derived), they performed poorly for autoimmune versus mimics and autoimmune versus viral comparisons in cohort 2 (Table 3).

Principal component analysis did not show differences between patients with autoimmune encephalitis who had been treated with corticosteroids before CSF acquisition versus untreated patients (Fig. 5)

Table 3: Panels derived from random forest analysis

Comparison	Constituent proteins	Median AUC in cohort 1	AUC in cohort 2
Viral vs mimics	A2MG/ASPG/SMS/P3IP1	1.00	0.96
Autoimmune vs mimics	C1QC/CH3L1/VAS1/C163A	0.93	0.44
Viral vs autoimmune	NCAN/FA12/CO8B/VIME/ASPG	0.85	0.57

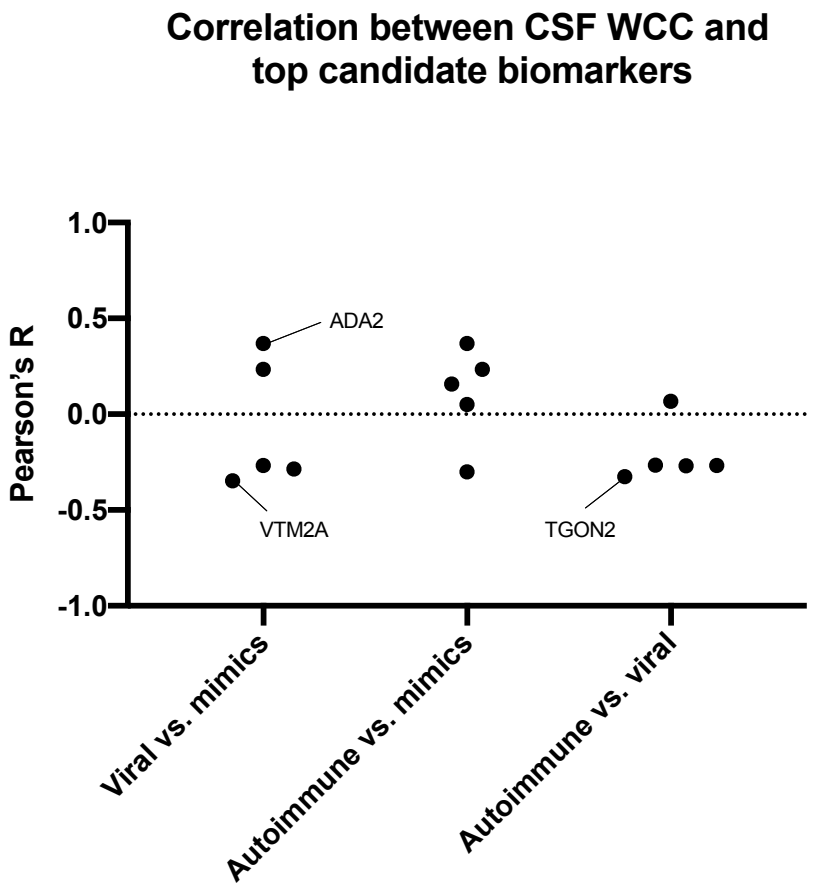


Figure 4: The top biomarker candidates do not correlate significantly with CSF WCC (analysis incorporates all aetiological groups from cohort 1 and 2 combined). The x axis shows the comparison for which each biomarker was significant. Those with the greatest positive or negative correlation are labelled.

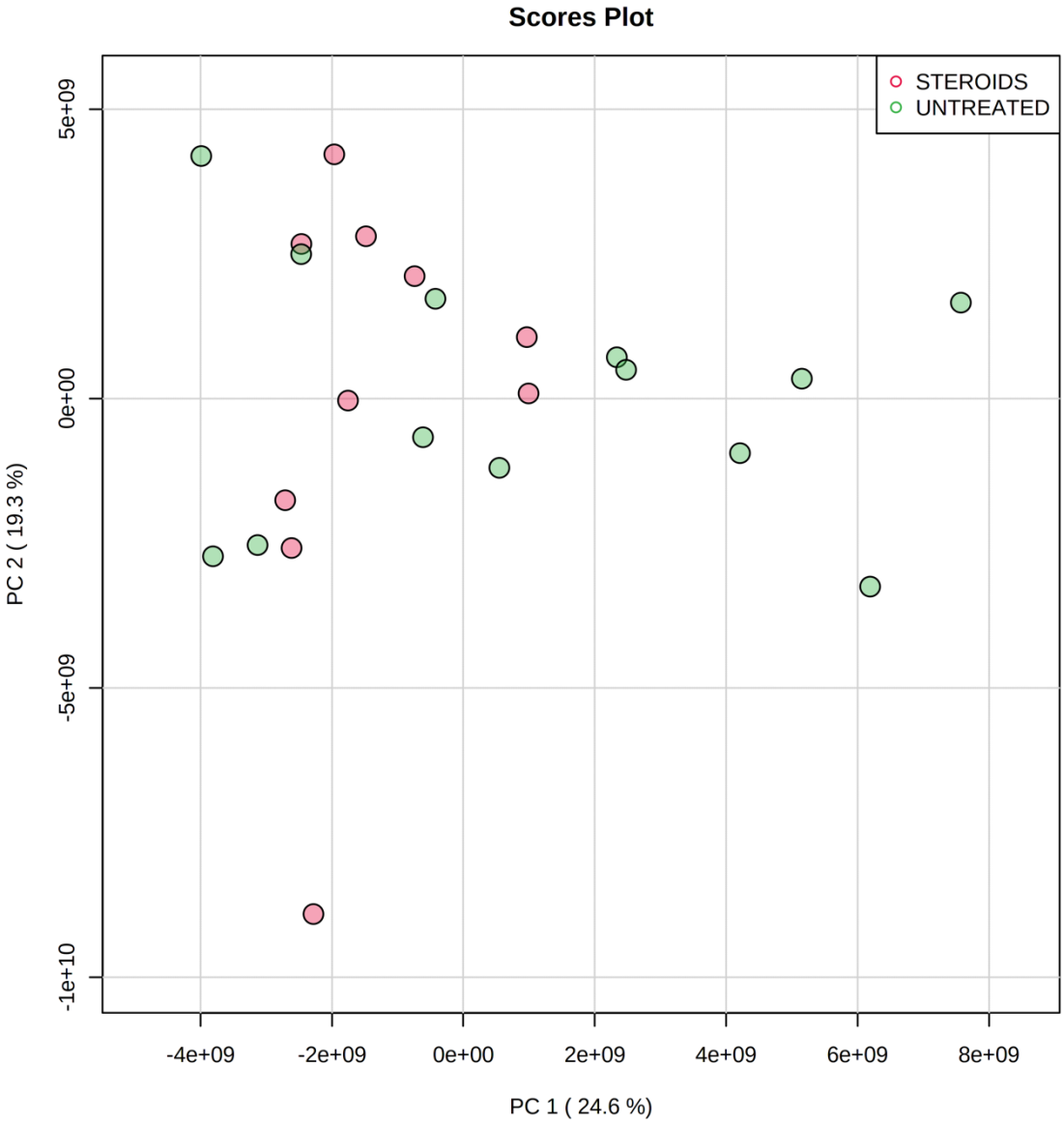


Figure 5. Principal component analysis (first two components) of proteomic profiles of patients with autoimmune encephalitis divided between those who had received corticosteroid treatment at the time of lumbar puncture (red) versus those who had not (green).

Correlations with outcome and MRI parameters

Proteomic analysis may reveal critical clues about disease mechanisms and severity in different forms of encephalitis. In order to investigate this further, I analysed the correlation between protein profiles and GOS score at last follow-up. In patients with NMDAR antibody encephalitis V-set immunoglobulin-domain-containing 4 (VSIG4) was strongly negatively correlated with GOS (Fig 6A). Vascular cell adhesion molecule 1 (VCAM1) was negatively correlated with GOS in patients with autoimmune encephalitis in general, with stronger correlations observed in those with NMDAR antibody encephalitis (fig. 6B).

In patients with HSV encephalitis, 30 proteins were negatively correlated with GOS, including paralemmin, interleukin 1 receptor 2 (IL-1R2) and fatty acid binding protein (heart).

4 patients with HSV encephalitis had data available for temporal lobe volumes and volume of T2 hyperintensity from MRI stereology. In these patients, 66 proteins had significant correlation with the total volume of T2 hyperintensity, notably the abundance of several immunoglobulin components (Fig 6C).

CSF WCC did not correlate significantly with either GOS or volume of T2 hyperintensity.

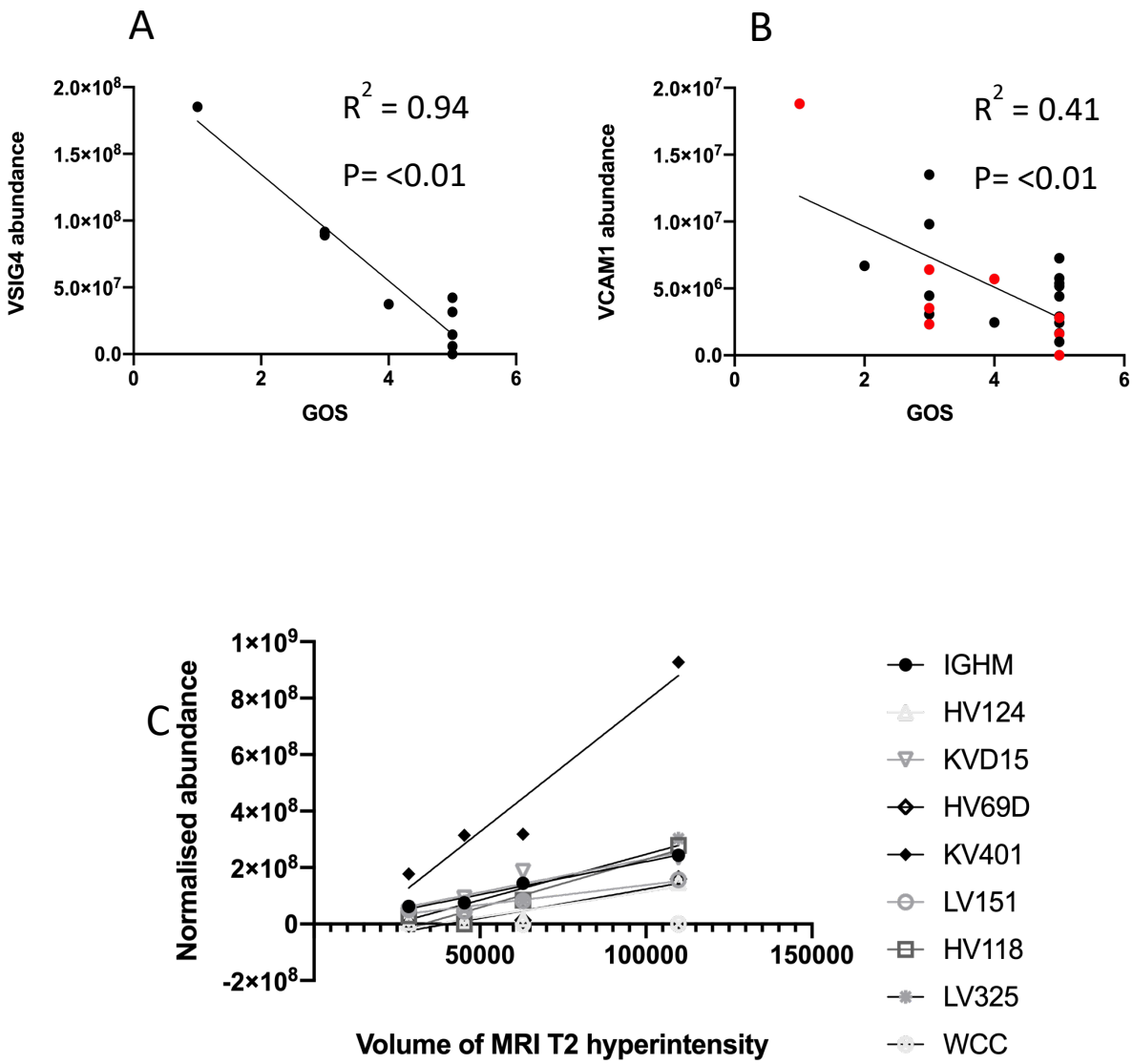


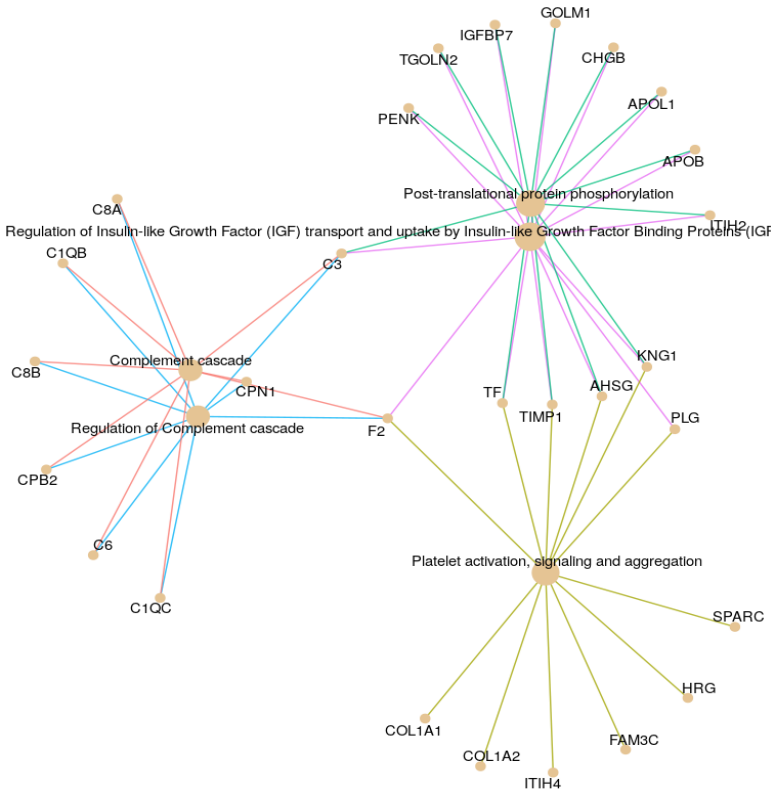
Figure 6. A: Correlation between VSIG normalised abundance and GOS in patients with NMDAR antibody encephalitis. B: Correlation between VCAM1 normalised abundance and GOS in patients with autoimmune encephalitis (patients with NMDAR antibody encephalitis in red). C: Correlation between immunoglobulin components and volume of T2 hyperintensity on MRI in four patients with HSV encephalitis.

Enrichment analysis

Using Reactome pathway analysis, I investigated pathway enrichment using significant results from pairwise analysis of encephalitis aetiology. The principal enriched pathways in viral encephalitis compared to autoimmune encephalitis included post-translational protein phosphorylation, insulin-like growth factor pathways, platelet activation, signalling and activation, complement cascade components and regulation of the complement cascade (Fig 7A).

Most of the proteins involved in the complement cascade were identified by mass spectrometry. C8b was identified as part of the random forest-derived panel differentiating autoimmune from viral in cohort 1, but not validated in cohort 2. Post hoc analysis of abundance of complement components by ANOVA revealed significantly higher abundance of components of the classical pathway including C1q (subunits A, B and C), complement components 2 and 5 in viral encephalitis and autoimmune encephalitis compared to mimics, with highest levels in viral encephalitis.

A



B

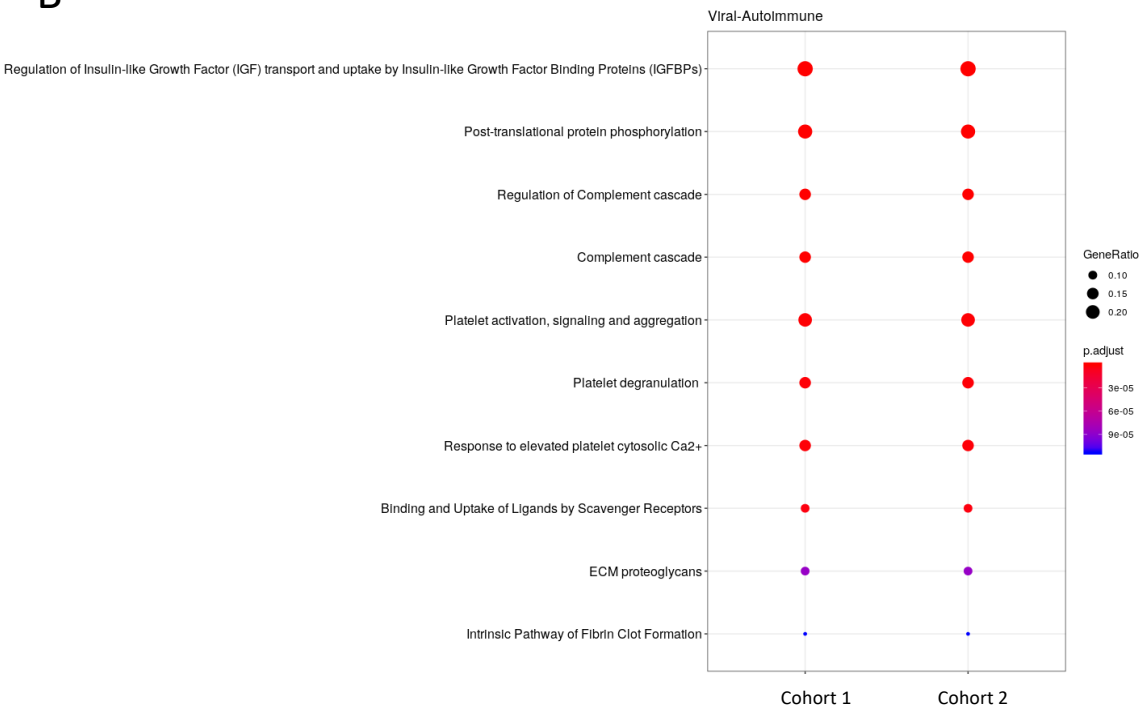


Figure 7. Geneset enrichment analysis for viral encephalitis versus autoimmune encephalitis. A: Network plot of enriched pathways. Central nodes represent pathway, with spokes representing enriched proteins. B: Dot plot of enriched pathways in Cohort 1 (left) and Cohort 2 (right). Colour represents adjusted p value; size of dot represents gene ratio (number of observed genes divided by number of expected genes for each pathway).

Discussion

I undertook an unbiased assessment of the proteome in CSF in patients with autoimmune and viral encephalitis, together with patients with conditions mimicking encephalitis, validated in a separate cohort. Combined analysis of these data revealed candidate protein biomarkers to distinguish between aetiological groups. In particular, discrimination between viral encephalitis and mimics was possible with a high degree of accuracy, even in the absence of CSF pleocytosis. Several proteins concerned with the immune response correlated closely with outcome in patients with autoimmune encephalitis. In patients with HSV encephalitis, the abundance of several immunoglobulin subunits correlated with the volume of T2 hyperintensity on MRI. Pathway enrichment analysis revealed a difference in abundance of components of the classical complement pathway, which were highest in viral encephalitis, lower in autoimmune encephalitis and lowest in mimics.

ADA2 was the most effective candidate biomarker distinguishing both viral and autoimmune encephalitis from mimics, and discriminated without error between viral encephalitis and mimics in cohort 2. ADA2 is an enzyme expressed in response to monocyte activation and is involved in purine metabolism, breaking down adenosine to inosine or 2'deoxyadenosine to 2'deoxyinosine. Genetic deficiency of ADA2 is associated with a form of severe combined immunodeficiency (Kendall et al. 2020). In this study ADA2 was clearly more abundant in CSF in encephalitis of any cause than in mimicking conditions and did not correlate well with CSF WCC, suggesting that its abundance is not dependent solely on leucocyte migration into the CSF space. Indeed, ADA2 abundance was able to discriminate between patients with

and without viral CNS infection in the absence of CSF pleocytosis, a situation which often poses a diagnostic dilemma. ADA2 abundance also discriminated well between patients with autoimmune encephalitis and mimics, suggesting that ADA2 tends to increase in inflammatory states whether in relation to pathogen or autoimmunity. In cohort 2, ADA2 abundance was able to distinguish between autoimmune and viral encephalitis, although this was not the case in cohort 1.

Several other single proteins were able to distinguish aetiological groups with high degrees of accuracy in both patient cohorts. Immunoglobulin components, both heavy and light chains, were identified and found to be significant in several analyses. Heavy chain constant region mu (the heavy chain of IgM antibody) was most significant in distinguishing viral and autoimmune encephalitis from mimics. The function of proteins VTM2A and 2B is poorly understood, although they have been linked to the regulation of cell differentiation in cancers and adipocyte regulation (Han et al. 2020). Lower VTM2B levels have been found in patients with postoperative delirium and genetic frontotemporal dementia, and lower gene expression has been reported in patients with neuropathic pain (Han et al. 2020; van der Ende et al. 2019; Tang et al. 2020). In this study, in both cohort 1 and cohort 2, abundance of both VTM2A and 2B were lower in viral and autoimmune encephalitis than mimics, and there was a trend to lower levels in viral than autoimmune encephalitis.

I investigated association of proteomic profiles with outcome, as measured by the GOS score at last follow-up. The GOS reflects functional outcome, and ability to live an independent life (McMillan et al. 2016). It is therefore highly clinically significant and reflects

the severity of brain injury. In NMDAR antibody encephalitis, VSIG4 abundance negatively correlated with GOS. VSIG4 is an immune regulatory protein expressed on resting tissue macrophages and is a powerful negative regulator of T cell proliferation and IL-2 production (Vogt et al. 2006), as well as an inhibitor of the alternative complement pathway via binding C3b (Wiesmann et al. 2006). IL-2 can act both as a T cell activator, and to promote the differentiation of T cells into regulatory T cells and prevent autoimmunity. Thus, high levels of VSIG4 may relate to an increase in auto-reactive T cell proliferation and activation. In autoimmune encephalitis generally, and particularly in NMDAR antibody encephalitis, VCAM1 levels correlated negatively with GOS. VCAM1 is a key mediator of leucocyte migration into the CNS, an important stage in the pathogenesis of CNS autoimmunity. In HSV encephalitis, proteins correlated with worse outcome included IL-1 receptor 2(IL-1R2), a decoy receptor for IL-1 expressed on neutrophils, monocytes and macrophages that acts to reduce IL-1β-dependent signalling. Expression of IL-1R2 is triggered by IL-1 pathway activation, and it is possible that detection of IL-1R2 is a proxy marker for IL-1 pathway activation, which is known to be correlated with worse outcome in HSV encephalitis (Michael et al. 2015).

In 4 patients with HSV encephalitis data were available from stereology of MRI scans, and I found correlations between several proteins and the volume of T2 hyperintensity, thought to be representative of the area of active inflammation and potential parenchymal injury. Particularly prominent were correlations with immunoglobulin chains, which did not correlate with CSF WCC. This may reflect the degree of humoral response to virus, although

it is increasingly recognised that inflammation in HSV encephalitis may represent an overzealous response and this may involve self-reactive antibodies.

This chapter has several limitations. Patients numbers were limited owing to the relatively low incidence of encephalitis and challenges in recruitment and sample collection in the acute setting, and the study design attempted to compensate for this by the division of clearly defined discovery and validation cohorts. Within each aetiological group, relatively heterogeneous groups were represented. Since the study aims were to distinguish groups requiring different therapeutic approaches (chiefly autoimmune encephalitis, with indication for immune therapy, from viral encephalitis and mimics) I combined patients with different autoantibodies or viruses. However, disease mechanisms in conditions such as ADEM, NMDAR antibody encephalitis and IgLON5 encephalitis differ significantly and this heterogeneity may have contributed to difficulties in validating candidate biomarkers for autoimmune encephalitis. Panels constructed via random forest analysis performed less well on validation than individual protein biomarkers in several analyses, suggesting that overfitting to the discovery cohort occurred.

Conclusion

This study in this chapter is the first to use mass spectrometry to investigate CSF proteome in autoimmune and viral encephalitis and demonstrates the power of unbiased -omic techniques to identify potential biomarkers and provide novel perspectives on pathogenesis in neurological disease. The putative biomarkers identified in this chapter provide

opportunities for validation in further, larger cohorts for diagnostic use, as well as avenues for exploration of pathogenic mechanisms in encephalitis. In the next chapter I will investigate a different approach to CSF analysis, exploring whether small molecule metabolites can provide further opportunities to distinguish aetiologies and reveal differences in pathophysiology between groups.

Chapter 5. Metabolomic analysis in cerebrospinal fluid

Introduction

Thus far I have examined the differences in gene transcripts in blood of patients with encephalitis and meningitis (Chapter 3), and then the differences in the abundances of proteins translated from mRNA in CSF (Chapter 4). Here, I will extend the study to the metabolite profiles of encephalitis and mimics. Metabolites are the small molecule products of the metabolic activity co-ordinated by the enzymatic activity of proteins, and metabolomics is the analysis of metabolic states, pathways and networks.

Various previous studies have used metabolomic techniques to investigate disease of the nervous system, including in bacterial meningitis (Chatterji et al. 2017), tuberculous meningitis (Li et al. 2017; Dai et al. 2017; van Laarhoven et al. 2018), VZV infection (Kuhn et al. 2018) and rabies (O'Sullivan et al. 2013), and have identified differences in amino acids, lipids, fatty acids and sugars in CNS infections. One small study has examined the metabolome in CSF of children with autoimmune encephalitis and other conditions causing acute CNS inflammation, finding differences in nitric oxide metabolism and the tryptophan-kynurenine pathway (Yan et al. 2020). However, to the best of my knowledge this study is the first to use metabolomic techniques to investigate both viral and autoimmune encephalitis in adults.

^{1}H NMR spectroscopy relies on the absorption and re-emission of electromagnetic radiation by magnetic nuclei placed in a magnetic field. As different molecules have different atomic structures the signatures of 1H 'chemical shifts' differ between metabolites allowing the different metabolite profiles between samples to be observed by NMR. Peak intensity or area under each ^{1}H signal present in the NMR spectrum is proportional to metabolite abundance. Therefore, NMR allows the analysis of biofluids in a rapid, non-invasive and non-destructive manner, providing quantitative results. Although it has relatively lower sensitivity than the other main metabolomic technique, mass spectrometry, the specificity of metabolite identification and reproducibility are major advantages of NMR.

The activity of metabolic pathways might be expected to differ between patients with infections of the CNS and those without, because of changes in the activity of supporting tissues such as choroid plexus and meninges, changes in the permeability of the blood-brain barrier and blood-CSF barrier, the metabolic activity of neurons and glial cells (to which CSF provides a source of nutrients) as well as metabolic activity of invading pathogens, particularly bacteria. In autoimmune disease changes in the CSF metabolome are less well established, although in multiple sclerosis metabolic profiles have been characterised in CSF and plasma (Jurynczyk, Probert, et al. 2017; Yeo et al. 2019; Lorefice et al. 2019).

Two small molecule metabolites, lactate and glucose, are already routinely measured in diagnostic laboratories. Glucose levels are typically reduced in bacterial meningitis, and in some cases of viral encephalitis and meningitis. Often the ratio of CSF to plasma glucose is calculated in order to account for the effect of blood glycaemia through passive and active

transport into the CSF. Lactate can be used as a supplementary diagnostic marker for bacterial infection, and is also used in the diagnosis of mitochondrial disorders, in which a defective respiratory chain leads to increased anaerobic metabolism and elevated lactate concentration.

I hypothesised that, given prior data showing that the metabolite profile of many infectious diseases can be characterised and differentiated, autoimmune conditions of the CNS may have their own distinguishable metabolomic signatures which could generate potential diagnostic biomarkers.

Methods

Patient groups and CSF collection

The cohort for metabolomic analysis is a subset of the cohort described in chapter 2, and identical split samples to those analysed in chapter 4 (proteomics). Following informed consent from patients or representatives, CSF was collected by lumbar puncture, using chlorhexidine skin preparation and lignocaine local anaesthetic, from patients with suspected encephalitis and stored in uncoated polypropylene tubes, frozen within 2 hours at -80°C.

Division of patients into aetiological groups is illustrated in Figure 1.

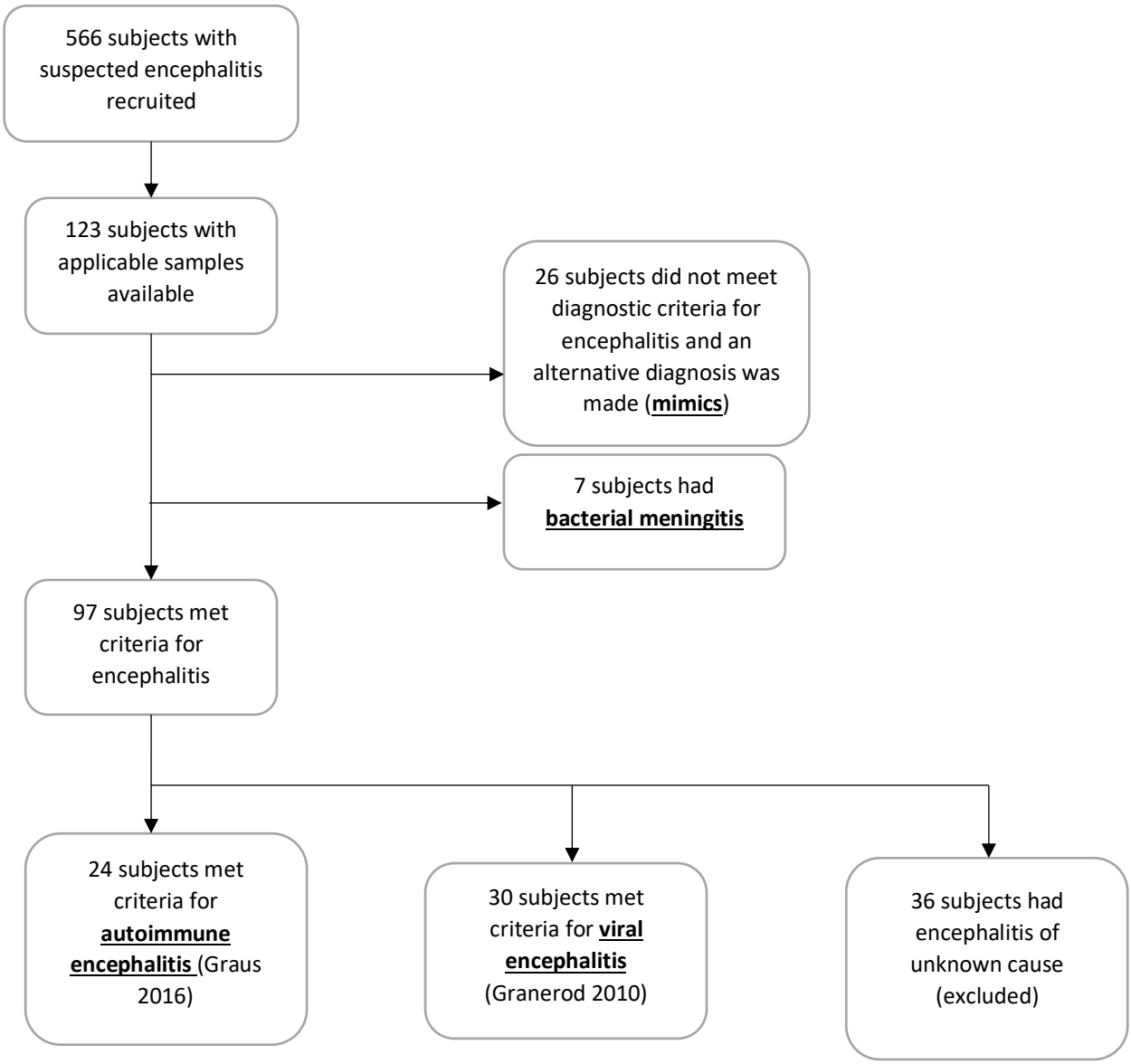


Figure 1. Flow chart illustrating cohort selection.

NMR Sample Preparation

Each 100 µL sample of thawed CSF was centrifuged at 1200xg for 10 minutes before aliquoting into polypropylene tubes. CSF was diluted to a final volume of 200 µl with a master mix containing 20 µL 1M Na_2HPO_4/NaH_2PO_4 (pH 7.4) in deuterium oxide, 80 µL double distilled water and 0.2 µL 1.2M sodium azide (final buffer concentration: 100mM phosphate, 10% ^2H_2O, 1.2mM sodium azide). Samples were vortexed for 1 minute and centrifuged at room temperature for 2 minutes at 25000xg. The samples were then transferred into 3mm outer diameter NMR tubes using a Pasteur pipette.

NMR Spectra

One dimensional ^1H NMR spectra were acquired on a 700 MHz NMR Bruker Avance IIIHD spectrometer equipped with a TCI cryoprobe (proton-optimized triple resonance NMR 'inverse' probe). Spectra were acquired at 25 °C using a standard (vendor supplied) CPMG pulse sequence. Spectral acquisition was carried out using TopSpin 3.5 (Bruker, UK) and automatically processed using vendor supplied routine (apk0.noe).

Metabolite Annotation

Spectra were assessed using community recommended quality control criteria, which included a flat baseline, water signal less than 0.4 ppm wide, and line-width half heights of representative beta anomeric glucose doublet all within one standard deviation (Sumner et al. 2007; Considine et al. 2019). Spectral regions (“buckets” or “bins”) were identified using

metabolite annotations included in Chenomx NMR Suite 8.2 with buckets attributed to multiple metabolites where peaks were found to overlap. Within each spectrum 182 buckets were annotated and quantified using TameNMR [github.com/PGB-LIV/tameNMR].

Statistical Analysis

Spectra were divided into four groups: viral, autoimmune, bacterial and mimics. Data were plotted, normalised and scaled using R (www.r-project.org). T tests, principal component analysis and partial-least-squares discriminant analysis (PLS-DA) were carried out using MetaboAnalyst 4.0 (http://www.metaboanalyst.ca), which uses R script. For t tests, correction for multiple analysis was performed using the Benjamini-Hochberg False-Discovery Rate (FDR) method, and a corrected p value of < 0.05 was considered statistically significant. One way ANOVA employed Fishers Least Significant Difference (LSD) for *post hoc* analysis in order to determine which groups gave rise to significant differences observed. For supervised multivariate modelling using PLSDA results were cross-validated to appraise model quality by sum of square (R2) and predicted sum of squares (Q2). A random selection of 70% of samples were used for model training with the remaining 30% used to test model quality. Enrichment and pathway analysis were performed using R and MetaboAnalyst (Chong et al. 2018) through metabolite set enrichment analysis and used metabocards from the Human Metabolome Database (accessed January 2020) and the Kyoto Encyclopedia of Genes and Genomes compound database (October 2019 version) (Okuda et al. 2008).

Results

Normalisation, scaling and outlier removal

Owing to the scarcity of published CSF NMR data and lack of established protocols, I investigated several methods of normalising the data in order to account for disease-independent differences between CSF samples from different patients or systematic differences in preparation (e.g. CSF collection or dilution).

In addition to more established approaches to normalisation of NMR data, I utilised laboratory measurements of CSF glucose concentration (performed by diagnostic laboratories in the collecting centres) in order to provide a normalisation constant for each spectrum. Initially, I identified the glucose_57 peak as showing maximum correlation with the other identified peaks (Fig. 2), between all spectra. Then, for each spectrum, I calculated a normalisation constant for each spectrum equal to the ratio of the recorded laboratory glucose assay result to the area under the glucose_57 peak (Fig. 3) Missing values for 10 samples were estimated as the median within-group value. The whole of each spectrum was then divided by its normalisation constant.

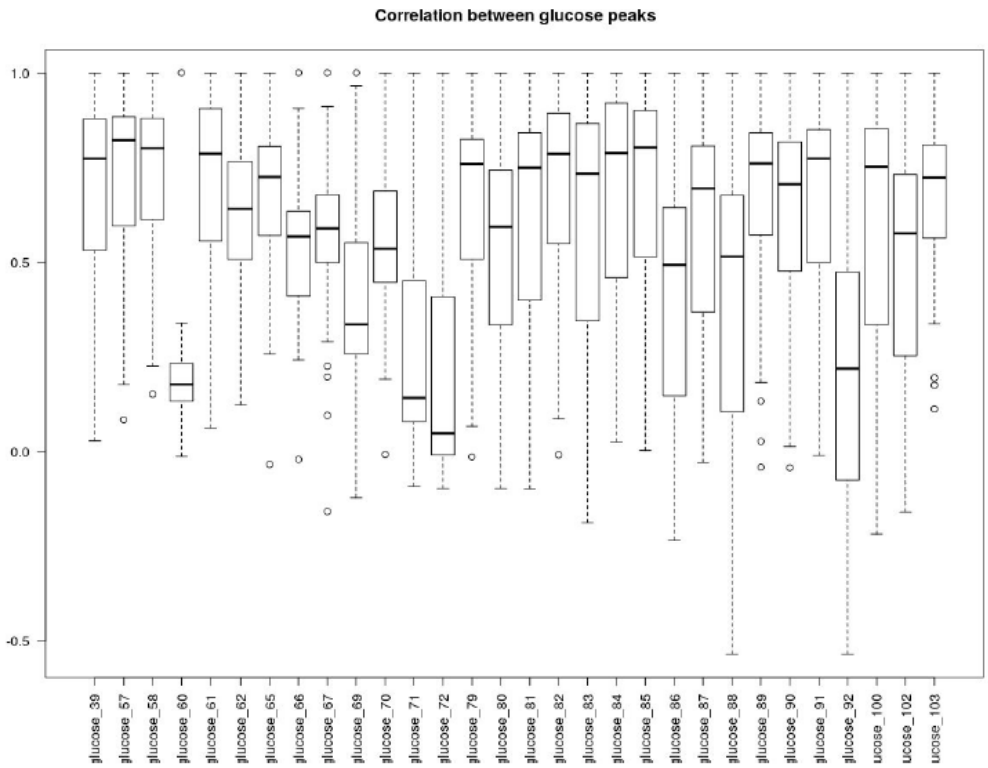


Figure 2. Correlation between peaks representing glucose

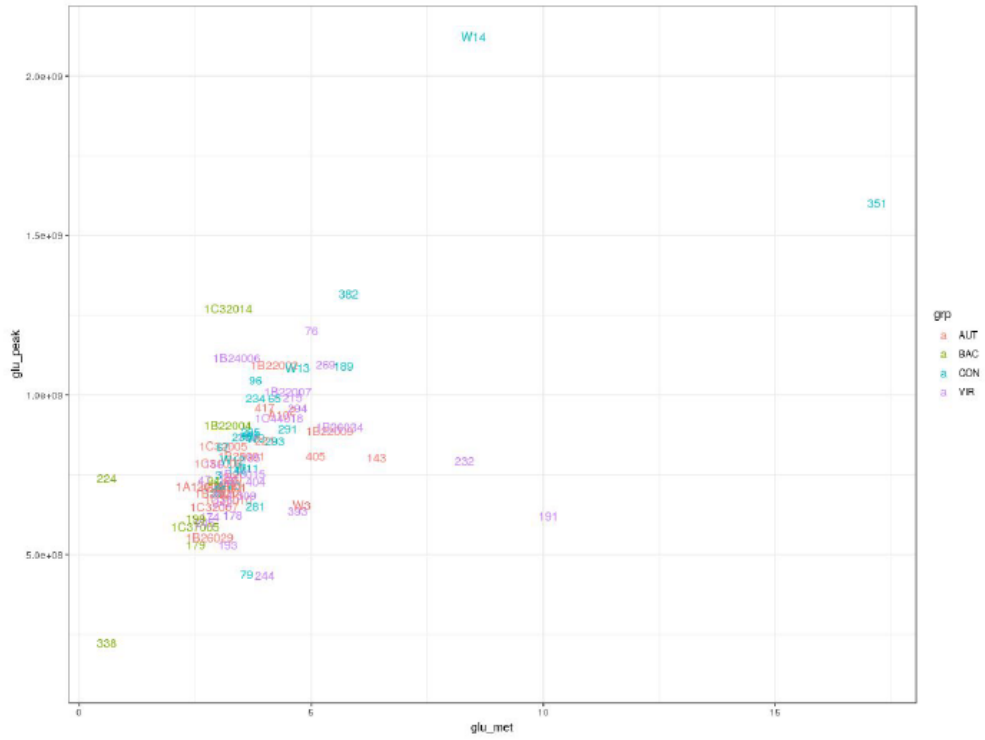


Figure 3. Scatter plot of the area under glucose_57 peak against glucose measured by assay in diagnostic laboratories

Spectra according to three methods of normalisation were plotted (Fig.4).

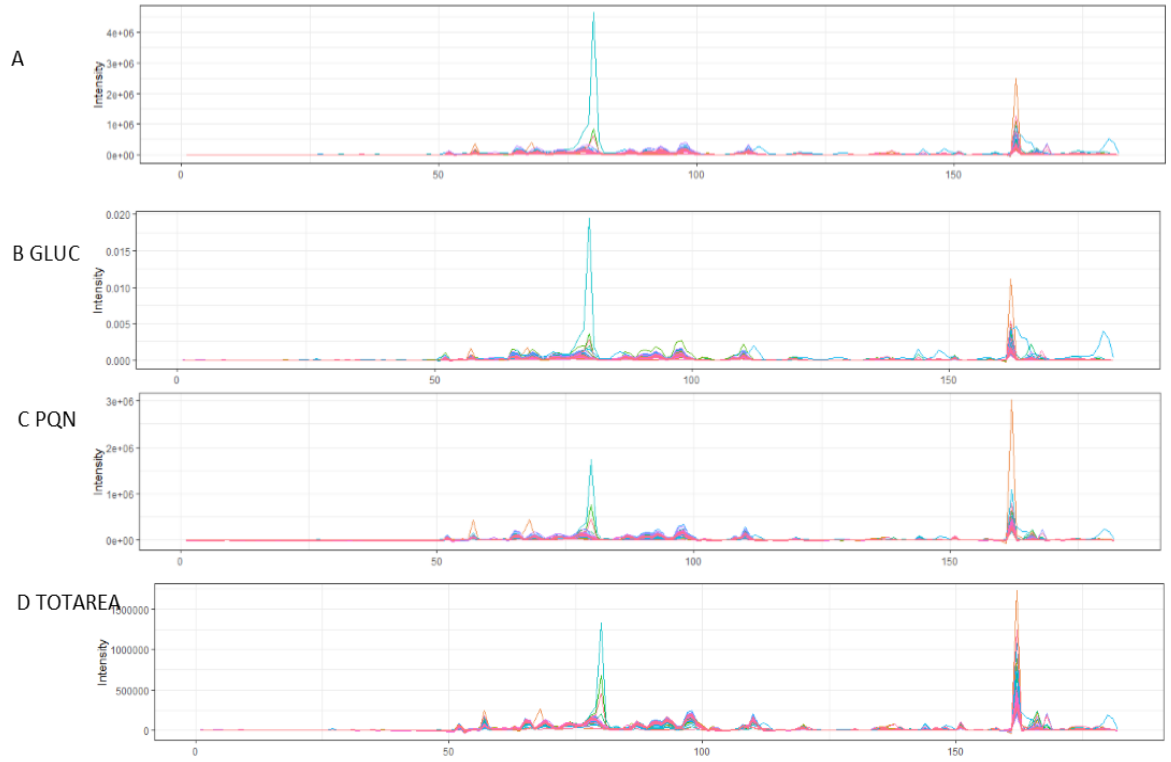


Figure 4. Overlayed ^{1}H NMR spectra of the entire cohort (binned).

A. Processed spectra before normalisation.
B. Spectra normalised by the ratio of laboratory glucose assay to the area under glucose_57 peak.
C. Spectra normalised by probabilistic quotient normalisation.
D. Spectra normalised by total peak area.

On inspection of the normalised plots there are several outlying spectra and clear variability within the cohort. Probabilistic quotient normalisation (PQN) (Dieterle et al. 2006) was most effective at reducing the most marked differences between spectra to allow robust comparison.

Following PQN the spectra were mean centred and divided by the standard deviation of each bin to scale each variable and allow for multivariate analysis. I subjected the resulting data to unsupervised multivariate analysis using principal component analysis (Fig. 5). This revealed tight clustering of all samples with the exception of 3 potential outliers. These samples were collected in the same centre in one part of the study. I therefore suspected that they were subject to a systematic error of processing (e.g. contamination in the laboratory or excessive freeze-thaw cycles). On examination of the spectra the outliers clearly showed broad peaks not present in the other samples which can be attributed to large macromolecules such as proteins present. These samples were therefore excluded from further analysis.

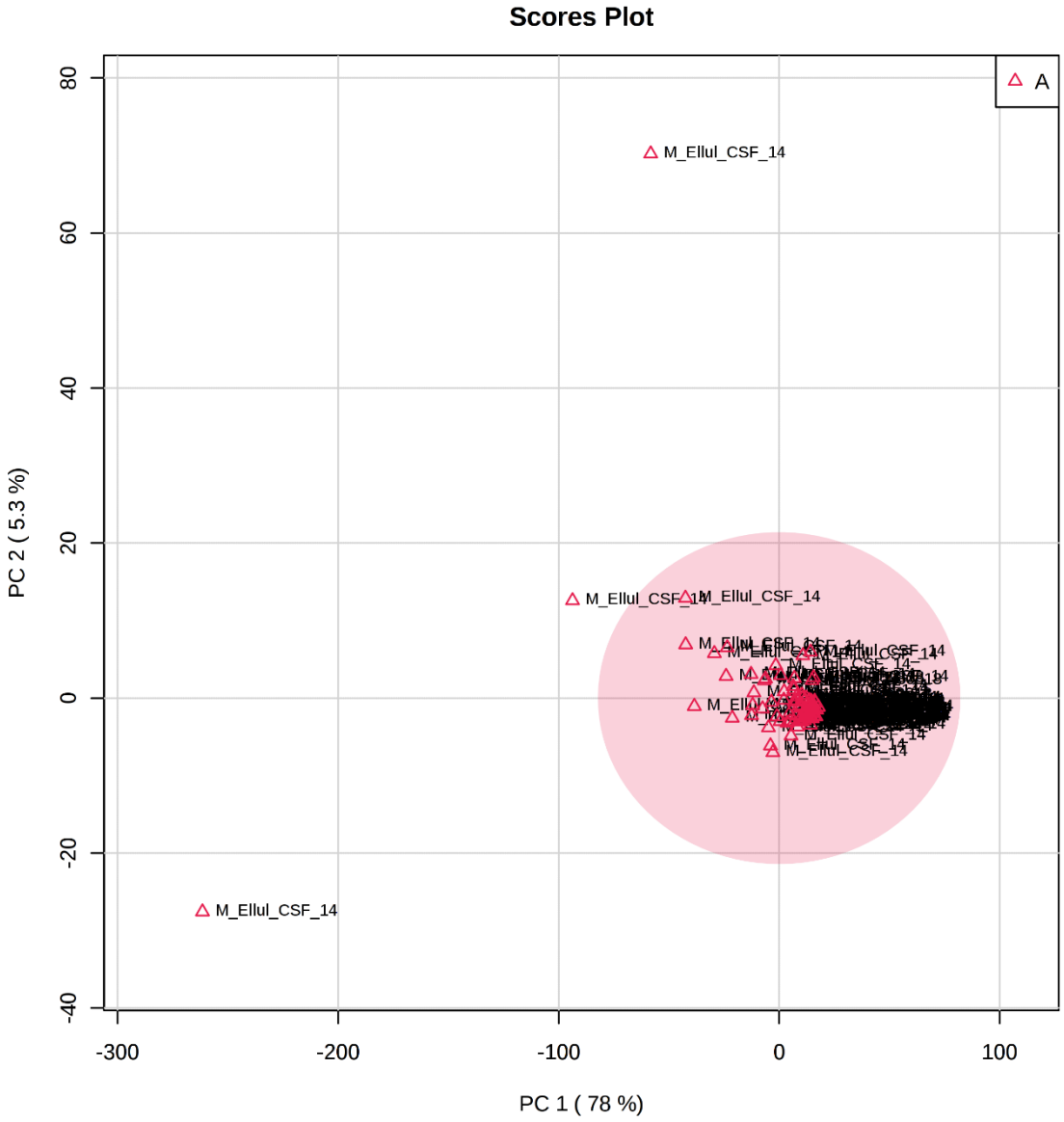


Figure 5. Principal component analysis showing 3 principal outliers.

Table 1: Clinical characteristics of metabolomics cohort of patients.					
	Autoimmune encephalitis n= 24	**Viral encephalitis n=30**	**Mimics n=26**	**Bacterial n=7**	**p value**
Age, median (range)	29 (6-73)	47 (1-91)	46 (18-80)	47 (31-72)	**0.020**
Female, n (%)	12 (50)	15 (50)	18 (69)	3 (45)	0.27
Fever	14 (58)	22 (73)	11 (42)	7 (100)	0.07
Seizures	10 (42)	11 (37)	8 (31)	0 (0)	0.72
Amnesia	10 (42)	6 (20)	7 (27)	3 (45)	0.21
Headache	13 (54)	15 (50)	18 (69)	6 (86)	0.32
Psychosis	3 (13)	0 (0)	2 (8)	0 (0)	0.16
Movement disorder	7 (29)	3 (10)	4 (15)	0 (0)	0.17
Immunosuppression at time of CSF sampling, n (%)	10 (42)	0 (0)	0 (0)	0 (0)	**<0.01**
Aciclovir at time of CSF sampling, n (%)	10 (42)	18 (60)	10 (38)	4 (57)	0.22
Brain imaging abnormality	16 (67)	21 (70)	3/21 (12)	4 (57)	**<0.01**
EEG abnormality	14/21 (67)	14/16 (88)	3/4 (12)	1 (14)	0.54
CSF pleocytosis	16 (73)	24 (80)	1 (4)	7 (100)	**<0.01**
Outcome (GOS score) at last follow-up- median (range)	5 (1-5)	4 (1-5)	5 (1-5)	4 (4-5)	0.090
Diagnoses	10 NMDA 3 VGKC (LGI-1 and CASPR2 negative in CSF) 6 ADEM 1 IgLON5 1 GAD 3 seronegative	19 HSV 7 VZV 3 enterovirus 1 adenovirus	12 headache disorders 5 seizures/epilepsy 2 confusion of unknown cause 1 concussion 3 toxic/metabolic encephalopathy 1 PRES 1 MoyaMoya 1 primary psychiatric disorder	3 TB meningitis 1 S. pneumoniae 3 purulent meningoencephalitis - organism not identified	

Patient characteristics

Table 1 shows the clinical features and diagnoses of included patients. Significant differences existed between groups in age (autoimmune encephalitis patients were younger), treatment with immune therapy (corticosteroids in all cases) before the time of lumbar puncture and CSF pleocytosis.

In order to explore the effect of differing sample collection and processing, sex of participants and exposure to steroid medication, I performed principal component analysis grouping by these features (Fig. 6). For the first two principal components there was no separation by these factors. In addition, univariate testing did not show any significant difference in any metabolite between these groups.

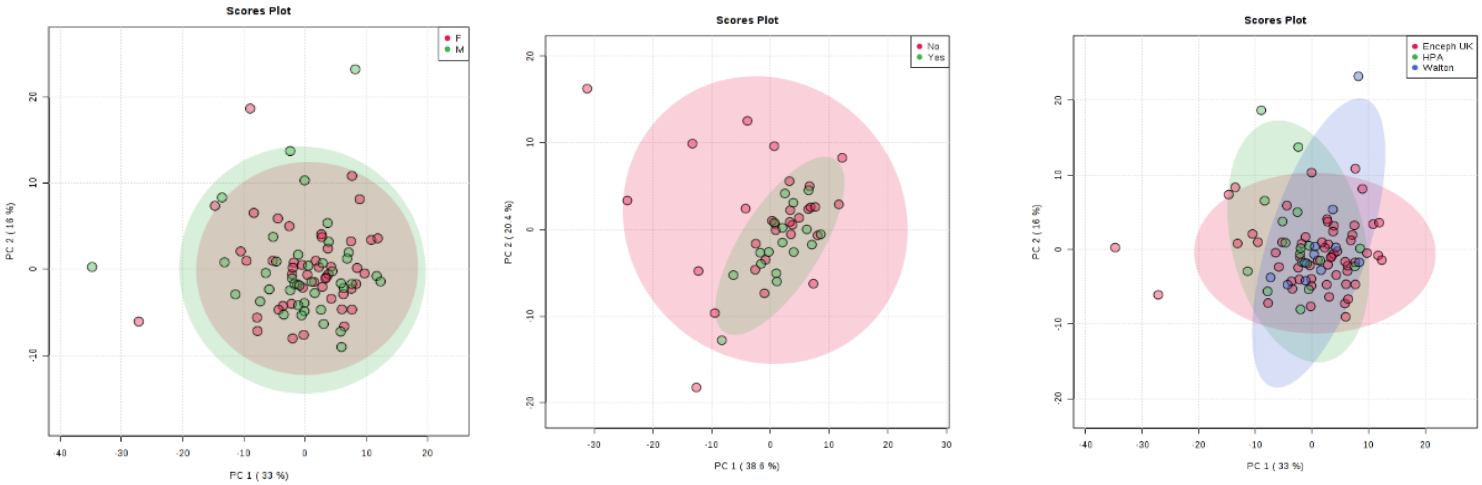


Figure 6. Principal component analysis of spectra grouped by sex (A), exposure to corticosteroids (B) and study recruited (C).

Table 2 shows the metabolites identified using the Chenomx software package from all spectra, and figure 7 shows the categories to which metabolite compounds belong. Unidentified metabolite peaks were removed from analysis.

Table 2. Identified metabolites presented by their common name as well as their unique human metabolome database (HMDB) identifiers.

Metabolite name	**HMDB code**
L-Phenylalanine	HMDB00159
L-Tyrosine	HMDB00158
L-Histidine	HMDB00177
D-Glucose	HMDB00122
D-Mannose	HMDB00169
3-Hydroxybutyrate	HMDB00357
Glucarate	HMDB00663
L-Lactate	HMDB00190
2-Hydroxyvaleric acid	HMDB00190
Creatinine	HMDB00562
3-Hydroxybutyrate	HMDB00357
Creatine	HMDB00064
L-Threonine	HMDB00167
myo-Inositol	HMDB00211
L-Proline	HMDB00162
L-Arginine	HMDB00517
Choline	HMDB00517
N-Nitrosodimethylamine	HMDB0031419
L-Lysine	HMDB00182
Citrate	HMDB0094
L-Glutamate	HMDB00148
Acetoacetate	HMDB0060
L-Glutamine	HMDB00641
Glycylproline	HMDB00721
Acetate	HMDB00042
L-leucine	HMDB00687
L-alanine	HMDB00161
Isopropyl alcohol	HMDB00863
Propylene glycol	HMDB001881
L-valine	HMDB00883
L-Isoleucine	HMDB00172
2-hydroxyisovaleric acid	HMDB00407

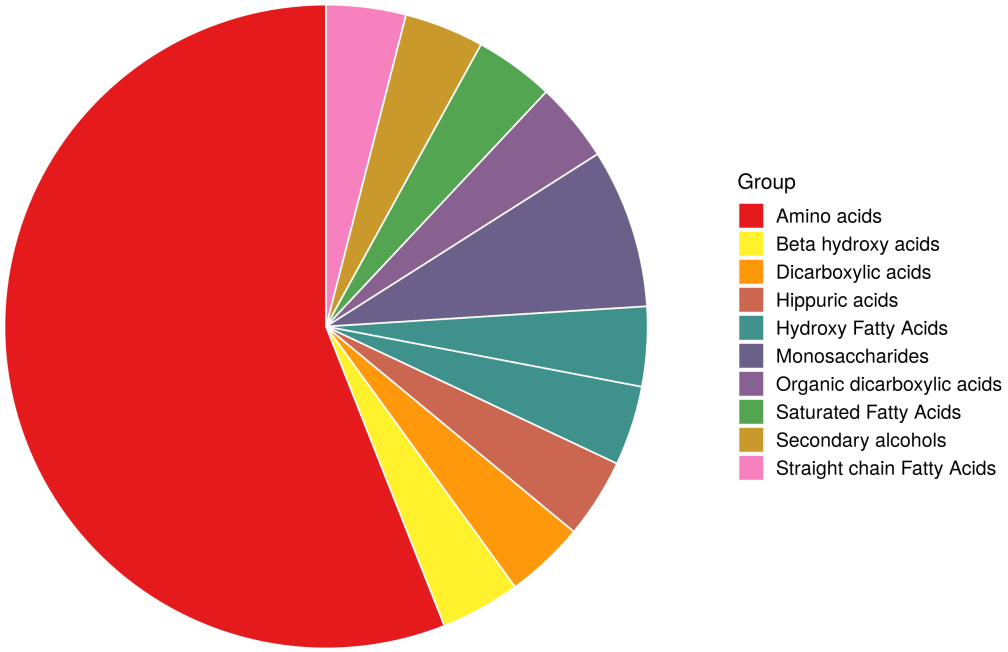


Figure 7. Metabolite categories for all identified compounds

Exogenous small molecules can also be detected by the NMR analysis. In particular, isopropyl alcohol is present in “Chloraprep”, the chlorhexidine containing antiseptic most commonly used in skin preparation for lumbar puncture. Propylene glycol is present in many skin products, cosmetic and medical, but may also occur as a product of anaerobic metabolism. Both isopropyl alcohol and propylene glycol have been detected in previous metabolomic studies of CSF (Mandal et al. 2012).

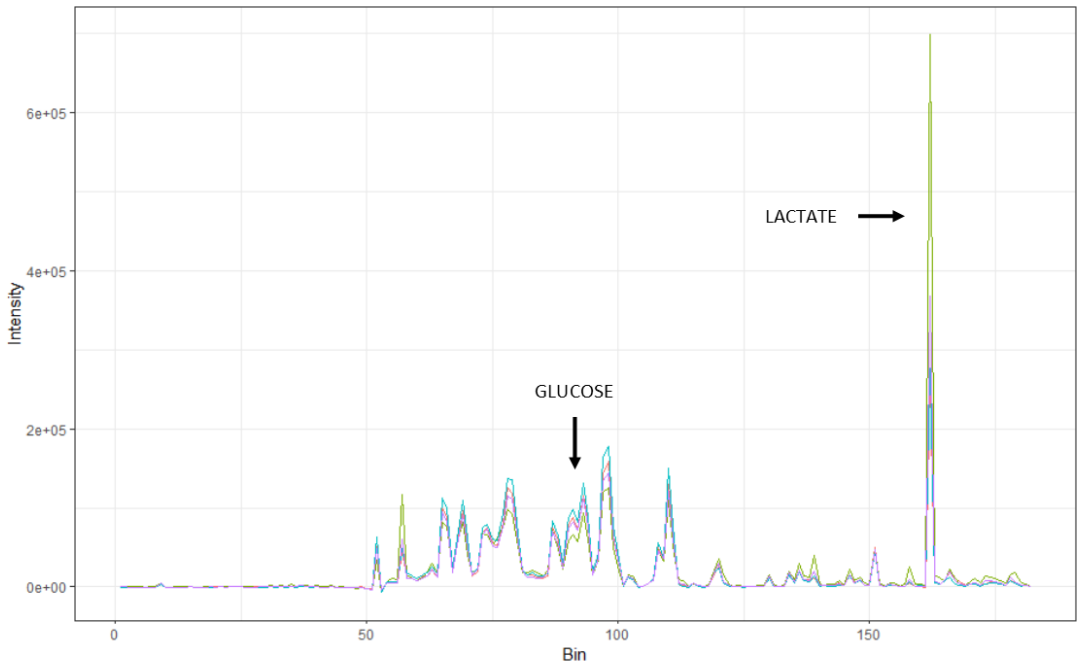


Figure 8. Overlayed median ^{1}H NMR spectra (binned) for samples from patients with autoimmune encephalitis (Red), bacterial meningoencephalitis (green), viral encephalitis (purple) and mimics (blue).

Figure 8 shows overlayed spectra for samples from patients with autoimmune, viral and bacterial encephalitis and mimics. Clear differences in peak intensity (and therefore metabolite abundance) can be seen in several regions of the spectrum including lactate and glucose peaks.

Metabolite profiling by aetiology of encephalitis

ANOVA including all four groups showed 63 of the 97 identified peaks with differential abundance between at least two groups (on the basis of corrected p value <0.05) (Table 3). The predominant differences were between bacterial samples and others, with higher concentrations of some metabolites in bacterial samples, although occasional metabolites showed differences between other groups (e.g. 2-hydroxyvalerate was higher in mimics and acetoacetate which was higher in the autoimmune group). Figure 9 shows example metabolite differences observed for lactate, alanine and 2-hydroxyvalerate.

Table 3. Significant metabolites using ANOVA with *post hoc* Fisher's least significant difference procedure. For each metabolite the metabolite peak with the lowest p-value is represented.

Metabolite peak	'raw' p.value	FDR p value	Fisher's LSD
Lactate_162	1.12E-08	8.58E-07	BAC - AUT; BAC - MIM; BAC - VIR
Ala_158	3.05E-08	9.87E-07	BAC - AUT; VIR - AUT; BAC - MIM; BAC - VIR; VIR - MIM
Valine_171	4.31E-06	0.00010442	BAC - AUT; VIR - AUT; BAC - MIM; BAC - VIR; VIR - MIM
lysine_121	8.49E-05	0.0013723	BAC - AUT; VIR - AUT; BAC - MIM; BAC - VIR; VIR - MIM
2-hydroxyvalerate_58	0.00021999	0.0030485	MIM - AUT; MIM - VIR
Leu_154	0.00038648	0.004686	BAC - AUT; BAC - MIM; BAC - VIR; VIR - MIM
Isoleucine_172	0.00082624	0.0087814	BAC - AUT; BAC - MIM; BAC - VIR; VIR - MIM
glucose_65	0.0015739	0.009166	MIM - AUT; MIM - BAC; MIM - VIR
glutamate_142	0.0016642	0.009166	BAC - AUT; BAC - MIM; BAC - VIR; VIR - MIM
phenylalanine_35	0.0017074	0.009166	BAC - AUT; VIR - AUT; BAC - MIM; BAC - VIR; VIR - MIM
citrate_130	0.0018149	0.009166	BAC - AUT; VIR - AUT; BAC - MIM; VIR - MIM
2-hydroxyisovalerate_179	0.001898	0.009166	BAC - AUT; AUT - MIM; BAC - MIM; BAC - VIR; VIR - MIM
creatinine_120	0.0019568	0.009166	BAC - AUT; VIR - AUT; BAC - MIM; VIR - MIM
Arg_152	0.0036029	0.012317	BAC - AUT; BAC - MIM; BAC - VIR
Gln_146	0.0046348	0.014049	BAC - AUT; BAC - MIM; BAC - VIR
acetoacetate_144	0.0059642	0.01607	AUT - MIM; AUT - VIR
2-hydroxybutyrate_178	0.006944	0.016874	BAC - AUT; BAC - MIM; VIR - MIM
choline_113	0.0075649	0.016874	BAC - AUT; BAC - MIM; VIR - MIM
tyrosine_43	0.029704	0.047234	BAC - AUT; BAC - MIM
3-hydroxybutyrate_55	0.031425	0.049164	BAC - MIM; BAC - VIR
phenylalanine_33	0.0323	0.049732	BAC - MIM; VIR - MIM

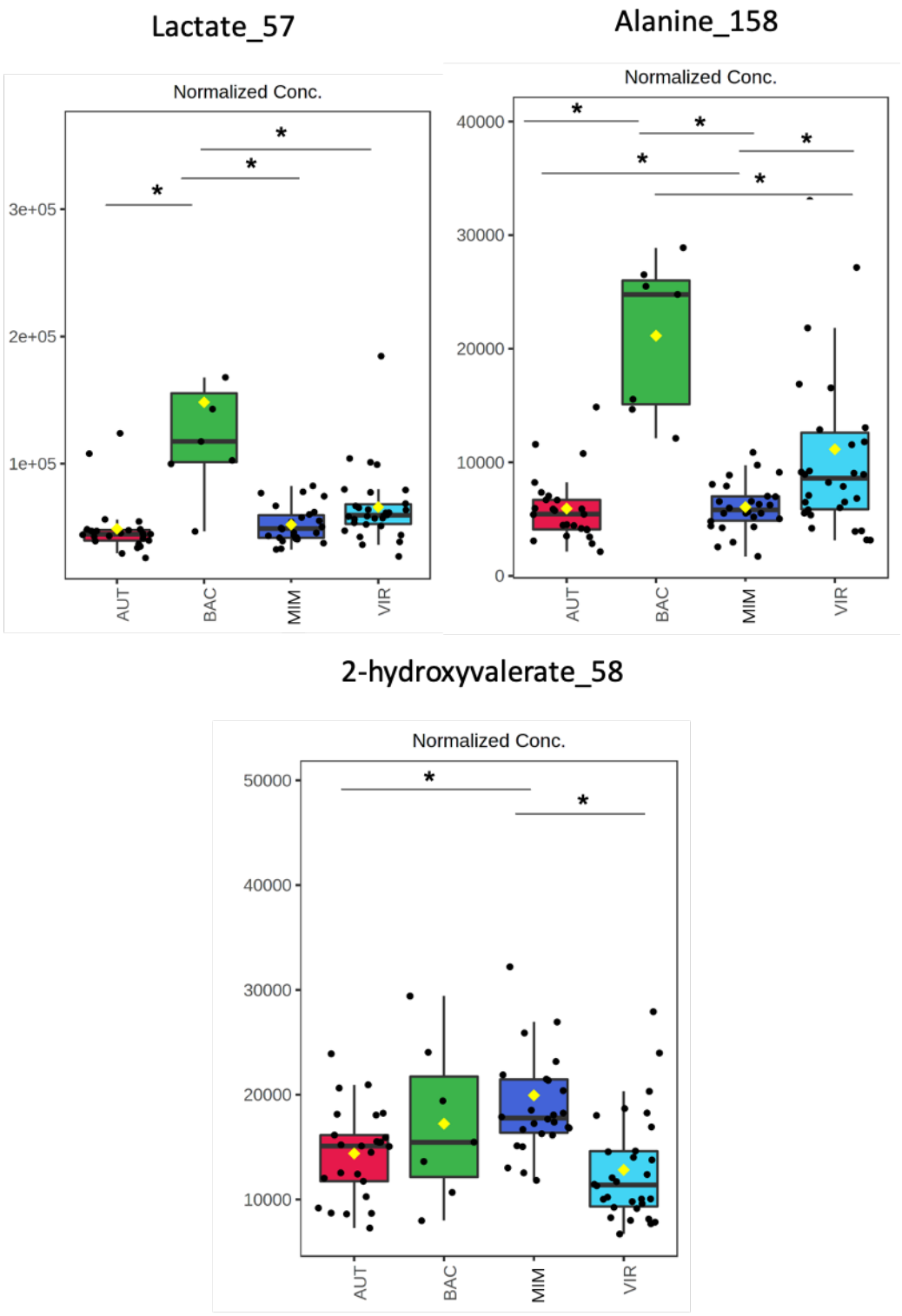


Figure 9: Three metabolites with lowest p-value as determined by ANOVA. Horizontal bars and asterisks represent significant differences between groups using Fisher's least significant difference procedure.

In order to identify single metabolites which showed significant differences between aetiological groups, I performed pairwise univariate analysis using t-tests. Single metabolites were found with significant differences in concentration between bacterial encephalitis and all the other groups, as well as between viral encephalitis and mimics. Only one metabolite (lactate, with two peaks) showed differences in concentration between viral and bacterial cases on this analysis. Table 4 shows metabolite peaks with lowest p-values for each pairwise comparison.

Table 4: Most effective metabolites on pairwise univariate analysis (t tests)

Comparison	**Metabolite peak**	**'raw' p.value**	**FDR p-value**	**ROC AUC**
Viral vs mimics	2-hydroxyvalerate_58	0.00011799	**0.021475**	0.83
Autoimmune vs mimics	2-hydroxyvalerate_58	8.9748E-4	0.13554	0.77
Bacterial vs mimics	Alanine_158	3.74E-11	**6.81E-09**	1.0
Viral vs bacterial	Lactate_57	0.00032931	**0.024157**	0.85
Viral vs autoimmune	Alanine_158	0.006916	0.42325	0.73
Autoimmune vs bacterial	Alanine_158	6.78E-10	**6.58E-08**	0.99

There were no significant single metabolites in the comparisons of autoimmune encephalitis vs mimics, or autoimmune encephalitis vs viral encephalitis. For these comparisons I went on to explore the effect of several metabolites in parallel in distinguishing aetiological groups (multivariate analysis).

There were no significant correlations of metabolites with outcome (GOS score) or clinical features such as the occurrence of seizures, headache, movement disorder or psychosis.

In order to investigate interactions between multiple metabolites between groups, I analysed the data using unsupervised principal component analysis and supervised partial least squares discriminant analysis (PLSDA).

Figure 10 shows principal component analysis including all four groups. Five principal components are required to explain 72.8% of the variance in the data. Since distinguishing four complex disease groups is unlikely within one principal component analysis, I used binary comparisons between two groups to elucidate the differences in metabolite profiles more clearly (Figure 11). Of these comparisons, the fewest principal components were needed to divide bacterial from mimics (3 principal components accounted for 70.6% of the variance). Although no two groups showed clear separation, viral and bacterial groups showed more variability between patients than the other groups.

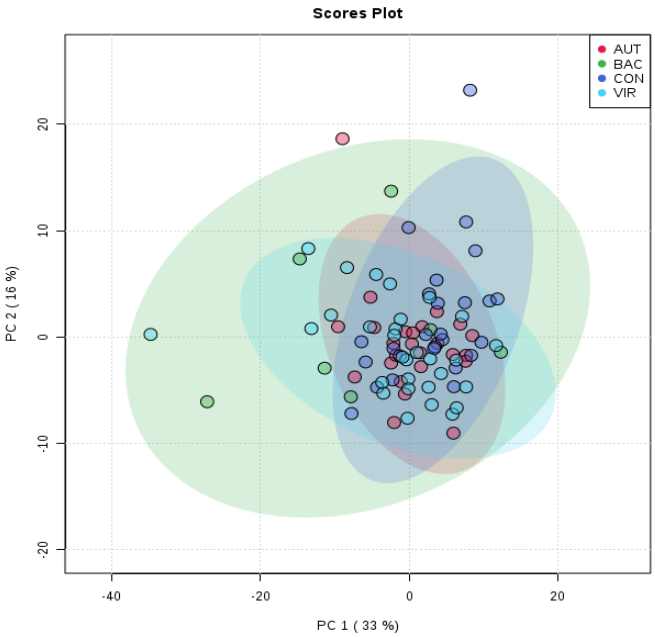


Figure 10. Principal component analysis for first two PCs showing aetiological groups. Shaded regions represent 95% confidence intervals.

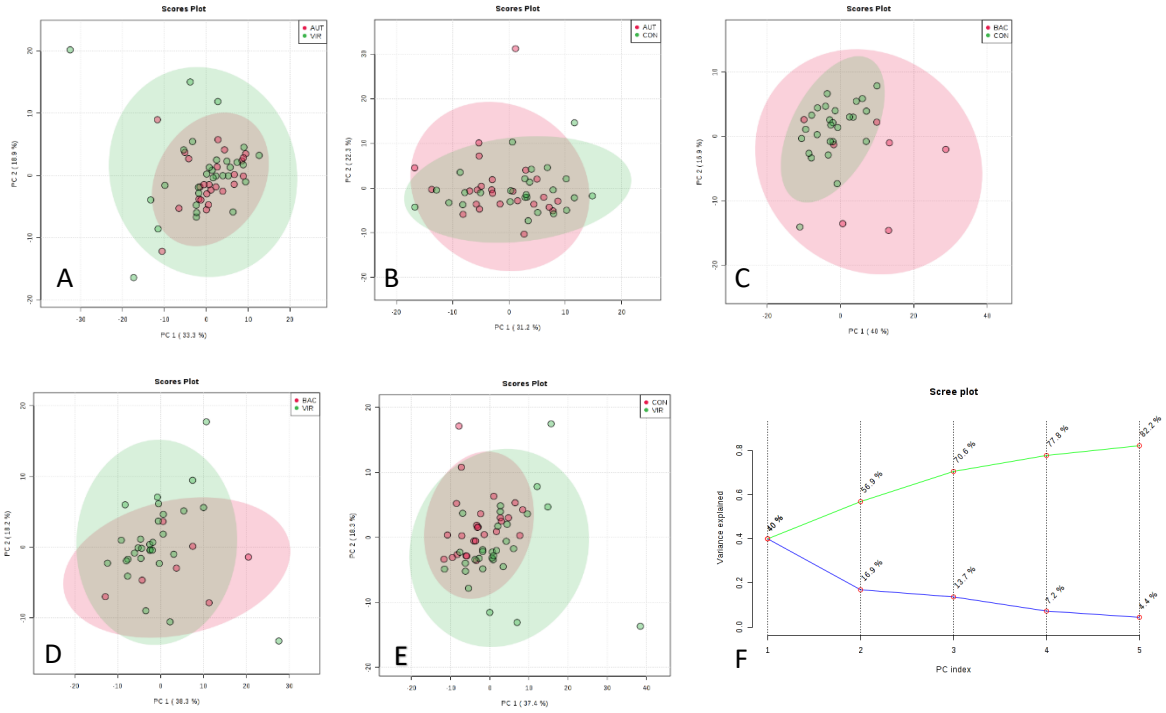


Figure 11. Principal component analysis of all samples grouped by aetiology. A: autoimmune (red) vs. viral (green), B: autoimmune (red) vs. mimics (green), C: bacterial (red) vs. mimics (green), D: bacterial (red) vs. viral (green), E: viral (green) vs. mimics (red). F: Percentage explained variance for the first five principal components

I used PLSDA to attempt to provide models incorporating several metabolites which could distinguish between the aetiological groups which proved more difficult to distinguish by univariate analysis. PLSDA models were validated by 10x cross validation by leave-one-out-cross-validation (LOOCV). Fig. 12 shows scores plots for PLSDA for these comparisons (autoimmune vs mimics and autoimmune vs viral), together with variable importance in the projection (VIP scores), an aggregate score for the coefficients of the metabolites making greatest contribution to the proposed model. R2 and Q2 scores are given for each model, and are low indicating a low to modest predictive power for the models calculated.

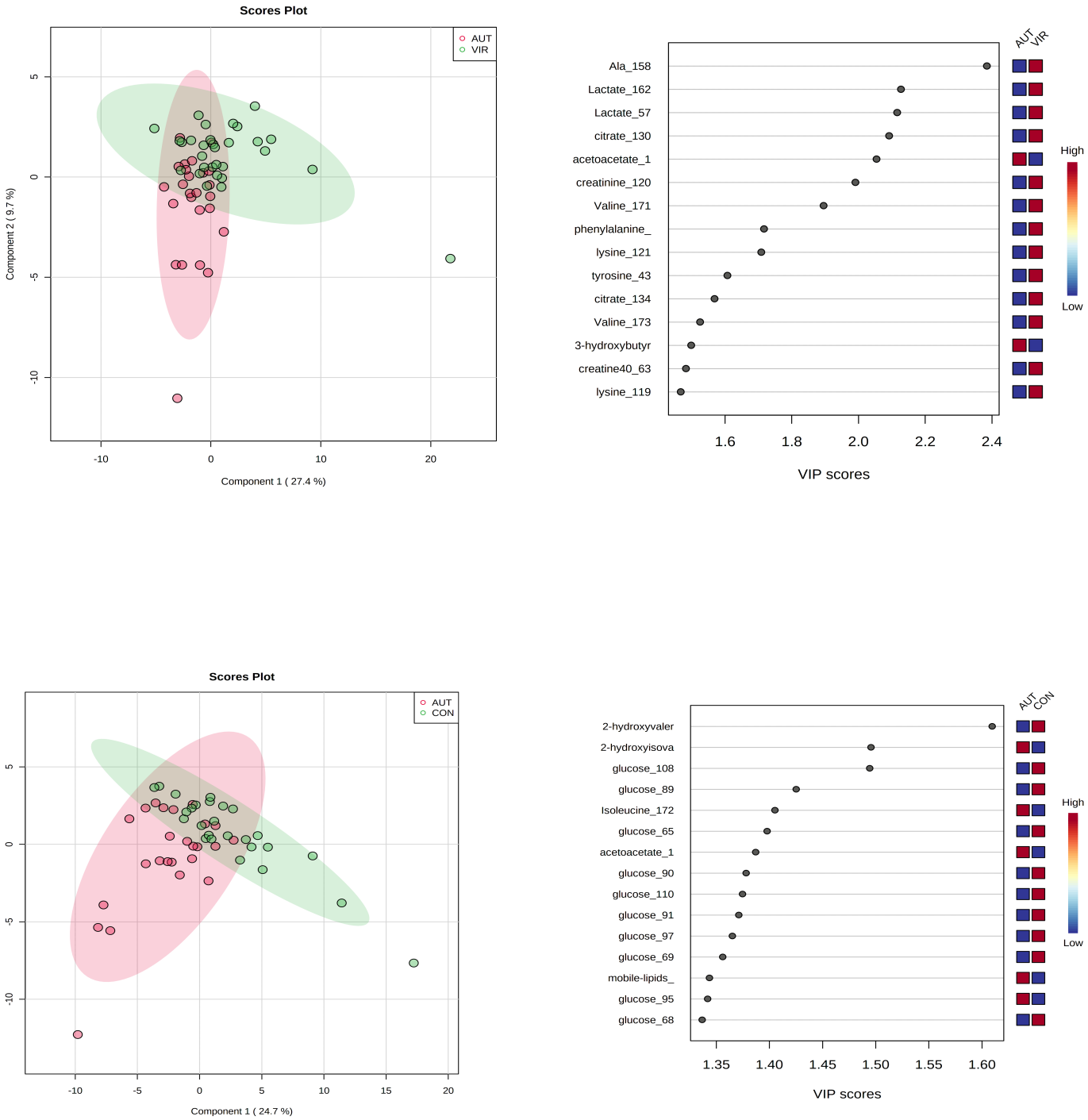


Figure 12. PLSDA analysis with scores plots and VIP scores. Upper: autoimmune (red) vs viral (green) (3 component-model R2=0.48 Q2=0.1). Lower: autoimmune (red) vs mimics (green) (4 component-model R2=0.54 Q2=0.15).

The PLSDA model (4 component-model R2=0.54 Q2=0.15) for autoimmune encephalitis vs mimics prominently included glucose (several peaks). This was a surprising result, since typically CSF glucose is not thought to vary between normal and autoimmune encephalitis. In order to explore this further, I investigated whether discrimination would be improved by looking at the ratio of glucose between CSF (from NMR) and plasma (as measured in the diagnostic laboratory from a blood sample taken at the time of lumbar puncture). The CSF:plasma glucose ratio is commonly used in practice, particularly in suspected cases of bacterial infection. Glucose ratio alone only distinguished significantly between bacterial meningitis and mimics, but not between encephalitis groups.

Next, I investigated the relationship between CSF glucose concentration and total CSF WCC. I hypothesised that metabolism of leucocytes partly accounted for the variation in CSF glucose concentration between groups. Figure 13A shows a weak negative correlation between total CSF WCC and the median CSF glucose peak for patients with autoimmune or viral encephalitis. Figure 13B is a boxplot of the median glucose concentration, which varies significantly between bacterial meningitis and the other groups. To investigate whether the number of leucocytes in CSF alone accounted for variation in glucose concentration, I then divided the glucose concentration for each sample by the total CSF WCC. The resulting ratio was significant for comparison between all groups (Fig. 13C). In particular, it discriminated between autoimmune encephalitis and mimics more effectively than CSF WCC alone (Fig.13 D-E)

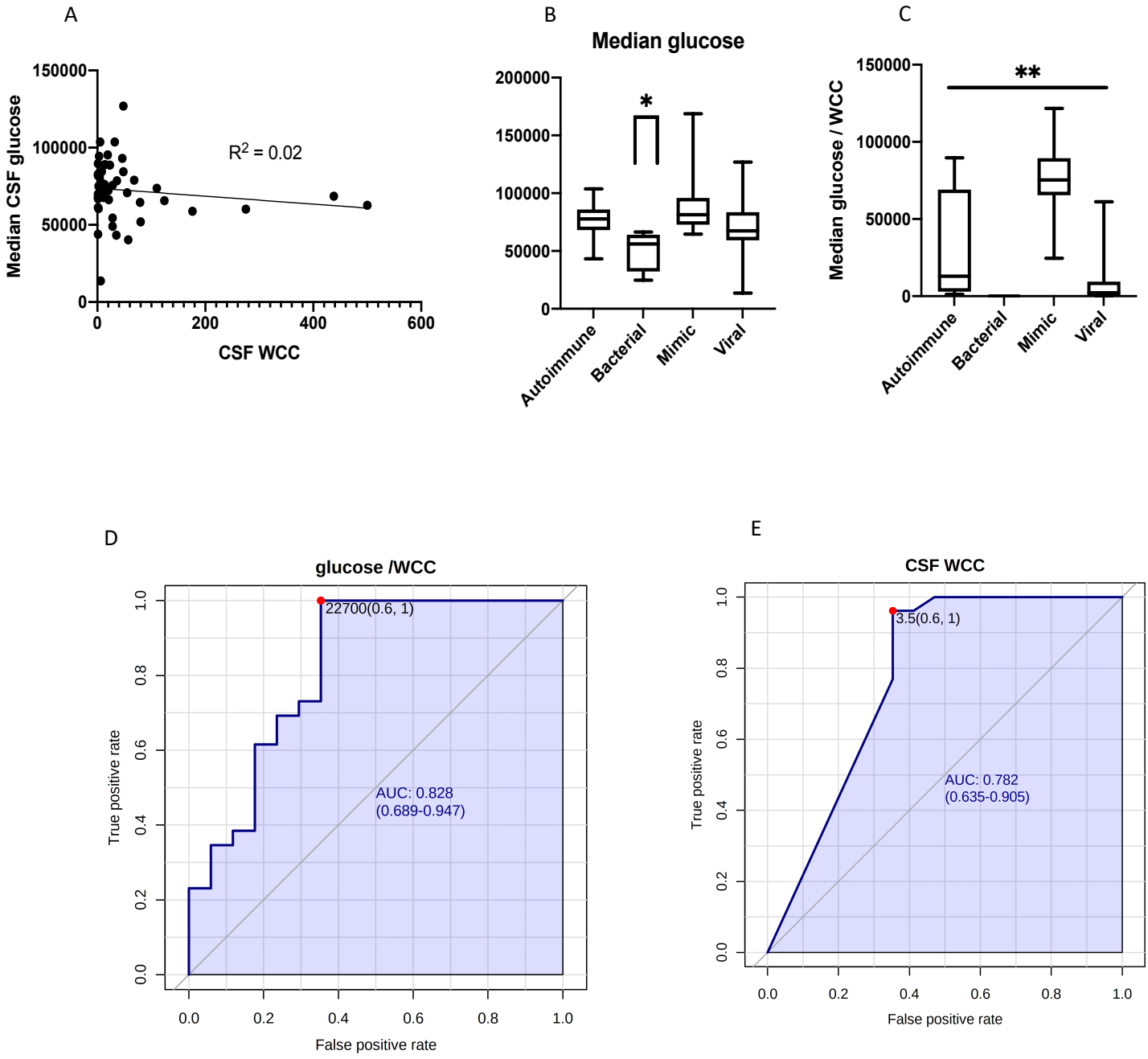


Figure 13. A: CSF WCC vs median CSF glucose peak for each sample (encephalitis only). B: Median glucose peak by aetiological group. C: Median glucose divided by CSF WCC by aetiological group (N.b. value for bacterial group is near zero). D: ROC analysis of glucose/CSF WCC for autoimmune encephalitis vs mimics. E: ROC analysis of CSF WCC alone for autoimmune encephalitis vs mimics.

Enrichment and pathway analysis

The metabolite profiles of viral encephalitis patients differed significantly from mimics on univariate analysis. In order to explore these differences, I looked for enrichment of pathways in the metabolite set. Metabolites with corrected p value <0.05 on univariate testing, or which were in the top 10 most significant contributors to the PLSDA model (according to VIP score) were included in enrichment analysis using the KEGG database.

Significantly enriched pathways are shown in Figure 14, together with categorisation of the significant metabolites and a simplified scheme of enriched metabolic pathways. Changes in amino acid processing are prominent, as are compounds related to the citric acid cycle.

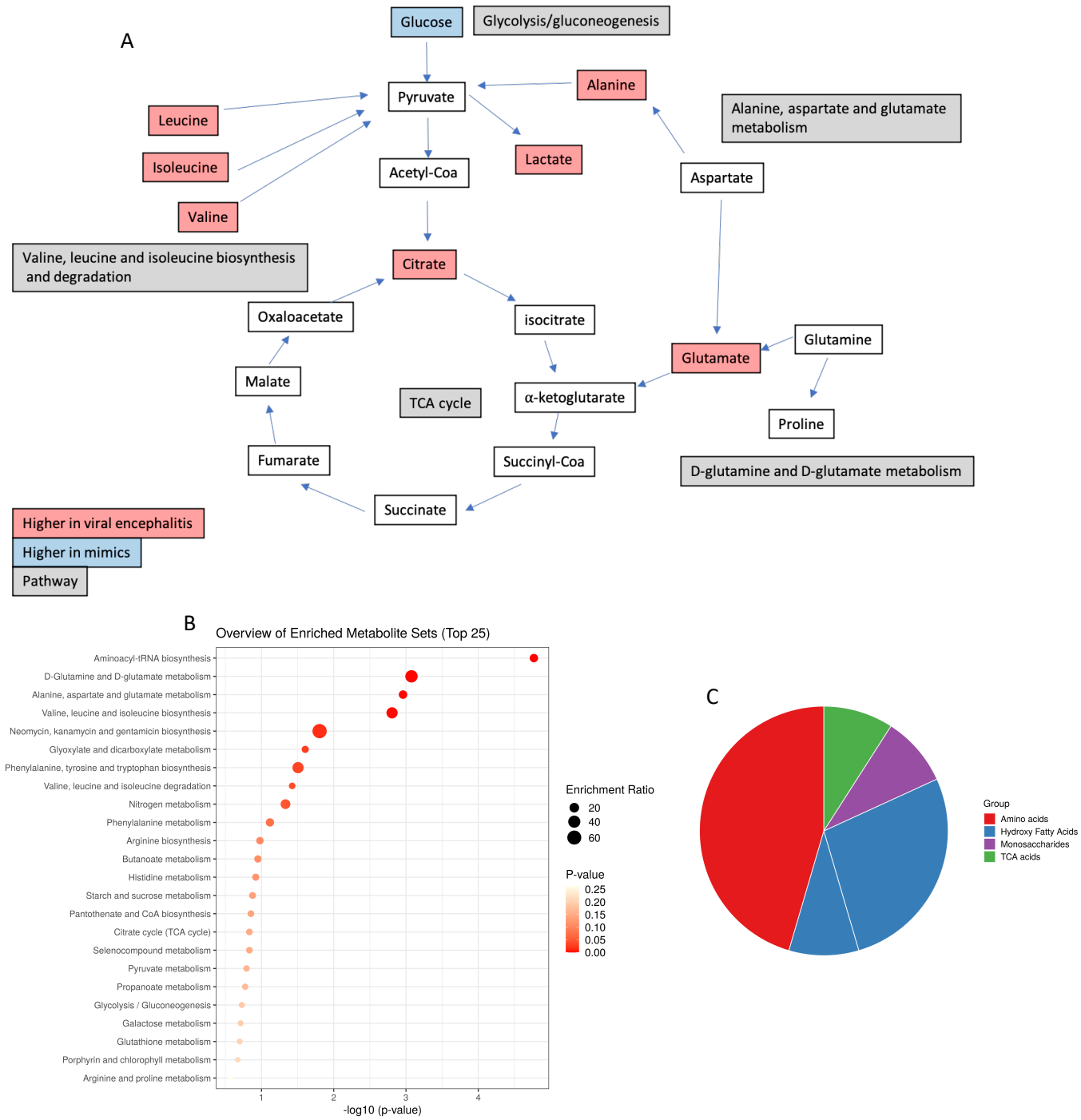


Figure 14. Enrichment analysis using metabolites showing significant difference between viral encephalitis and mimics. A: key metabolic pathways with differences between viral encephalitis and mimics. B: Enriched metabolite sets between viral encephalitis and mimics, colour corresponding to p value, size of dot corresponding to enrichment ratio (number of metabolites represented as a ratio to the number of metabolites expected for each pathway). C: Pie chart showing categories of metabolites varying between viral encephalitis and mimics.

Discussion

I used ^{1}H NMR spectroscopy to analyse the CSF of 87 patients with encephalitis, meningitis and mimics. Having identified 32 compounds I detected significant differences on univariate analysis between patients with bacterial meningitis and others, which were able to distinguish bacterial meningitis from encephalitis and mimics effectively. Viral encephalitis could be distinguished from mimics by differences in 2-hydroxyvalerate concentration. Autoimmune encephalitis was more challenging to distinguish from other groups, and multivariate models generated through PLSDA did not show good discrimination. However, differences in glucose concentration between autoimmune encephalitis and mimics showed promise as a biomarker, as a ratio with the CSF total WCC. Pathway analysis showed that the metabolic pathways connected with several amino acids, particularly glutamate, alanine and valine, as well as glycolysis and the citric acid cycle, are key to the metabolic profile of viral encephalitis when compared to mimicking conditions.

Amino acid concentrations may reflect their role in energy metabolism, cell signalling, the metabolism of pathogens in the case of bacterial meningitis, or as a breakdown product connected to inflammation. Glutamate, which was found in higher concentrations in viral and bacterial meningitis, also functions as a neurotransmitter and excitotoxicity mediated by glutamate forms part of the pathogenesis of CNS inflammatory conditions, particularly in association with seizure activity. Alanine, the concentration of which was clearly significantly increased in bacterial and viral encephalitis, is used as an energy source, via pyruvate, but also forms an important part of many structural macromolecules.

2-hydroxyvalerate is a hydroxy fatty acid which can act as an energy source or in the maintenance of cell membranes. Its significance in CSF is unclear, but its concentration was lower in bacterial meningitis and viral and autoimmune encephalitis than mimicking conditions. It has been found in increased levels in plasma in patients with mitochondrial dysfunction and acidosis, suggesting a role in anaerobic metabolism (Asano et al. 1988).

Glucose concentrations in CSF are well known to decrease in bacterial infection, and the results of metabolomic analysis confirmed this. However, reduction in CSF glucose in viral and autoimmune conditions are less well explored in the literature. NMR showed reductions in CSF glucose concentrations in autoimmune and viral encephalitis versus mimics. Since in autoimmune encephalitis the metabolic activity of leucocytes might be expected to account for consumption of CSF glucose, I investigated the ratio of glucose to the total CSF WCC, which proved the most effective biomarker for distinguishing autoimmune encephalitis from mimics, performing better than CSF WCC alone. It is possible that although not all cases of autoimmune encephalitis demonstrate CSF pleocytosis, the metabolic activity of leucocytes is increased. Alternatively, the decrease in glucose concentration may represent increased energy demand of neurones or glia. Since both CSF glucose and WCC are currently routinely measured as part of basic CSF analysis, this would represent a simple and attractive biomarker.

The analysis of metabolome in CSF is experimentally novel and the limits of normality, as well as patterns of metabolic disruption in disease states, are still being explored. Most metabolomics studies have analysed plasma or urine which differ greatly in metabolite

concentration and homeostatic mechanisms. There is a need for improved standard operating procedures in the collection and preparation of samples for NMR analysis, since metabolite concentrations are highly vulnerable to disruption by laboratory handling, exposure to containers, temperature control and freeze-thaw cycles. In the setting of multicentre studies, as in this cohort, variability between laboratory practices represents a potential confounding factor. Reducing confounding factors between groups is important, and in this cohort there were significant differences in age between patients with autoimmune encephalitis and others, as well as differences in exposure to treatments between groups, although these were not evident on principal component analysis. The groups themselves were heterogeneous, and the sample size relatively small, which may have limited the detection of more subtly varying metabolites. Enrichment analysis is limited by incomplete databases, including KEGG, which rely on existing literature and can therefore contain bias. The small number of studies using NMR in CSF also poses a challenge to identify all molecules present. Further studies are required to elucidate all the endogenous metabolites in CSF observable by NMR.

Conclusion

Metabolite profiles in CSF differ between types of encephalitis and mimicking conditions. These differences principally reflect the activity of pathways concerned with amino acid metabolism and energy production.

These findings, together with analysis of the CSF proteome and gene expression in blood, form a comprehensive snapshot of disruptions to the homeostatic environment in encephalitis. The transcription of genes to form mRNA, which is then translated to build proteins which interact with small molecules, represents a rich source of potential integrative networks which may yield further potential biomarkers, targets for adjunctive treatments or insights into mechanisms of disease.

Chapter 6. Integration of transcriptome, proteome and metabolome

Introduction

In previous chapters I have described the analysis of gene expression, protein abundance and metabolite profiles of patients with encephalitis and mimicking conditions. In addition to providing candidate biomarkers to distinguish aetiological groups, these analyses can shed light on the ways in which disease mechanisms differ between pathologies.

Integrating data from different biological levels can work synergistically and can provide more information than separate analysis, both for biomarker discovery and pathogenic mechanisms. This multiomic integration approach can involve looking for correlations between variables in different datasets, analysing biological pathways and networks with contributions from genes, proteins and metabolites, or accessing publicly available data repositories on the disease of study or related conditions.

However, the computational integration of datasets presents challenges. Since each complex dataset is a snapshot of a certain physiological or pathological state, differing datasets may not be directly comparable. The ideal situation for comparison of datasets is a "split sample" approach, where parts of the same biofluid sample is used for several -omic analyses, as in the present study where CSF was used for both proteomic and metabolomic profiling, although the transcriptomic dataset is distinct in both sample collection and tissue

compartment. The analysis of pathways and networks relies on the state of knowledge about biological systems, which are represented in searchable databases and software.

Few studies have applied truly multiomic approaches in the setting of brain inflammation and infection. One study combined metabolomics with genomics to outline links between cerebral tryptophan metabolic pathways and outcome in tuberculous meningitis (van Laarhoven et al. 2018). Other authors have studied the interaction between microbiome and neurological disease using combined -omic approaches (Xu et al. 2020). One study used analysis of multiple genomic, transciptomic and proteomic datasets to explore the hypothesised role of viruses in the pathogenesis of Alzheimer's disease (Readhead et al. 2018).

In this chapter I will examine the similarities and differences between gene expression in blood and protein abundances in CSF as they vary between viral encephalitis, autoimmune encephalitis and mimicking conditions. I will explore panels of genes associated with the host immune response derived from key proteins identified from proteomic analysis. Through joint pathway analysis, I will look for biological processes which are associated with genes, proteins and metabolites identified as important in viral encephalitis. In addition, in this chapter, I will summarise and compare the most effective biomarkers identified from all of the analyses of earlier chapters.

Methods

Datasets

For these comparisons and enrichment/network analyses I used the datasets arising the transcriptomic, proteomic and metabolomic analyses from chapters 3, 4 and 5. For proteomic and metabolomic data, quality control, normalisation and scaling were identical to the approaches used in the relevant chapters.

For transcriptomic data, since this analysis is exploratory rather than an attempt to identify biomarkers, I used more permissive filtering in order to analyse data for genes coding for proteins and networks identified from proteomic analysis. Therefore features with a signal:background ratio of <1.5 were excluded (compared to 2.5 ratio threshold in chapter 3). Genes that did not show expression in at least one array were removed.

The cohort for fold changes between groups and cluster analysis was the same as those in the relevant chapters. However, for correlation between protein abundance in CSF and gene expression in blood, I limited the cohort to the samples where both transcriptomic and proteomic analysis were performed, and where the protein in question was identified.

Statistical analysis

Gene and protein feature hierarchical clustering used Euclidean distance and Ward's linkage method.

Enrichment and pathway analysis were performed using MetaboAnalyst (Chong et al. 2018) through metabolite set enrichment analysis and used metabocards from the Human Metabolome Database (accessed January 2020) and the KEGG compound database (October 2019 version) (Kanehisa et al. 2004). Genes were converted to entrez identifiers (IDs). Proteins were converted to Entrez IDs using Uniprot (Pundir et al. 2017). Topology analysis was performed by assessing the importance of each molecule (node) based on the number of pathways that connect to that node (degree centrality). p values from genes, proteins and metabolites were given equal weight.

Results

Correlations between gene expression in blood and protein abundance in CSF

Initially I investigated whether the profiles of candidate biomarkers identified from proteomic analysis of CSF (chapter 4) would be mirrored in their gene expression in blood, which might indicate that the proteins we were measuring in CSF had actually spilled over from the blood, perhaps through a leaky blood brain barrier. I identified the genes coding for each of the candidate protein biomarkers, and calculated the fold change between aetiological groups, performing cluster analysis by gene (Fig 1a). I then calculated the fold change in the respective proteins and plotted these in the same order (Fig 1b).

The gene expression profiles between groups differ from the pattern of protein abundances in CSF. Only *CECR1* (ADA2) and *VSTM2A* (VTM2A) show similar changes between groups, being most upregulated/abundant in viral encephalitis and mimics respectively. Several genes show inverse patterns between groups to their relevant proteins, e.g. SST (somatostatin) showed highest abundance in CSF in mimics, while its gene expression in blood was upregulated in viral encephalitis.

Since adenosine deaminase 2 (ADA2) was the most significant candidate protein biomarker identified by mass spectrometry (chapter 3), I analysed the correlation between *CECR1* gene expression in blood and ADA2 abundance in CSF within each patient where both values were measured. This showed a very weak positive correlation (Fig. 1C).

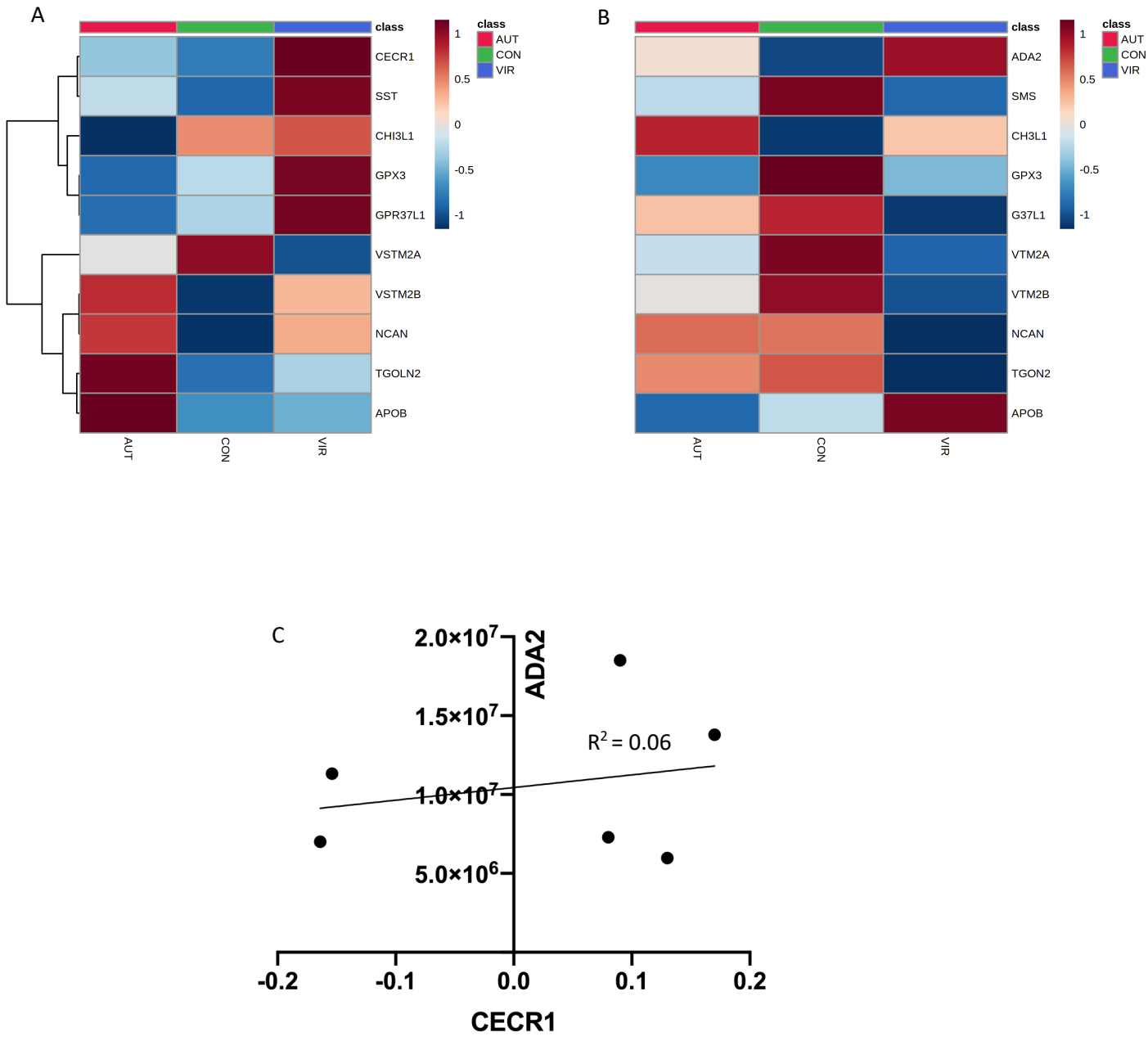


Figure 1. Correlations between the top candidate biomarkers from proteomic analysis and their coding genes in blood. A: fold change in gene expression between aetiological groups in blood (coloured bar indicates groups, red=autoimmune encephalitis, green=mimics, blue=viral encephalitis). B: fold change in abundance of corresponding proteins in CSF (order for genes and proteins is the same). C: Scatter plot of *CECR1* gene expression in blood against ADA2 abundance in CSF.

Gene expression patterns within immune networks identified from proteomic analysis

In Chapter 4 I identified several significant proteins associated with host immune responses, both showing differences in abundance between aetiological groups, and showing correlations with outcome or MRI features. In particular, cell adhesion molecules (VCAM1), interleukin signalling (IL-1R2), chemokine signalling and Th2 pathway responses and immunoglobulin production.

I therefore identified proteins involved in KEGG pathways for leucocyte transepithelial migration, chemokine signalling pathways, cell adhesion molecules and cytokine-cytokine receptor interactions. Where genes coding for proteins involved in these pathways were identified in the transcriptomic dataset, I analysed fold changes between aetiological groups and performed cluster analysis (Fig. 2).

Many of the genes identified were upregulated in mimics, including chemokine pathway genes such as *CXCL1*, *CCL2*, *CXCL10*, and interleukins including *IL7*, *IL13*, *IL2* and *IL1B*. *VCAM1*, which I found to be negatively correlated with outcome in autoimmune encephalitis, was also upregulated in mimics.

Two genes, *EGF* and *IL25*, were upregulated in autoimmune encephalitis compared to other groups. Genes upregulated in viral encephalitis included *ICAM1*, *CX3CL1* and *CCL3*.

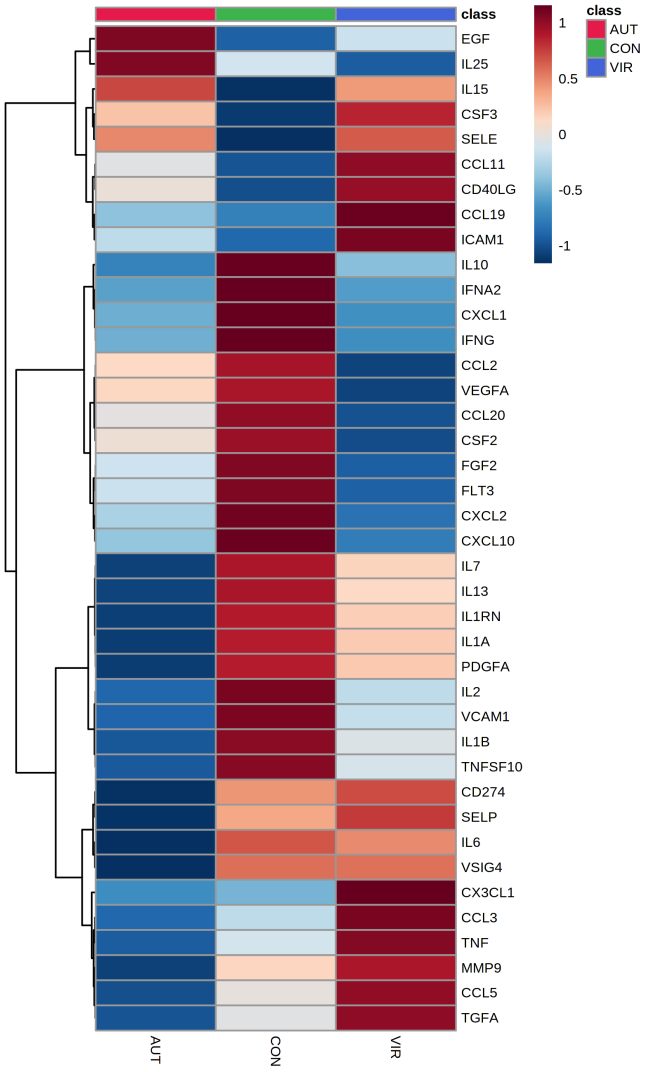


Figure 2. Fold change in gene expression in blood of an immune panel derived from pathways identified in proteomic analysis. Red: autoimmune encephalitis, green: mimics, blue: viral encephalitis.

Joint pathway analysis of the transcriptomic, proteomic and metabolomic profile of viral encephalitis compared with mimics.

The datasets from chapters 3, 4 and 5 provide three different perspectives on the inflammatory environment in encephalitis. Combining several datasets can illuminate pathways or networks that are particularly important in mechanisms of disease. I therefore used a joint pathway analysis approach to combine transcriptomic, proteomic and metabolomic data. I decided to investigate pathways involved in viral encephalitis, as compared to mimics. This comparison provided sufficient genes, proteins and metabolites with significant differences between groups to allow for a meaningful pathway analysis.

I identified 43 genes which made significant contributions to the sparse partial least squares analysis (sPLSDA) comparing viral encephalitis with mimics (Chapter 3). I converted these gene identifiers to Entrez IDs. From the proteomic dataset (Chapter 4), I identified 185 proteins which showed significant differences between groups on univariate (*limma*) analysis in cohort 1. I converted these proteins to gene Entrez IDs using Uniprot software (Pundir et al. 2017). Twelve metabolites which showed significant differences between viral encephalitis and mimics on t test were also included in the analysis, coded by their HMDB identifiers (Chapter 5).

Figure 3 shows the significance of enrichment of the pathway (y axis) against the estimated impact of the differentially regulated genes, proteins and metabolites on the activity of that pathway (also represented in table 1). Complement and coagulation cascades were the

most significantly affected pathways, while others concerned with immune responses, including specific infections such as *Staphylococcus aureus* and *Bordetella pertussis* were identified, reflecting those host responses common to several infections. Antigen processing and presentation and cell adhesion molecules were also significantly affected. Pathways concerned with the metabolism of amino acids, including alanine, aspartate, glutamate and phenylalanine, showed high impact but did not reach significance owing to the small number of molecules affected within relatively large networks.

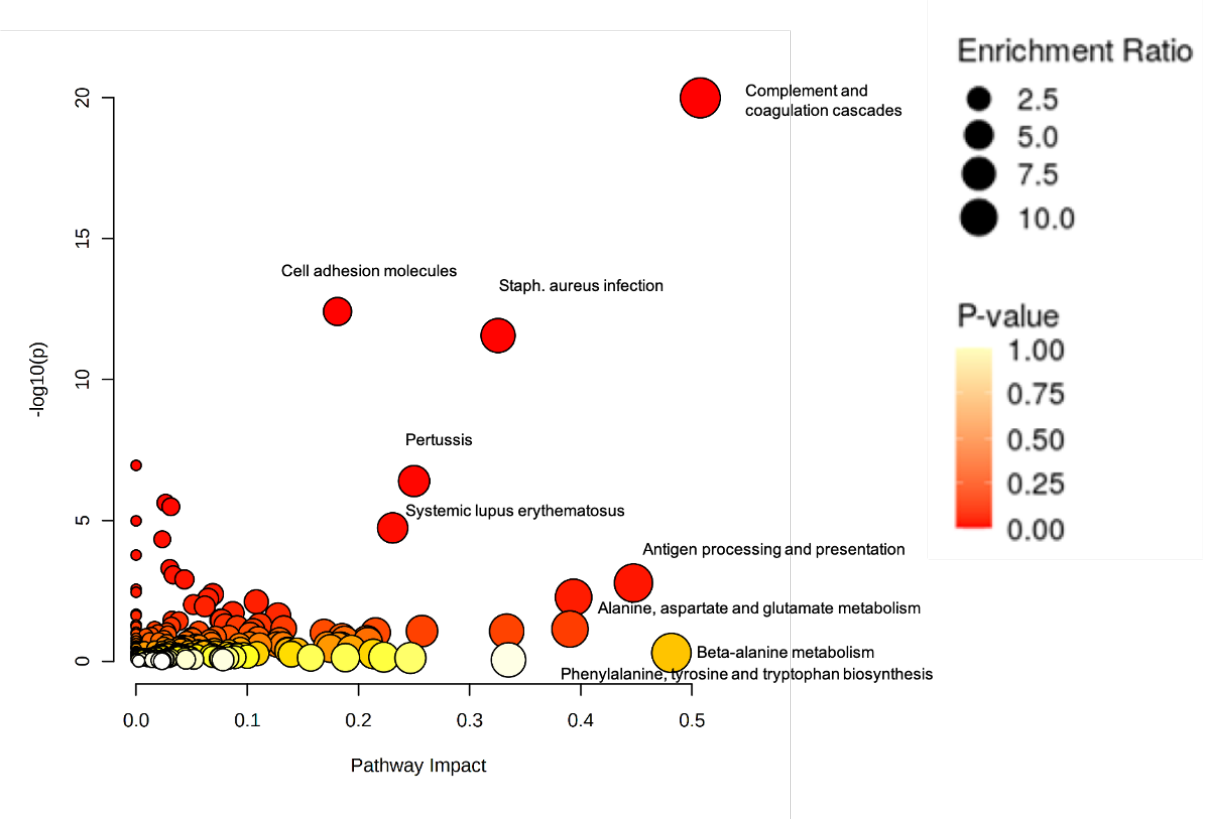
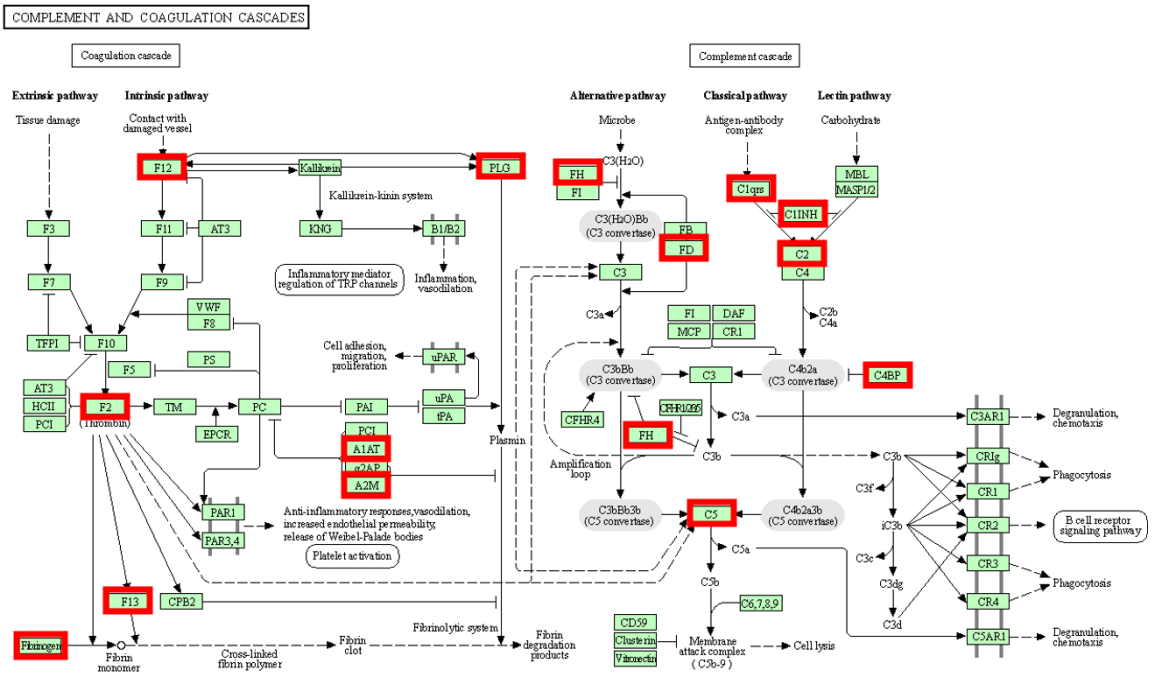


Figure 3. Pathways enriched in viral encephalitis compared with mimics, according to joint pathway analysis incorporating transcriptomic, proteomic and metabolomic datasets.

Table 1. Pathway enrichment in viral encephalitis compared with mimics					
Pathway name	**Nodes in pathway**	**Hits**	**Raw p**	**FDR**	**Pathway Impact**
Complement and coagulation cascades	80	19	1.01E-20	3.35E-18	0.50746
Cell adhesion molecules (CAMs)	147	17	3.87E-13	6.40E-11	0.18113
Staphylococcus aureus infection	98	14	2.76E-12	3.05E-10	0.32558
Phagosome	153	12	1.11E-07	9.21E-06	0
Pertussis	86	9	4.03E-07	2.66E-05	0.25
Central carbon metabolism in cancer	106	9	2.39E-06	0.00013184	0.026549
Prion diseases	38	6	3.28E-06	0.0001549	0.03125
Type I diabetes mellitus	46	6	1.03E-05	0.00042794	0
Systemic lupus erythematosus	136	9	1.85E-05	0.00067923	0.23077
Mineral absorption	87	7	4.68E-05	0.0015494	0.023529
Protein digestion and absorption	142	8	0.00016935	0.0050959	0
Viral myocarditis	60	5	0.00050356	0.01389	0.030303
Allograft rejection	39	4	0.00085641	0.021805	0.033333
Graft-versus-host disease	43	4	0.0012421	0.029367	0.043478
Antigen processing and presentation	78	5	0.001663	0.036697	0.44737

Figure 4a shows the most significantly affected pathway in viral encephalitis, complement and coagulation cascades, illustrating the proteins upregulated in viral encephalitis. Both the coagulation cascade and complement cascade are upregulated. Within the coagulation cascade, factors 12 and 13, factor 2 (thrombin), fibrinogen and plasminogen are all upregulated in viral encephalitis. Alpha 1 antitrypsin and alpha 2 macroglobulin, both protease inhibitors, are also upregulated. Complement factors involved in both the classical and alternative pathway are upregulated.

A



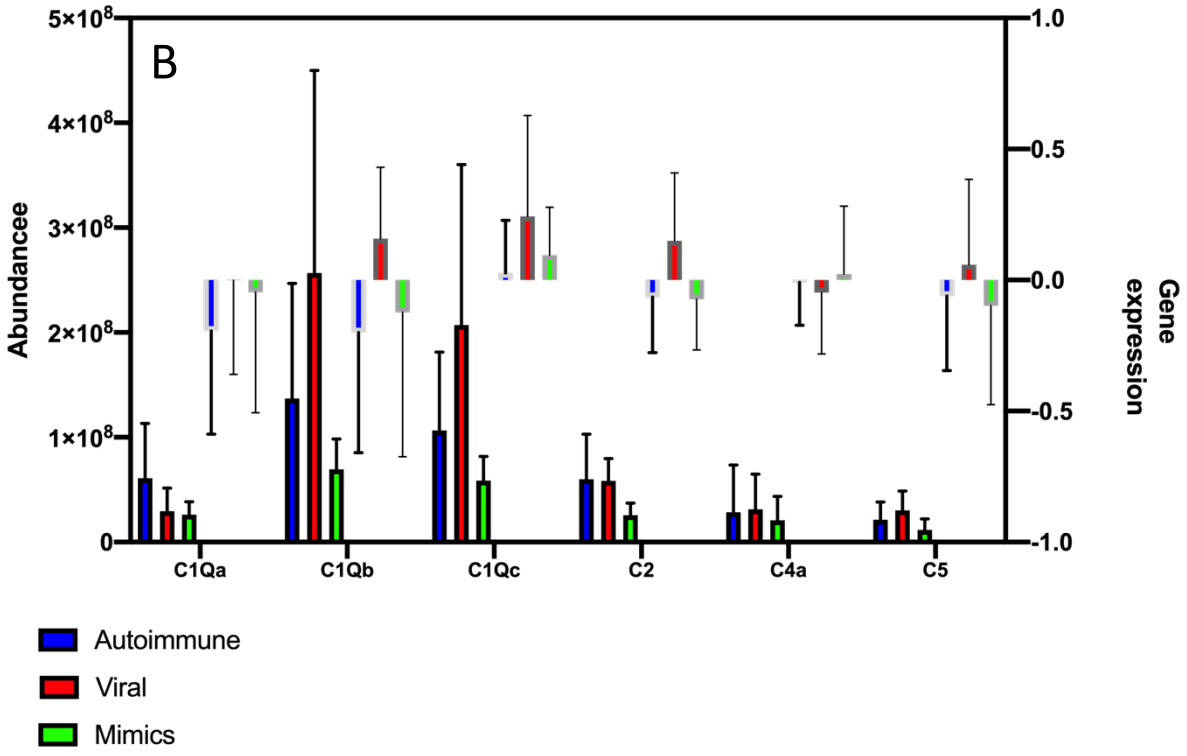


Figure 4. Complement and coagulation cascades in viral encephalitis compared with mimics. A: pathway diagram reproduced from KEGG. Upregulated components in viral encephalitis are highlighted in red. B: complement component protein abundance in CSF and gene expression in blood. Lower bars (and left y-axis) represent protein abundance; upper bars (and right y-axis) represent gene expression.

I decided to investigate complement component gene expression profiles alongside differences in protein abundance between groups. Fig. 4b shows the abundance of 6 complement components which were identified by mass spectrometry (lower bars), alongside their gene expression fold change (upper bars). In general, the abundance of the proteins in CSF is mirrored by the expression of the coding gene in blood, with all components other than C1Q subunit A, which showed highest abundance in autoimmune encephalitis in CSF. In general, both gene expression and protein abundance were highest in viral encephalitis.

Comparison of candidate biomarkers from all datasets

In chapters 3, 4 and 5 I looked for biomarkers which could distinguish viral encephalitis from autoimmune encephalitis and mimics. Each approach yielded potential biomarkers, and within each cohort (or, for the proteomic cohort, within a separate validation cohort), I tested each candidate biomarker for accuracy of group prediction by area under the ROC curve (AUC). Table 2 shows the candidate biomarkers with the highest AUC for each pairwise comparison.

Table 2. Most effective candidate biomarkers for each pairwise comparison.

Comparison	Biomarker class	Biomarker name	FDR	ROC AUC
Viral vs mimics	Protein	Adenosine deaminase 2	2.84×10^{-5}	1
Autoimmune vs mimics	Protein	Adenosine deaminase 2	0.0062	0.84
Viral vs autoimmune	Gene panel	*TMEM40*/*ATP7A*	n/a	0.85

ADA2 was the most effective biomarker overall, and by mass spectrometry this result was validated in a separate cohort (chapter 4). There were fewer effective biomarkers to distinguish autoimmune from viral encephalitis, but the most effective by ROC analysis was a gene panel incorporating *TMEM40* (transmembrane protein 40) and *ATP7A* (Copper-transporting ATPase 1) (chapter 3).

In chapter 2 I developed models based on clinical and basic laboratory investigations to distinguish viral and autoimmune encephalitis from other groups. For viral encephalitis the model included CSF protein, the presence of abnormal neuroimaging and fever. For autoimmune encephalitis, the model included young age, absence of headache, presence of memory problems and presence of movement disorder.

Since ADA2 was the most effective biomarker overall, I investigated whether incorporating ADA2 abundance in CSF into these models would improve discrimination between groups on ROC analysis. Adding ADA2 to the model distinguishing viral encephalitis from others improved AUC from 0.85 to 0.96 (Fig. 5). However, adding ADA2 to the model distinguishing autoimmune encephalitis from others did not improve AUC, in fact it decreased from 0.93 to 0.91, although this was not a statistically significant difference.

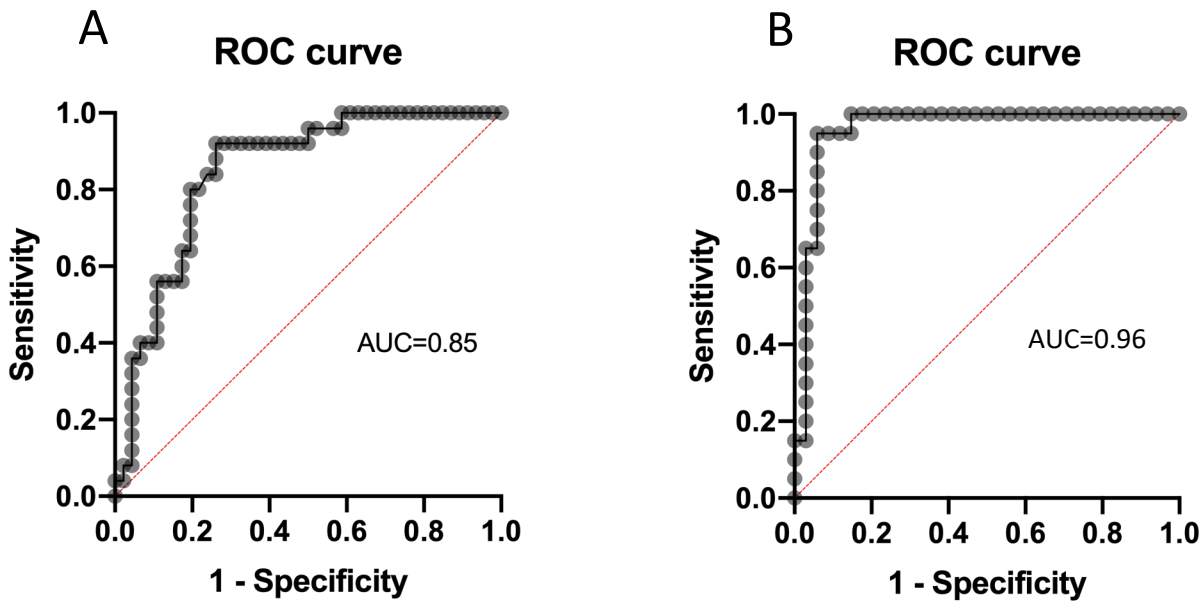


Figure 5. A: ROC analysis of model for identifying cases of viral encephalitis incorporating CSF protein, presence of abnormal neuroimaging and presence of fever. B: ROC analysis of the same model with the addition of ADA2 abundance in CSF.

Discussion

In this exploratory chapter I have brought together the three datasets from transcriptomic, proteomic and metabolomic analyses to investigate links and differences between them. I found that there were significant differences between the expression profiles of genes in blood, and the abundance of the coded proteins in CSF. The candidate protein biomarkers identified from proteomic analysis (Chapter 4) did not correlate well with their coding genes in blood. An immune panel of genes based on findings from proteomic analysis shows trends towards upregulation of genes in encephalitis, although most genes were upregulated in mimics. Joint pathway analysis including genes, proteins and metabolites with significant differences between viral encephalitis and mimics revealed significant enrichment in a number of pathways involved in host immune responses, with the complement and coagulation cascades showing the most significant enrichment and highest pathway impact. Further investigation of complement profiles showed that components, principally of the classical pathway, were more abundant in viral encephalitis than autoimmune encephalitis and mimics, and these differences were mirrored in gene expression in blood.

Comparing biomarker candidates from chapters 3, 4 and 5, ADA2 was the most effective biomarker for distinguishing encephalitis from mimics, for both viral and autoimmune encephalitis. For distinguishing autoimmune encephalitis from viral encephalitis, a gene panel incorporating *TMEM40* and *ATP7A* was most effective, although discrimination was still suboptimal. ADA2 significantly improved the clinical model for discriminating viral encephalitis from other groups, although did not affect the model for discriminating autoimmune encephalitis from others.

There are several reasons why protein abundances in CSF may not correlate with expression of genes in blood. The transcriptomic profiles of leucocytes in CSF and blood, even when strictly paired in time in the same patient, are known to differ significantly (Brynedal et al. 2010). Proteins present in CSF constitute those produced by cells within the CSF compartment, but also those present by active or passive transport from blood or from the brain parenchyma (Romeo et al. 2005). In the setting of inflammation or infection, blood-brain barrier and blood-CSF barrier permeability tends to increase, and some proteins may become more abundant in CSF, although this effect is not linear or entirely predictable (Michael et al. 2020). Additionally, changes in gene expression may not predictably lead to correlating changes in protein abundance in a biofluid, particularly if mRNA transcripts are degraded, or protein lifespan is variable. Hence the profiles of key biomarker candidates from proteomic analysis in Chapter 4 were generally not mirrored in gene expression profiles in blood.

Strikingly, exploratory analysis of a panel of immune response genes showed a trend towards upregulation of many genes in encephalitis mimics. This group included patients with delirium, headache disorders, alcohol withdrawal and other common conditions which may present in a similar way to encephalitis. It is possible that peripheral infection or inflammation was present, reflected in the observed upregulation of markers of inflammation such as *IL10*, *CXCL1* and *VCAM1*. Conversely, genes found to be upregulated in viral encephalitis, such as *CX3CL1*, *CCL11*, *ICAM*, *CCL3* and *MMP9* echo the findings of previous studies of brain infection and inflammation (Hulshof et al. 2003; Michael et al. 2020; Michael et al. 2016; Michael et al.

2015; Vilela et al. 2011). IL-25, upregulated in autoimmune encephalitis, is known to be involved in promotion of Th2 responses, although some evidence suggests that it contributes to regulation of Th17 responses, and therefore the suppression of autoimmunity (Kleinschek et al. 2007).

Combining transcriptome, proteome and metabolome in joint pathway analysis showed that several host immune response pathways are enriched in viral encephalitis compared with mimicking conditions, with the complement and coagulation cascades showing the greatest pathway impact. Most of the pathways identified were significantly affected by gene and protein changes; pathways involving amino acid metabolism were also identified, although the impact on these was limited by the smaller number of molecules involved.

Comparison of candidate biomarkers revealed that the most effective single marker for discriminating encephalitis from mimicking conditions was ADA2. In Chapter 4 I explored this protein in detail and showed that its abundance was significantly increased in viral encephalitis. In one proteomic cohort, the abundance was shown to be significantly higher in viral encephalitis than autoimmune, but this finding was not reproduced in the second cohort. ADA2 therefore appears to be a marker for CNS infection or inflammation, although with a trend to higher abundance in cases of infection. Integration of ADA2 into a clinical model, developed in Chapter 2 in the same cohort, for identification of cases of viral encephalitis showed a significant improvement in the model's power (AUC 0.85 to 0.96). This serves as a simulation for the effect the biomarker could provide in clinical practice, over and above the clinician's assessment of the clinical scenario. In autoimmune encephalitis, ADA2 did not

provide the same benefit. However, the clinical model was already fairly effective (AUC 0.93), perhaps artificially so given the presence of a larger number of NMDAR antibody encephalitis presence in this group, with younger age and movement disorder, than might ordinarily be seen in clinical practice.

The combining of these distinct datasets has several limitations, meaning that caution is needed when drawing conclusions from these analyses. Since -omic datasets are a single snapshot of a dynamic and rapidly changing environment, timing of sample collection is critical. Although metabolomic and proteomic data are derived from "split samples", collection of blood from RNA extraction in some patients occurred several hours or days apart from CSF collection, and this could have contributed to the poor correlation between group differences in blood transcriptome versus CSF proteome. The patient cohorts were not completely overlapping, with lower patient numbers in the transcriptomic cohort (see Chapter 2). Pathway analysis is limited by the scope of data repositories such as KEGG, which are confined to published datasets and pathway associations.

Conclusion

Through uniting transcriptomic, proteomic and metabolomic profiles it is possible to identify pathways and networks which show common changes between interdependent genes, proteins and metabolites. However, the comparison of these complex datasets is limited by differences in biofluid compartment, cohort differences and differences in sample collection, as well as technical limitations of the analysis and computational processing. Comparison of candidate biomarkers between techniques showed that ADA2 is overall the most effective marker for distinguishing encephalitis of any cause from mimics, and significantly improved discrimination of patients with viral encephalitis from others compared with clinical features alone.

Chapter 7. General discussion

Summary of key findings

In this thesis I aimed to use transcriptomic, proteomic and metabolomic methods to look for new biomarkers to improve diagnosis in encephalitis, to predict clinical outcome and to investigate disease mechanisms. These aims have been successfully met.

In chapter 2 I characterised a clinical cohort of patients with encephalitis and mimicking conditions showing that it is generally reflective of the epidemiology of encephalitis in the UK, and that the patients with mimicking conditions represent broadly the differential diagnosis of encephalitis in clinical practice (Granerod, Ambrose, et al. 2010). I showed that within this cohort, models based on clinical features and basic laboratory criteria could discriminate cases of viral and autoimmune encephalitis to some degree, albeit with classification errors, reflecting the diagnostic process of treating clinicians. These findings supported the need for biomarkers to improve diagnostic discrimination.

In order to address this clinical problem, I analysed gene expression in whole blood from 40 patients with encephalitis and mimicking conditions using DNA microarray (chapter 3). No significant single gene differences were identified between autoimmune encephalitis, viral encephalitis and mimicking conditions. However, small gene panels developed through multivariate analysis could distinguish these groups with acceptable accuracy. Multivariate analysis identified several genes of the HLA group which were upregulated in both

autoimmune and viral encephalitis compared to mimics. The most significant differences identified overall were between patients with bacterial meningitis and other groups, with 285 genes showing differences in transcript abundance. Pathway enrichment analysis of bacterial meningitis versus other groups showed highly significant enrichment of gene sets connected to innate immunity and neutrophil degranulation.

Since gene expression in blood is distant from the inflammatory environment in encephalitis, and RNA stabilised blood samples were limited, I next analysed proteomic profiles in CSF from 79 patients with encephalitis and mimicking conditions through liquid chromatography/mass spectrometry (chapter 4). In order to validate the results, I split the cohort into discovery (cohort 1) and validation (cohort 2) arms. Using this approach, the most effective validated biomarker was ADA2, which was identified in cohort 1 and distinguished viral encephalitis from mimics without error in cohort 2. ADA2 also distinguished autoimmune encephalitis from mimics, although with lower accuracy. In autoimmune encephalitis, and particularly NMDAR antibody encephalitis, VSIG4 and VCAM1 negatively correlated with clinical outcome. In 4 patients with HSV encephalitis, the abundance of several immunoglobulin components correlated with the volume of white matter T2 hyperintensity on MRI. Pathway enrichment analysis revealed key differences in complement cascade components, regulation of insulin-like growth factor, post-translational protein phosphorylation and platelet activation, signalling and aggregation between viral and autoimmune encephalitis.

In CSF samples from 87 patients, using split samples from those used in proteomic analysis, I investigated whether profiles of small molecule metabolites could distinguish between groups, as analysed by ^{1}H NMR (chapter 5). Viral and autoimmune encephalitis differed from mimics by concentrations of 2-hydroxyvalerate. Distinguishing autoimmune encephalitis from other groups was challenging, although the ratio of CSF glucose to the CSF white cell concentration differed between autoimmune encephalitis and mimics. Comparing metabolic pathways enriched in viral encephalitis with mimics highlighted processing pathways of several amino acids, glycolysis and the citric acid cycle.

Having explored the unique signatures of each aetiological group through these three techniques, I proceeded to integrate the datasets to look for common pathways and correlations which could shed further light on disease mechanisms in encephalitis (chapter 6). Comparing single transcripts with proteins, gene expression in blood did not correlate well with protein abundance in CSF. Exploratory analysis of the expression of genes connected with the host immune response showed a trend towards upregulation in mimics as compared to encephalitis in many genes, perhaps reflecting peripheral inflammation or infection. Joint pathway analysis of all three datasets, incorporating differences between viral encephalitis and mimicking conditions, showed that the complement and coagulation cascades were the most enriched pathways. Detailed analysis of the upregulated components showed that the classical pathway of complement fixation showed the greatest differences in abundance in CSF, and that these differences were mirrored by upregulation of complement component genes in blood.

Comparison of candidate biomarkers identified from each of the three techniques showed that ADA2 was the most effective biomarker overall, discriminating viral and autoimmune encephalitis from mimicking conditions. I therefore incorporated ADA2 abundance into the clinical models I had developed for identifying cases of autoimmune and viral encephalitis. ADA2 substantially improved the discrimination of viral encephalitis from other groups. However, it did not improve the identification of cases of autoimmune encephalitis.

Candidate biomarkers for diagnosis

The most effective biomarker identified, ADA2, arose from analysis of the CSF proteome (chapter 4). ADA2 was more abundant in both viral and autoimmune encephalitis than in mimics, with the highest abundance in viral cases. It could therefore function as a marker of CNS inflammation, whether infectious or autoimmune, although it also showed promise in the identification of viral encephalitis specifically.

Although ADA2 is important as a regulator of the immune response, its abundance did not correlate with overall CSF WCC. Strikingly, ADA2 abundance was significantly different between cases of viral encephalitis and mimics even in those cases with a normal CSF WCC. In 5-10% of patients with HSV encephalitis the CSF may be completely normal; this scenario is more common early in the illness, in immunosuppressed patients and in children, particularly infants (Raschilas et al. 2002; Solomon et al. 2011). These patients therefore

represent a significant diagnostic problem and ADA2 concentration may represent a useful adjunctive biomarker in this clinical situation.

ADA2 has been suggested in previous studies as a potential diagnostic biomarker in tuberculous meningitis, pneumonia, and as a potential therapeutic target in cardiovascular disease, since adenosine has an important role in myocardial contractility (Kutryb-Zajac et al. 2020; Eintracht et al. 2000; Hatzistilianou et al. 2002). The adenosine deaminases (ADA1 and ADA2) catalyse the deamination of adenosine to inosine, and deoxyadenosine to deoxyinosine, as part of the purinergic pathway (Zavialov et al. 2005). ADA2 is predominantly expressed by monocytes and is thought to contribute to CD4+ T cell activation, stimulation of macrophage proliferation and neutrophil function (Zavialov et al. 2010). Genetic deficiency of ADA2 is associated with a form of severe monogenic disorder of immune system dysregulation (Kendall et al. 2020). Clinical features include vasculitis, hepatosplenomegaly and lymphadenopathy with an accompanying profound immunodeficiency, suggesting that its role in the regulation of immune responses is crucial.

Confidence in the validity of ADA2 as a biomarker is provided by its validation between two separate cohorts (chapter 4, cohorts 1 and 2) and the strength of its performance in both cohorts. Its potential as a biomarker of inflammation has biological plausibility, given its known function as a regulator of the immune response, and it has been identified as a putative biomarker in several diseases similar to encephalitis in pathophysiology. When I incorporated ADA2 into a clinical model for the identification of cases of viral encephalitis, it improved the performance of the model (chapter 6), which suggests that the biomarker may

be of real clinical use, over and above diagnosis based on standard clinical features. How such biomarkers can be developed further for clinical use is discussed in the "Limitations and Future Directions" section below.

CSF proteomic analysis has several theoretical advantages over the other two techniques which may have contributed to its greater success in biomarker identification. Proteins are the effector molecules of the host immune responses, and analysis of CSF gives a close proxy to the site of inflammation in encephalitis. Additionally, the number of variables generated by the mass spectrometry analysis- around a thousand- allowed more scope for the identification of several strong candidate markers than NMR spectroscopy but did not present the problems with multiple comparisons encountered in microarray analysis with the identification of tens of thousands of genes.

Although ADA2 was also the best discriminator of autoimmune encephalitis from mimics, it was not validated as a candidate biomarker to discriminate between autoimmune and viral encephalitis and the addition of ADA2 did not improve the clinical model for identifying autoimmune cases (chapter 6). Several other candidate biomarkers for the identification of autoimmune encephalitis were identified, including the combination of two genes, *TMEM40* (transmembrane protein 40) and *ATP7A* (Copper-transporting ATPase 1), which was the best discriminator of autoimmune from viral encephalitis overall. *ATP7A* has been linked to the bactericidal function of macrophages (White et al. 2009), but the biological significance of the *TMEM40* protein product is less clear, although it has been identified as a biomarker for several cancers (Zhang, Huang, et al. 2019). The ratio of CSF glucose to CSF white cell

concentration also emerged as a putative marker for autoimmune encephalitis versus mimics, distinguishing these groups more effectively than CSF WCC alone. This is a potentially attractive biomarker as both components are measured as part of the standard diagnostic process.

Identifying biomarkers for autoimmune encephalitis was challenging, as many of the candidates demonstrated a spectrum of upregulation with autoimmune cases showing an intermediate level, between viral encephalitis and mimics. This situation was demonstrated for both ADA2 and CSF glucose/WCC ratio. Therefore, although these biomarkers are useful for pairwise discrimination between groups, identifying autoimmune encephalitis cases from all others is challenging. A multistep algorithm involving exclusion of infection, followed by discrimination of autoimmune cases from mimics would be one possible approach to mitigate this problem.

In both gene expression and metabolomic analysis, several biomarkers were identified to discriminate patients with an encephalitic presentation caused by bacterial meningitis from other groups of patients, including a large number of single genes and amino acid metabolites. The gene with the greatest fold change between bacterial meningitis and other groups, *ARG1* (arginase-1), codes for an important regulator of macrophage and neutrophil activity, which is released by activated neutrophils and metabolises arginine, thus reducing the activity of nitric oxide synthase (Haydar et al. 2021; Jacobsen et al. 2007). Although patients with meningitis exhibiting clear septic features are usually clinically distinct from patients with encephalitis, in children, older adults or those with immunosuppression signs

of sepsis may be less evident (McGill et al. 2016). Biomarkers to distinguish these patients from encephalitis or mimicking conditions could be clinically valuable. Further investigation will be required to identify whether these biomarkers can distinguish between systemic sepsis and bacterial meningitis.

Candidate biomarkers for outcome

Proteomic analysis identified biomarkers correlating with outcome in both autoimmune and viral encephalitis (chapter 4). In HSV encephalitis, IL-1R2 expression correlated with worse outcome. IL-1R2 acts as a decoy receptor for IL-1β, and therefore is predominantly anti-inflammatory. IL-1 signalling is known to be of importance in the pathogenesis of HSV encephalitis, and in general contributes to immune-mediated pathology. The ratio of IL-1β: IL-1RA in CSF has been shown previously to correlate negatively with outcome in encephalitis by the Liverpool Brain Infections Group (Michael et al. 2015), and this finding seems to contradict the paradigm that antagonism of IL-1 signalling is beneficial in HSV encephalitis. It is possible that detection of IL-1R2 is acting as a proxy marker for IL-1 signalling (IL-1α and β were not detected by mass spectrometry), or IL-1R2 may represent a remnant of earlier IL-1 pathway activity which had subsided by the time of sampling.

In autoimmune encephalitis two proteins, VSIG4 and VCAM1, correlated with worse outcome. The correlation was particularly marked in cases of NMDAR antibody encephalitis. The course of NMDAR antibody encephalitis is unpredictable and variable. Optimal first- and second-line treatment strategies are unclear and clinical trials are lacking (Nosadini et al.

2015), although a trial of intravenous immunoglobulin for autoimmune encephalitis, led from Liverpool, will shortly begin recruitment. Biomarkers that can predict clinical outcome at presentation would be valuable in NMDAR antibody encephalitis to guide the intensity of early immune therapy, and potentially the indication for empirical oophorectomy in females (Anderson et al. 2021). VSIG4 is a phagocytic receptor and negative regulator of IL-2 production and T cell proliferation (Vogt et al. 2006). Since IL-2 tends to promote the differentiation of T cells into regulatory T cells and prevention of autoimmunity, high levels of VSIG4 may relate to increased auto-reactive T cell proliferation and activation. VSIG4 has previously been identified as a candidate biomarker for primary CNS lymphoma (Waldera-Lupa et al. 2020). VCAM1 is a key adhesion molecule for the migration of leucocytes across the blood-brain barrier and it is upregulated, together with ICAM1, in the setting of inflammation. Previous studies have demonstrated that VCAM and ICAM correlate with increased blood-brain barrier permeability in encephalitis (Michael et al. 2015).

In four patients with HSV encephalitis, I analysed correlations between proteins identified by mass spectrometry and volume of T2 hyperintensity on MRI, a marker for the extent of inflammation, measured by stereology as part of a previous study by the Liverpool group (Defres et al. 2017). Amongst a number of significant proteins identified, several immunoglobulin subunits were strikingly correlated with the volume of abnormal signal on MRI, even though the number of subjects studied was small. A biomarker which correlates with volume of inflamed brain would be desirable for several purposes: monitoring of progression of inflammation may help to guide treatment, particularly administration of corticosteroids as an adjunctive therapy, as is being tested in an ongoing clinical trial co-

ordinated from Liverpool (NCT03084783). The ability to assess brain inflammation without scanning may also be useful where MRI scanning is contraindicated or impractical because of mechanical ventilation or agitation. It might also give a more rapid assessment of whether treatments are effective: currently it typically takes several days before the effects of treatment for brain inflammation can be seen on imaging (Heine et al. 2015).

Throughout this thesis I have explored pathways and networks which are enriched in certain disease states. The complement cascade, and related proteins involved in coagulation pathways, emerged as significant both from the proteomic comparison between viral and autoimmune encephalitis in chapter 4, and joint pathway analysis between viral encephalitis and mimics in chapter 6. The fact that this pathway emerged from two analytical approaches provides confidence in its validity. The complement system is a set of immune proteins comprising an ancient first-line defence against pathogens (Conigliaro et al. 2019). The crucial role of the complement cascade in the intrathecal space in viral encephalitis mirrors findings in tick-borne encephalitis (Veje et al. 2019), and West Nile virus encephalitis, when complement interaction with microglia was found to relate to memory impairment in mice (Vasek et al. 2016). In a rat model of HSV encephalitis, complement components, particularly complexes containing C3 and C4, were increased in serum and CSF (Lange et al. 2017). Indeed, complement activation itself contributes to disruption of the blood brain barrier, as previously shown in the setting of neuropsychiatric systemic lupus erythematosus (Duarte-Delgado et al. 2019), thus potentially playing a key role in the pathogenesis of viral encephalitis, as well as altering the balance of the CSF proteome (Michael et al. 2020). In the current study, abundance of complement components in CSF

was mirrored in upregulation of complement genes in blood (chapter 6). Although most complement components are primarily produced in the liver, complement genes are also expressed in macrophages and upregulation of these genes occurs in acute infection (Martin 2007).

Limitations and future directions

Multiomic analysis, as employed in this thesis, has several inherent limitations. Since this approach harnesses the power of high-throughput laboratory techniques to generate large datasets, careful computational analysis is required to generate relevant and replicable results (Mirza et al. 2019). Since extremely large numbers of variables are analysed, particularly in transcriptomic and proteomic analysis, there is a potential for overfitting models to data. Throughout this thesis I have corrected carefully for multiple comparisons, and in the proteomic cohort, where the most significant results were generated, all candidates were validated in a separate cohort to provide confidence that overfitting did not occur. Nonetheless, the emphasis of this thesis is on hypothesis and candidate generation for future validation and clinical evaluation. All the techniques used are rarely employed in the diagnostic laboratory, and results may be less reliable than standard laboratory techniques, particularly in the case of microarray and mass spectrometry analyses (Yates et al. 2009). Orthogonal validation is therefore required before biomarkers can be taken forward as candidates for clinical testing, for example by ELISA for proteins, and by real-time PCR (RT-PCR) or multiplex RT-PCR for genes or panels of genes.

Patient numbers in this cohort were relatively limited owing to the relatively low incidence of encephalitis and the challenges inherent to recruitment and sample collection in the acute setting, although numbers compare favourably with other studies in the field where valuable results have been obtained (Chapter 1, literature reviews). In general, patient numbers met those specified by power calculations (Chapter 2), although numbers of autoimmune patients in proteomic and metabolomic analyses fell short. This could have contributed to difficulty identifying biomarkers for the autoimmune group. Within each aetiological group, relatively heterogeneous disease entities were represented. Since the key difference in management, whether to administer immune therapy, lies between autoimmune and viral encephalitis, this distinction makes clinical sense. However, disease mechanisms differ between different forms of viral and autoimmune encephalitis, and heterogeneity within the groups may have made the identification of biomarkers for discrimination less likely.

I did not include patients with encephalitis of unknown cause in this study cohort, although these patients had been recruited into the original clinical study, since in order to identify biomarkers clear diagnostic groups are required. However, analysing samples from these patients using the identified biomarkers would allow stratification into groups, which could then be analysed for differences in clinical features and response to therapy. Several novel methods of pathogen and antibody identification, such as metagenomic sequencing and antigen microarray, are now available and could be employed to search for causes in

patients with encephalitis of unknown cause, potentially identifying novel antibodies or pathogens (Wilson et al. 2019; Wingren et al. 2008).

The key biomarkers identified would be practical candidates for use at the bedside or in diagnostic laboratories. Single protein biomarkers, such as ADA2, can be used as an ELISA or incorporated into a simple kit, such as a lateral flow assay, similar to a urinary pregnancy test. This method has been employed in the diagnosis of tuberculosis and cryptococcal meningitis (Siddiqi et al. 2019; Rajasingham et al. 2019). This approach does not require a diagnostic laboratory and is simple and pragmatic to translate to resource poor settings. A lateral flow assay designed to measure CSF ADA2 would be more straightforward, and potentially more accurate, for the diagnosis of encephalitis than CSF WCC, which can be very challenging to obtain accurately in diagnostic laboratories, especially in less developed countries. Gene panels, such as that identified to distinguish autoimmune from viral encephalitis, can be analysed accurately by multiplex RT-PCR (Hawkins et al. 2017). This technique can be incorporated into a bedside device for rapid testing (taking around 30 minutes) without the need for a diagnostic laboratory (Inoue et al. 2021). Biomarkers based on gene expression in blood also have the theoretical advantage of avoiding the need for lumbar puncture, should a gene panel be validated in the future.

The next step for validation of putative biomarkers is validation in an independent, prospectively recruited cohort of patients with encephalitis, with a larger number of

patients and detailed clinical outcome measurement. This cohort should recruit patients with suspected encephalitis, employing biomarkers in a blinded fashion to stratify patients into groups at presentation, for example using a combination of ADA2 ELISA and a multiplex RT-PCR assay in blood. Patients should then undergo a standardised and thorough diagnostic workup and the final diagnosis reached should be compared with the results of novel biomarker tests. It would be of particular interest to include immunosuppressed patients, in whom immune responses may be attenuated, to ensure the biomarkers are still effective in this group. If biomarkers were destined for use in lower- and middle-income countries it would be important to conduct validation studies on appropriate populations reflecting local ethnicity and disease exposure. Follow-up and convalescent samples should be taken to monitor the variation of markers over the natural history of the disease, particularly for markers associated with outcome in HSV and NMDAR antibody encephalitis.

The Liverpool Brain Infections Group has extensive experience of translating findings of diagnostic biomarker studies into validated commercially produced test kits. For example, they have developed a range of biomarker antibody tests for Japanese encephalitis in collaboration with partners in Asia; this includes rapid dot-blot and ELISA formats developed with commercial partners, Venture Technologies, Singapore and XCyton Diagnostics Limited in India (Solomon et al. 1998; Lewthwaite et al. 2010). They have also developed host mRNA diagnostic assays for diagnosis of brain infections. Using samples from the multicentre observational UK Meningitis Study (McGill et al. 2018), the group identified 5 highly discriminatory host mRNA transcripts in the blood of adults to distinguish patients with bacterial meningitis from its mimics (filed for patent No. GB1606537.7 14th April 2016). This

is now being developed evaluated further for commercial development with Fast-Track Diagnostics Ltd, and Siemens Healthineers (Griffiths 2019).

Further potential investigations include analysis of genomic variation between individuals with encephalitis and controls, particularly in relation to variations in immune response. Polymorphisms in genes connected with innate immunity, such as toll-like receptor 3 mutations, are known to convey susceptibility to viral encephalitis (Mortaz et al. 2017), but other polymorphisms associated with adaptive immunity, and perhaps connected with the pathophysiology of autoimmune encephalitis, have not been adequately explored. In the setting of this cohort, there is an opportunity to integrate genomic analysis with transcriptomic, proteomic and metabolomic datasets, potentially revealing the interaction of dynamic immune responses in disease with variations in the host genome.

In summary, this thesis has shown that an integrated multiomic approach to investigating patients with encephalitis did identify new biomarkers which can potentially distinguish different encephalitis aetiologies and point to underlying disease mechanisms. The Liverpool Brain Infections Group is well placed to validate these candidate biomarkers and develop diagnostic assays to use clinically for patient benefit.

References

Ahmad S., Singh P., Sharma A., Arora S., Shriwash N., Rahmani A., Almatroodi S., Manda K., Dohare R., and Syed M. 2019. 'Transcriptome Meta-Analysis Deciphers a Dysregulation in Immune Response-Associated Gene Signatures during Sepsis', *Genes*, 10: 1005.

Al-Obaidi M. M. J., and Desa M. N. M. 2018. 'Mechanisms of Blood Brain Barrier Disruption by Different Types of Bacteria, and Bacterial-Host Interactions Facilitate the Bacterial Pathogen Invading the Brain', *Cell Mol Neurobiol*, 38: 1349-68.

Alamowitch S., Graus F., Uchuya M., Reñé R., Bescansa E., and Delattre J. Y. 1997. 'Limbic encephalitis and small cell lung cancer. Clinical and immunological features', *Brain*, 120 (Pt 6): 923-8.

Almasy E., Szederjesi J., Grigorescu B. L., Badea I., Petrisor M., Manasturean C., Negrea V., Timar A. E., Coman O., Azamfirei L., Santini A., and Copotoiu S. M. 2020. 'The Diagnostic and Prognostic Role of Vascular Endothelial Growth Factor C in Sepsis and Septic Shock', *J Crit Care Med (Targu Mures)*, 6: 152-58.

Anderson D., Nathoo N., Henry M., Wood G., Smyth P., and McCombe J. 2021. 'Oophorectomy in NMDA receptor encephalitis and negative pelvic imaging', *Practical Neurology*, 21: 57-60.

Angel T. E., Jacobs J. M., Smith R. P., Pasternack M. S., Elias S., Gritsenko M. A., Shukla A., Gilmore E. C., McCarthy C., Camp D. G., 2nd, Smith R. D., and Warren H. S. 2012. 'Cerebrospinal fluid proteome of patients with acute Lyme disease', *J Proteome Res*, 11: 4814-22.

Arino H., Armangue T., Petit-Pedrol M., Sabater L., Martinez-Hernandez E., Hara M., Lancaster E., Saiz A., Dalmau J., and Graus F. 2016. 'Anti-LGI1-associated cognitive impairment: Presentation and long-term outcome', *Neurology*, 87: 759-65.

Armangue T., Spatola M., Vlagea A., Mattozzi S., Carceles-Cordon M., Martinez-Heras E., Llufriu S., Muchart J., Erro M. E., Abraira L., Moris G., Monros-Gimenez L., Corral-Corral I., Montejo C., Toledo M., Bataller L., Secondi G., Arino H., Martinez-Hernandez E., Juan M., Marcos M. A., Alsina L., Saiz A., Rosenfeld M. R., Graus F., and Dalmau J. 2018. 'Frequency, symptoms, risk factors, and outcomes of autoimmune encephalitis after herpes simplex encephalitis: a prospective observational study and retrospective analysis', *Lancet Neurol*, 17: 760-72.

Asano K., Miyamoto I., Matsushita T., Murakami Y., Minoura S., Wagatsuma T., and Oshima M. 1988. 'Succinic acidemia: a new syndrome of organic acidemia associated with congenital lactic acidosis and decreased NADH-cytochrome c reductase activity', *Clin Chim Acta*, 173: 305-12.

Asano T., Koizumi S., Takagi A., Hatori T., Kuwabara K., Fujino O., and Fukunaga Y. 2011. 'Identification of a novel biomarker candidate, a 4.8-kDa peptide fragment from a neurosecretory protein VGF precursor, by proteomic analysis of cerebrospinal fluid from children with acute encephalopathy using SELDI-TOF-MS', *BMC Neurol*, 11: 101.

Aung H. H., Tsoukalas A., Rutledge J. C., and Tagkopoulos I. 2014. 'A systems biology analysis of brain microvascular endothelial cell lipotoxicity', *BMC systems biology*, 8: 80-80.

Aurelius E., Johansson B., Sköldenberg B., and Forsgren M. 1993. 'Encephalitis in immunocompetent patients due to herpes simplex virus type 1 or 2 as determined by type-specific polymerase chain reaction and antibody assays of cerebrospinal fluid', *J Med Virol*, 39: 179-86.

Bartholomeus E., De Neuter N., Lemay A., Pattyn L., Tuerlinckx D., Weynants D., Van Lede K., van Berlaer G., Bulckaert D., Boiy T., Vander Auwera A., Raes M., Van der Linden D., Verhelst H., Van Steijn S., Jonckheer T., Dehoorne J., Joos R., Jansens H., Suls A., Van Damme P., Laukens K., Mortier G., Meysman P., and Ogunjimi B. 2019. 'Diagnosing enterovirus meningitis via blood transcriptomics: an alternative for lumbar puncture?', *Journal of Translational Medicine*, 17: 282.

Baumgartner A., Rauer S., Mader I., and Meyer P. T. 2013. 'Cerebral FDG-PET and MRI findings in autoimmune limbic encephalitis: correlation with autoantibody types', *J Neurol*, 260: 2744-53.

Bereczki E., Branca R. M., Francis P. T., Pereira J. B., Baek J. H., Hortobagyi T., Winblad B., Ballard C., Lehtio J., and Aarsland D. 2018. 'Synaptic markers of cognitive decline in neurodegenerative diseases: a proteomic approach', *Brain*, 141: 582-95.

Bertrand A., Leclercq D., Martinez-Almoyna L., Girard N., Stahl J. P., and De-Broucker T. 2017. 'MR imaging of adult acute infectious encephalitis', *Med Mal Infect*, 47: 195-205.

Bonnet J., Garcia C., Leger T., Couquet M. P., Vignoles P., Vatunga G., Ndung'u J., Boudot C., Bisser S., and Courtioux B. 2019. 'Proteome characterization in various biological fluids of Trypanosoma brucei gambiense-infected subjects', *J Proteomics*, 196: 150-61.

Brown K. A., Melby J. A., Roberts D. S., and Ge Y. 2020. 'Top-down proteomics: challenges, innovations, and applications in basic and clinical research', *Expert Rev Proteomics*, 17: 719-33.

Brynedal B., Khademi M., Wallström E., Hillert J., Olsson T., and Duvefelt K. 2010. 'Gene expression profiling in multiple sclerosis: a disease of the central nervous system, but with relapses triggered in the periphery?', *Neurobiol Dis*, 37: 613-21.

Chatterji T., Singh S., Sen M., Singh A. K., Agarwal G. R., Singh D. K., Srivastava J. K., Singh A., Srivastava R. N., and Roy R. 2017. 'Proton NMR metabolic profiling of CSF reveals distinct differentiation of meningitis from negative controls', *Clin Chim Acta*, 469: 42-52.

Chong J., Soufan O., Li C., Caraus I., Li S., Bourque G., Wishart D. S., and Xia J. 2018. 'MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis', *Nucleic Acids Res*, 46: W486-w94.

Cinque P., Cleator G. M., Weber T., Monteyne P., Sindic C. J., and van Loon A. M. 1996. 'The role of laboratory investigation in the diagnosis and management of patients with suspected herpes simplex encephalitis: a consensus report. The EU Concerted Action on Virus Meningitis and Encephalitis', *J Neurol Neurosurg Psychiatry*, 61: 339-45.

Comabella M., Fernández M., Martin R., Rivera-Vallvé S., Borrás E., Chiva C., Julià E., Rovira A., Cantó E., Alvarez-Cermeño J. C., Villar L. M., Tintoré M., and Montalban X. 2010. 'Cerebrospinal fluid chitinase 3-like 1 levels are associated with conversion to multiple sclerosis', *Brain*, 133: 1082-93.

Conigliaro P., Triggianese P., Ballanti E., Perricone C., Perricone R., and Chimenti M. S. 2019. 'Complement, infection, and autoimmunity', *Curr Opin Rheumatol*, 31: 532-41.

Considine E. C., and Salek R. M. 2019. 'A Tool to Encourage Minimum Reporting Guideline Uptake for Data Analysis in Metabolomics', *Metabolites*, 9: 43.

Cooper J., Kierans C., Defres S., Easton A., Kneen R., and Solomon T. 2016. 'Diagnostic Pathways as Social and Participatory Practices: The Case of Herpes Simplex Encephalitis', *PLoS One*, 11: e0151145.

Cordeiro A. P., Silva Pereira R. A., Chapeaurouge A., Coimbra C. S., Perales J., Oliveira G., Sanchez Candiani T. M., and Coimbra R. S. 2015. 'Comparative proteomics of cerebrospinal fluid reveals a predictive model for differential diagnosis of pneumococcal, meningococcal, and enteroviral meningitis, and novel putative therapeutic targets', *BMC Genomics*, 16 Suppl 5: S11.

Cox J., Hein M. Y., Luber C. A., Paron I., Nagaraj N., and Mann M. 2014. 'Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ', *Mol Cell Proteomics*, 13: 2513-26.

Crooke S. T., Witztum J. L., Bennett C. F., and Baker B. F. 2018. 'RNA-Targeted Therapeutics', *Cell Metab*, 27: 714-39.

Cunningham A. L., Diefenbach R. J., Miranda-Saksena M., Bosnjak L., Kim M., Jones C., and Douglas M. W. 2006. 'The cycle of human herpes simplex virus infection: virus transport and immune control', *J Infect Dis*, 194 Suppl 1: S11-8.

Cyster J. G., Hartley S. B., and Goodnow C. C. 1994. 'Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire', *Nature*, 371: 389-95.

Dai Y. N., Huang H. J., Song W. Y., Tong Y. X., Yang D. H., Wang M. S., Huang Y. C., Chen M. J., Zhang J. J., Ren Z. Z., Zheng W., and Pan H. Y. 2017. 'Identification of potential metabolic biomarkers of cerebrospinal fluids that differentiate tuberculous

meningitis from other types of meningitis by a metabolomics study', *Oncotarget*, 8: 100095-112.

Dalmau J., and Graus F. 2018. 'Antibody-Mediated Encephalitis', *N Engl J Med*, 378: 840-51.

Dalmau J., Graus F., Villarejo A., Posner J. B., Blumenthal D., Thiessen B., Saiz A., Meneses P., and Rosenfeld M. R. 2004. 'Clinical analysis of anti-Ma2-associated encephalitis', *Brain*, 127: 1831-44.

Defres S., Keller S. S., Das K., Vidyasagar R., Parkes L. M., Burnside G., Griffiths M., Kopelman M., Roberts N., and Solomon T. 2017. 'A Feasibility Study of Quantifying Longitudinal Brain Changes in Herpes Simplex Virus (HSV) Encephalitis Using Magnetic Resonance Imaging (MRI) and Stereology', *PLoS One*, 12: e0170215.

del Saz S. V., Sued O., Falco V., Aguero F., Crespo M., Pumarola T., Curran A., Gatell J. M., Pahissa A., Miro J. M., and Ribera E. 2008. 'Acute meningoencephalitis due to human immunodeficiency virus type 1 infection in 13 patients: clinical description and follow-up', *J Neurovirol*, 14: 474-9.

Dieterle F., Ross A., Schlotterbeck G., and Senn H. 2006. 'Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics', *Anal Chem*, 78: 4281-90.

Duarte-Delgado N. P., Vásquez G., and Ortiz-Reyes B. L. 2019. 'Blood-brain barrier disruption and neuroinflammation as pathophysiological mechanisms of the diffuse manifestations of neuropsychiatric systemic lupus erythematosus', *Autoimmun Rev*, 18: 426-32.

Dubey D., Pittock S. J., Kelly C. R., McKeon A., Lopez-Chiriboga A. S., Lennon V. A., Gadoth A., Smith C. Y., Bryant S. C., Klein C. J., Aksamit A. J., Toledano M., Boeve B. F., Tillema J. M., and Flanagan E. P. 2018. 'Autoimmune encephalitis epidemiology and a comparison to infectious encephalitis', *Ann Neurol*, 83: 166-77.

Eintracht S., Silber E., Sonnenberg P., Koornhof H. J., and Saffer D. 2000. 'Analysis of adenosine deaminase isoenzyme-2 (ADA(2)) in cerebrospinal fluid in the diagnosis of tuberculosis meningitis', *J Neurol Neurosurg Psychiatry*, 69: 137-8.

Ellul M. A., Benjamin L., Singh B., Lant S., Michael B. D., Easton A., Kneen R., Defres S., Sejvar J., and Solomon T. 2020. 'Neurological associations of COVID-19', *Lancet Neurol*, 19: 767-83.

Ellul M. A., Griffiths M. J., Iyer A., Avula S., Defres S., Baborie A., Vincent A., Martin N. G., Sadarangani M., Pollard A. J., Solomon T., and Kneen R. 2016. 'Anti-N-Methyl-D-Aspartate Receptor Encephalitis In A Young Child With Histological Evidence On Brain Biopsy Of Coexistent Herpes Simplex Virus Type 1 Infection', *The Pediatric Infectious Disease Journal*, 35: 347-49.

Ellul M. A., Wood G., Tooren H. V. D., Easton A., Babu A., and Michael B. D. 2020. 'Update on the diagnosis and management of autoimmune encephalitis', *Clin Med (Lond)*, 20: 389-92.

Engelhardt B. 2006. 'Molecular mechanisms involved in T cell migration across the blood–brain barrier', *Journal of Neural Transmission*, 113: 477-85.

Engelhardt B., Carare R. O., Bechmann I., Flügel A., Laman J. D., and Weller R. O. 2016. 'Vascular, glial, and lymphatic immune gateways of the central nervous system', *Acta Neuropathol*, 132: 317-38.

Erkkinen M. G., and Berkowitz A. L. 2019. 'A Clinical Approach to Diagnosing Encephalopathy', *Am J Med*, 132: 1142-47.

Erwin-Cohen R. A., Porter A. I., Pittman P. R., Rossi C. A., and DaSilva L. 2017. 'Human transcriptome response to immunization with live-attenuated Venezuelan equine encephalitis virus vaccine (TC-83): Analysis of whole blood', *Hum Vaccin Immunother*, 13: 169-79.

Esiri M. M. 1982. 'Herpes simplex encephalitis. An immunohistological study of the distribution of viral antigen within the brain', *J Neurol Sci*, 54: 209-26.

Fraisier C., Papa A., Granjeaud S., Hintzen R., Martina B., Camoin L., and Almeras L. 2014. 'Cerebrospinal fluid biomarker candidates associated with human WNV neuroinvasive disease', *PLoS One*, 9: e93637.

Gibson R. M., Rothwell N. J., and Le Feuvre R. A. 2004. 'CNS injury: the role of the cytokine IL-1', *Vet J*, 168: 230-7.

Giraudeau P. 2020. 'NMR-based metabolomics and fluxomics: developments and future prospects', *Analyst*, 145: 2457-72.

Glaser C. A., Gilliam S., Schnurr D., Forghani B., Honarmand S., Khetsuriani N., Fischer M., Cossen C. K., and Anderson L. J. 2003. 'In search of encephalitis etiologies: diagnostic challenges in the California Encephalitis Project, 1998-2000', *Clin Infect Dis*, 36: 731-42.

Goenka A., Michael B. D., Ledger E., Hart I. J., Absoud M., Chow G., Lilleker J., Lunn M., McKee D., Peake D., Pysden K., Roberts M., Carrol E. D., Lim M., Avula S., Solomon T., and Kneen R. 2014. 'Neurological manifestations of influenza infection in children and adults: results of a National British Surveillance Study', *Clin Infect Dis*, 58: 775-84.

Gomez-Baena G., Bennett R. J., Martinez-Rodriguez C., Wnek M., Laing G., Hickey G., McLean L., Beynon R. J., and Carrol E. D. 2017. 'Quantitative Proteomics of Cerebrospinal Fluid in Paediatric Pneumococcal Meningitis', *Sci Rep*, 7: 7042.

Granerod J., Ambrose H. E., Davies N. W., Clewley J. P., Walsh A. L., Morgan D., Cunningham R., Zuckerman M., Mutton K. J., Solomon T., Ward K. N., Lunn M. P., Irani S. R.,

Vincent A., Brown D. W., and Crowcroft N. S. 2010. 'Causes of encephalitis and differences in their clinical presentations in England: a multicentre, population-based prospective study', *Lancet Infect Dis*, 10: 835-44.

Granerod J., Cousens S., Davies N. W., Crowcroft N. S., and Thomas S. L. 2013. 'New estimates of incidence of encephalitis in England', *Emerg Infect Dis*, 19: 1455-562.

Granerod J., Cunningham R., Zuckerman M., Mutton K., Davies N. W., Walsh A. L., Ward K. N., Hilton D. A., Ambrose H. E., Clewley J. P., Morgan D., Lunn M. P., Solomon T., Brown D. W., and Crowcroft N. S. 2010. 'Causality in acute encephalitis: defining aetiologies', *Epidemiol Infect*, 138: 783-800.

Graus F., Escudero D., Oleaga L., Bruna J., Villarejo-Galende A., Ballabriga J., Barcelo M. I., Gilo F., Popkirov S., Stourac P., and Dalmau J. 2018. 'Syndrome and outcome of antibody-negative limbic encephalitis', *Eur J Neurol*, 25: 1011-16.

Graus F., Titulaer M. J., Balu R., Benseler S., Bien C. G., Cellucci T., Cortese I., Dale R. C., Gelfand J. M., Geschwind M., Glaser C. A., Honnorat J., Hoftberger R., Iizuka T., Irani S. R., Lancaster E., Leypoldt F., Pruss H., Rae-Grant A., Reindl M., Rosenfeld M. R., Rostasy K., Saiz A., Venkatesan A., Vincent A., Wandinger K. P., Waters P., and Dalmau J. 2016. 'A clinical approach to diagnosis of autoimmune encephalitis', *Lancet Neurol*, 15: 391-404.

Griffiths M. 2019. 'MICA: Development of a novel host blood test using TRanscripts to Identify bacterial Meningitis - TRIM test study'. https://gtr.ukri.org/projects?ref=MR%2FR015406%2F1#/tabOverview.

Griffiths M. J., Ooi M. H., Wong S. C., Mohan A., Podin Y., Perera D., Chieng C. H., Tio P. H., Cardosa M. J., and Solomon T. 2012. 'In enterovirus 71 encephalitis with cardio-respiratory compromise, elevated interleukin 1beta, interleukin 1 receptor antagonist, and granulocyte colony-stimulating factor levels are markers of poor prognosis', *J Infect Dis*, 206: 881-92.

Griffiths M. J., Shafi M. J., Popper S. J., Hemingway C. A., Kortok M. M., Wathen A., Rockett K. A., Mott R., Levin M., Newton C. R., Marsh K., Relman D. A., and Kwiatkowski D. P. 2005. 'Genomewide analysis of the host response to malaria in Kenyan children', *J Infect Dis*, 191: 1599-611.

Griffiths M. J., Turtle L., and Solomon T. 2014. 'Japanese encephalitis virus infection', *Handb Clin Neurol*, 123: 561-76.

Hamzeiy H., and Cox J. 2017. 'What computational non-targeted mass spectrometry-based metabolomics can gain from shotgun proteomics', *Curr Opin Biotechnol*, 43: 141-46.

Han Y., Chen W., Song Y., Yuan Y., Li Z., Zhou Y., Liu T., Han D., Mi X., Li M., Wang G., Zhong L., Zhou J., and Guo X. 2020. 'Proteomic Analysis of Preoperative CSF Reveals Risk Biomarkers of Postoperative Delirium', *Front Psychiatry*, 11: 170.

Harris L., Griem J., Gummery A., Marsh L., Defres S., Bhojak M., Das K., Easton A., Solomon T., and Kopelman M. 2020. 'Neuropsychological and psychiatric outcomes in encephalitis: A multi-centre case-control study', *PLoS One*, 15: e0230436.

Hatanpaa K. J., and Kim J. H. 2014. 'Neuropathology of viral infections', *Handb Clin Neurol*, 123: 193-214.

Hatzistilianou M., Hitoglou S., Gougoustamou D., Rekliti A., Tzouvelekis G., Nanas C., Catriu D., and Kotsis O. 2002. 'Serum procalcitonin, adenosine deaminase and its isoenzymes in the aetiological diagnosis of pneumonia in children', *Int J Immunopathol Pharmacol*, 15: 119-27.

Hawkins S. F. C., and Guest P. C. 2017. 'Multiplex Analyses Using Real-Time Quantitative PCR', *Methods Mol Biol*, 1546: 125-33.

Haydar D., Gonzalez R., Garvy B. A., Garneau-Tsodikova S., Thamban Chandrika N., Bocklage T. J., and Feola D. J. 2021. 'Myeloid arginase-1 controls excessive inflammation and modulates T cell responses in Pseudomonas aeruginosa pneumonia', *Immunobiology*, 226: 152034.

Heine J., Prüss H., Bartsch T., Ploner C. J., Paul F., and Finke C. 2015. 'Imaging of autoimmune encephalitis – Relevance for clinical practice and hippocampal function', *Neuroscience*, 309: 68-83.

Horstmann D. M., and Yamada N. 1968. 'Enterovirus infections of the central nervous system', *Res Publ Assoc Res Nerv Ment Dis*, 44: 236-53.

Hu J., Zou F., and Wright F. A. 2005. 'Practical FDR-based sample size calculations in microarray experiments', *Bioinformatics*, 21: 3264-72.

Hulshof S., van Haastert E. S., Kuipers H. F., van den Elsen P. J., De Groot C. J., van der Valk P., Ravid R., and Biber K. 2003. 'CX3CL1 and CX3CR1 expression in human brain tissue: noninflammatory control versus multiple sclerosis', *J Neuropathol Exp Neurol*, 62: 899-907.

Ichiyama T., Shoji H., Takahashi Y., Matsushige T., Kajimoto M., Inuzuka T., and Furukawa S. 2008. 'Cerebrospinal fluid levels of cytokines in non-herpetic acute limbic encephalitis: comparison with herpes simplex encephalitis', *Cytokine*, 44: 149-53.

Inoue T., Chihara Y., Kiba K., Hirao S., Tanaka M., Yoneda T., Saka T., Beppu K. I., Furubayashi K., Takashima M., Nagai H., Kobayashi H., and Fujimoto K. 2021. 'Rapid multiplex PCR assay for simultaneous detection of Neisseria gonorrhoeae and Chlamydia trachomatis in genitourinary samples: A 30-minute assay', *J Microbiol Methods*, 180: 106103.

Irani S. R., Gelfand J. M., Bettcher B. M., Singhal N. S., and Geschwind M. D. 2014. 'Effect of rituximab in patients with leucine-rich, glioma-inactivated 1 antibody-associated encephalopathy', *JAMA Neurol*, 71: 896-900.

Irani S. R., Michell A. W., Lang B., Pettingill P., Waters P., Johnson M. R., Schott J. M., Armstrong R. J., A S. Z., Bleasel A., Somerville E. R., Smith S. M., and Vincent A. 2011. 'Faciobrachial dystonic seizures precede Lgi1 antibody limbic encephalitis', *Ann Neurol*, 69: 892-900.

Jacobsen L. C., Theilgaard-Mönch K., Christensen E. I., and Borregaard N. 2007. 'Arginase 1 is expressed in myelocytes/metamyelocytes and localized in gelatinase granules of human neutrophils', *Blood*, 109: 3084-7.

Jmor F., Emsley H. C., Fischer M., Solomon T., and Lewthwaite P. 2008. 'The incidence of acute encephalitis syndrome in Western industrialised and tropical countries', *Virol J*, 5: 134.

Johnson J. G., and Jenkins M. K. 1994. 'The role of anergy in peripheral T cell unresponsiveness', *Life Sci*, 55: 1767-80.

Jurynczyk M., Messina S., Woodhall M. R., Raza N., Everett R., Roca-Fernandez A., Tackley G., Hamid S., Sheard A., Reynolds G., Chandratre S., Hemingway C., Jacob A., Vincent A., Leite M. I., Waters P., and Palace J. 2017. 'Clinical presentation and prognosis in MOG-antibody disease: a UK study', *Brain*, 140: 3128-38.

Jurynczyk M., Probert F., Yeo T., Tackley G., Claridge T. D. W., Cavey A., Woodhall M. R., Arora S., Winkler T., Schiffer E., Vincent A., DeLuca G., Sibson N. R., Isabel Leite M., Waters P., Anthony D. C., and Palace J. 2017. 'Metabolomics reveals distinct, antibody-independent, molecular signatures of MS, AQP4-antibody and MOG-antibody disease', *Acta Neuropathol Commun*, 5: 95.

Kamei S., Taira N., Ishihara M., Sekizawa T., Morita A., Miki K., Shiota H., Kanno A., Suzuki Y., Mizutani T., Itoyama Y., Morishima T., and Hirayanagi K. 2009. 'Prognostic value of cerebrospinal fluid cytokine changes in herpes simplex virus encephalitis', *Cytokine*, 46: 187-93.

Kanehisa M., Goto S., Kawashima S., Okuno Y., and Hattori M. 2004. 'The KEGG resource for deciphering the genome', *Nucleic Acids Res*, 32: D277-80.

Kang J. H., Ho J. D., Chen Y. H., and Lin H. C. 2009. 'Increased risk of stroke after a herpes zoster attack: a population-based follow-up study', *Stroke*, 40: 3443-8.

Kawashima H., Oguchi M., Ioi H., Amaha M., Yamanaka G., Kashiwagi Y., Takekuma K., Yamazaki Y., Hoshika A., and Watanabe Y. 2006. 'Primary biomarkers in cerebral spinal fluid obtained from patients with influenza-associated encephalopathy analyzed by metabolomics', *Int J Neurosci*, 116: 927-36.

Kendall J. L., and Springer J. M. 2020. 'The Many Faces of a Monogenic Autoinflammatory Disease: Adenosine Deaminase 2 Deficiency', *Curr Rheumatol Rep*, 22: 64.

Keun H. C., and Athersuch T. J. 2011. 'Nuclear magnetic resonance (NMR)-based metabolomics', *Methods Mol Biol*, 708: 321-34.

Kilgore P. E., Kruszon-Moran D., Seward J. F., Jumaan A., Van Loon F. P., Forghani B., McQuillan G. M., Wharton M., Fehrs L. J., Cossen C. K., and Hadler S. C. 2003. 'Varicella in Americans from NHANES III: implications for control through routine immunization', *J Med Virol*, 70 Suppl 1: S111-8.

Kimura A., Yoshikura N., Koumura A., Hayashi Y., and Inuzuka T. 2015. 'B-cell-activating factor belonging to the tumor necrosis factor family (BAFF) and a proliferation-inducing ligand (APRIL) levels in cerebrospinal fluid of patients with meningoencephalitis', *J Neurol Sci*, 352: 79-83.

Kleinschek M. A., Owyang A. M., Joyce-Shaikh B., Langrish C. L., Chen Y., Gorman D. M., Blumenschein W. M., McClanahan T., Brombacher F., Hurst S. D., Kastelein R. A., and Cua D. J. 2007. 'IL-25 regulates Th17 function in autoimmune inflammation', *J Exp Med*, 204: 161-70.

Kraig E., Pierce J. L., Clarkin K. Z., Standifer N. E., Currier P., Wall K. A., and Infante A. J. 1996. 'Restricted T cell receptor repertoire for acetylcholine receptor in murine myasthenia gravis', *J Neuroimmunol*, 71: 87-95.

Kuhn M., Sühs K. W., Akmatov M. K., Klawonn F., Wang J., Skripuletz T., Kaever V., Stangel M., and Pessler F. 2018. 'Mass-spectrometric profiling of cerebrospinal fluid reveals metabolite biomarkers for CNS involvement in varicella zoster virus reactivation', *J Neuroinflammation*, 15: 20.

Kulohoma B. W., Marriage F., Vasieva O., Mankhambo L., Nguyen K., Molyneux M. E., Molyneux E. M., Day P. J. R., and Carrol E. D. 2017. 'Peripheral blood RNA gene expression in children with pneumococcal meningitis: a prospective case–control study', *BMJ Paediatrics Open*, 1: e000092.

Kutryb-Zajac B., Mierzejewska P., Slominska E. M., and Smolenski R. T. 2020. 'Therapeutic Perspectives of Adenosine Deaminase Inhibition in Cardiovascular Diseases', *Molecules*, 25: 4652.

Lamour S. D., Alibu V. P., Holmes E., and Sternberg J. M. 2017. 'Metabolic Profiling of Central Nervous System Disease in Trypanosoma brucei rhodesiense Infection', *J Infect Dis*, 216: 1273-80.

Lange S., Bergström T., Johansson E., Oshalim M., and Lönnroth I. 2017. 'Reaction of complement factors and proteasomes in experimental encephalitis', *J Neurovirol*, 23: 313-18.

Lê Cao K.-A., Boitard S., and Besse P. 2011. 'Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems', *BMC Bioinformatics*, 12: 253.

Le K. T. T., Matzaraki V., Netea M. G., Wijmenga C., Moser J., and Kumar V. 2019. 'Functional Annotation of Genetic Loci Associated With Sepsis Prioritizes Immune and Endothelial Cell Pathways', *Frontiers in Immunology*, 10: 1949-49.

Lee P. Y., Osman J., Low T. Y., and Jamal R. 2019. 'Plasma/serum proteomics: depletion strategies for reducing high-abundance proteins for biomarker discovery', *Bioanalysis*, 11: 1799-812.

Lee S. H., Shin J. H., Song J. H., Leem A. Y., Park M. S., Kim Y. S., Chang J., and Chung K. S. 2017. 'Clinical implications of the plasma EphA2 receptor level in critically ill patients with septic shock', *Sci Rep*, 7: 17612.

Lejuste F., Thomas L., Picard G., Desestret V., Ducray F., Rogemond V., Psimaras D., Antoine J. C., Delattre J. Y., Groc L., Leboyer M., and Honnorat J. 2016. 'Neuroleptic intolerance in patients with anti-NMDAR encephalitis', *Neurol Neuroimmunol Neuroinflamm*, 3: e280.

Lewthwaite P., Shankar M. V., Tio P. H., Daly J., Last A., Ravikumar R., Desai A., Ravi V., Cardosa J. M., and Solomon T. 2010. 'Evaluation of two commercially available ELISAs for the diagnosis of Japanese encephalitis applied to field samples', *Trop Med Int Health*, 15: 811-8.

Leypoldt F., Hoftberger R., Titulaer M. J., Armangue T., Gresa-Arribas N., Jahn H., Rostasy K., Schlumberger W., Meyer T., Wandinger K. P., Rosenfeld M. R., Graus F., and Dalmau J. 2014. 'Investigations on CXCL13 in anti-N-methyl-D-aspartate receptor encephalitis: a potential biomarker of treatment response', *JAMA Neurol*, 72: 180-6.

Li Z., Du B., Li J., Zhang J., Zheng X., Jia H., Xing A., Sun Q., Liu F., and Zhang Z. 2017. 'Cerebrospinal fluid metabolomic profiling in tuberculous and viral meningitis: Screening potential markers for differential diagnosis', *Clin Chim Acta*, 466: 38-45.

Lill M., Taba P., Kõks S., Soomets U., Lutsar I., Schalkwyk L., and Fernandes C. 2013. 'Peripheral blood RNA gene expression profiling in patients with bacterial meningitis', *Frontiers in Neuroscience*, 7: 7-33.

Lorefice L., Murgia F., Fenu G., Frau J., Coghe G., Murru M. R., Tranquilli S., Visconti A., Marrosu M. G., Atzori L., and Cocco E. 2019. 'Assessing the Metabolomic Profile of Multiple Sclerosis Patients Treated with Interferon Beta 1a by (1)H-NMR Spectroscopy', *Neurotherapeutics*, 16: 797-807.

Mailles A., De Broucker T., Costanzo P., Martinez-Almoyna L., Vaillant V., and Stahl J. P. 2012. 'Long-term outcome of patients presenting with acute infectious encephalitis of various causes in France', *Clin Infect Dis*, 54: 1455-64.

Makuch M., Wilson R., Al-Diwani A., Varley J., Kienzler A. K., Taylor J., Berretta A., Fowler D., Lennox B., Leite M. I., Waters P., and Irani S. R. 2018. 'N-methyl-D-aspartate receptor antibody production from germinal center reactions: Therapeutic implications', *Ann Neurol*, 83: 553-61.

Malter M. P., Helmstaedter C., Urbach H., Vincent A., and Bien C. G. 2010. 'Antibodies to glutamic acid decarboxylase define a form of limbic encephalitis', *Ann Neurol*, 67: 470-8.

Mancini M., and Vidal S. M. 2018. 'Insights into the pathogenesis of herpes simplex encephalitis from mouse models', *Mamm Genome*, 29: 425-45.

Mandal R., Guo A. C., Chaudhary K. K., Liu P., Yallou F. S., Dong E., Aziat F., and Wishart D. S. 2012. 'Multi-platform characterization of the human cerebrospinal fluid metabolome: a comprehensive and quantitative update', *Genome Med*, 4: 38.

Mandel-Brehm C., Retallack H., Knudsen G. M., Yamana A., Hajj-Ali R. A., Calabrese L. H., Tihan T., Sample H. A., Zorn K. C., Gorman M. P., Madan Cohen J., Sreih A. G., Marcus J. F., Josephson S. A., Douglas V. C., Gelfand J. M., Wilson M. R., and DeRisi J. L. 2019. 'Exploratory proteomic analysis implicates the alternative complement cascade in primary CNS vasculitis', *Neurology*, 93: e433-e44.

Martin B. K. 2007. 'Transcriptional control of complement receptor gene expression', *Immunol Res*, 39: 146-59.

Mason S., Reinecke C. J., and Solomons R. 2017. 'Cerebrospinal Fluid Amino Acid Profiling of Pediatric Cases with Tuberculous Meningitis', *Front Neurosci*, 11: 534.

Mason S., van Furth A. M., Mienie L. J., Engelke U. F., Wevers R. A., Solomons R., and Reinecke C. J. 2015. 'A hypothetical astrocyte-microglia lactate shuttle derived from a (1)H NMR metabolomics analysis of cerebrospinal fluid from a cohort of South African children with tuberculous meningitis', *Metabolomics*, 11: 822-37.

Maurer M. H. 2010. 'Proteomics of brain extracellular fluid (ECF) and cerebrospinal fluid (CSF)', *Mass Spectrom Rev*, 29: 17-28.

McGill F., Griffiths M. J., Bonnett L. J., Geretti A. M., Michael B. D., Beeching N. J., McKee D., Scarlett P., Hart I. J., Mutton K. J., Jung A., Adan G., Gummery A., Sulaiman W. A. W., Ennis K., Martin A. P., Haycox A., Miller A., and Solomon T. 2018. 'Incidence, aetiology, and sequelae of viral meningitis in UK adults: a multicentre prospective observational cohort study', *Lancet Infect Dis*, 18: 992-1003.

McGill F., Heyderman R. S., Michael B. D., Defres S., Beeching N. J., Borrow R., Glennie L., Gaillemin O., Wyncoll D., Kaczmarski E., Nadel S., Thwaites G., Cohen J., Davies N. W., Miller A., Rhodes A., Read R. C., and Solomon T. 2016. 'The UK joint specialist societies guideline on the diagnosis and management of acute meningitis and meningococcal sepsis in immunocompetent adults', *J Infect*, 72: 405-38.

McMillan T., Wilson L., Ponsford J., Levin H., Teasdale G., and Bond M. 2016. 'The Glasgow Outcome Scale - 40 years of application and refinement', *Nat Rev Neurol*, 12: 477-85.

Michael B., Menezes B. F., Cunniffe J., Miller A., Kneen R., Francis G., Beeching N. J., and Solomon T. 2010. 'Effect of delayed lumbar punctures on the diagnosis of acute bacterial meningitis in adults', *Emerg Med J*, 27: 433-8.

Michael B. D., Bricio-Moreno L., Sorensen E. W., Miyabe Y., Lian J., Solomon T., Kurt-Jones E. A., and Luster A. D. 2020. 'Astrocyte- and Neuron-Derived CXCL1 Drives Neutrophil

Transmigration and Blood-Brain Barrier Permeability in Viral Encephalitis', *Cell Rep*, 32: 108150.

Michael B. D., Griffiths M. J., Granerod J., Brown D., Davies N. W., Borrow R., and Solomon T. 2016. 'Characteristic Cytokine and Chemokine Profiles in Encephalitis of Infectious, Immune-Mediated, and Unknown Aetiology', *PLoS One*, 11: e0146288.

Michael B. D., Griffiths M. J., Granerod J., Brown D., Keir G., Wnek G., Cox D. J., Vidyasagar R., Borrow R., Parkes L. M., and Solomon T. 2015. 'The Interleukin-1 Balance During Encephalitis Is Associated With Clinical Severity, Blood-Brain Barrier Permeability, Neuroimaging Changes, and Disease Outcome', *J Infect Dis*, 213: 1651-60.

Mirza B., Wang W., Wang J., Choi H., Chung N. C., and Ping P. 2019. 'Machine Learning and Integrative Analysis of Biomedical Big Data', *Genes*, 10: 87.

Misra U. K., Tan C. T., and Kalita J. 2008. 'Viral encephalitis and epilepsy', *Epilepsia*, 49 Suppl 6: 13-8.

Moore M. 1982. 'Centers for Disease Control. Enteroviral disease in the United States, 1970-1979', *J Infect Dis*, 146: 103-8.

Mortaz E., Adcock I. M., Tabarsi P., Darazam I. A., Movassaghi M., Garssen J., Jamaati H., and Velayati A. 2017. 'Pattern recognitions receptors in immunodeficiency disorders', *Eur J Pharmacol*, 808: 49-56.

Mu J., Yang Y., Chen J., Cheng K., Li Q., Wei Y., Zhu D., Shao W., Zheng P., and Xie P. 2015. 'Elevated host lipid metabolism revealed by iTRAQ-based quantitative proteomic analysis of cerebrospinal fluid of tuberculous meningitis patients', *Biochem Biophys Res Commun*, 466: 689-95.

Nahmias A. J., Whitley R. J., Visintine A. N., Takei Y., and Alford C. A., Jr. 1982. 'Herpes simplex virus encephalitis: laboratory evaluations and their diagnostic significance', *J Infect Dis*, 145: 829-36.

Njunge J. M., Oyaro I. N., Kibinge N. K., Rono M. K., Kariuki S. M., Newton C. R., Berkley J. A., and Gitau E. N. 2017. 'Cerebrospinal fluid markers to distinguish bacterial meningitis from cerebral malaria in children', *Wellcome Open Res*, 2: 47.

Nosadini M., Mohammad S. S., Ramanathan S., Brilot F., and Dale R. C. 2015. 'Immune therapy in autoimmune encephalitis: a systematic review', *Expert Rev Neurother*, 15: 1391-419.

O'Sullivan A., Willoughby R. E., Mishchuk D., Alcarraz B., Cabezas-Sanchez C., Condori R. E., David D., Encarnacion R., Fatteh N., Fernandez J., Franka R., Hedderwick S., McCaughey C., Ondrush J., Paez-Martinez A., Rupprecht C., Velasco-Villa A., and Slupsky C. M. 2013. 'Metabolomics of cerebrospinal fluid from humans treated for rabies', *J Proteome Res*, 12: 481-90.

O'Sullivan B. J., Steele T., Ellul M. A., Kirby E., Duale A., Kier G., Crooks D., Jacob A., Solomon T., and Michael B. D. 2016. 'When should we test for voltage-gated potassium channel complex antibodies? A retrospective case control study', *J Clin Neurosci*, 33: 198-204.

Okuda S., Yamada T., Hamajima M., Itoh M., Katayama T., Bork P., Goto S., and Kanehisa M. 2008. 'KEGG Atlas mapping for global analysis of metabolic pathways', *Nucleic Acids Res*, 36: W423-6.

Ou. 2013. 'An iTRAQ approach to quantitative proteome analysis of cerebrospinal fluid from patients with tuberculous meningitis', *BioScience Trends*, 7: 186-92.

Palfreeman A., Fisher M., Ong E., Wardrope J., Stewart E., Castro-Sanchez E., Peto T., Rogstad K., Sheather J., Gazzard B., Pillay D., O'Brien J., Delpech V., Lowbury R., Fleet R., Azad Y., Lyall H., Hardie J., Adegbite G., Rooney G., and Whitehead R. 2009. 'Testing for HIV: concise guidance', *Clin Med (Lond)*, 9: 471-6.

Pan L., Liu F., Zhang J., Li J., Jia H., Huang M., Liu X., Chen W., Ding Z., Wang Y., Du B., Wei R., Sun Q., Xing A., and Zhang Z. 2019. 'Genome-Wide miRNA Analysis Identifies Potential Biomarkers in Distinguishing Tuberculous and Viral Meningitis', *Front Cell Infect Microbiol*, 9: 323.

Pohl D., Alper G., Van Haren K., Kornberg A. J., Lucchinetti C. F., Tenembaum S., and Belman A. L. 2016. 'Acute disseminated encephalomyelitis: Updates on an inflammatory CNS syndrome', *Neurology*, 87: S38-45.

Pollak T. A., Lennox B. R., Muller S., Benros M. E., Pruss H., Tebartz van Elst L., Klein H., Steiner J., Frodl T., Bogerts B., Tian L., Groc L., Hasan A., Baune B. T., Endres D., Haroon E., Yolken R., Benedetti F., Halaris A., Meyer J. H., Stassen H., Leboyer M., Fuchs D., Otto M., Brown D. A., Vincent A., Najjar S., and Bechter K. 2020. 'Autoimmune psychosis: an international consensus on an approach to the diagnosis and management of psychosis of suspected autoimmune origin', *Lancet Psychiatry*, 7: 93-108.

Pundir S., Martin M. J., and O'Donovan C. 2017. 'UniProt Protein Knowledgebase', *Methods Mol Biol*, 1558: 41-55.

Rajasingham R., Wake R. M., Beyene T., Katende A., Letang E., and Boulware D. R. 2019. 'Cryptococcal Meningitis Diagnostics and Screening in the Era of Point-of-Care Laboratory Testing', *J Clin Microbiol*, 57: e01238-18.

Ramberger M., Berretta A., Tan J. M. M., Sun B., Michael S., Yeo T., Theorell J., Bashford-Rogers R., Paneva S., O'Dowd V., Dedi N., Topia S., Griffin R., Ramirez-Franco J., El Far O., Baulac S., Leite M. I., Sen A., Jeans A., McMillan D., Marshall D., Anthony D., Lightwood D., Waters P., and Irani S. R. 2020. 'Distinctive binding properties of human monoclonal LGI1 autoantibodies determine pathogenic mechanisms', *Brain*, 143: 1731-45.

Raschilas F., Wolff M., Delatour F., Chaffaut C., De Broucker T., Chevret S., Lebon P., Canton P., and Rozenberg F. 2002. 'Outcome of and prognostic factors for herpes simplex encephalitis in adult patients: results of a multicenter study', *Clin Infect Dis*, 35: 254-60.

Ratuszny D., Suhs K. W., Novoselova N., Kuhn M., Kaever V., Skripuletz T., Pessler F., and Stangel M. 2019. 'Identification of Cerebrospinal Fluid Metabolites as Biomarkers for Enterovirus Meningitis', *Int J Mol Sci*, 20: 337.

Readhead B., Haure-Mirande J. V., Funk C. C., Richards M. A., Shannon P., Haroutunian V., Sano M., Liang W. S., Beckmann N. D., Price N. D., Reiman E. M., Schadt E. E., Ehrlich M. E., Gandy S., and Dudley J. T. 2018. 'Multiscale Analysis of Independent Alzheimer's Cohorts Finds Disruption of Molecular, Genetic, and Clinical Networks by Human Herpesvirus', *Neuron*, 99: 64-82.e7.

Reo N. V. 2002. 'NMR-based metabolomics', *Drug Chem Toxicol*, 25: 375-82.

Ritchie M. E., Phipson B., Wu D., Hu Y., Law C. W., Shi W., and Smyth G. K. 2015. 'limma powers differential expression analyses for RNA-sequencing and microarray studies', *Nucleic Acids Res*, 43: e47.

Rohart F., Gautier B., Singh A., and KA L. C. 2017. 'mixOmics: An R package for 'omics feature selection and multiple data integration', *PLoS Comput Biol*, 13: e1005752.

Rohlwink U. K., Figaji A., Wilkinson K. A., Horswell S., Sesay A. K., Deffur A., Enslin N., Solomons R., Van Toorn R., Eley B., Levin M., Wilkinson R. J., and Lai R. P. J. 2019. 'Tuberculous meningitis in children is characterized by compartmentalized immune responses and neural excitotoxicity', *Nat Commun*, 10: 3767.

Roizman B., and Taddeo B. 2007. 'The strategy of herpes simplex virus replication and takeover of the host cell.' in A. Arvin, G. Campadelli-Fiume, E. Mocarski, P. S. Moore, B. Roizman, R. Whitley and K. Yamanishi (eds.), *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis* (Cambridge University Press

Romeo M. J., Espina V., Lowenthal M., Espina B. H., Petricoin E. F., 3rd, and Liotta L. A. 2005. 'CSF proteome: a protein repository for potential biomarker identification', *Expert Rev Proteomics*, 2: 57-70.

Rumboldt Z. 2008. 'Imaging of topographic viral CNS infections', *Neuroimaging Clin N Am*, 18: 85-92; viii.

Santoro J. D., and Chitnis T. 2019. 'Diagnostic Considerations in Acute Disseminated Encephalomyelitis and the Interface with MOG Antibody', *Neuropediatrics*, 50: 273-79.

Saraya A. W., Wacharapluesadee S., Petcharat S., Sittidetboripat N., Ghai S., Wilde H., and Hemachudha T. 2016. 'Normocellular CSF in herpes simplex encephalitis', *BMC Res Notes*, 9: 95.

Schiess N., and Pardo C. A. 2008. 'Hashimoto's encephalopathy', *Ann N Y Acad Sci*, 1142: 254-65.

Schmidt A., Bühler R., Mühlemann K., Hess C. W., and Täuber M. G. 2011. 'Long-term outcome of acute encephalitis of unknown aetiology in adults', *Clinical Microbiology and Infection*, 17: 621-26.

Schuurman H. J., Van Loveren H., Rozing J., and Vos J. G. 1992. 'Chemicals trophic for the thymus: risk for immunodeficiency and autoimmunity', *Int J Immunopharmacol*, 14: 369-75.

Sengupta N., Mukherjee S., Tripathi P., Kumar R., Suryavanshi A., and Basu A. 2015. 'Cerebrospinal Fluid Biomarkers of Japanese Encephalitis', *F1000Res*, 4: 334.

Siddiqi O. K., Birbeck G. L., Ghebremichael M., Mubanga E., Love S., Buback C., Kosloff B., Ayles H., Atadzhanov M., Dheda K., and Koralnik I. J. 2019. 'Prospective Cohort Study on Performance of Cerebrospinal Fluid (CSF) Xpert MTB/RIF, CSF Lipoarabinomannan (LAM) Lateral Flow Assay (LFA), and Urine LAM LFA for Diagnosis of Tuberculous Meningitis in Zambia', *J Clin Microbiol*, 57: e00652-19.

Skoldenberg B., Forsgren M., Alestig K., Bergstrom T., Burman L., Dahlqvist E., Forkman A., Fryden A., Lovgren K., Norlin K., and et al. 1984. 'Acyclovir versus vidarabine in herpes simplex encephalitis. Randomised multicentre study in consecutive Swedish patients', *Lancet*, 2: 707-11.

Soares C. N., Brasil P., Carrera R. M., Sequeira P., de Filippis A. B., Borges V. A., Theophilo F., Ellul M. A., and Solomon T. 2016. 'Fatal encephalitis associated with Zika virus infection in an adult', *Journal of Clinical Virology*, 83: 63-65.

Solomon T., Michael B. D., Smith P. E., Sanderson F., Davies N. W., Hart I. J., Holland M., Easton A., Buckley C., Kneen R., and Beeching N. J. 2011. 'Management of suspected viral encephalitis in adults--Association of British Neurologists and British Infection Association National Guidelines', *J Infect*, 64: 347-73.

Solomon T., Thao L. T., Dung N. M., Kneen R., Hung N. T., Nisalak A., Vaughn D. W., Farrar J., Hien T. T., White N. J., and Cardosa M. J. 1998. 'Rapid diagnosis of Japanese encephalitis by using an immunoglobulin M dot enzyme immunoassay', *J Clin Microbiol*, 36: 2030-4.

Stahl J. P., Azouvi P., Bruneel F., De Broucker T., Duval X., Fantin B., Girard N., Herrmann J. L., Honnorat J., Lecuit M., Mailles A., Martinez-Almoyna L., Morand P., Piroth L., and Tattevin P. 2017. 'Guidelines on the management of infectious encephalitis in adults', *Med Mal Infect*, 47: 179-94.

Steiner I., and Kennedy P. G. 2015. 'Acute disseminated encephalomyelitis: current knowledge and open questions', *J Neurovirol*, 21: 473-9.

Steiner I., Kennedy P. G., and Pachner A. R. 2007. 'The neurotropic herpes viruses: herpes simplex and varicella-zoster', *Lancet Neurol*, 6: 1015-28.

Sumner L. W., Amberg A., Barrett D., Beale M. H., Beger R., Daykin C. A., Fan T. W., Fiehn O., Goodacre R., Griffin J. L., Hankemeier T., Hardy N., Harnly J., Higashi R., Kopka J., Lane A. N., Lindon J. C., Marriott P., Nicholls A. W., Reily M. D., Thaden J. J., and Viant M. R. 2007. 'Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI)', *Metabolomics*, 3: 211-21.

Tang S., Jing H., Huang Z., Huang T., Lin S., Liao M., and Zhou J. 2020. 'Identification of key candidate genes in neuropathic pain by integrated bioinformatic analysis', *J Cell Biochem*, 121: 1635-48.

Tiberti N., Lejon V., Mumba Ngoyi D., Matovu E., Enyaru J., Walter N., Fouda C., Lutumba P., Kristensson K., Bisser S., Mathu Ndung'u J., Büscher P., and Sanchez J.-C. 2015. 'Increased acute immune response during the meningo-encephalitic stage of Trypanosoma brucei rhodesiense sleeping sickness compared to Trypanosoma brucei gambiense', *Translational Proteomics*, 6: 1-9.

Titulaer M. J., McCracken L., Gabilondo I., Armangue T., Glaser C., Iizuka T., Honig L. S., Benseler S. M., Kawachi I., Martinez-Hernandez E., Aguilar E., Gresa-Arribas N., Ryan-Florance N., Torrents A., Saiz A., Rosenfeld M. R., Balice-Gordon R., Graus F., and Dalmau J. 2013. 'Treatment and prognostic factors for long-term outcome in patients with anti-NMDA receptor encephalitis: an observational cohort study', *Lancet Neurol*, 12: 157-65.

Titulaer M. J., Soffietti R., Dalmau J., Gilhus N. E., Giometto B., Graus F., Grisold W., Honnorat J., Sillevis Smitt P. A., Tanasescu R., Vedeler C. A., Voltz R., and Verschuuren J. J. 2011. 'Screening for tumours in paraneoplastic syndromes: report of an EFNS task force', *Eur J Neurol*, 18: 19-e3.

Turtle L., Griffiths M. J., and Solomon T. 2012. 'Encephalitis caused by flaviviruses', *QJM*, 105: 219-23.

Tüzün E., Zhou L., Baehring J. M., Bannykh S., Rosenfeld M. R., and Dalmau J. 2009. 'Evidence for antibody-mediated pathogenesis in anti-NMDAR encephalitis associated with ovarian teratoma', *Acta Neuropathol*, 118: 737-43.

Tyler K. L. 2004. 'Herpes simplex virus infections of the central nervous system: encephalitis and meningitis, including Mollaret's', *Herpes*, 11 Suppl 2: 57a-64a.

Valcour V., Chalermchai T., Sailasuta N., Marovich M., Lerdlum S., Suttichom D., Suwanwela N. C., Jagodzinski L., Michael N., Spudich S., van Griensven F., de Souza M., Kim J., and Ananworanich J. 2012. 'Central nervous system viral invasion and inflammation during acute HIV infection', *J Infect Dis*, 206: 275-82.

van der Ende E. L., Meeter L. H., Stingl C., van Rooij J. G. J., Stoop M. P., Nijholt D. A. T., Sanchez-Valle R., Graff C., Öijerstedt L., Grossman M., McMillan C., Pijnenburg Y. A. L., Laforce R., Jr., Binetti G., Benussi L., Ghidoni R., Luider T. M., Seelaar H., and van Swieten J. C. 2019. 'Novel CSF biomarkers in genetic frontotemporal dementia identified by proteomics', *Ann Clin Transl Neurol*, 6: 698-707.

van Laarhoven A., Dian S., Aguirre-Gamboa R., Avila-Pacheco J., Ricaño-Ponce I., Ruesen C., Annisa J., Koeken V., Chaidir L., Li Y., Achmad T. H., Joosten L. A. B., Notebaart R. A., Ruslami R., Netea M. G., Verbeek M. M., Alisjahbana B., Kumar V., Clish C. B., Ganiem A. R., and van Crevel R. 2018. 'Cerebral tryptophan metabolism and outcome of tuberculous meningitis: an observational cohort study', *Lancet Infect Dis*, 18: 526-35.

van Sonderen A., Schreurs M. W. J., Wirtz P. W., Sillevis Smitt P. A. E., and Titulaer M. J. 2016. 'From VGKC to LGI1 and Caspr2 encephalitis: The evolution of a disease entity over time', *Autoimmunity Reviews*, 15: 970-74.

Vasek M. J., Garber C., Dorsey D., Durrant D. M., Bollman B., Soung A., Yu J., Perez-Torres C., Frouin A., Wilton D. K., Funk K., DeMasters B. K., Jiang X., Bowen J. R., Mennerick S., Robinson J. K., Garbow J. R., Tyler K. L., Suthar M. S., Schmidt R. E., Stevens B., and Klein R. S. 2016. 'A complement-microglial axis drives synapse loss during virus-induced memory impairment', *Nature*, 534: 538-43.

Veje M., Studahl M., and Bergström T. 2019. 'Intrathecal complement activation by the classical pathway in tick-borne encephalitis', *J Neurovirol*, 25: 397-404.

Venkatesan A., Michael B. D., Probasco J. C., Geocadin R. G., and Solomon T. 2019. 'Acute encephalitis in immunocompetent adults', *Lancet*, 393: 702-16.

Vilela M. C., Campos R. D., Mansur D. S., Rodrigues D. H., Lacerda-Queiroz N., Lima G. K., Rachid M. A., Kroon E. G., Campos M. A., and Teixeira A. L. 2011. 'Role of IL-4 in an experimental model of encephalitis induced by intracranial inoculation of herpes simplex virus-1 (HSV-1)', *Arq Neuropsiquiatr*, 69: 237-41.

Vogt L., Schmitz N., Kurrer M. O., Bauer M., Hinton H. I., Behnke S., Gatto D., Sebbel P., Beerli R. R., Sonderegger I., Kopf M., Saudan P., and Bachmann M. F. 2006. 'VSIG4, a B7 family-related protein, is a negative regulator of T cell activation', *J Clin Invest*, 116: 2817-26.

Waldera-Lupa D. M., Poschmann G., Kirchgaessler N., Etemad-Parishanzadeh O., Baberg F., Brocksieper M., Seidel S., Kowalski T., Brunn A., Haghikia A., Gold R., Stefanski A., Deckert M., Schlegel U., and Stühler K. 2020. 'A Multiplex Assay for the Stratification of Patients with Primary Central Nervous System Lymphoma Using Targeted Mass Spectrometry', *Cancers (Basel)*, 12: 1732.

Weil A. A., Glaser C. A., Amad Z., and Forghani B. 2002. 'Patients with suspected herpes simplex encephalitis: rethinking an initial negative polymerase chain reaction result', *Clin Infect Dis*, 34: 1154-7.

White C., Lee J., Kambe T., Fritsche K., and Petris M. J. 2009. 'A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity', *J Biol Chem*, 284: 33949-56.

Whitley R. J. 2009. 'A 70-year-old woman with shingles: review of herpes zoster', *Jama*, 302: 73-80.

Whitley R. J., Kimberlin D. W., and Roizman B. 1998. 'Herpes simplex viruses', *Clin Infect Dis*, 26: 541-53; quiz 54-5.

Whitley R. J., Soong S. J., Dolin R., Galasso G. J., Ch'ien L. T., and Alford C. A. 1977. 'Adenine arabinoside therapy of biopsy-proved herpes simplex encephalitis. National Institute of Allergy and Infectious Diseases collaborative antiviral study', *N Engl J Med*, 297: 289-94.

Wiesmann C., Katschke K. J., Yin J., Helmy K. Y., Steffek M., Fairbrother W. J., McCallum S. A., Embuscado L., DeForge L., Hass P. E., and van Lookeren Campagne M. 2006. 'Structure of C3b in complex with CRIg gives insights into regulation of complement activation', *Nature*, 444: 217-20.

Wilson M. R., Sample H. A., Zorn K. C., Arevalo S., Yu G., Neuhaus J., Federman S., Stryke D., Briggs B., Langelier C., Berger A., Douglas V., Josephson S. A., Chow F. C., Fulton B. D., DeRisi J. L., Gelfand J. M., Naccache S. N., Bender J., Dien Bard J., Murkey J., Carlson M., Vespa P. M., Vijayan T., Allyn P. R., Campeau S., Humphries R. M., Klausner J. D., Ganzon C. D., Memar F., Ocampo N. A., Zimmermann L. L., Cohen S. H., Polage C. R., DeBiasi R. L., Haller B., Dallas R., Maron G., Hayden R., Messacar K., Dominguez S. R., Miller S., and Chiu C. Y. 2019. 'Clinical Metagenomic Sequencing for Diagnosis of Meningitis and Encephalitis', *N Engl J Med*, 380: 2327-40.

Wingren C., and Borrebaeck C. A. 2008. 'Antibody microarray analysis of directly labelled complex proteomes', *Curr Opin Biotechnol*, 19: 55-61.

Winter P. M., Dung N. M., Loan H. T., Kneen R., Wills B., Thu le T., House D., White N. J., Farrar J. J., Hart C. A., and Solomon T. 2004. 'Proinflammatory cytokines and chemokines in humans with Japanese encephalitis', *J Infect Dis*, 190: 1618-26.

Wong S. H., Jenkinson M. D., Faragher B., Thomas S., Crooks D., and Solomon T. 2010. 'Brain biopsy in the management of neurology patients', *Eur Neurol*, 64: 42-5.

Xu C., Jia Q., Zhang L., Wang Z., Zhu S., Wang X., Liu Y., Li M., Zhang J., Wang X., Zhang J., Sun Q., Wang K., Zhu H., and Duan L. 2020. 'Multiomics Study of Gut Bacteria and Host Metabolism in Irritable Bowel Syndrome and Depression Patients', *Front Cell Infect Microbiol*, 10: 580980.

Yan J., Kuzhiumparambil U., Bandodkar A., Bandodkar S., Dale R. C., and Fu S. 2020. 'Cerebrospinal fluid metabolites in tryptophan-kynurenine and nitric oxide pathways: biomarkers for acute neuroinflammation', *Dev Med Child Neurol*, 63: 552-59.

Yang Y., Mu J., Chen G., Zhan Y., Zhong J., Wei Y., Cheng K., Qin B., You H., and Xie P. 2015. 'iTRAQ-based quantitative proteomic analysis of cerebrospinal fluid reveals NELL2 as a potential diagnostic biomarker of tuberculous meningitis', *Int J Mol Med*, 35: 1323-32.

Yarlagadda S. G., and Perazella M. A. 2008. 'Drug-induced crystal nephropathy: an update', *Expert Opin Drug Saf*, 7: 147-58.

Yates J. R., Ruse C. I., and Nakorchevsky A. 2009. 'Proteomics by mass spectrometry: approaches, advances, and applications', *Annu Rev Biomed Eng*, 11: 49-79.

Yeo T., Probert F., Jurynczyk M., Sealey M., Cavey A., Claridge T. D. W., Woodhall M., Waters P., Leite M. I., Anthony D. C., and Palace J. 2019. 'Classifying the antibody-negative NMO syndromes: Clinical, imaging, and metabolomic modeling', *Neurol Neuroimmunol Neuroinflamm*, 6: e626.

Yeshokumar A. K., Gordon-Lipkin E., Arenivas A., Cohen J., Venkatesan A., Saylor D., and Probasco J. C. 2017. 'Neurobehavioral outcomes in autoimmune encephalitis', *J Neuroimmunol*, 312: 8-14.

Ygberg S., Fowler A., and Wickstrom R. 2016. 'Cytokine and Chemokine Expression in CSF May Differentiate Viral and Autoimmune NMDAR Encephalitis in Children', *J Child Neurol*, 31: 1450-56.

Zavialov A. V., and Engstrom A. 2005. 'Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity', *Biochem J*, 391: 51-7.

Zavialov A. V., Gracia E., Glaichenhaus N., Franco R., Zavialov A. V., and Lauvau G. 2010. 'Human adenosine deaminase 2 induces differentiation of monocytes into macrophages and stimulates proliferation of T helper cells and macrophages', *J Leukoc Biol*, 88: 279-90.

Zhang P., Zhang W., Lang Y., Qu Y., Chen J., and Cui L. 2019. '(1)H nuclear magnetic resonance-based metabolic profiling of cerebrospinal fluid to identify metabolic features and markers for tuberculosis meningitis', *Infect Genet Evol*, 68: 253-64.

Zhang Q., Huang D., Zhang Z., Feng Y., Fu M., Wei M., Zhou J., Huang Y., Liu S., and Shi R. 2019. 'High expression of TMEM40 contributes to progressive features of tongue squamous cell carcinoma', *Oncol Rep*, 41: 154-64.

Zheng L., Boehme S. A., Critchfield J. M., Zuniga-Pflucker J. C., Freedman M., and Lenardo M. J. 1994. 'Immunological tolerance by antigen-induced apoptosis of mature T lymphocytes', *Adv Exp Med Biol*, 365: 81-9.

Appendix. Diagnostic Criteria

Autoimmune encephalitis

(Adapted from consensus criteria (Graus et al. 2016))

Possible autoimmune encephalitis

Diagnosis can be made when all three of the following criteria have been met:

1. Subacute onset (rapid progression of less than 3 months) of working memory deficits (short-term memory loss), altered mental status*, or psychiatric symptoms

2. At least one of the following:

• New focal CNS findings

• Seizures not explained by a previously known seizure disorder

• CSF pleocytosis (white blood cell count of more than five cells per mm3)

• MRI features suggestive of encephalitis†

3. Reasonable exclusion of alternative causes

*Altered mental status defined as decreased or altered level of consciousness, lethargy, or personality change. †Brain MRI hyperintense signal on T2-weighted fluid-attenuated inversion recovery sequences highly restricted to one or both medial temporal lobes (limbic encephalitis), or in multifocal areas involving grey matter, white matter, or both compatible with demyelination or inflammation.

Definite autoimmune limbic encephalitis

Diagnosis can be made when all four* of the following criteria have been met:

1. Subacute onset (rapid progression of less than 3 months) of working memory deficits, seizures, or psychiatric symptoms suggesting involvement of the limbic system

2. Bilateral brain abnormalities on T2-weighted fluid-attenuated inversion recovery MRI highly restricted to the medial temporal lobes†

3. At least one of the following:

• CSF pleocytosis (white blood cell count of more than five cells per mm3)

• EEG with epileptic or slow-wave activity involving the temporal lobes

4. Reasonable exclusion of alternative causes

*If one of the first three criteria is not met, a diagnosis of definite limbic encephalitis can be made only with the detection of antibodies against cell-surface, synaptic, or onconeural proteins.

†18Fluorodeoxyglucose (18F-FDG) PET can be used to fulfil this criterion. Results from studies from the past 5 years suggest that 18F-FDG-PET imaging might be more sensitive than MRI to show an increase in FDG uptake in normal-appearing medial temporal lobes.

Definite acute disseminated encephalomyelitis

Diagnosis can be made when all five of the following criteria have been met:

1. A first multifocal, clinical CNS event of presumed inflammatory demyelinating cause

2. Encephalopathy that cannot be explained by fever

3. Abnormal brain MRI:

• Diffuse, poorly demarcated, large (>1–2 cm) lesions predominantly involving the cerebral white matter

• T1-hypointense lesions in the white matter in rare cases • Deep grey matter abnormalities (eg, thalamus or basal ganglia) can be present

4. No new clinical or MRI findings after 3 months of symptom onset

5. Reasonable exclusion of alternative causes

Probable NMDAR antibody encephalitis

Diagnosis can be made when all three of the following criteria have been met:

1. Rapid onset (less than 3 months) of at least four of the six following major groups of symptoms:

• Abnormal (psychiatric) behaviour or cognitive dysfunction

• Speech dysfunction (pressured speech, verbal reduction, mutism)

• Seizures

• Movement disorder, dyskinesias, or rigidity/abnormal postures

• Decreased level of consciousness

• Autonomic dysfunction or central hypoventilation

2. At least one of the following laboratory study results:

• Abnormal EEG (focal or diffuse slow or disorganised activity, epileptic activity, or extreme delta brush)

• CSF with pleocytosis or oligoclonal bands

3. Reasonable exclusion of other disorders (appendix)

Diagnosis can also be made in the presence of three of the above groups of symptoms accompanied by a systemic teratoma

Definite NMDAR antibody encephalitis

Diagnosis can be made in the presence of one or more of the six major groups of symptoms and IgG anti-GluN1 antibodies,† after reasonable exclusion of other disorders (appendix)

Patients with a history of herpes simplex virus encephalitis in the previous weeks might have relapsing immune-mediated neurological symptoms (post-herpes simplex virus encephalitis). †Antibody testing should include testing of CSF. If only serum is available, confirmatory tests should be included (eg, live neurons or tissue immunohistochemistry, in addition to cell-based assay).

Hashimoto's encephalopathy

Diagnosis can be made when all six of the following criteria have been met:

1. Encephalopathy with seizures, myoclonus, hallucinations, or stroke-like episodes

2. Subclinical or mild overt thyroid disease (usually hypothyroidism)

3. Brain MRI normal or with non-specific abnormalities

4. Presence of serum thyroid (thyroid peroxidase,

thyroglobulin) antibodies*

5. Absence of well characterised neuronal antibodies in

serum and CSF

6. Reasonable exclusion of alternative causes

*There is no disease-specific cutoff value for these antibodies (detectable in 13% of healthy individuals).

Viral encephalitis

Adapted from consensus criteria (Granerod, Cunningham, et al. 2010)

Virus	Confirmed	Possible	Excluded
Adenovirus	ADV detected in CSF/brain specimens by PCR;OR ADV-specific intrathecal antibody response; OR Autopsy neuropathology demonstrates basophilic nuclear inclusions in neurons and glia containing ADV DNA/antigen, variable inflammation, necrosis and haemorrhage; AND No other explanatory pathogen or cause found	Serological evidence of primary ADV infection ; OR ADV DNA/antigen detected in a blood, respiratory, urine or faecal sample (excluding adenovirus 40 & 41 in faecal samples)	ADV DNA negative on CSF specimen taken 3–7 days after symptom onset ; AND No ADV-specific intrathecal antibody response# at least 7–10 days after symptom onset ; OR Meets confirmed or probable CD for another cause and does not meet the possible CD for ADV
Enterovirus	Enteroviruses detected in any CSF/ brain specimens OR Enterovirus-specific intrathecal antibody response AND *If available, a*utopsy neuropathology demonstrates poliomyelitis/polioencephalitis AND No other explanatory pathogen or cause found	Laboratory detection of organism outside the CNS ; OR Serological evidence of recent infection	Meets confirmed or probable CD for another cause and does not meet the possible CD for enterovirus

Herpes simplex virus	HSV DNA/antigen detected in any CSF/brain specimens ; OR HSV-specific intrathecal antibody response; OR Autopsy neuropathology demonstrates panencephalitis with HSV DNA/antigen present in eosinophilic neuronal inclusions	Serological evidence suggestive but not conclusive (IgM positive) of primary HSV infection or positive sample other than CNS site	HSV DNA negative on CSF specimen taken 3–7 days after symptom onset ; AND No HSV-specific intrathecal antibody response at least 7–10 days after symptom onset ; OR Meets confirmed or probable CD for another cause and does not meet the possible CD for HSV
Varicella zoster virus	VZV DNA/antigen detected in any CSF/brain specimens ; OR VZV-specific intrathecal antibody response; OR Autopsy neuropathology demonstrates panencephalitis with nuclear inclusions containing VZV DNA/antigen	Serological evidence suggestive but not conclusive (IgM positive) of primary VZV infection or virus detection at a site other than the CNS	VZV DNA negative on CSF specimen taken 3–7 days after symptom onset ; AND No VZV-specific intrathecal antibody response at least 7–10 days after symptom onset ; OR Meets confirmed or probable CD for another cause and does not meet the possible CD for VZV

Publications during PhD period

Key publications relating to encephalitis marked with † and reproduced hereafter.

†Ellul MA, Griffiths MJ, Iyer A, Avula S, Defres S, Baborie A, Vincent A, Martin NG, Sadarangani M, Pollard AJ, Solomon T, Kneen R.
Anti-N-Methyl-D-Aspartate Receptor Encephalitis in a Young Child with Histological Evidence on Brain Biopsy of Co-existent Herpes Simplex Virus Type 1 Infection.
Pediatr Infect Dis J. 2016;35(3):347-9.

O'Sullivan BJ, Steele T, Ellul MA, Kirby E, Duale A, Kier G, Crooks D, Jacob A, Solomon T, Michael BD.
When should we test for voltage-gated potassium channel complex antibodies? A retrospective case control study.
J Clin Neurosci. 2016;33:198-204.

Ellul MA, Soares CN, Solomon T.
Zika virus and Guillain-Barre syndrome.
J R Coll Physicians Edinb. 2016;46(2):103-105.

Soares CN, Brasil P, Carrera RM, Sequeira P, de Filippis AB, Borges VA, Theophilo F, Ellul MA, Solomon T.
Fatal encephalitis associated with Zika virus infection in an adult.
J Clin Virol. 2016;83:63-5.

Bonello M, Jacob A, Ellul MA, Barker E, Parker R, Jefferson S, Alusi S.
IgLON5 disease responsive to immunotherapy.
Neurol Neuroimmunol Neuroinflamm. 2017;4(5):e383.

Ellul MA, Griffiths MJ.
Decoding the association between herpes simplex virus and antibody-mediated encephalitis.
Dev Med Child Neurol. 2017;59(8):776-777.

Ellul MA, Cross TJ, Larner AJ.
Asterixis.
Pract Neurol. 2017;17(1):60-62.

Mehta R, Soares CN, Medialdea-Carrera R, Ellul M, da Silva MTT, Rosala-Hallas A, Jardim MR, Burnside G, Pamplona L, Bhojak M, Manohar R, da Silva GAM, Adriano MV, Brasil P, Nogueira RMR, Dos Santos CC, Turtle L, de Sequeira PC, Brown DW, Griffiths MJ, de Filippis AMB, Solomon T.
The spectrum of neurological disease associated with Zika and chikungunya viruses in adults in Rio de Janeiro, Brazil: A case series.
PLoS Negl Trop Dis. 2018;12(2):e0006212.

†Ellul M, Solomon T.
Acute encephalitis - diagnosis and management.
Clin Med (Lond). 2018;18(2):155-159.

Swaminathan M, Ellul MA, Cross TJ.
Hepatic encephalopathy: current challenges and future prospects.
Hepat Med. 2018;10:1-11.

†*Van Den Tooren H, *Ellul MA, Davies NW, Easton A, Vincent A, Solomon T, Michael BD; CoroNerve Studies Group.
Standing on the shoulders of giants: 100 years of neurology and epidemic infections.
J Neurol Neurosurg Psychiatry. 2020;91(11):1129-1131. (* Joint first authors)

Brito Ferreira ML, Militão de Albuquerque MFP, de Brito CAA, de Oliveira França RF, Porto Moreira ÁJ, de Morais Machado MÍ, da Paz Melo R, Medialdea-Carrera R, Dornelas Mesquita S, Lopes Santos M, Mehta R, Ramos E Silva R, Leonhard SE, Ellul M, Rosala-Hallas A, Burnside G, Turtle L, Griffiths MJ, Jacobs BC, Bhojak M, Willison HJ, Pena LJ, Pardo CA, Ximenes RAA, Martelli CMT, Brown DWG, Cordeiro MT, Lant S, Solomon T.
Neurological disease in adults with Zika and chikungunya virus infection in Northeast Brazil: a prospective observational study.
Lancet Neurol. 2020;19(10):826-839.

†Ellul MA, Wood G, Tooren HVD, Easton A, Babu A, Michael BD.
Update on the diagnosis and management of autoimmune encephalitis.
Clin Med (Lond). 2020;20(4):389-392.

Varatharaj A, Thomas N, Ellul MA, Davies NWS, Pollak TA, Tenorio EL, Sultan M, Easton A, Breen G, Zandi M, Coles JP, Manji H, Al-Shahi Salman R, Menon DK, Nicholson TR, Benjamin LA, Carson A, Smith C, Turner MR, Solomon T, Kneen R, Pett SL, Galea I, Thomas RH, Michael BD; CoroNerve Study Group.
Neurological and neuropsychiatric complications of COVID-19 in 153 patients: a UK-wide surveillance study.
Lancet Psychiatry. 2020;7(10):875-882**.**

†Ellul MA, Benjamin L, Singh B, Lant S, Michael BD, Easton A, Kneen R, Defres S, Sejvar J, Solomon T.
Neurological associations of COVID-19.
Lancet Neurol. 2020;19(9):767-783.

†Ellul M, Varatharaj A, Nicholson TR, Pollak TA, Thomas N, Easton A, Zandi MS, Manji H, Solomon T, Carson A, Turner MR, Kneen R, Galea I, Pett S, Thomas RH, Michael BD; CoroNerve Steering Committee.
Defining causality in COVID-19 and neurological disorders.
J Neurol Neurosurg Psychiatry. 2020;91(8):811-812.

Leonhard SE, Halstead S, Lant SB, Militão de Albuquerque MFP, de Brito CAA, de Albuquerque LBB, Ellul MA, de Oliveira França RF, Gourlay D, Griffiths MJ, de Miranda Henriques-Souza AM, de Morais Machado MÍ, Medialdea-Carrera R, Mehta R, da Paz Melo R, Mesquita SD, Moreira ÁJP, Pena LJ, Santos ML, Turtle L, Solomon T, Willison HJ, Jacobs BC, Brito Ferreira ML.
Guillain-Barre syndrome during the Zika virus outbreak in Northeast Brazil: An observational cohort study.
J Neurol Sci. 2021;420:117272.