Detection and diagnosis of acute viral encephalitis

Thesis submitted in accordance with the requirements of the University of Liverpool

for the Degree of Doctor in Philosophy by Benedict Daniel Michael

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Liverpool or any other University or Institute of learning.

Signature: _____

Date: _____

Abstract

Introduction

Acute viral encephalitis is a severe form of brain inflammation due to sporadic infection, typically with herpes simplex virus, or epidemic/pandemic infections. Epidemiological data are particularly important for pandemic viruses. Although new reporting approaches are often considered, no real-time clinical data collection tool has been developed. These data are dependent on diagnosis of individual cases. However, the aspects of management that result in delays and missed diagnoses are not clear and it is not known if interventions can improve sample collection and diagnosis. Whilst the importance of cytokines and associated mediators is increasingly recognised, signatures associated with specific aetiologies have not been established. Also, it is not known whether these mediators correlate with clinical severity and outcome, or their impact on blood-brain barrier permeability.

Methods

I undertook a national surveillance study through neurology networks, and investigated alternative notification approaches. I undertook a multicentre cross-sectional study of clinical investigation, studied viral load and assessed the impact of a lumbar puncture pack. I used bead array to assess mediator profiles and assessed the albumin ratio and viral load, in samples from a Health Protection Agency study. I examined profiles with respect to aetiology, disease severity and outcome and compared this with histopathology tissue and a blood-brain barrier model.

Results

In the context of a pandemic influenza virus, existing mechanisms identified limited cases, and a smartphone application was developed to collect real-time data. Delays in lumbar puncture and sub-optimal sample collection were identified, in association with a lower viral load. A lumbar puncture pack improved sample collection. Mediator profiles differed between those with an infectious versus immune-mediated aetiology, and those of unknown aetiology best reflected infectious; particularly myeloperoxidase, in part relating to neutrophils in cerebrospinal fluid and parenchyma. The interleukin1 antagonists, IL1RA and IL10, were associated with coma and outcome; and IL10 with reduced blood-brain barrier permeability. Adhesion molecules may counteract this, in both clinical samples and the model.

Conclusions

Current limitations of detection may be augmented with novel real-time technologies. Diagnosis is limited by delayed and sub-optimal sample collection, which can be improved with a simple pack. Mediators profiles may assist in the distinction of infectious from immune-mediated encephalitis, and cytokines that act against IL1 correlated with clinical severity and outcome. This may be more closely associated with outcome than viral load, although this may reflect sample timing. These findings should direct future research to develop approaches for improved diagnostics and adjunctive therapies.

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Abbreviations

ADEM Acute Disseminated Encephalomyelitis

ALT Alanine transaminase

App' Smartphone application software

AQP4 Aquaporin4

BBB Blood-Brain Barrier

CD Cluster of Differentiation

CDC Centers for Disease Control

CMV Cytomegalovirus

CNS Central Nervous System

CSF Cerebrospinal Fluid

CT Computed Tomography

DFA Discriminant Function Analysis

DMEM Dulbecco's Modified Eagle's Medium

DNA Deoxyribonucleic Acid

EBV Epstein Barr Virus

EDSS Expanded Disability Status Scale

EEG Electroencephalography

ELISA Enzyme-linked immunosorbent Assay

GBS Guillain-Barre Syndrome

G-CSF Granulocyte Colony Stimulating Factor

GCS Glasgow Coma Scale

GM-CSF Granulocyte Macrophage Colony Stimulating Factor

GOS Glasgow Outcome Scale

GP General Practitioner

HBEC Human Brain Endothelial Cells

HIV Human Immunodeficiency Virus

HPA Health Protection Agency

HSV Herpes Simplex Virus

HSVE Herpes Simplex Virus Encephalitis

ICU Intensive Care Unit

IFN Interferon

IV Intravenous

IVIg Intravenous Immunoglobulin

ICAM Intercellular Adhesion Molecule

IL Interleukin

JEV Japanese Encephalitis Virus

LP Lumbar Puncture

MCP Monocyte Chemotactic Protein

MC+S Microscopy Culture and Sensitivity

MIP Monocyte Inflammatory Protein

MMP Matrix Metalloproteinase

MOI Multiplicity of infection

MPO Myeloperoxidase

MRI Magnetic Resonance Imaging

NMDA-R N-Methyl D-Aspartate Receptor

NMO Neuromyelitis Optica

PCR Polymerase Chain Reaction

RANTES Regulated on Activation Normal T-cell Expressed and Secreted

RNA Ribonucleic acid

TB Tuberculosis

TEER Transendothelial Electrical Resistance

TNFα Tumour Necrosis Factor Alpha

TRAIL TNFα-Related Apoptosis-Inducing Ligand

VCAM Vascular Cell Adhesion Molecule

VEGF Vascular Endothelial Growth Factor

VGKC Voltage-Gated Potassium Channel

VZV Varicella Zoster Virus

WCC White Cell Count

WCNN Walton Centre for Neurology and Neurosurgery

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1. Introduction

1.1 Definition of encephalitis

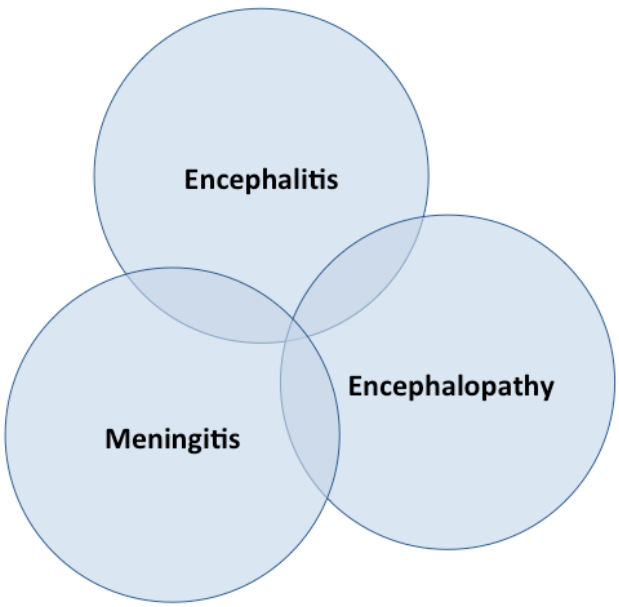
'Encephalitis' is inflammation of the brain parenchyma (Jmor et al 2008). Therefore, fundamentally this diagnosis can only be established by histopathological examination of brain tissue. However, as this is obviously only possible either post-mortem, or only justified ante-mortem in the minority of patients who will have a brain biopsy, proxy markers of brain inflammation are routinely used (Michael et al 2010). Whilst there is some role for neuroimaging, it is the presence of an elevated leucocyte count in the cerebrospinal fluid [CSF] that is most often used clinically to establish the presence of inflammation and culture and molecular analysis of the CSF that identifies the aetiology (Solomon et al 2007). In contrast 'Encephalopathy' is the constellation of clinical features reflecting impaired function of the central nervous system [CNS], including changes in cognition, consciousness, personality and behaviour; for which there is a broad range of differential diagnoses, including systemic metabolic, toxic, endocrine disturbances, neoplastic or vascular processes and, infection outside of the CNS. Whilst encephalitis can present with an encephalopathic picture, this is distinguished from encephalitis by the absence of evidence of inflammation in the CSF, on imaging or determined by histopathological examination (Michael et al 2010) [Table 1]. Although meningitis is distinguished from encephalitis when there is fever, neck stiffness, and nausea/vomiting in the absence of alterations in consciousness, there can often be clinical and even a degree of histopathological overlap, and the term 'meningoencephalitis' is used [Figure 1]. Although encephalitis is relatively rare, its importance lies in that fact that for many

forms treatment is effective if started promptly; in contrast, delays in treatment can be devastating.

Table 1. Definitions of encephalopathy and encephalitis

Encephalopathy	Clinical syndrome of altered mental status (manifesting as reduced consciousness or altered cognition, personality, or behaviour) Has many causes including systemic infection, metabolic encephalopathies, toxins, hypoxia, trauma, vasculitic, or central nervous system infection
Encephalitis	Inflammation of the brain Strictly a pathological diagnosis; but surrogate clinical markers often used, including inflammatory change in the cerebrospinal fluid or parenchymal inflammation on imaging Causes include viruses, small intracellular bacteria that directly infection the brain parenchyma and some parasites Can also occur without direct brain infection, for example in acute disseminated encephalomyelitis, or antibody-associated encephalitis

Figure 1. Inter-relationship between encephalitis, encephalopathy and meningitis



1.2 Classification of encephalitis

The causes of encephalitis can be defined as those due to direct infection of the CNS, para- or post-infectious causes, non-infectious antibody-associated causes, and a proportion in whom no cause is identified [Figure 2]. Infectious causes include numerous viruses, bacteria [especially intracellular bacteria such as *Mycoplasma pneumoniae*], parasites and fungi [Table 2; Table 3]. Acute disseminated encephalomyelitis (Mofenson et al 2009) after influenza is an example of a post-infectious encephalitis. Non-infectious causes include antibody-associated encephalitis, which may or may not be paraneoplastic. Most viral encephalitis is acute, but sub-acute and chronic presentations are characteristic of particular pathogens, especially in the immunocompromised.

Figure 2. Aetiological classifications of encephalitis

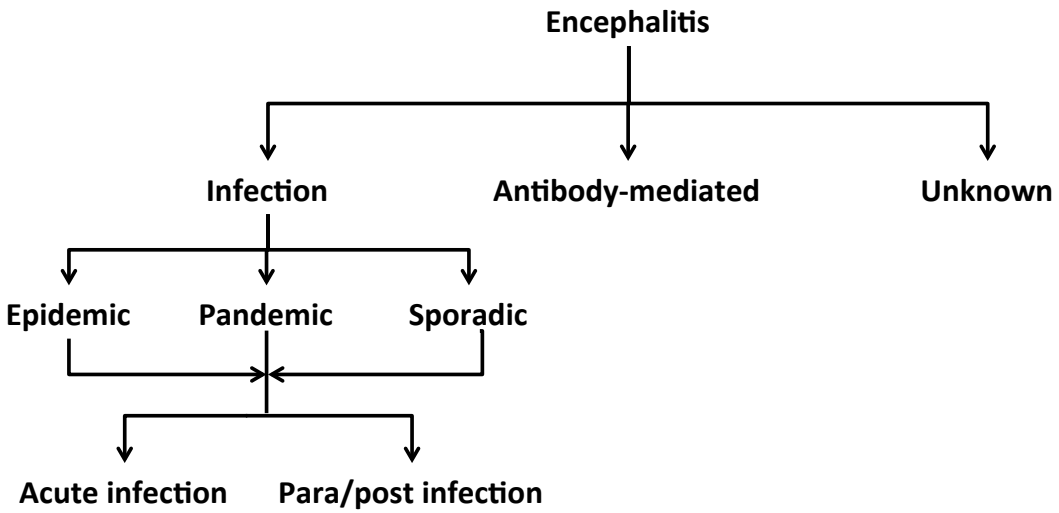


Table 2. Causes of acute viral encephalitis with geographical clues

Groups	Viruses	Comments
Sporadic causes (not geographically restricted) listed by group		
Herpes viruses (family *Herpesviridae*)		
	Herpes simplex virus type 1	Most commonly diagnosed sporadic encephalitis
	Herpes simplex virus type 2	Causes meningitis in adults (esp. recurrent); Meningoencephalitis occurs typically in the immunocompromised. Also causes a radiculitis.
	Varicella zoster virus	Post-infective cerebellitis, or acute infective encephalitis or vasculopathy
	Epstein-Barr virus	Encephalitis in the immunocompromised
	Cytomegalovirus	Encephalitis in the immunocompromised; also retinitis or radiculitis; often neutrophilic CSF with low glucose
	Human herpes virus 6 & 7	Febrile convulsions in children (after roseola); encephalitis in immunocompromised
Enteroviruses (family *Picornaviridae*)		
	Enterovirus 70	Epidemic haemorrhagic conjunctivitis, with CNS involvement
	Enterovirus 71	Epidemic hand foot and mouth disease, with aseptic meningitis, brainstem encephalitis, myelitis
	Poliovirus	Myelitis
	Coxsackieviruses, Echoviruses, Parechovirus	Mostly aseptic meningitis
Paramyxoviruses (family *Paramyxoviridae*)		
	Measles virus	Causes acute post-infectious encephalitis, subacute encephalitis and subacute sclerosing panencephalitis
	Mumps virus	Parotitis, orchitis or pancreatitis may occur before, during or after meningoencephalitis
Others (rarer causes)		
	Influenza viruses, adenovirus, parvovirus B19, lymphocytic choriomeningitis virus, rubella virus,	
Arthropod-borne and zoonotic viruses*		

Tick-borne encephalitis virus	Travel in Eastern Europe, Former USSR; tick bite; upper limb flaccid paralysis
Dengue viruses (types 1-4)	Causes fever, arthralgia, rash and haemorrhagic disease, occasional CNS disease
Alphaviruses (family *Togaviridae*)	
Western, Eastern and Venezuelan equine encephalitis viruses	Found in the Americas; encephalitis of horses and humans
Chikungunya virus	Asia Pacific, Africa
Bunyaviruses	
Lacrosse virus	Encephalitis in America
Coltiviruses	
Colorado tick fever virus	North America
Rhabdoviruses	
Rabies, virus other lyssaviruses	Non-arthropod-borne zoonitic viruses transmitted by dogs, cats, bats, depending on location
Chandipura virus	Transmitted by sandflies, causing outbreaks in India
Henipah Viruses	
Nipah virus	Transmitted in faeces of fruit bats in Malaysia, Bangladesh

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Note viral causes of chronic encephalitis such as JC viruses are not included here

*Most are zoonotic - i.e. animals rather than humans are the main natural hosts, the exceptions being dengue and chikungunya viruses

Adapted from (Solomon et al 2013; Solomon et al 2010)

Table 3. Non-viral causes of encephalitis and its mimics

ENCEPHALITIS	MIMICS
CNS INFECTIONS	
Bacteria	
Small bacteria (mostly intracellular)	
Mycoplasma pneumoniae	*Mycobacterium tuberculosis*
Chlamydophila	*Streptococcus pneumoniae*
Rickettsiae (including scrub typhus, Rocky Mountain spotted fever)	*Haemophilus influenza*
Ehrlichiosis (anaplasmosis)	*Neisseria meningitidis*
Coxiella burnetti (Q fever)	
Bartonella hensellae (cat scratch fever)	*Salmonella typhi*
Tropherema whipplei (Whipple's disease)	
Brucella (Brucellosis)	
Listeria monocytogenes	
Spirochetes	
Trepenoma pallidum (Syphilis)	*Leptospira*
Borrelia burgdorferi (Lyme neuroborreliosis)	
Borrelia recurrentis (relapsing fever)	
Other bacteria	
Nocardiosis	Sub-acute bacterial endocarditis
Actinomycosis	Parameningeal infection
	Abscess / empyema
Parasites	
Trypanosoma brucei gambiense and *Trypanosoma brucei rhodesiense* (sleeping sickness)	Malaria
Naegleria fowleri, Balamuthia mandrellis (Amoebic encephalitis)	Cysticercosis
Angiostrongylus cantonensis (rat lung worm)	
	Trichinosis
Fungi	

Coccidiomycosis
Histoplasmosis
North American blastomycosis

Cryptococcosis

PARA/POST INFECTIOUS CAUSES

Inflammatory

Acute disseminated encephalomyelitis (ADEM)
Acute haemorrhagic leukoencephalopathy (AHLE)
Acute necrotising encephalitis (ANE) in children
Bickerstaff's encephalitis

Toxic / Metabolic

Reye's syndrome

Systemic infection

Septic encephalopathy
Shigellosis

NON-INFECTIOUS CAUSES

Vascular

Vasculitis
Systemic lupus erythematosis
Behcet's disease
Subarachnoid & subdural haemorrhage
Ischaemic cerebrovascular accidents

Neoplastic

Paraneoplastic encephalitis

Primary brain tumour
Metastases

Metabolic encephalopathy

Hepatic encephalopathy
Renal encephalopathy
Hypoglycaemia
Toxins (alcohol, drugs)
Hashimoto's disease

	Septic encephalopathy
	Mitochondrial diseases
Other	
Antibody-associated encephalitis: VGKC complex or NMDA receptor	Drug reactions
Encephalitis lethargica	Epilepsy
Haemophagocytic Lymphohistiocytosis (HLH) usually children	
	Functional disorder

* Almost every infectious and non-infectious condition can occasionally present with an encephalitis-like illness.
In this table some of the important aetiologies are classified into whether they cause an encephalitis, with inflammatory changes seen histopathologically in the brain parenchyma, or encephalopathy without inflammatory changes in the parenchyma, although for some aetiologies this is based on limited evidence

Abbreviations: VGKC, voltage gated potassium channel; NMDA, N-Methyl-D-Aspartic acid

1.3 Epidemiology

The global reported incidence of encephalitis varies according to the location, population studied, and differences in case definitions and research methods; however, the reported incidence in western settings ranges from 0.7-13.8 per 100,000 for all ages, being approximately 0.7-12.6 per 100,000 for adults and 10.5-13.8 per 100,000 children (Jmor et al 2008; Granerod et al 2010). Recent capture-recapture methods have reported an incidence in the UK of 5.23-8.66/100,00 per year (Granerod et al 2013). Previous surveillance data are suspected to reflect under-reporting to disease surveillance organisations, and this may be particularly important during epidemic and pandemic outbreaks (Solomon et al 2007). Moreover, robust epidemiological data are potentially further limited by sub-optimal clinical identification of individual cases (Bell et al 2009).

Causes of viral encephalitis are defined as sporadic or epidemic. Herpes simplex virus [HSV] encephalitis is the most common sporadic cause of viral encephalitis and the most frequently diagnosed viral encephalitis in industrialised nations, with an annual incidence of 1 in 250,000 to 500,000 (Whitley et al 2006). The age specific incidence is bimodal, with peaks in the young and the elderly. Most HSV encephalitis is due to HSV-1, but about 10% is caused by HSV-2. The latter typically occurs in immunocompromised individuals and neonates, in whom it can cause a disseminated infection. HSV is an alpha herpes DNA virus; whereas HSV type 2 is transmitted sexually, the majority of the population are exposed to HSV type 1 during childhood and almost all have been infected by adulthood through transmission by droplet spread (Whitley et al 2006). The virus crosses the oral mucous membrane and travels by

retrograde axonal transport along the trigeminal nerve to then establish latency in the trigeminal ganglion. Periodically the virus reactivates and travels by antegrade axonal transport to be shed; this usually occurs asymptomatically, but in a small proportion will be manifest as a herpes labialis, the common 'cold sore' (Michael et al 2014). Rarely the virus will replicate in the brain resulting in encephalitis; it is not clear if this follows directly on from further retrograde axonal transport after reactivation in the trigeminal ganglion or whether this is due to reactivation of virus latent within the brain. Indeed, a proportion of people who die without any evidence of any neurological disease will have the nucleic acid of this highly neurotropic virus within the brain parenchyma (Wozniak et al 2005). The exact immunological mechanisms contributing to the re-activation from latency are yet to be fully elucidated, but it appears that certain Toll-like receptors and associated cytokines may be important in preventing peripheral and central re-activation (Zhang et al 2007).

Several studies have documented the potential mimics of HSV encephalitis (Whitley et al 1989; Chataway et al 2004; Bell et al 2009). Whitley *et al.* demonstrated that of 432 patients undergoing brain biopsy for presumed HSV encephalitis 195 [45%] had the diagnosis proven histologically and in a further 95 patients [22%] an alternative, often treatable, diagnosis was established (Whitley et al 1989). However, the clinical presenting features of these two groups were very similar. Chataway *et al*. found that of those patients initially considered to have HSV encephalitis, inflammatory aetiologies such as acute disseminated encephalomyelitis [ADEM] or multiple sclerosis were the most frequent mimics (Chataway et al 2004). In a retrospective single-site study, in collaboration with Bell *et al*; I showed the broad range of final diagnoses in patients initially treated undergoing a lumbar puncture [LP] for possible

encephalitis in a single secondary care hospital in the UK (Bell et al 2009). However, the range of final diagnoses made in patients who undergo investigation and treatment for *suspected* encephalitis is not known, and this has not been studied across more than one centre; therefore the sensitivity and specificity of clinical features also remains unknown.

The second most frequently identified cause of sporadic viral encephalitis is varicella zoster virus [VZV], which is the most common viral cause of CNS infection and is particularly important in the immunocompromised (Gilden et al 2004).

The aetiology of epidemic viral encephalitis varies greatly by geographical location. Whilst many of these aetiologies are predominantly limited to the developing world setting, occasional cases have been reported of dengue encephalopathy, rabies, Japanese encephalitis, and Eastern equine encephalitis, in the developed world and West Nile virus has been responsible for severe epidemics in the USA and is emerging in parts of southern Europe (Solomon et al 2004). In addition the recent emergence of novel influenza strains, such as H1N1, has raised concerns of the potential for further epidemic outbreaks of encephalitis in both developed and developing world settings (Ekstrand et al 2010). However, most publications have been limited to case reports and small case series, as global disease surveillance data has been sub-optimal (Cisse et al 2010). Some have started to explore crowd-sourcing techniques to try to augment infectious disease surveillance, but studies have been limited (Shaman et al 2012).

Among the other causes, encephalitis associated with antibodies to the voltage-gated potassium channel [VGKC]-complex, or N-methyl-D-aspartate antibody [NMDA] receptors are increasingly recognised (Granerod et al 2010).

1.4 Clinical features

The differential diagnosis of acute encephalitis is broad, encompassing infectious, para-infectious immune-mediated, autoimmune, metabolic, vascular, neoplastic, paraneoplastic, and toxic aetiologies as well as brain dysfunction due to systemic sepsis (Davies et al 2006; Solomon et al 2007).

Fever and abnormal mental status, often with severe headache, nausea and vomiting, are the classical clinical features of infectious encephalitis. Eighty-five [91%] of 93 adults with HSV-1 encephalitis in one study were febrile on admission (Raschilas et al 2002); even those not febrile on admission will typically have a history of febrile illness. Disorientation [76%], speech disturbances [59%] and behavioural changes [41%] were the most common features, and one-third of patients had seizures. Alterations in higher mental function include lethargy, drowsiness, confusion, disorientation and coma.

With the advent of polymerase chain reaction [PCR] applied to CSF more subtle presentations of HSV encephalitis have been recognised (Fodor et al 1998). These include low-grade pyrexia rather than a high fever and behavioural changes which can be mistaken for psychiatric illness, or the consequences of drugs or alcohol, occasionally with tragic consequences. In one study, chronic alcohol abuse was one of several features associated with delays in initiating treatment (Poissy et al 2009).

Seizures can sometimes be the initial presenting feature of a patient with encephalitis. Seizures are more common in patients presenting with encephalitic processes affecting the cerebral cortex. These are more often infectious in aetiology, as opposed to encephalitic processes predominantly affecting the sub-cortical white matter, such as ADEM. However, intractable seizures, often in the absence of fever, are also common in antibody–associated encephalitides (Vincent et al 2010).

The relatively higher rate of acute symptomatic seizures in patients with encephalitis due to HSV type 1 may be result from the predilection of HSV to areas of high epileptogenic potential: the mesial temporal lobes, particularly the hippocampus, and, to a lesser extent, the orbitofrontal cortices (Misra et al 2008; Michael et al 2012). This concurs with the findings that *ex vivo* rat brain tissue infected with HSV type 1 show acute electrographic evidence of seizure activity and longer-term hippocampal changes with neuronal loss, predominantly in the cornu ammonis 3 area (Chen et al 2004). Both these findings suggest that the hippocampus is uniquely susceptible. In addition the necrotising nature of HSV encephalitis, the extent of leucocyte infiltration, the degree of cortical involvement, and potentially the pro-inflammatory cytokine cascade may all be important in promoting seizures (Misra et al 2008). Acute symptomatic seizures predict a worse outcome, as do a lower Glasgow coma scale [GCS] score, delays in commencing aciclovir treatment and older age (Whitley et al 2006). Following HSV encephalitis, 40–65% of patients develop later unprovoked seizures (Whitley et al 2006). However, there is no evidence to support primary prophylaxis with anti-epileptic drugs during the acute encephalitic illness (Pandey 2014). Further work is needed to delineate the complex mechanisms by which HSV

establishes neurotropism, evades the host immune system and results in acute symptomatic seizures and later unprovoked epileptic seizures.

1.5 Diagnostic features for specific aetiologies

The history is important in defining the spectrum of agents potentially responsible for encephalitis as this is influenced by age, immunocompetence, geography and exposure. Geographical restrictions are particularly significant for arthropod-borne infections.

As the features for HSV are non-specific, most patients with *suspected* HSV encephalitis prove to have a different diagnosis (Whitley et al 1989; Bell et al 2009). Although olfactory hallucinations are described in HSV encephalitis, they are not a reliable predictor. The finding of labial herpes has no diagnostic specificity for HSV encephalitis and is merely a marker of critical illness.

Encephalitis caused by VZV at the time of primary infection [chickenpox] may follow the rash at an interval of days or weeks; though it occasionally occurs before the rash, or even in patients with no rash (Jemsek et al 1983; Dworkin et al 2007). Adults over 20 years old; the immunocompromised; or those with cranial dermatome involvement or disseminated skin disease are at increased risk of encephalitis following chickenpox. The presentation may be acute or sub-acute with fever, headache, altered consciousness, ataxia and seizures. Sometimes there is a brainstem encephalitis associated with Ramsay Hunt syndrome (De La Blanchardiere et al 2000). Rashes are seen in other encephalitides; for example a maculopapular or

vesicular rash may be present in some rickettsial infections. Lesions on the hands, feet and mouth are seen in enteroviral infections, such as that caused by Enterovirus 71.

Sometimes the pattern of neurological deficit can provide clues to the possible aetiology. Autonomic dysfunction, myoclonus and cranial neuropathies can indicate a brainstem encephalitis, which is seen in listeriosis, brucellosis, tuberculosis [TB], and some viral CNS infections. Tremors or other movement disorders occur with thalamic or other basal ganglia involvement. This is seen in some flavivirus infections, such as West Nile virus and Japanese encephalitis virus, and alphavirus infection such as Eastern equine encephalitis virus and chikungunya (Solomon et al 2004; Harvala et al 2009). An encephalitis with an acute flaccid paralysis is characteristic of polio, and other enteroviruses, such as Enterovirus 71, as well as flaviviruses.

Recognising encephalitis in the elderly can be especially difficult because they are more likely than younger people to have other causes of neurological presentations, such as stroke. Additionally, they are at increased risk of systemic causes for altered cerebral function, such as systemic sepsis. However, as HSV encephalitis is more common in the elderly than younger adults, it is especially important that the diagnosis is considered promptly in such patients (Solomon et al 2007).

Viral encephalitis needs to be urgently distinguished from antibody-associated encephalitis as the treatment is very different and early intervention may significantly improve outcome (Vincent et al 2004; Buckley et al 2005, Irani et al 2010; Solomon et al 2012). For VGKC-complex antibody-associated encephalitis the median age at presentation is 65 years and the male to female ratio is 2:1 (Irani et al 2010), although

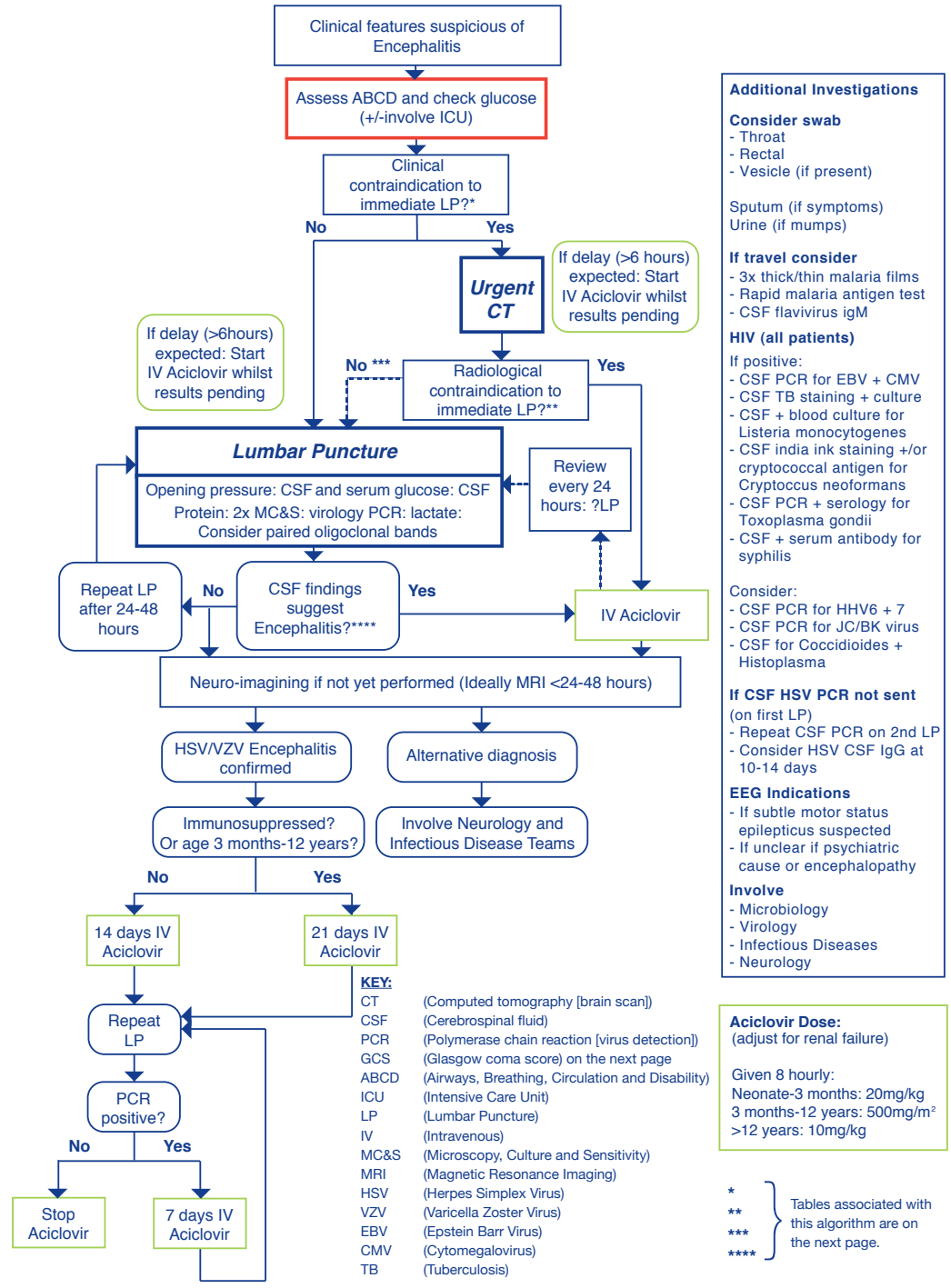
children are now beginning to be identified. It is uncommon for adult patients to have fever or headache. Instead they usually have profound disorientation and confusion with seizures and anterograde and retrograde amnesia. Low plasma sodium is found in about 60%. A recently described faciobrachial dystonic seizure syndrome may precede the onset of the encephalitis by a few weeks and appears to be pathognomonic for this condition (Irani et al 2010). For NMDA-receptor antibody-associated encephalitis the median age at presentation is 25 years and the male to female ratio is 1:2. These patients often have headache, and sometimes fever, as one of the earliest symptoms and there may be evidence of a prodromal viral infection. The subsequent illness then appears to have two phases. The first phase is characterised by seizures, confusion, amnesia and psychosis. Days to a few weeks later, the patients develop involuntary movements, classically choreathetosis and orofacial dyskinesia, fluctuations in conscious level, dysautonomia and, in some, episodes of central hypoventilation. As a result, despite current best therapy, the median length of hospital stay is 160 days [range 16-850] and many patients require admission to the ICU for assisted ventilation.

However, there is marked heterogeneity in the clinical presentation and many of the clinical features overlap between the various aetiologies of encephalitis. Therefore, urgent investigation to narrow this broad differential diagnosis is pivotal to early appropriate treatment.

1.6 Investigation

A LP is an essential investigation in the management of patients with suspected encephalitis both to confirm the diagnosis and to rule out other causes. There has been considerable controversy over the role of computed tomography [CT] and LP in patients with suspected CNS infection, in particular whether a CT is needed before an LP (Kneen et al 2002; van Crevel et al 2002). In patients with suspected encephalitis, an early CT scan has two clear roles: suggesting the diagnosis of viral encephalitis and indicating an alternative diagnosis. An initial CT scan soon after admission will show a suggestive abnormality in about 25-80% of patients with HSV encephalitis, though it is not, on its own, diagnostic (Raschilas et al 2002; Behzad-Behbahani et al 2003). An important role of CT, in some patients, is to exclude shift of brain compartments, due to mass lesions and/or oedema, which might make a subsequent LP dangerous. In patients with brain shift, a reduction of the CSF pressure below the lesion following a LP could precipitate herniation of the brainstem or cerebellar tonsils (Kneen et al 2002; Hasbun et al 2001). However, it is now recognised that clinical signs are sufficient to establish whether the LP can be performed with out being delayed for neuroimaging, and this is supported in national guidelines (Kneen et al 2012; Solomon et al 2012) [Figure 3].

Figure 3. The algorithm from the national Association of British Neurologists and British Infection Association encephalitis guideline, which describes the gold standard diagnostic and treatment pathway.



In adults with HSV encephalitis the CSF opening pressure is typically moderately elevated; there is a moderate CSF pleocytosis [tens to hundreds of cells per mm^3], a mildly elevated CSF protein, and normal CSF:plasma glucose ratio (McGrath et al 1997; Raschilas et al 2002; Kennedy et al 2005). Occasionally, polymorphonuclear cells predominate or the CSF may even be normal, especially early in the illness. In approximately 5-10% of adults with proven HSV encephalitis initial CSF findings may be normal with no pleocytosis and a negative HSV PCR (Raschilas et al 2002; Steiner et al 2005; Whitley et al 2006; Poissy et al 2009). The figure is even higher in the immunocompromised and in children, especially infants. However, if the first CSF is normal in patients with HSV encephalitis, a second CSF examination 24-48 hours is likely to be abnormal with a positive HSV PCR (Raschilas et al 2002; Steiner et al 2005).

A series of studies have shown the apparent difficulty in measuring plasma glucose at the same time as CSF glucose, but without the former, interpretation of the CSF results is very difficult (Kneen et al 2002; Bell et al 2009). HSV encephalitis can be haemorrhagic, and the CSF red cell count is elevated in approximately 50% of cases (Koskiniemi et al 1984). An acellular CSF is also described in encephalitis caused by other viruses, including VZV, EBV, and cytomegalovirus [CMV]; it occurs more frequently in the immunocompromised (Studahl et al 2000).

Although the list of viral causes of encephalitis is long, HSV 1 and 2, VZV and enteroviruses are the most commonly identified causes of viral encephalitis in immunocompetent individuals in Europe and the United States of America (Donoso

Mantke et al 2006; Glaser et al 2006; Solomon et al 2007; Granerod et al 2010). Our ability to diagnose encephalitis caused by herpes viruses & enteroviruses has been improved greatly by developments in PCR methodology (Read et al 1997; Tyler 2004). CSF PCR for HSV between day 2 and10 of illness has overall sensitivity and specificity of >95% for HSV encephalitis in immunocompetent adults (Cinque et al 1996; Steiner et al 2005). Although HSV PCR may be negative in the first few days of the illness. a second CSF taken 3–7 days later will often be HSV positive, even if aciclovir treatment has been started (Weil et al 2002; Tunkel et al 2008). However, others have identified a lower proportion of patients in whom a pathogen is identified if there are delays in performing the LP, particularly beyond 5 days from symptom onset (Davies et al 2005). It is unclear if this reflects a falling viral load in the CSF, and there has been conflicting findings as to whether this is a marker of disease severity and prognosis (Wildemann et al 1997; Domingues et al 1998).

Further microbiological investigations are based on specific epidemiological factors [age; animal, insect, and sexual contacts; immune status; occupation; recreational activities; geography and a recent travel history; season of the year; and vaccination history] and clinical findings [hepatitis, lymphadenopathy, rash, respiratory tract infection, retinitis, urinary symptoms and neurological syndrome] (Whitley et al 2002; Kennedy et al 2005; Sejvar et al 2006).

In a patient with suspected encephalitis with the appropriate clinical features serum VGKC-complex antibodies are tested in addition to imaging to identify an associated tumour (Vincent et al 2004). In NMDA-receptor antibody-associated encephalitis the CSF is frequently abnormal especially in the first phase with lymphocytosis [up to 500

cells/mm^3 has been reported] and detectable NMDA antibodies. CSF is not essential to make the diagnosis as the antibodies are also present in the serum, but paired samples are informative. All patients need investigation for an associated tumour, which in women, is almost always an ovarian teratoma (Dalmau et al 2011).

1.7 Treatment

Aciclovir is a nucleoside analogue with strong antiviral activity against HSV and related herpes viruses including VZV. Two randomised trials have shown that aciclovir [10mg/kg three times a day] improves the outcome in adults with HSV encephalitis reducing mortality to less than 20-30%; whereas patients who receive no antiviral medication have a mortality rate in excess of 70% (Skoldenberg et al 1984; Whitley et al 1986). Even with aciclovir treatment the outcome is often still poor, especially in patients with advanced age, a reduced coma score, or delays of more than 48 hours between hospital admission and starting treatment (McGrath et al 1997; Raschilas et al 2002). Because HSV encephalitis is the most commonly diagnosed viral encephalitis in industrialised countries, treatment with aciclovir is usually started once the initial CSF and/or imaging findings suggest viral encephalitis, without waiting for confirmation of HSV by PCR. However, in contrast to patients with meningococcal septicaemia, where a delay of minutes in initiation of treatment may be fatal, patients with encephalopathy and only mild confusion, investigation with a lumbar puncture before considering treatment is pragmatic, especially given the very wide differential diagnosis and relative the rarity of HSV encephalitis (Proulx et al 2005). Moreover, empirical use of antimicrobial agents can prematurely halt the

diagnostic pathway because clinicians feel falsely reassured; this delays the identification of other aetiologies for which different treatments might be appropriate.

Experience from paediatric practice has shown that the use of presumptive antiviral treatment for all patients with encephalopathy, without regard to the likely diagnosis, is not beneficial (Kneen et al 2010). However, if there is a strong clinical suspicion of encephalitis and potential delay in performing a LP, or the patient is rapidly deteriorating, then aciclovir should be started sooner. In HSV encephalitis the CSF usually remains positive by PCR for several days after starting aciclovir treatment; therefore where the LP is delayed, later CSF sampling can still confirm the diagnosis (Tunkel et al 2008). However, the viral load has been found to decrease rapidly after starting treatment with aciclovir (Kamei et al 2004). Therefore, attempts to assess the utility of the CSF viral load in predicting prognosis would need to take the timing of both symptom-onset and treatment being commenced before the LP.

Although aciclovir is a relatively safe drug it has important side effects. It is predominantly excreted by the kidneys, where it can cause renal impairment through crystalluria resulting in obstructive nephropathy (Sawyer et al 1988). This reversible nephropathy usually manifests after 4 days of intravenous therapy and can affect up to 20% of patients (Pacheco et al 2005).

Duration of treatment in the original randomised trials of aciclovir for HSV encephalitis was 10 days. However, reports of clinical relapse after 10 days treatment were published subsequently (VanLandingham et al 1988; Dennett et al 1996).

Although an on-going immune-mediated and inflammatory reaction to the infection is now thought by many to be the major pathogenic process of relapse, (VanLandingham et al 1988; Skoldenberg et al 2006) there is evidence for continuing viral replication in some cases (Raschilas et al 2002; Yamada et al 2003). As a consequence most clinicians now use at least 14-21 days intravenous treatment in confirmed cases and national guidelines advocate repeating a CSF examination at 14-21 days, and continuing treatment until the CSF is negative of virus by PCR (Kneen et al 2012; Solomon et al 2012).

For most patients with suspected HSV encephalitis, presumptive aciclovir treatment is started on the basis of a clinical picture and initial CSF findings consistent with viral encephalitis (Kneen et al 2012; Solomon et al 2012). Where the initial CSF reveals an alternative diagnosis, such as bacterial infection, the aciclovir can be safely stopped. However, as discussed, the initial CSF PCR can be negative in HSV encephalitis, especially if it is taken very early in the illness, or late in the illness after virus has been cleared, particularly if treatment has been started. Thus aciclovir treatment is not stopped on the basis of a single negative CSF PCR only, where HSV encephalitis is still suspected clinically.

The role of steroids in the treatment of HSV encephalitis is not established (Openshaw et al 2005). Even before antiviral drugs became available, many clinicians considered that corticosteroids were beneficial in HSV encephalitis, though others disagreed (Upton et al 1971; Habel et al 1972). Since the advent of aciclovir, corticosteroids have often be used, especially in patients with marked cerebral oedema, brain shift or

raised intracranial pressure, but their role remains controversial because, as well as reducing swelling, corticosteroids have strong immunomodulatory effects, which in theory could facilitate viral replication. Although, this has not been identified in murine models and a retrospective analysis of 45 patients with HSV encephalitis showed that older age, lower GCS on admission, and lack of administration of corticosteroids were significant independent predictors of a poor outcome (Kamei et al 2005; Meydig-Lamade et al 2003; Thompson et al 2000). Although there has not yet been a randomised placebo-controlled trial. Indeed it may be that a targeted neuroimmunological modulation may be able to reduce the pro-inflammatory response in the CNS without increasing viral load, although this has not yet been explored in HSV encephalitis.

1.8 Case Example

The key requirement for detection of acute viral encephalitis is an early LP and appropriate CSF analysis. This case example was chosen from my clinical experience during my neurological infectious disease training as it illustrates potentially important aspects of clinical management that may be associated with delays in suspicion of the diagnosis and delayed investigation that may reduce rates of detection.

A 43 year old right-handed successful deputy director of a London-based company was found to be acting in an irritable and uncharacteristically aggressive manner at work. Around mid-morning he was absent from his desk and is finally found by his

colleagues around the back of the office building in which he worked. At this time he is said to have been looking for a fire. Although his work colleagues told us that there was no evidence of this, the gentleman is adamant that he could intermittently smell smoke. His colleagues settled him back at his desk and he was said to appear more calm. In the early afternoon he was again absent from his desk. This time his colleagues were unable to find him anywhere on the premises and he was not answering his mobile telephone.

His location was finally established as he eventually answered his mobile telephone and, with some considerable encouragement, he was able to describe his surroundings. It became apparent that he was in a service station some 150 miles from the office.

An ambulance was directed to his location and he was admitted to the local district general hospital. His GCS score was determined to be 14/15 [Eyes: 4/4; Motor: 6/6; Verbal: 4/5]. The admitting doctor, who also established that his abbreviated mental test score was 8/10, confirmed this. The initial neurological examination did not identify any other abnormality. There was no evidence of meningism or rash and no stigmata of alcoholic liver disease or intravenous drug abuse. He was found to have a slightly elevated serum white cell count and C-reactive protein, normal urea and electrolytes and a normal arterial blood gas result. A ward test of his urine suggested the possibility of some blood 1+ and leucocytes 1+ and a formal microscopy and culture of his urine was requested. He was started on oral trimethoprim, admitted under the general surgical team on-take and referred for a urological opinion.

The next day, whilst awaiting urological review, he was reported to be acting in an increasingly bizarre manner, which was disturbing to both the nursing staff and other patients on the ward. He appeared to have strange ideas about the nursing staff, claiming that they are refusing to give him painkillers for his headache, despite having been given both oral paracetamol and tramadol. The security service and junior doctor on-call were contacted and he was given an intramuscular injection of haloperidol. The junior doctor noticed that the patient has been recorded as having an intermittent low-grade pyrexia for the last few hours, although he was not febrile on admission clerking or post-take ward round. His GCS at this time is recorded as 13/15 [Eyes: 4; Motor: 5; Verbal: 4]. In the context of fever, headache and confusion, the junior doctor suspected a possible CNS infection, such as meningitis. He took a peripheral blood culture, started intravenous cefotaxime and telephoned the medical registrar on-call for help with a performing a LP, but was advised to get a CT scan of the patients head first.

They were unable to get the CT scan until the next morning, which was performed without intravenous contrast, and is reported as normal. Once the CT report had been read, a LP was arranged to obtain CSF, this was performed approximately 24 hours after intravenous antibiotics have been started; the results of which are:

- Opening pressure: Not done
- CSF White cell count 45 [85% lymphocytes, 15% neutrophils]
- CSF Red blood cells 4
- CSF Protein 0.65g/dl
- CSF Glucose 52mg/dl
- Plasma Glucose: Not sent

- CSF Microscopy: No organisms seen
- CSF Bacterial culture: Awaited
- CSF Virology: Not Sent

On the basis of the lymphocyte predominance, intravenous aciclovir 10mg/kg eight hourly was started. However, as there was a mixed lymphocyte and neutrophil picture and there were delays in performing the LP after starting antibiotics, the team decided to continue the cefotaxime also.

On the morning of day 6 of admission, the patient was found to have a GCS of 7/15 [Eyes: 2; Verbal: 2; Motor: 3]. His pupils were equal and reactive and his plantars were flexor; he was thought to have bilateral papilloedema. He had a repeat CT scan, again without intravenous contrast, which did not identify any specific focal abnormality, although there was some suggestion of effacement of the sulci, possibly more of the right hemisphere.

The on-call neurology registrar was contacted who established, through discussion with the nursing staff, that the GCS has actually been fluctuating between 6-13/15 over the last 1-2 hours and that he had been having some subtle twitching movements of the lower part of the right side of his face. An urgent electroencephalogram [EEG] was arranged, a human immunodeficiency virus [HIV] test was requested and the team were asked to ask the microbiology laboratory to find the CSF sample and send it for viral PCR at the virology laboratory. The EEG confirmed subtle motor status epilepticus; intravenous lorazepam and then phenytoin was given and the patient was transferred to the intensive care unit. At this time the CSF culture was reported as

negative and therefore the cefotaxime is stopped. After 36 hours his consciousness improved; initially to a GCS of 14/15 with mild confusion. The following day, the result from the PCR analysis of his CSF became available, which was negative for HSV types 1 and 2, VZV and enterovirus. The aciclovir was continued, he was transferred to the high dependency unit and the LP was repeated on day 8:

- Opening pressure: 28cm H_2O
- CSF White cell count 32 [90% Lymphocytes, 10% neutrophils]
- CSF Red blood cells 6
- CSF Protein 0.83g/dl
- CSF:Serum Glucose ratio 78%
- CSF Microscopy: No organisms seen
- CSF Bacterial culture: Negative
- CSF Virology:
    - Antibody-positive for HSV type 1 [serum:CSF albumin ratio confirms intrathecal production]
    - PCR Positive for HSV type 1 [result available after antibody]

His HIV antigen and antibody test were negative, his GCS returned to 15/15 and he was discharged after 12 days of intravenous aciclovir. An MRI scan identified bilateral but asymmetrical high signal on T2 weighted images affecting the mesial temporal lobes and, to a lesser extent, the orbitofrontal cortex.

At 12 month follow-up he reported impaired short-term memory and frequent headaches, both of which are limiting his ability to work. He is continuing to suffer with simple-partial seizures every couple of months; although this is some improvement on the previous frequency and he had no further complex partial seizures on combination antiepileptic drug therapy.

This case highlights the common clinical pathway for many patients with suspected HSV encephalitis, with delays in suspicion, delay in investigation, sub-optimal CSF investigations performed, and extensive CNS inflammation on imaging, with consequent neurological morbidity. However, it is unclear currently to what extent poor outcome reflects delays in diagnosis, viral load, or the uncontrolled inflammatory response.

1.9 Inflammation in viral encephalitis

The central nervous system has been considered an 'immunologically privileged site' since the phrase was first coined by Billingham and Boswell in 1953 (Rothwell et al 2002). However, recent years have seen important advances in our understanding of significance of immunological processes within the CNS both in pathophysiological conditions and in healthy individuals; and now our understanding of the dynamic interplay between various cells within the CNS and the wider immune system is expanding (Gibson et al 2004). These improvements in our understanding have been driven by advances in proteomic and transcriptomic techniques, novel neuroimaging

techniques and development of animal and cell culture models (Sener et al 2001; Griffiths et al 2012; Patabendige et al 2012).

The potential for pathophysiological impact of immunological processes is of particular importance in the CNS as, although there is some room for a degree of neuronal plasticity, there is limited scope for functional repair, and many patients are left with significant sequelae following viral encephalitis (Easton et al 2007).

1.9.1 The role of the blood-brain barrier

There are three barriers that are pertinent when considering leucocyte penetration into the brain parenchyma. Firstly, the blood-brain barrier [BBB], which represents the largest surface area for exchange, formed by the juxtaposition of astrocyte foot processes and endothelial cells forming gliovascular units. Secondly, the endothelial-ependymal interaction at the choroid plexuses, which are the sites of CSF production in the lateral, third and fourth ventricles. Thirdly, the CSF-brain barrier formed by the pia covering the surface of the brain parenchyma (Abbott et al 2006). Therefore, studies comparing CSF and serum cytokines and associated mediators are assessing the second of these three barriers and only represent a proxy marker of parenchymal processes. Penetration of peripheral leucocytes trafficking into the brain parenchyma is regulated by the tight junctions between the endothelial cells and astrocytic foot processes, which cover 90% of the blood-brain barrier, in addition to pericytes and smooth muscle in larger vessels (Patabendige et al 2012). These tight junctions, which form the zona occludens, are composed of transmembrane cytoplasmic proteins, such as ocludin and junction adhesion molecules, linked to an actin based cytoskeleton,

which has the ability for rapid regulation (Abbott et al 2010). The astrocytic foot processes appear to be important for the development and maintenance of tight junctions and enhancement of the barrier function of endothelial cells. Small gaseous and lipophilic molecules such as oxygen and ethanol diffuse through the lipid membranes whereas hydrophilic molecules, including peptides, are transported by specific membrane transporters present on the luminal and abluminal membranes (Abbott et al 2006). As opposed to peripheral vasculature, the brain endothelium has a much lesser endocytosis and transcytosis activity. Nevertheless, the BBB has a pivotal role in supplying the brain with essential nutrients, removing toxic metabolites, and regulating ionic constituents of interstitial fluid to maintain a homeostatic microenvironment (Abbott et al 2010; Patabendige et al 2012).

The BBB can rapidly increase in permeability and promote leucocyte trafficking following signals arising from the brain parenchyma or the immune system; notably protein kinase C, cAMP, G protein coupled events, calcium and cytokines/chemokines/growth factors (Persidsky et al 2006). Activated compounds in the CSF and serum also facilitate leucocyte migration, including a broad range of cytokines/chemokines, leukotriene and eicosanoids such as prostaglandin E2 (Rothwell et al 2002). The nature of the leucocyte populations recruited is strongly influenced by the, now over 50, chemokines that have been identified to be present following the production of cytokines. Generally the 'CXC' chemokines, such as CXCL14 [Macrophage Inflammatory Protein-2], are responsible for the recruitment of neutrophils and the 'CCL' chemokines, such as CCL5 [Regulated on Activation, Normal T Cell Expressed and Secreted: RANTES] and CCL 2 [Monocyte Chemotactic Protein: MCP-1], are responsible for the recruitment of monocytes and lymphocytes (Griffith et

al 2014). Additionally these chemokines have specific binding sites on brain microvessels. Transmembrane glycoproteins also play an important role, including vascular cell adhesion molecule [VCAM] and intercellular adhesion molecule [ICAM] (Rothwell et al 2002). Additionally, the presence of neutrophils on the BBB endothelium further disrupts the BBB and promotes permeability through changes in the cytoskeleton and disassembly of the junction complex due to increased phosphorylation of tyrosine residues and decreased expression of zonale occludens-1 in addition to other processes. Significant differences have been identified in the relative concentration of various CSF constituents, particularly peptides and relative macrophage populations, between ventricular and lumbar CSF, although this may be diminished in association with increased BBB and blood-CSF permeability, as is seen in meningoencephalitis (Bradner et al 2014; Sommer et al 2002).

1.9.2 Resident neuroimmunologically active cells

In addition to those immune effector cells that migrate into the CNS, within the CNS there is already the presence of microglia, which are derived from the macrophage/monocyte lineage cells within the bone marrow. These are resident effectors of immune function capable of a rapid response to neuronal injury, such as that due to Wallerian degeneration, excitotoxic processes, osmotic changes, ischaemia and infection (Love et al 2008). Nevertheless, microglia express fewer surface antigens during health than other resident tissue macrophages and therefore are unable to promote a primary T cell mediated response. Although there is rapid up-regulation of antigen expression upon microglial activation, including up-regulation of class I and II major histocompatibility antigens, CD4, and amyloid precursor protein,

but this may be less than that seen in other resident tissue macrophages in other organs (Rothwell et al 2002). Upon activation, microglia also change to an amoeboid morphology, significantly increase phagocytic capacity and production of cytokines/chemokines (Love et al 2008). Additionally, within the perivascular areas of the meninges and choroid plexuses macrophages are present in healthy individuals.

1.9.3 The role of cytokine/chemokines

There is growing evidence that cytokines and chemokines are key pro- and anti-inflammatory mediators in the CNS (Kamei et al 2009; Marcotte et al 2013; Michael et al 2013). These small peptides act predominantly in an autocrine and paracrine fashion to exert a wide range of effects, which include promoting and retarding both microglial activation and BBB permeability and leucocyte recruitment/activation, in addition to having roles in apoptosis, and excitotoxic injury (Labus et al 2014). Analysis of the action of these peptides is made difficult as a single cytokine can enact opposing effects dependent on the timing and concentration of the peptide and the presence or absence of promoting or inhibitory peptides at the time of expression (Boutin et al 2003; Pinteaux et al 2009). Therefore, the investigator must develop models for analysing the relative importance of multiple cytokines/chemokines and associated co-regulators in a single sample to be able to accurately draw assumptions about activity (Boutin et al 2003; Michael et al 2013). For example, the prototypical pro-inflammatory cytokine IL-1 exists as IL-1α and IL-1β and can act through two distinct receptors, IL-1 RI and IL-1-RII, and additionally has a naturally occurring receptor antagonist, IL-1Ra (Alheim et al 1998; Boutin et al 2003). In addition TNFα acts through two distinct receptors, TNFRI and TNFRII (Wright et al 1994). Therefore,

previous studies of clinical samples which have been limited to the assessment of a narrow range of mediators, typically using enzyme-linked immunosorbent assay [ELISA] which requires both significant time and sample volumes, have been unable to draw robust conclusions (Ichiyama et al 2008). For example, the largest study of these mediators in viral encephalitis assessed patients with enteroviral infection and found that both the pro-inflammatory cytokine IL1β and its antagonist were associated with greater clinical disease severity and complications (Griffiths et al 2012). Therefore, it is likely that the relative concentrations of cytokines/chemokines is of greater importance. This approach has been found to be associated with disease severity and outcome in patients with encephalitis due to Japanese encephalitis virus and cerebral malaria (Day et al 1999; Winter et al 2004). However, these studies assessed simple ratios between 2 cytokines and, whilst useful, more inclusive multivariate approaches are needed to better understand the interaction of these mediators in determining disease severity and outcome, and for a better understanding of pathophysiology. These techniques have started to be taken forward in studies of CNS inflammation caused by chronic viral infection and autoantibodies (Marcotte et al 2013; Michael et al 2013).

1.9.4 Assesment of inflammatory mediators

Due to the demonstrable variation of these mediators over time in response to local and peripheral insults, much research has focused on animal or cell-culture models of disease, which can allow repeated sampling and analysis over time. These has proven an effective tool to investigate cytokine-mediated responses in other neurological diseases, such as the middle-cerebral artery occlusion murine model of

cerebrovascular accidents and has led on to the development of clinical trials of the use of therapeutic, targeted cytokine antagonist administration (Emsley et al 2005; de Bilbao et al 2009). However, this is more limited in the field of neurological infection: There is currently no robust, clinically applicable animal model for acute HSV encephalitis, as all require primary intranasal infection rather than the intra-neuronal reactivation, retrograde neuronal transport and early [possibly even pre-encephalitis] brain parenchymal infection (Ellermann-Eriksen et al 2005). Therefore, despite the limitations of sample time points, clinical samples from patients with viral encephalitis arguably remain the most accurate by which to investigate the neuroimmunological response.

1.10 Scope of this thesis

Acute viral encephalitis is a severe form of CNS inflammation with high morbidity and mortality despite current antiviral treatments. However, the epidemiology of viral encephalitis is not completely understood, especially during pandemic viral infections; whether novel technological approaches can be developed with the potential to improve epidemiological tracking is not known. Epidemiological studies are further hampered by sub-optimal identification of individual cases of acute viral encephalitis, although it is not well known what aspects of clinical management result in delays in diagnosis and missed diagnoses. It is also not clear what impact these delays may have on the viral load, both in terms of the implications for likelihood of viral detection and prognosis. Moreover the potential for simple, practical interventions to increase the diagnosis of acute viral encephalitis at both an individual and disease surveillance level has not been fully explored. In addition, it has long been

recognised that there is significant inflammation of the CNS in acute viral encephalitis and there is growing evidence that the cytokine/chemokine-mediated response may be important. However, it is not known if this response is different between the aetiologies of acute encephalitis or which aspects of this response correlate with disease severity and outcome. An improved understanding of these innate pathophysiological processes may improve the diagnosis of acute viral encephalitis and, ultimately, pave the way for research to develop adjunctive anti-inflammatory therapies. Therefore, this thesis will set out to address the following questions:

1. What is the epidemiology of acute viral encephalitis in the UK, in the context of a pandemic influenza infection?
2. Can novel technologies be developed which can collect data on acute viral encephalitis that have the potential to enhance disease reporting?
3. What aspects of clinical management of acute viral encephalitis are important contributors to sub-optimal detection of the condition?
4. Do delays in performing the LP, one key aspect of the management, affect the viral load, and hence the ability to make a diagnosis?
5. Can the introduction of a simple LP pack improve sample collection and therefore rates of diagnosis of acute viral encephalitis?
6. Do cytokine/chemokine responses in acute encephalitis differ between those with viral or immune-mediated aetiologies? If so, can such differences be used to help us begin to classify those in whom no aetiology is established?
7. Are these cytokine/chemokine-mediated responses associated with disease severity and outcome, which might thus point towards new avenues for treatment?

2. Materials and Methods

2.1 Cohorts

2.1.1 Pilot cohorts to develop techniques

To explore the technique of cytometric bead array and to better understand multivariate statistical interpretation of these data, pilot work was conducted on samples from two cohorts of patients with CNS inflammation.

To prepare for the main Health Protection Agency [HPA] cohort in which I would undertake an assessment of mediator profiles between patients with CNS inflammation due to viruses in comparison to antibodies, I accessed patient cohorts with chronic CNS inflammation related to viral infection or antibody associated disease. I wanted first to assess whether mediator profiles could be used to distinguish between aetiological groups, and secondly whether, within a cohort with chronic viral infection, mediator profiles were associated with disease severity.

To determine if cytokine and associated mediator profiles could distinguish between the aetiology of two clinically similar syndromes that may or may not be due to a distinct antibody I identified samples from patients with chronic CNS inflammation due to the antibody, anti-aquaporin 4. These were recruited consecutively in to the Neuromyelitis [NMO]-UK study and these were compared with samples from patients attending the same clinic but who were negative for the aquaporin 4 antibody and in whom a diagnosis of multiple sclerosis was established. Cases were grouped into the diagnostic definitions as “Aquaporin 4 Ab positive NMO” or “Multiple sclerosis” based

on the established criteria (McDonald et al 2001; Wingerchuk et al 2006). Serum samples were initially chosen at random and differences in the key marker of disease severity, the extended disability severity scale [EDSS], was minimised between the groups. Serum samples were collected at recruitment and stored at -70^{o}C and freeze thaw cycles were minimised.

To assess whether cytokines and associated mediators could be predicative of disease severity, samples from patients with chronic CNS inflammation and chronic viral infection were analysed. These 98 patients were recruited through the longitudinal components of the CNS HIV Antiretroviral Effects Research [CHARTER] and HIV Neurobehavioral Research Center research programs. Cases were identified by screening nearly 3,500 visits from 430 participants who were assessed between August 1999 and December 2008. Cases were selected in if they had at least three consecutive visits and availability of at least 6 mL of blood and CSF specimens stored at -80°C. Cases were excluded if they had severe neurocognitive co-morbidities [e.g., history of severe traumatic brain injury, mental retardation, as described by published criteria (Antinori A 2007)]. All participants had a third visit, which was used to verify the stability of the neurocognitive grouping. For the work of this project, only the first two visits were used in analyses.

These two studies provided the opportunity to explore the technique of cytometric bead array, with a focus on optimising the accuracy of data generated. In addition, these data were explored using multivariate statistical analyses, including those described below, which allowed for assessment of the impact of mediator concentrations in the context of the concentration of associated mediators. Both these

approaches were pivotal to the work I conducted with samples and data from the HPA cohort of acute encephalitis. Data from this pilot work on chronic CNS inflammation is not presented in the body of this thesis, but the published manuscripts arising from this are included in the 'related publications' section.

2.1.2 Health Protection Agency Cohort

Patients were recruited prospectively through the HPA study of Encephalitis in England, which identified 217 patients with acute encephalitis from 20 centres over 2 years [2004-2006], the methodology is described in detail previously (Granerod J 2010). In brief, the case definition included any person of any age admitted to hospital with encephalopathy [altered consciousness that persisted for longer than 24 hours, including lethargy, irritability, or a change in personality and behaviour] and with two or more of the following: fever or history of fever [≥38^{o}C] during the presenting illness; seizures and/or focal neurological findings [with evidence of brain parenchyma involvement]; CSF pleocytosis [>4cells/μL]; EEG findings indicative of encephalitis; and abnormal results of neuroimaging CT or MRI suggestive of encephalitis. All patients underwent an extensive panel of post-clinical CSF testing for RNA, DNA and antibodies to establish the aetiology which was determined by immune status, travel history and, when no aetiology was established, further second-line testing as directed by HPA expert panel review, described in detail in chapter 8.

2.1.2.1 Markers of clinical and outcome severity

The markers of clinical disease severity in the HPA cohort (chapter 8 and 9) were the admission GCS score and the presence of seizures, as these features have been identified to be associated with worse outcome in previous studies (Solomon et al 2007; Michael et al 2012). A normal GCS [15/15] was defined as good and an altered GCS [≤14/15] as poor. Outcome was determined in the both the viral load study (chapter 6) and HPA cohort (chapter 8 and 9) by the Glasgow outcome scale [GOS] score recorded at hospital discharge, with a good outcome defined as a GOS of 5/5 [minor-no disability] and a poor outcome defined as a GOS of ≤4/5 [moderate disability-death], as described previously (Kamei et al 2009). CSF white cell count and differential were recorded where available. CSF samples collected within the first 48 hours of symptoms were excluded from subgroup analysis of white cell count [WCC] correlations, as these may be falsely low during this period (Solomon et al 2012).

2.1.3 Neurohistopathology cohort

Histopathological samples were obtained from brain biopsy and post-mortem material of patients with acute encephalitis identified from the Walton Centre Neurohistopathology database. These cases were HSV encephalitis [n=2], immune-mediated/paraneoplastic encephalitis [n=4], encephalitis with unknown aetiology despite biopsy [n=2]. These slides underwent immunohistochemistry with haematoxylin and eosin to assess for neutrophils and common surface markers reflecting leucocyte subsets CD3 [T cell], CD4 [T helper], CD8 [cytotoxic T cell], CD79a [B cell], CD68 [monocytes/macrophage/microglia]. The population of each subset was

quantified by light microscopy at both the immediate perivascular space and the surrounding parenchyma [<150um from adjacent Virchow-Robin space]. This process was repeated across three brain regions within the temporal and frontal lobes. CD68 positive cells were only recorded if identified in amoeboid morphology reflecting either activated microglia or macrophage/monocyte cells.

2.1.4 Blood brain barrier model

The BBB model has been developed to assess the impact of viral infections on the permeability of this key barrier between the brain and leucocytes and inflammatory peptides in the serum. It is an *in vitro* model composed of human brain endothelial cells [HBEC] cultured on a semipermeable filter and primary human astrocytes. The co-culture model allowed for samples to be taken at multiple time-points from both the luminal, reflecting the blood, and abluminal sides, reflecting the brain side, of the model. This therefore allowed for a direct comparison of mediator profiles and kinetics between proxy markers of intrathecal/parenchymal and intravascular spaces that was not possible in clinical samples. At multiple time points it was possible to assess a proxy marker of the integrity of the barrier by measuring the resistance of electrical conduction across the model, termed the transendothelial electrical resistance [TEER]. The composition of BBB model is described in detail elsewhere (Patabendige 2012). In brief, to set up the co-culture BBB model, primary human astrocytes [catalogue number: 1800; ScienCell Research Laboratories, USA] at passage 2-5 were grown in poly-D-lysine-coated 12-well plates at 1.0×10^5 cells/well and were allowed to grow to confluence. Immortalised HBEC-5i cells were grown in

collagen-coated T75 flasks and maintained in Dulbecco's Modified Eagle's Medium [DMEM, Life Technologies] and seeded on collagen-coated Corning Transwell-Clear inserts at 1.0 x 10^5 cells/insert; then were transferred to a 12-well plate containing confluent astrocytes. TEER across the model was determined using electrodes connected to an EVOM2 epithelial voltohmmeter [World Precision Instruments, Hertfordshire, UK]. The resistance of cells grown on Transwell filter inserts was corrected for resistance across an empty collagen-coated Transwell insert, and multiplied by surface area, to give TEER in ohms x cm^2 [$\Omega.cm^2$]. Experiments were performed when HBEC-5i cells were confluent, typically within 3 days of seeding and when TEER across the monolayer was over 50 $\Omega.cm^2$. The model was infected with HSV from the abluminal 'brain' side at a multiplicity of infection [MOI] of 0.01. Virus diluent media [DMEM with 2% FBS] was used for mock-infected controls. TEER measurements and samples were taken immediately post infection and at time points 6, 12, 24 hrs from both the luminal and abluminal sides for cytokine analysis.

2.2 Measurement of mediators

2.2.1 Mediators in pilot cohorts

Serum and CSF samples were stored at -80^{o}C and were not thawed until the time of assay where possible; throughout freeze-thaw cycles were minimised. Cytokines and associated mediators for this analysis were measured using commercially available immunoassays according to the instructions of the manufacturer [Procarta ® Immunoassay kit, Panomic Solutions, Affymetrix ® Milano, Itay]. Where possible mediators were measured using multiplex cytometric bead array. Where this was not possible single-plex kits or ELISA were used [EMD Millipore, Billerica, MA and

Quantikine; R&D Systems, Minneapolis, MN USA]. In summary, multiplex cytometric bead array data were generated through determination of the fluorescence intensity using a Bio-Rad platform [BioPlex Manager 4.1, Bio-Rad Laboratories ©, Hertfordshire, UK]. Standard curves were adjusted at the points of fluorescence intensity saturation, at the extremes of the scale, to generate a sigmoid standard curve with 6-8 fluorescence intensity points. The concentration of each mediator was assessed relative to this fluorescence intensity on the same standard curve for each sample. To ensure precision of mediators assessments, in both cohorts, samples were analysed in duplicate with the mean value established, and measurements repeated when coefficients of variation greater than 20% or outliers that were more than 3 standard deviations from the mean were found.

To avoid undetectable levels of mediators or missing data biasing, only mediators that were detected in at least 80% of the cohort were included in the analysis. As sample storage, and particularly the number of freeze-thaw cycles, is recognised to have a significant and deleterious impact on these mediators in the CSF and serum , mediator concentrations were log transformed and then median centred for each patient, using established methodology as described previously (Griffiths et al 2012). This allows for the assessment of the concentration of each mediator relative to the standard median mediator concentration within each sample.

2.2.2 Mediators in Health Protection Agency Cohort

2.2.2.1 Cytometric bead array

I assessed thirty-eight mediators [cytokines, chemokines and associated co-mediators] using a commercial cytometric magnetic-bead array assay system [Procarta ® Immunoassay kit, Panomic Solutions, Affymetrix ® Milano, Italy] in accordance with the manufacturers' instructions. The reliability in comparison with conventional ELISA has been confirmed (Khan, Smith et al. 2004). The mediators assessed were identified from previous literature and chosen to reflect in part leucocyte proliferation/differentiation [granulocyte-colony stimulating factor [G-CSF], granulocyte-macrophage-colony stimulating factor [GM-CSF], granzyme B, myeloperoxidase [MPO], leptin and eSelectin]; leucocyte chemotaxis [CCL2 [MCP1]], CCL3 [monocyte inflammatory protein 1a], CCL5 [regulated on activation normal T cell expressed and secreted], CXCL9, CXCL10 [inducible protein 10]]; adhesion molecules [vascular cell adhesion molecule [VCAM], intracellular adhesion molecule [ICAM]]; markers of blood-brain barrier permeability [vascular endothelial growth factor [VEGF]a, and matrix metalloproteinases [MMP] 1,2,3,7,8,9,12,13]; antiviral peptides [interferon [IFN] α, β, ο, γ, TNFα and its soluble receptors [TNFR1 and TNFR2] and TNFa-related apoptosis inducing ligand [TRAIL]]; and broadly pro-inflammatory interleukins [1α, 1β, 6, 8, 17a]; and broadly anti-inflammatory interleukins [IL1-receptor antagonist [IL1RA], IL4, IL10]. Fluorescence intensity was determined using a Bio-Rad platform [BioPlex Manager 4.1, Bio-Rad Laboratories ©, Hertfordshire, UK]. Again, standards and samples were analysed in duplicate and the mean value used in analysis, measurements with coefficients of variation greater than 20% were removed. Again, standard curves were adjusted at the points of

fluorescence intensity saturation, to generate a sigmoid standard curve with 6-8 fluorescence intensity points. To avoid undetectable levels of mediators or missing data biasing the analysis, only mediators that were detected in at least 80% of the cohort were included in the analysis (Griffiths et al 2012; Michael et al 2013). To minimise the potential impact of sample storage, mediator concentrations were median centred and normalised for each patient, using established methodology as described previously (Michael et al 2013). In addition to analysis of each mediator the pro- to anti-inflammatory balance was also assessed using the IL6:IL4, IL1:IL1RA and IL1:IL10 ratios as described (Day et al 1999; Gibson et al 2004; Winter et al 2004). The concentration of TNFα was assessed in isolation and as a ratio with its soluble decoy receptors TNFR1 and 2, as a proxy marker of the concentration of TNFα that is soluble and active (McCoy et al 2008; Rodrigues et al 2009).

2.2.2.2 Blood-brain barrier permeability

I assessed the CSF:serum albumin ratio using radial immunodiffusion as described previously, in accordance with the manufacturer's instructions [Binding Site, Birmingham, UK©]. I analysed the relationship between this ratio and the CSF albumin concentration in isolation so that analysis could be undertaken using patients where only CSF samples were available without paired serum (Reiber et al 1995).

2.2.2.3 Quantitative real-time polymerase chain reaction

To determine the viral load in the CSF of patients in both the viral load study (chapter 6) and for the HPA cohort studies (chapters 8 and 9) I conducted real-time qPCR using a validated commercial assay [Artus HSV type 1 and 2, and VZV LC-PCR, Qiagen Group], and processed this using a Light-Cycler analyser [Roche Diagnostics™ 2007].

2.3 Statistics

2.3.1 One-way hierarchical cluster analysis, nearest neighbour analysis and proximity matrix heat-maps

I conducted this analysis to generate a visual representation of the interaction of all mediators with each other in each of the diagnostic groups. Firstly, overall mediator data from all groups collectively underwent a one-way hierarchical cluster analysis. This groups mediators with each other in Euclidean space, with those for which the variations in concentration most closely correlates being most closely grouped. Then mediator data from each of the aetiological groups underwent nearest neighbour analysis, using Pearson's correlation coefficient, to generate proximity matrices [representing the strength of correlation between mediators], with the mediators ordered as grouped in the original cluster analysis, using SPSS [SPSS Inc © 2011]; a correlation was expressed from a positive correlation of 1 to a negative correlation of -1. From these matrices, heat-maps were generated using SigmaPlot [SigmaPlot ©, Systat Software Inc., San Jose, California, USA], with a positive correlation of 1 represented as red, a negative correlation of -1 as blue and no correlation as 0, as described previously (Szodoray et al 2007). This approach was explored in the cohort

of patients with chronic CNS inflammation due to antibodies and, following publication of this methodology, was applied to the HPA cohort (Michael et al 2013).

2.3.2 Supervised univariate and multivariate discrimination function analysis

The Mann Whitney U test was performed to assess for differences in the median concentration of each mediator between the groups, based on aetiology, clinical severity or outcome using SPSS [SPSS Inc © 2011]. Nevertheless the pilot studies confirmed the importance of assessing the relative concentration of mediators in addition to assessing mediators in isolation (Marcotte et al 2013; Michael et al 2013). Therefore, predictive multivariate models were developed with the complete set of normalised mediators in concert using a step-wise discriminant function analysis [DFA]. This technique further assists in aetiological group classification by generating a predictive model based not just the absolute concentration of the selected mediators, but also on a numerical weighting that is applied to selected mediators that best discriminate between the diagnostic groups within the model. Each mediator is assessed in turn to determine the accuracy with which the model will determine appropriate group allocation. The mediator at each step that best minimises the Wilk's Lambda entered, with a value of 1 as no correlation and 0 as optimal appropriate group allocation. If a mediator had a maximum significance of F of 0.05, equivalent to a p value of 0.05, then it would be added to the model and if a mediator reduced the F to 0.1, equivalent to a p value of 0.1, it would be removed from the model. This approach has the potential to include none, some, or even all mediators in the generated model and preferentially selects those which best determine the appropriate group allocation. This was used to select the group of mediators that

maximally differentiated between the diagnostic groups. Selected analytes then underwent a leave-one-out cross-validation to protect against over-fitting and type 1 error. The resultant power of each analyte is expressed using by a Wilk's Lambda coefficient, from perfect discriminatory power [0.0] to no discriminatory power [1.0]. This technique further assists in group classification by generating a predictive model based not just the absolute concentration of the selected mediators, but also on a numerical weighting that is applied to selected mediators that best discriminate between the diagnostic groups within the model. Statistical significance was defined throughout as $p<0.05$.

2.4 Ethical Approval

2.4.1 Pilot cohorts

For the pilot work assessing inflammatory mediators in the context of chronic viral infection, the protocol was approved by the Institutional Review Board for the CHARTER Study Group, and the University of California San Diego Human Research Protection Program approved the study. For the pilot work assessing inflammatory mediators in antibody-mediated CNS inflammation, ethical approval was obtained as part of the national NMO-UK study funded by the National Commissioning Group [UK].

2.4.2 Epidemiological surveillance study

For the study of the epidemiological surveillance of encephalitis in relation to an influenza pandemic [chapter 3], approval was obtained from the Clinical Research and

Academic Committee of the British Neurological Surveillance Unit and the British Paediatric Neurological Surveillance Unit, under the criteria of 'surveillance' as determined by the National Patient Safety Agency.

2.4.3 Sub-optimal investigation studies

For the multi-centre retrospective cohort study of clinical investigation [chapter 4], ethical approval was obtained from the clinical audit and research departments of the hospitals at which data were collected: University Hospital Aintree NHS Foundation Trust, the Countess of Chester NHS Foundation Trust, Mid-Cheshire NHS Foundation Trust, Warrington and Halton NHS Foundation Trust, Whiston and St Helens NHS Foundation Trust, Central Manchester University Hospitals NHS Foundation Trust, University Hospital South Manchester NHS Foundation Trust, North Manchester General Hospital, Pennine Acute Hospitals NHS Trust, and East Cheshire NHS Trust. For the analysis of the impact on viral load [chapter 6] samples were analysed in accordance with the protocol for optimising standard operating procedures for the Liverpool Specialist Virology Laboratory. For the study of the impact of the LP pack [chapter 7] audit approval was obtained from the Clinical Audit and Research Department of the Royal Liverpool Hospital NHS Foundation Trust.

2.4.4 Cytokine and associated mediator studies

Samples were analysed from patients recruited in the HPA study of Encephalitis in England, which received approval from the North and East Devon Multicentre Research Ethics Committee [Reference: 05/Q2102/22]. This sub-study was approved

by the HPA Encephalitis Study Steering Group and also approved by the Pan-Manchester Research and Development Group for the University of Manchester.

3. Detection at population level- Limits of current epidemiological surveillance of encephalitis in relation to an influenza pandemic

3.1 Introduction

The most common sporadic cause of encephalitis is HSV type 1, representing an uncommon complication of a common infection (Granerod et al 2010). Whilst globally acute viral encephalitis is often due to epidemic pathogens, such as Japanese encephalitis virus, the epidemiology of encephalitis in relation to a pandemic viral infection is poorly understood, particularly in the UK (Surana et al 2011).

The emergence of novel influenza A:H1N1 in 2009 was associated with the appearance of case reports and series describing related neurological manifestations (Centers for Disease Control and Prevention 2009; Ekstrand et al 2010; Farooq et al 2012; Khandaker et al 2012). While there is no robust evidence that H1N1 is more likely than other subtypes to cause neurological manifestations, it may be associated with more severe neurological syndromes (Ekstrand et al 2010).

Influenza infection is associated with a wide variety of acute neurological presentations, of which febrile seizures and encephalopathy are the most commonly reported (Glaser et al 2012; Khandaker et al 2012). Approximately three quarters of all influenza-related neurological manifestations occur in children (Glaser et al 2012). In Japan, influenza is the most commonly identified pathogen in acute encephalopathy,

and it is a notifiable condition (Okabe et al 2000; Hoshino et al 2012). In the USA and Australia, between 6-19% of children hospitalised with influenza infection have neurological manifestations (Ekstrand et al 2010; Farooq et al 2012; Khandaker et al 2012). With the increasing availability of MRI and newer imaging modalities, the generic term "influenza-related encephalopathy/encephalitis" has been sub-classified into a range of specific acute encephalopathy syndromes [AES] e.g. acute necrotizing encephalopathy [ANE] (Mizuguchi et al 1995; Akins et al 2010). Fewer specific AES have been described in adults but acute haemorrhagic leukoencephalopathy [AHL] appears to be important (Cisse et al 2010). Identification and description of AES is useful to help predict the prognosis and ultimately will improve our understanding of the pathophysiology of these disorders. For example, the delineation of ANE and the description of familial cases led to the discovery of inherited mutations in the Ran-binding Protein 2 [RANBP2] gene (Neilson et al 2009).

However, most previous publications of encephalitis and associated complications of pandemic infections has been limited to case reports and small series, from single centres. In addition, comparisons across these has been made more difficult as standardised case definitions have not been used and most have been restricted to adults or children.

Therefore, I led a multicentre surveillance study, through national surveillance units, to examine the neurological manifestations of influenza following the H1N1 pandemic in adults and children across the UK applying standardised case definitions. This study collected data on the spectrum of manifestations seen and their sequelae, describes selected cases of specific AES in more detail, proposes a modification of the

current classification system, and explores the strengths and weaknesses of this surveillance programme (Akins et al 2010).

3.2 Methods

A national multicentre surveillance study of children and adults was performed through the British paediatric neurology surveillance unit [BPNSU- http://www.bpnsu.co.uk], and British neurological surveillance unit [BNSU- http://www.theabn.org/what-we-do/bnsu.html], respectively, between February 2011 to February 2013; the surveillance allowed reporting of current cases, or those in the preceding 6 months. Cases were notified by doctors in response to a monthly email to all members of the British Paediatric Neurology Association and the Association of British Neurologists respectively. In addition the regional microbiology laboratories for adults and paediatrics in the Northwest Neurological Infections Network was screened for patients undergoing investigation for influenza. In addition, colleagues at a large specialist children's hospital in London and large regional neurology service in Manchester who are part of the Brain Infections UK Group and who were collaborating on related studies on encephalitis were recruited to identify patients.

Cases were notified if they had an acute neurological presentation occurring within 1 month of proven influenza infection: defined as positive PCR for influenza RNA from throat or nasal swab, respiratory secretions, serum or CSF. Clinical case definitions were adapted from those used previously for CNS infections based on the presenting clinical features, CSF and imaging findings [Table 4] (Michael et al 2010; Kelly et al

2012). Children were defined as those aged under 18 years.

Table 4. Case definitions for influenza-related acute neurological illnesses

Encephalopathy only - alteration in consciousness including behavioural changes with no evidence of inflammation in the CNS.
Encephalitis – encephalopathy (as above) with evidence of CNS inflammation (CSF white cell count >4/ml, or MRI consistent with inflammation).
Meningism only – Meningism (headache with neck stiffness and/or photophobia) without evidence of altered consciousness, and no evidence of inflammation on imaging or CSF analysis (i.e. white cell count <5/ml)
Meningitis - Meningism (as detailed above) in a conscious patient, with a CSF white cell count of 5–20 or 20–1000/ml with a lymphocyte predominance
Meningoencephalitis – Meningism and encephalopathy (as detailed above) with evidence of CNS inflammation (as detailed above)
Guillain-Barré/Fisher syndrome - ascending sensory-motor flaccid, areflexic paralysis or the triad of ophthalmoplegia, ataxia and areflexia

Following online notification of each case, a standardised data collection pro forma was sent to the notifying clinician to request detailed clinical information including the presentation, investigations and the Glasgow outcome score (Jennett and Bond 1975). This study fell within the remit of anonymous surveillance and therefore did not require formal ethical approval .

3.3 Results

During this 2 year period the overall response rate was 36% from BPNA members [n=311], and 49% from ABN members [n=1022].

Through the BPNSU 21 doctors reported having seen a case and were sent the data capture proforma; 6 of these then replied apologising for "hitting the wrong button". Of the remaining 15 only 10 questionnaires were returned via email; 5 did not reply to repeated emails. The screen of the regional paediatric microbiology laboratory identified 4 additional cases; data for 7 further cases were referred from Evelina Children's Hospital, London. Therefore, in total data were available for 21 children.

The BNSU surveillance screen identified 5 doctors who reported having seen a case and were sent the data capture proforma; only 2 returned this completed. A further 2 cases were identified from the regional neurology hub, Department of Neurology, Hope Hospital, Manchester. Therefore, data were available for 4 adult cases. Therefore, of the total 25 cases identified 13 [52%] were identified through active screening of regional clinical and laboratory data and only 12 [48%] through the 2 year national adult and paediatric surveillance programme.

All 25 notified cases met the inclusion criteria: 21 [84%] children and 4 [16%] adults. Twelve children had encephalopathy [1 with movement disorder], 8 had encephalitis and 1 had meningoencephalitis. Two adults had encephalopathy with movement disorder, 1 had encephalitis, and 1 had Guillain-Barre syndrome [GBS]. For 7 patients, 6 children and 1 adult, the encephalopathy syndrome was documented; 4 acute

necrotising encephalopathy [ANE], 1 acute infantile encephalopathy predominantly involving the frontal lobes [AIEF], 1 haemorrhagic shock and encephalopathy syndrome [HSES] and 1 acute haemorrhagic leukoencephalopathy [AHL].

The median [range] age of the children was 4 years [9 months – 14 years] and median age of the adults was 42 years [26 – 48 years]. The overall male:female ratio was 1:1.5. Pre-existing neurological diagnoses were present in 6 [28%] children; one each with San-Filippo syndrome, Trisomy 21, Wolf-Hirschhorn syndrome, and idiopathic generalised epilepsy, and 2 with developmental delay and epilepsy of unknown aetiology. No cases were immunosuppressed. Because this was a surveillance programme, ethnicity and travel history, which might be unique identifiers, were not recorded. Details of clinical presentation, investigations, diagnosis, treatment and outcome are summarised in Table 5.

Table 5. Clinical presentation, investigations, diagnosis, treatment and outcome of patients with neurological manifestations of influenza infection

Case	Age	Sex	Acute Clinical Presentation (and Any Neurological Comorbidity)	Influenza Subtype Respiratory Secretions	Laboratory Values[a]	Head CT	Brain MRI	Clinical Case Definition	Diagnosis	Oseltamivir Treatment	Adjunctive Treatment	Days in ICU	GOS[b] (Pre-) and Post-illness
1	9 mo	F	Fever, vomiting, hypotonia, seizures (known microcephaly, epilepsy, & developmental delay)	A(H1N1)	Not performed	Low attenuation periventricular white matter	T2 hyperintensity of bilateral thalamus, dorsal midbrain, & pons with associated diffusion restriction. Mild swelling of thalami.	Encephalitis	ANE	Given	Nil relevant	2	(5) 3
2	10 mo	M	Coryza, croup, and encephalopathy (known trisomy 21)	A(H1N1)	Not performed	Normal	Not performed	Encephalopathy	Acute benign encephalopathy	Given	Nil relevant	3	(5) 5
3	12 mo	F	Coryza and status epilepticus (known Wolf-Hirschhorn syndrome)	A(H1N1)	Not performed	Not performed	Not performed	Encephalopathy	Acute benign encephalopathy	Given	Nil relevant	1	(4) 4
4	12 mo	M	Coryza, dyspnea, encephalopathy, circulatory shock	A(H1N1)	Normal	Normal	Bilateral signal abnormality & restricted diffusion in frontal & medial parietal cortex	Encephalitis	AIEF	Given	Nil relevant	25	(5) 3
5	13 mo	M	Fever and status epilepticus	A(H1N1)	Normal	Normal	Not performed	Encephalopathy	Acute benign encephalopathy	Given	Nil relevant	0	(5) 5
6	15 mo	M	Fever & status epilepticus (known developmental delay & epilepsy)	A(H1N1)	Not performed	Not performed	Evidence of subtle cortical dysplasia on preillness MRI	Encephalopathy	Acute benign encephalopathy	Given	Dex	12	(4) 4
7	18 mo	F	Fever, encephalopathy, & right-sided focal seizures	B	Clotted specimen, culture: no growth	Normal	Left cerebral generalized subcortical & thalamic restricted diffusion secondary to seizures	Encephalopathy	Acute benign encephalopathy	Given	Nil relevant	13	(5) 4
8	19 mo	F	Fever and status epilepticus	B	Normal	Normal	Normal	Encephalopathy	Acute benign encephalopathy	Given	Nil relevant	1	(4) 4
9	2 y	M	Fever, vomiting, encephalopathy, coagulopathy	A(H1N1)	Not performed	Thalami, basal ganglia, and brain stem swelling	Bilateral symmetrical lesions in thalami, putamina, cerebral, & cerebellar white matter & brain stem with marked cerebral edema	Encephalitis	ANE	Given	Man, MP, IVIG	3	(5) 1
10	3 y	F	Coryza, fever, seizures, coagulopathy, & circulatory shock	A(H1N1)	Not performed	Diffuse cerebral edema	Not performed	Encephalitis	HSES	Given	Man	1	(5) 1
11	4 y	F	Coryza & prolonged generalized seizure	A(H1N1)	Normal	Normal	Bilateral T2 hyperintensity of dentate nuclei, pons, midbrain, thalami, & subcortical white matter of both cerebral hemispheres.	Encephalitis	ANE	Given	Nil relevant	4	(5) 5

Case	Age	Sex	Acute Clinical Presentation (and Any Neurological Comorbidity)	Influenza Subtype Respiratory Secretions	Laboratory Values[a]	Head CT	Brain MRI	Clinical Case Definition	Diagnosis	Oseltamivir Treatment	Adjunctive Treatment	Days in ICU	GOS[b] (Pre-) and Post-illness
12	4 y	F	Fever, lethargy, rash, and vomiting	B	Normal	Not performed	Not performed	Encephalopathy	Acute benign encephalopathy	Given	Nil relevant	0	(5) 5
13	5 y	F	Coryza & status epilepticus	A(H1N1)	Normal	Normal	Normal	Encephalopathy	Acute benign encephalopathy	Given	Nil relevant	3	(5) 3
14	6 y	M	Coryza, fever, & seizures (known previous febrile seizures)	A(H1N1)	Normal	Occipital lobe calcification	Bilateral T2 hyperintensity & mild occipital focal atrophy related to previous ischemic insult	Encephalopathy	Acute benign encephalopathy	Given	Nil relevant	2	(5) 5
15	8 y	F	Coryza, fever, encephalopathy, & cranial nerve VI palsy	A(H1N1)	Normal	Not performed	T2 hyperintensity in pons, posteromedial thalami, & the right external capsule	Encephalitis	ANE	Given	MP, IVIG	0	(5) 4
16	10 y	M	Encephalopathy, & status epilepticus	A(H1N1)	wcc 16, rcc 1, protein 2.09, glucose 3.8, no growth	Normal	Normal	Encephalitis	Acute benign encephalitis	Given	IVIG	14	(5) 4
17	11 y	M	Coryza, fever, vomiting, encephalopathy, & seizures	B	wcc 184, rcc 140, protein 1.02, glucose 4.2, no growth	Normal	Normal	Encephalitis	Acute benign encephalitis	Given	Nil relevant	3	(5) 5
18	12 y	F	Fever, headache, encephalopathy, and photophobia	A(H1N1)	wcc 900, rcc 1200, protein 4.8, glucose <0.1, culture: *S. pneumoniae*	Normal	Cerebellar tonsillar herniation with ischemia at the craniocervical junction	Meningo-encephalitis	Meningoencephalitis	Given	Dex	5	(5) 1
19	13 y	F	Fever, headache, irritability, & encephalopathy	A(non-H1N1)	Normal	Normal	Normal	Encephalopathy	Acute benign encephalopathy	Given	MP, IVIG	0	(5) 5
20	13 y	F	Fever, headache, & acute facial and upper limb dyskinesia (known Sanfilippo syndrome)	A(H1N1)	wcc 0, protein & glucose normal, low CSF pyridoxal 5-phosphate, normal CSF neurotransmitters	Not performed	Changes consistent with Sanfilippo syndrome, no acute lesions	Encephalopathy & movement disorder	Acute dyskinesia with low CSF pyridoxal 5-phosphate	Not given	Pyridoxal phosphate (resolution)	0	(3) 3
21	14 y	F	Coryza & status epilepticus (known idiopathic generalized epilepsy)	A(H1N1)	Not performed	Normal	Not performed	Encephalopathy	Acute benign encephalopathy	Given	Nil relevant	1	(4) 4
22	26 y	F	Fever, headache, irritability, & intermittent resting tremor right hand, upper limb rigidity	A(H1N1)	Normal	Normal	Normal	Encephalopathy & movement disorder	Acute benign encephalopathy with movement disorder	Given	Nil relevant	67	(5) 5
23	42 y	M	Fever, encephalopathy, circulatory shock, & cranial diabetes insipidus	A(H1N1)	Not performed	Significant cerebral edema	Not performed	Encephalitis	AHL	Given	HC	9	(5) 1

Case	Age	Sex	Acute Clinical Presentation (and Any Neurological Comorbidity)	Influenza Subtype Respiratory Secretions	Laboratory Values[a]	Head CT	Brain MRI	Clinical Case Definition	Diagnosis	Oseltamivir Treatment	Adjunctive Treatment	Days in ICU	GOS[b] (Pre-) and Post-illness
24	42 y	M	Fever, headache, bilateral upper limb tremor & rigidity, orofacial bradykinesia	A(H1N1)	Normal	Normal	Not performed	Encephalopathy & movement disorder	Acute benign encephalopathy with movement disorder	Given	MP	60	(5) 4
25	48 y	F	Ascending flaccid paralysis	A(H1N1)	Normal	Normal	Normal	Guillain-Barré syndrome	Guillain-Barré syndrome	Not given	IVIG	28	(5) 4

Abbreviations: AHL, acute hemorrhagic leukoencephalopathy; AIEF, acute infantile encephalopathy predominantly affecting the frontal lobes; ANE, acute necrotizing encephalopathy; CSF, cerebrospinal fluid; CT, computed tomography; Dex, dexamethasone; GOS, Glasgow Outcome Scale; HC, hydrocortisone; HSES, hemorrhagic shock and encephalopathy syndrome; ICU, intensive care unit; IVIG, intravenous immunoglobulin; Man, mannitol; MP, methylprednisolone; MRI, magnetic resonance imaging; Nil, none; rcc, red cell count; wcc, white cell count.

[a] CFS, white and red cell count/mm^3; protein, g/L; glucose, mmol/L.

[b] Glasgow Outcome Scale: 1–2 = very poor outcome persistent vegetative state or death); 3–4 = poor outcome; 5 = no-mild disability [16].

3.3.1 Acute clinical features

At presentation, fever and/or respiratory symptoms were present in all patients, 12 [48%] had seizures and 3 [12%] had circulatory shock. An acute movement disorder was seen in three cases: two adults had parkinsonism and the child with San Fillippo syndrome had acute non-epileptic myoclonus and dystonia.

3.3.2 Investigations

Influenza A was detected by PCR in the respiratory secretions of 21 [84%] cases, of which 20 [95%] were the H1N1 [2009] subtype, and influenza B was detected in the respiratory secretions of 4 [16%] cases. Two cases had co-infection with *Streptococcus pneumoniae,* a child with pneumococcal meningitis [case 18] and an adult with pneumococcal sepsis [case 23], both of whom died

All patients with an acute encephalopathy syndrome had a metabolic screen and were tested for plasma ammonia, liver function, renal function, electrolytes and glucose levels. Serum was tested for anti-N-methyl-D-aspartate receptor antibodies in 5 children, voltage-gated potassium channel antibodies in 3 children: all were negative. RANBP2 mutation was tested for 2 children with ANE and was positive in one [case 15]. The adult with GBS was not tested for the presence of antiganglioside antibodies.

Cerebrospinal fluid was examined for 18 [72%] patients; 7 had clinical contraindications to a LP. CSF was abnormal in 4 [22%] cases: 3 had pleocytosis [median [range], 184 [16-900] x 10^6 cells/L], and one child [case 20] with a pre-

existing neurodevelopmental disorder had an unexplained low-pyridoxal 5-phosphate. All CSF samples underwent Gram stain and bacterial culture; 1 [case 18] grew *Streptococcus pneumoniae* and all others were negative. CSF was tested for influenza RNA by PCR in 10 [53%] cases: all were negative. Oligoclonal bands were tested in 3 patients: all were negative.

Electroencephalography [EEG] was undertaken in 12, [8 children]: 8 were abnormal; 6 showed diffuse slowing of the background activity in keeping with an encephalopathy [one also had some additional focal right temporal changes]; 2 were low amplitude, impoverished recordings in keeping with severe bihemispheric dysfunction. None showed epileptiform activity.

Cerebral imaging was performed in 23; 3 MRI, 6 CT, and 14 had both. Abnormalities included recognised acute encephalopathy syndromes in 5, and non-specific changes including cerebral oedema or diffusion restriction, in 5 cases.

3.3.3 Outcome

Twenty [80%] required admission to an intensive care unit: the median [range] length of stay was 3 [1-67] days. Oseltamivir was commenced in 23 [92%] patients. Presumptive antibiotic therapy with a third generation cephalosporin for suspected meningitis was started for 18 [72%] patients, 9 of whom also received clarithromycin. Twelve [48%] were also treated empirically with aciclovir. Only eight [32%] had a good outcome, 13 [52%] had a poor outcome, and 4 [16%] had a very poor outcome, all of whom died.

3.3.4 Case examples of acute encephalopathy syndromes

Below are further descriptions illustrating the particular clinical features of 4 patients with specific AES, to help clinicians recognise such rare complications in the future.

Case 4 - Acute infantile encephalopathy predominantly affecting the frontal lobes [AIEF]

A 1 year old boy presented with a 2-day coryzal illness. On examination he was floppy, with respiratory distress, circulatory shock and decorticate posturing. A plain chest radiograph demonstrated patchy changes bilaterally. CT brain scan was normal. Intensive care was required and antimicrobial treatment included cefotaxime, clarithromycin, aciclovir and oseltamivir.

Respiratory secretions were positive by PCR for influenza A:H1N1. Alanine transaminase [ALT] was raised [1319 IU/L]. The MRI brain scan [day 10] demonstrated cerebral atrophy and bilateral symmetrical restricted diffusion involving in the frontal cortex. He had severe sequelae: tracheostomy insertion, and at six months he was unable to sit unaided or use his hands for purposeful activities and was mute. Bulbar function was abnormal and he required a percutaneous gastrostomy.

Case 9 - Acute necrotising encephalopathy [ANE]

A previously well 2 year old boy presented with a 2-day history of pyrexia, diarrhoea and vomiting. Over several hours, his GCS dropped to 4/15 and he developed signs of raised intracranial pressure. He was intubated, ventilated, and given a single dose of intravenous mannitol. He received intravenous ceftriaxone, aciclovir, and oseltamivir.

A CT brain scan on admission demonstrated oedema of the thalami, basal ganglia and brainstem.

He developed acute renal and liver failure [ALT 13,713 IU/L] with coagulopathy. PCR of respiratory secretions was positive for influenza A:H1N1. An MRI brain scan on day 5 demonstrated multiple symmetrical lesions in the thalami. He died despite treatment with intravenous methylprednisolone and immunoglobulin. Investigations to look for an underlying metabolic, genetic and autoimmune cause were negative.

Case 10 - Haemorrhagic shock and encephalopathy syndrome [HSES]

A 3 year old girl presented with a 2-day coryzal illness, and a brief generalised tonic-clonic seizure. She was drowsy with refractory circulatory shock. Initial antimicrobial treatment included ceftriaxone and aciclovir. During intubation she became bradycardic and required cardiopulmonary resuscitation.

She developed acute renal and hepatic failure, coagulopathy, hypernatraemia and lactic acidosis. ALT was markedly raised [3192 IU/L]. A CT brain scan on admission demonstrated diffuse cerebral oedema. She received a single dose of mannitol and antimicrobial cover was broadened to include cefotaxime, clarithromycin, osteltamivir as well as aciclovir. Twelve hours after presentation, she had signs of brainstem death and bilateral retinal haemorrhages: treatment was withdrawn. PCR of her respiratory secretions subsequently identified influenza A:H1N1.

Case 23 – Acute haemorrhagic leukoencephalopathy [AHL]

A 42 year old woman with well-controlled asthma presented with a 1-day history of

left-sided pleuritic chest pain, dyspnoea and pyrexia. A plain chest radiograph demonstrated left lower lobe consolidation. She was commenced on continuous positive airway pressure and intravenous co-amoxiclav and clarithromycin; subsequently piperacillin/tazobactam and oseltamivir were added. She deteriorated, requiring vasopressors and invasive ventilation.

Admission blood cultures grew *S. pneumoniae* and urine PCR for pneumococcal antigen was positive. Antibiotic cover was narrowed to intravenous benzylpenicillin according to sensitivities, and hydrocortisone was added. Her throat swab was positive for influenza A:H1N1. CT-brain scan on admission demonstrated significant cerebral oedema, and EEG reflected severe encephalopathy. She was treated with mannitol and mechanical hyperventilation. Three days later a further CT-brain scan demonstrated worsening cerebral oedema. She lost brainstem reflexes and care was withdrawn. Post-mortem examination demonstrated AHL.

3.4 Discussion

Influenza-related neurological manifestations were first reported in 1918 (Jeliffe et al 1918). There were then few reports until the 1990s, when increasing descriptions of influenza-related acute encephalopathy emerged predominantly in Japan (Okabe et al 2000). Since the emergence of novel influenza A:H1N1 there has been considerable interest outside Japan too. The first report described 4 children from the USA with seizures and/or encephalopathy, who all recovered (Centers for Disease Control and

Prevention 2009). Subsequently, paediatric case series have described a variety of neurological manifestations ranging in severity from febrile seizures to specific acute encephalopathy syndromes associated with poor outcomes (Farooq et al 2012; Kawashima et al 2012; Khandaker et al 2012; Okumura et al 2013). The corresponding adult literature is predominantly confined to single case reports (Cisse et al 2010). There is limited evidence that influenza A:H1N1 is associated with more severe forms of acute encephalopathy compared with pre-2009 influenza subtypes, although this may reflect reporter and publication bias (Ekstrand et al 2010). The estimated incidence of neurological manifestations has been estimated at 1.2 per 100,000 symptomatic H1N1 infections (Glaser et al 2012). However, good epidemiological data are limited, due in part to a lack of standardised case definitions, such as the generic term 'influenza-associated encephalopathy/encephalitis' or relying on clinical features alone (Ekstrand 2012; Glaser et al 2012). Moreover, studies have been limited small numbers reported from single centres, and no previous systematic surveillance programme has been reported.

As previous case series have typically been retrospective and restricted to children, we applied clinical case definitions to undertake the first surveillance study through British adult and paediatric neurological surveillance units. Most cases in our study presented during the period when influenza A:H1N1 was the predominant subtype in circulation, which thus accounts for the majority of isolates in our cohort (Health Protection Agency 2011). Neurological manifestations were commonly observed in children with pre-existing neurological diagnoses, an unexplained but well-reported phenomenon (Yildizdas et al 2010; Kawashima et al 2012; Khandaker et al 2012; Okumura et al 2013). In keeping with previous studies, we identified specific AES

including ANE, AIEF and HSES, more frequently in children (Hoshino et al 2012). We saw GBS in one adult, consistent with previous reports (Sivadon et al 2009). In contrast to the majority of previous reports, our cohort represents a more severe spectrum of neurological manifestations; 17 [68%] had poor outcome or died and 20 [80%] required ICU management (Centers for Disease Control and Prevention 2009; Ekstrand et al 2010; Yildizdas et al 2010; Farooq et al 2012). The patients who had a particularly poor outcome were those with AES. Of our 4 cases with ANE, 3 had typical clinical features. ANE, first described by Mizuguchi in 1995, is characterised by bilateral, symmetrical necrotic lesions affecting the thalami (Mizuguchi et al 1995). While influenza is the most common associated pathogen, human herpes virus 6 has also been frequently described (Mizuguchi et al 1995; Hoshino et al 2012). Supportive investigations for ANE include elevated serum aminotransferases, a raised CSF protein without a pleocytosis and characteristic neuroimaging. The child with atypical neuroimaging [case 15] had more prominent brainstem lesions. However, a previous influenza-related encephalopathy prompted testing for the RANBP2 mutation. She tested positive for a previously described RANBP2 mutation [p.Ile656Val], as did her mother and maternal grandmother. Mutations of RANBP2, a nuclear pore protein, are associated with familial and recurrent ANE characterized by autosomal dominant inheritance and incomplete penetrance (Neilson et al 2009). Particular markers of a poor prognosis in ANE include haemorrhagic lesions or cavitation on neuroimaging, although these were not found in those with poor outcomes in our study (Wong et al 2006).

The other paediatric AES cases we identified [HSES and AIEF] had typical presentations. HSES was first described by Levin in 1983, as a high-mortality

condition presenting with hyperpyrexia, encephalopathy, diarrhoea, circulatory shock, multi-system dysfunction and coagulopathy (Levin et al 1983). Neuroimaging typically demonstrates cerebral oedema (Mizuguchi et al 2007). Various pathogens have been linked to the condition including rotavirus and adenovirus (Rinka et al 2008). AIEF, first described by Yamanouchi in 2006, is characterised by acute fever, encephalopathy, seizures and radiological changes in the frontal lobes (Yamanouchi et al 2006). Frontal lobe dysfunction, such as speech regression typifies the sequelae, as in our case. AIEF has been reported in association with human herpes virus 6, measles and pre-2009 influenza subtypes however we are not aware of any other cases reported in association with influenza A:H1N1 (Yamanouchi et al 2006).

AES are rare in adults: we identified one case with AHL. AHL is characterised by acute encephalopathy with diffuse haemorrhagic necrosis and perivenular demyelination of the brain and spinal cord, with characteristic EEG and MRI findings (Alemdar et al 2006; Lann et al 2010). It has been associated with several infections, including *Mycoplasma pnuemoniae* and Epstein-Barr virus (Catalan et al 2009; Befort et al 2010). A previous reported case with H1N1 was associated with poor outcome, similar to that in our case (Cisse et al 2010).

Some milder AES which have been associated with influenza previously such as acute encephalopathy with biphasic seizures and late reduced diffusion [AESD] and mild encephalitis/encephalopathy with reversible splenial lesion [MERS] were not reported in our study, perhaps because our methodology favoured cases of particular clinical interest and severity. All encephalopathic cases in our cohort who did not fulfil criteria for a specific AES were classified as “acute benign encephalopathy”, and all but

one [case 13] had minimal residual effects. Using data from our study, and additional reports in the published literature, we propose an expanded version of the categorisation of neurological manifestations of influenza developed by Akins et al [Table 6] (Yamanouchi et al 2006; Takanashi et al 2009; Akins et al 2010; Bartynski et al 2010; Cisse et al 2010). This expanded categorical table illustrates in particular how prognosis varies across the increasing spectrum of recognised AES.

Table 6 Proposed classification of neurological manifestations of influenza

	Acute Onset—Cytokine Storm	Subacute Onset—Adaptive Immune Responses	Late Onset—Unknown Pathophysiology
	Febrile seizures	Guillain-Barré syndrome	Post–viral parkinsonism
	Acute movement disorder	Transverse myelitis	Encephalitis Lethargica
	Acute benign encephalopathy/encephalitis	Acute disseminated encephalomyelitis (ADEM)	
	Acute encephalopathy syndromes (AESs): Mild encephalitis/encephalopathy with reversible splenial lesion (MERS)	Myositis	
	Posterior reversible encephalopathy syndrome (PRES)	Cerebellitis	
	Acute necrotizing encephalopathy (ANE)		
	Acute encephalopathy with biphasic seizures and late reduced diffusion (AESD)		
	Acute infantile encephalopathy predominantly affecting the frontal lobes (AIEF)		
Increasing neurological sequelae and mortality	Acute shock with encephalopathy and multiorgan failure (ASEM)[a]		
	Acute hemorrhagic leukoencephalopathy (AHL)		

[a] Proposed revised nomenclature for hemorrhagic shock and encephalopathy syndrome (HSES).

Influenza viruses do not appear to demonstrate neurotropism and viral RNA is rarely identified in the CSF (Fujimoto et al 1998; Ito et al 1999). There is unlikely to be a single mechanism underlying the pathophysiology of the wide spectrum of neurological manifestations of influenza. Some have suggested that duration of influenza-like illness preceding neurological manifestations may afford distinction into two broad categories: a. acute – in association with an innate immune response and a 'cytokine storm' and b. sub-acute – with an adaptive, cell-mediated response

(Akins et al 2010). Increased concentrations of pro-inflammatory cytokines have been found in the serum and CSF of children with neurological manifestations of influenza (Fujimoto et al 1998; Ichiyama et al 2004). Adaptive immune-mediated pathophysiology is associated with several sub-acute neurological syndromes, including GBS (Costiniuk et al 2011). Although none of those in our cohort had evidence of inborn errors of metabolism, the thermolabile phenotype of carnitine palmitoyltransferase II may precipitate dysfunctional fatty acid oxidation during fever with influenza; such variations may be more common in East Asians (Chen et al 2005).

Co-infection with *S. pneumoniae* was observed in two patients [cases 18 & 23]; both developed malignant cerebral oedema and died. Pneumococcal/influenza respiratory co-infection is a recognised distinct clinical entity associated with poor outcome (Palacios et al 2009; Launes et al 2012). While the pathophysiology underlying the synergy between the two organisms is poorly understood, proposed factors include the role of influenza virulence factors in epithelial damage and subsequent facilitated entry of pneumococcus, as well as up-regulation of the inflammatory response (McCullers et al 2006).

There are no randomised trials informing the management of AES. Early steroid treatment in ANE is thought to be beneficial, but the efficacy of intravenous immunoglobulin remains uncertain (Okumura et al 2009). However, due to the rarity of these individual conditions and sub-optimal disease surveillance for identification and recruitment to treatment trials, these have not been undertaken.

Influenza vaccine is efficacious in preventing influenza infection in children over 2-

years of age, and also in reducing hospitalisation (Jefferson et al 2008; Gilca et al 2011). None of our cohort were vaccinated against influenza A:H1N1. However, 8 had clinical indications for vaccination, including the 6 with pre-existing neurological diagnoses and 2 with asthma [cases 13 and 23]; 3 of these died, and the remaining 5 all had poor outcomes (Sammon et al 2012). Currently there is no real-time platform to assess where patients are and are not receiving vaccinations. Nevertheless, it is pivotal in the monitoring the effectiveness of national vaccination programmes to have these data.

A limitation of this study is that the reporting system relies on the willingness of individual clinicians to notify cases; a handful UK centres reported all the cases while most centres reported none. The majority of cases notified to this study were through colleagues who were involved in parallel studies of CNS infections, for whom this is a specialist interest, rather than through the surveillance programme. Therefore, cases notified are heavily biased for the Northwest of England and London. In addition, our methodology is likely to have led towards a bias towards more severe cases, because the reporting was via neurologists rather than general clinicians, and the majority required ITU support and/or died.

Public Health England collects data on a 4 monthly basis from general practitioner [GP] practices (and/or Primary Care Trust Flu co-ordinators) in England through the ImmForm website (Begum et al 2013). Although 84% of GP practices responded to the most recent annual survey (2012/2013), weekly collections are only available in a sub-group of 60% of these, and there are several other limitations. Data collection requires manual uploading in the majority of cases and is not in real-time, therefore

there will be delays which may be of significance for emerging infections, it does not record all vaccinations performed by other healthcare providers (e.g. pharmacies, antenatal and other specialist clinics etc), data are not available for those not registered with a GP practice, or for those who refuse the vaccine, also data are only collected for specific groups relating to pre-determined 'at-risk' groups at need of vaccination and are not able to provide wider data of relevance to the establishment of herd immunity. In addition, such approaches require review with structural changes within the NHS, such as the abolition of Strategic Health Authorities, and Primary Care Trusts, the development of Clinical Commissioning Groups and changes in software used by individual GP practices to store data. Although there is a move towards automation of these processes, these have been instituted to answer one specific question, i.e. has the WHO target of $\geq$75% of at-risk individuals been vaccinated for influenza has been met in this timeframe? Therefore, the mandatory need for GP practices to have methods to submit this data led to the development of these automated procedures. Thus, like our influenza study, this was dependent on the willingness of individual clinicians and practices to submit data. In addition, these data are limited to England and do not reflect both those with suspected and proven clinical disease in addition to those at risk who do not receive vaccination.

Recent studies have attempted to augment current influenza surveillance mechanisms using new technological approaches, such as 'crowd-sourcing' using Google © 'Flu Trends data (Ginsberg et al 2009; Arnold 2013). The success of future epidemiological work will be dependent, to a large extent, on effective and robust surveillance systems and the use of standardised case definitions.

In conclusion, in this paediatric and adult UK study that I helped to co-ordinate, we attempted to use predetermined diagnostic criteria applied to clinical data collected through both national neurological surveillance networks. This was only able to identify a small number of cases over a 2 year period despite pandemic infection. The majority of cases were identified through colleagues with a specialist interest, which may be biased towards those with more severe manifestations. This study identified a spectrum of neurological manifestations of influenza. Specific AES had a worse outcome and were reported more frequently in children. These data are used to propose a new classification of influenza-related neurological manifestations. Nevertheless, the current methodological approaches for disease surveillance studies such as this are limited by sub-optimal notification, and the data collected are biased by geography, interest areas and disease severity. Moreover, data collection is delayed following the acute event and no real-time reporting systems for encephalitis are in use in the UK. Therefore, I took this experience forward to explore in more detail the current disease reporting systems pertinent to acute viral encephalitis to determine whether novel approaches could be developed with the potential to augment real-time reporting.

4. Detection of encephalitis at the population level- Improving disease reporting through novel technological approaches

4.1 Background

Improved understanding of the distribution of viral causes of encephalitis and the development of novel causes requires robust and readily utilised identification and tracking mechanisms; and this is fundamental for adequate public health policy planning (Sintchenko et al 2009). In addition, a clear understanding of current clinical practice norms is necessary to audit clinical care, identify areas of concern and develop interventions to improve care quality (Ivers et al 2012).

Whilst many countries have national disease surveillance programmes for major communicable diseases, or 'notifiable diseases', the work of our group and others has identified several areas of sub-optimal notification, even in developed countries. For example, there were only 18 cases of acute encephalitis notified to the HPA in 2007 (Health Protection Agency 2007). However, during a 3 month study assessing patients in the same year, which is described in more detail in the next chapter, in only 10 adult hospitals I identified 13 cases of encephalitis (Michael et al 2010). Further example of suboptimal notification of acute CNS infections is seen for acute viral meningitis, as between 2004-2006 there was approximately 1381 notifications per year (Health Protection Agency 2007). During this same period in a single 950-bed

standard, general NHS teaching hospital, we identified 68 hospitalised patients with viral meningitis (Michael et al 2010). Although a crude estimate, if extrapolated based on nationally published data across the 159,386 NHS inpatient beds over this same time period, the estimated number of cases would be nearly three times larger [n=3,802 cases] (King's Fund 2010). Indeed several studies have reported that the true incidence of meningitis in the UK may be 10-14 times higher than notified figures (Chadwick et al 1995; Brabazon et al 2004; Chadwick et al 2004).

Nevertheless, microbiological notification of specific organisms responsible for acute CNS infections maybe somewhat better, as there are clear notification standard operating procedures in most laboratories. However, this may falsely skew epidemiological data to favour particular organisms of interest. Data are limited for acute viral encephalitis. But for meningitis, for example, in 2011, 263 [48.8%] of all acute meningitis notifications to the HPA were due to *Neisseria meningitidis* (Health Protection Agency 2011). Whereas, in disease-specific, rather than organism-specific, surveillance programmes in developed countries only around 13% of all meningitis cases are due to *Neisseria meningitidis* (Thigpen et al 2011). Furthermore, all bacteria may only account for up to 26% of meningitis (Magazzini et al 2012). In parallel, specialty-focused surveillance programmes may bias data to cases of greater or lesser disease severity, influenced by geography and specialist interest as I demonstrated with neurological inflammation related to influenza infection (Goenka et al 2013).

The US Center for Disease Control and Prevention [CDC] has brought together several reporting systems, including for HIV and tuberculosis [TB], both important causes of CNS infection, into a single place through the National Electronic Disease Surveillance

System (Center for Disease Control and Prevention 2013). However, this approach still requires each individual to upload their data and does not collect data automatically from doctors on the front line of patient care.

Suboptimal reporting from doctors on the front line may potentially be due to lack of awareness that the condition is a notifiable one, lack of knowledge of the procedure for notification and/or limited resources to facilitate the notification process, such as limited time. In addition assessments of current clinical practice are typically limited to small retrospective audits of case notes in individual hospitals. Therefore, they only provide delayed information, which is specific to a locality rather than reflecting wider healthcare practice (Bell et al 2009).

To start to address some of these issues and to enhance infectious disease surveillance, novel technological approaches, such as 'crowd-sourcing' are being used increasingly. In this context, crowd-sourcing is effectively collecting the input of data from a wide section of the public via the internet. For example, two recent papers report attempts to use Google © trends data to assess influenza incidence and compared this with CDC data. One identified good correlation between Google © influenza search trends and CDC notifications and a latter paper describes how these historical data were used to develop a mathematical model to predict influenza outbreaks 7 weeks prior to the occurrence (Ginsberg et al 2009; Shaman et al 2012) However, whilst this approach utilises large datasets, which are required to generate such models, the veracity of the data is less robust than that submitted by clinicians.

In the UK other approaches have been taken to attempt to augment disease

surveillance of pandemic infections (Health Protection Agency 2011). For example, 'Flusurvey' which is a European Union-funded project developed by the London School of Hygiene and Tropical Medicine which accesses internet-based surveillance of influenza in the general UK population (London School of Hygiene and Tropical Medicine 2014). In addition, 'Flu Watch' is a Medical Research Council/Wellcome funded community-based cohort surveillance system in England run by University College London (University College London 2014). However, both these approaches are geographically-restricted and rely on the good will of individuals to submit data for the benefit of the project or reflect a small cohort of participants respectively. Moreover, these modalities do not collect data in real-time.

Medical technology, such as 'Apps' for smartphones are revolutionising the way doctors practice medicine, increasing dissemination of research and making clinical guidelines more widely available (British Medical Journal Publishing Group 2012). However, the majority of these support only unidirectional traffic of information [e.g. simply dissemination of guidelines or monitoring of an individual's health parameters, such as blood pressure recordings, and relaying this information to their individual doctor] (Boulos et al 2011). A structured Medline search was conducted for papers between 1985-current in any language using the search terms: "smartphone" OR "phone" OR "application" WITH "disease surveillance" OR "epidemiology". A further search was conducted using the above search terms in the Apple App Store ©. However, neither approach identified any applications, which allow for the bidirectional flow of data from a central database to clinicians [i.e. clinical guidelines] and from clinicians to a central database [i.e. disease notifications].

Therefore, as a proof-of-principle project, I set out to determine if it was possible to develop a novel smartphone application that was useful for the individual doctor's practice but also allowed for bidirectional information traffic and had the potential to address both of these issues; firstly to make it easy for doctors to report cases of acute viral encephalitis and other major infectious diseases and secondly to collect data on the management of these patients [https://sites.google.com/site/clickclinicarx/].

4.2 Construction and content

To increase the applicability of the app across doctors from a wide range of specialities and regions, the application brought together the clinical guidelines from across guidelines groups in a single place and is made freely available for the iPhone. These include guidelines from large national and international bodies such as the National Institute for Health and Clinical Excellence, HPA, CDC, the Resuscitation Council, the World Health Organization [WHO] and many others. Depending on the size of each guideline, they are either stored in whole or as a clinical summary of the key points as a PDF. Therefore, the clinician does not require internet access at the time they wished to get the information. Where guidelines were given in summary format or further multimedia material is available, a hyperlink to the full online guideline is provided. The app does not require any additional effort from the doctor than simply reading the guidelines they want to read anyway. Indeed, by making them readily available and clearly indexed, it is easier for the doctor to use the app to read a section of the guidelines than it is to perform an internet search, then find the website, then find the document, then download the PDF, then scroll through to find the page

of interest.

In reading guidelines on the application, the app has the potential to generate a real-time global disease reporting database based on the usage. Also from a brief question that appears on the bottom of the screen when viewing a specific disease, more detailed information on either important epidemiological or clinical care quality issues can be collected. One example is that when a doctor is viewing a guideline on H1N1 influenza, our central database is made aware that the guideline is being read and the doctor's grade and hospital. Moreover, on clicking yes in the 'question box' at the bottom of the screen, such as "Are you prescribing chemoprophylaxis?" we are immediately also notified that the doctor has answered the question and their name, specialty, GPS location and, if allowed, email address, medical registration number and also whether the doctor is happy to be contacted for research purposes regarding this case. In answering the question this 'question box' disappears providing more space for the guideline to be viewed [Figure 4]. The doctor only needs to input their personal information once at the point of downloading the application [Figure 5]. Therefore, the application makes it possible to immediately track the distribution of disease, the spread of outbreaks and their management. The question at the bottom of the screen is specific for each disease, or sub-section within the disease guideline.

For example, for influenza:

- When reviewing the front page, the question is "are you seeing a patient with H1N1?"
- When reviewing the pages on disease severity within the influenza document the question is "does this patient have CNS involvement?"
- When reviewing the treatment table within the influenza document the question is "are you prescribing antivirals?"

Figure 4. Guideline and database views on ClickClinica ©, with questions being answered

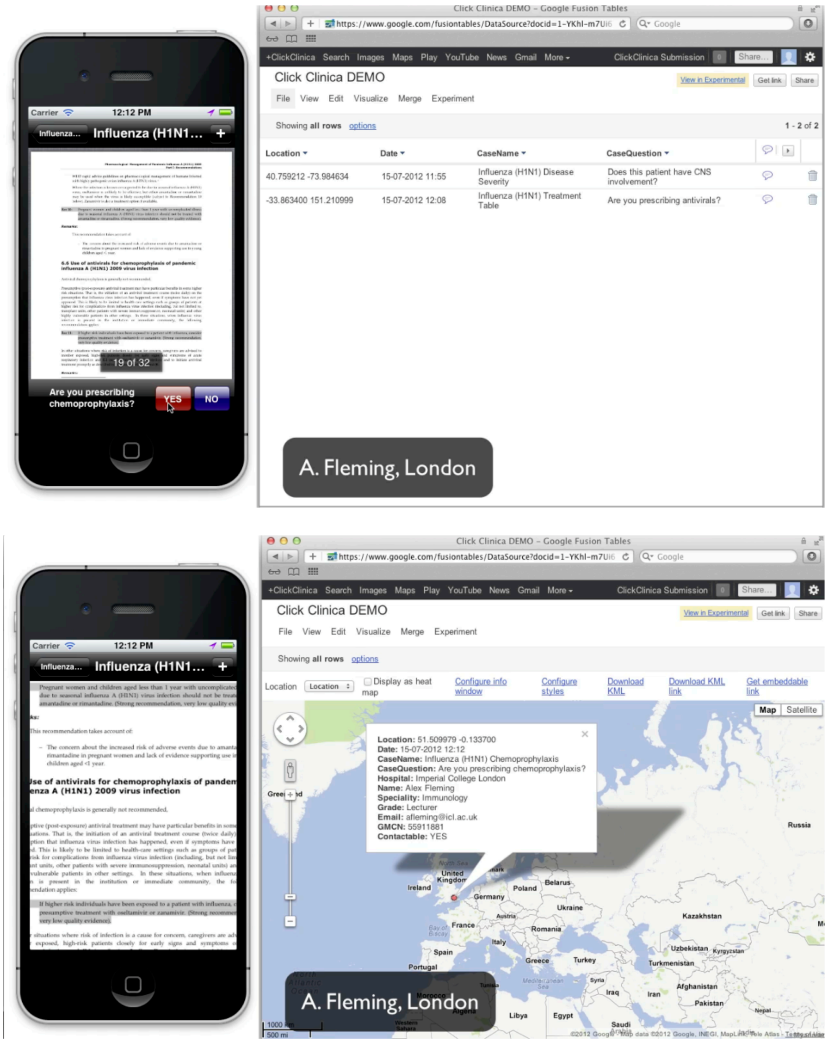
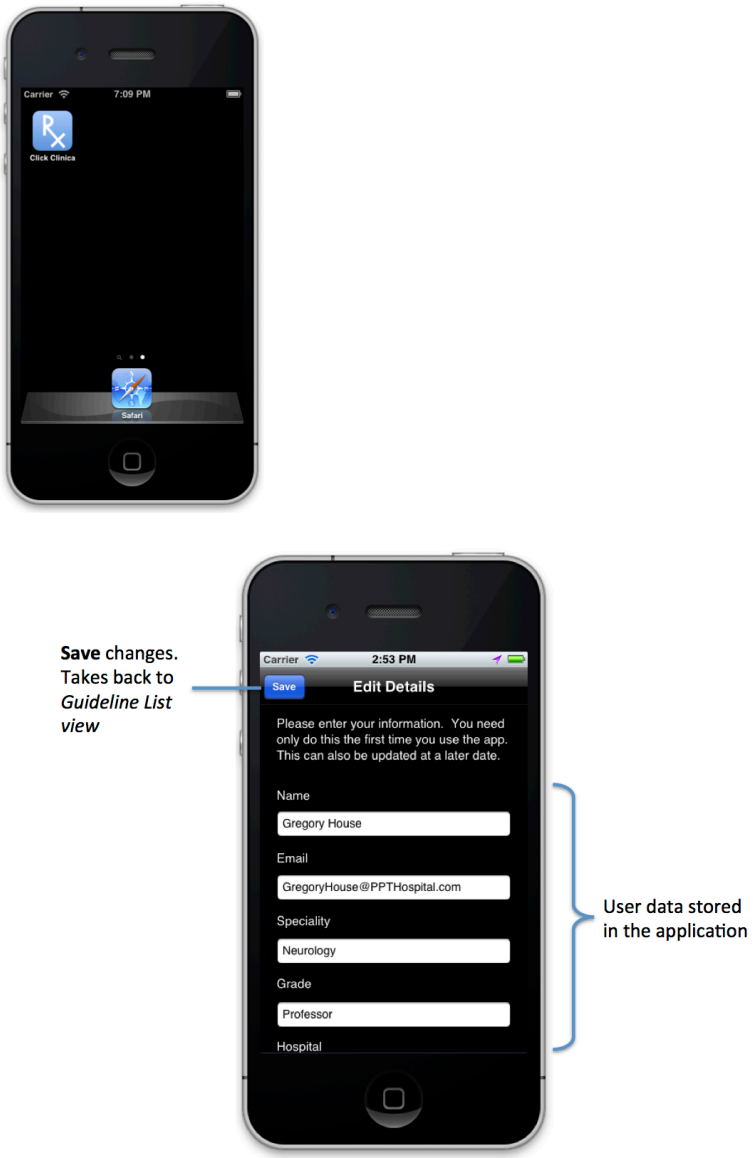


Figure 5. Launching the ClickClinica © application and initial registration



Whilst these questions are currently set by me centrally, as the project expands I am developing collaborations with other research institutions to use our platform for them to include research questions of interest to their particular field. The data collected regarding this is made freely available to them to access in real-time through a dedicated terminal, including questions submitted by users. When the app is used in a situation where a clinician does not have an internet connection the notification is stored within the application and subsequently submitted to the central database automatically when an internet connection is established.

By obtaining comprehensive data on the usage of the app, I am able to include guidelines that reflect usage in future updates. In addition the application contains a 'Flag it up' button, which allows users to suggest guidelines they would like to see in future updates of the application.

To encourage use of the application, I have included guidelines to all the major acute medical presentations that are covered in competing paid-for applications and handbooks. Also, I have made it possible for clinicians to print, email or bookmark the guidelines directly from their smartphone. This also helps to foster guideline dissemination and the creation of a personalised app. To encourage users to answer the research questions we have included internal ClickClinica © certificates that can be used as evidence of involvement in research for their portfolio of continuing professional development. Also at any time the user can view their personalised index of recorded cases.

4.3 Utility and Discussion

The database generated from the usage of the app can also assess new, dynamic and targeted questions. When a new guideline is released [e.g. for a new pandemic infection] this app has the potential to track, in real-time, the global uptake and usage of the guideline within a cohort of doctors. It can also assess whether the recommendations in that guideline [e.g. vaccine delivery] are being followed and where cases with key markers of severe disease [e.g. neurological involvement] are occurring. This is a potential resource of clinical information not only for guideline-producing bodies but also for clinical governance and public health institutions.

In the first 4 weeks the app was downloaded by >1000 users and received >600 specific disease notifications. The app did not receive any external funding and did not undergo a marketing campaign. Potential users were made aware of the app through the Apple App Store, within the 'free medical applications' category. In an effort to reduce spurious notifications, the app was designed such that it is equally efficient for the user remove the 'question box' from the screen by choosing to not notify as it is if they notify. Of the notifications, 578 have included an email address and 405 of these notifications have included consent to be contacted to provide further information for research purposes on the case they have notified.

Data quality is assessed firstly by the veracity of the user's details [e.g. the data are treated as the weakest possible evidence of a disease notification when the report only contains the user's name]. The data are treated as most robust when a notification contains the user's name, official NHS email address and General Medical

Council number, which are crosschecked with the register. Furthermore, downstream data quality assessment is undertaken through an automated process to exclude multiple disease notifications during a short period of time [e.g. if the same disease is notified repeatedly within 10 minutes or multiple diseases are notified within 5 minutes] as such situations are likely to reflect users simply reading the guidelines for their interest or initially to 'test out' the app, rather than in relation to a specific patient. Nevertheless, I have endeavoured to minimise these types of inappropriate notifications, as it is equally efficient, from a user's point of view, to not notify, as it is to notify.

Specific disease notifications have come from a broad range of clinical specialities, including General Practice [25%], Emergency Medicine [15%], General Medicine [14%], and Anaesthetics, Paediatrics, Surgery, Infectious Diseases, Psychiatry, Cardiology, and Neurology.

Also, downloads and notifications have been coming from across the globe, including some resource-poor countries [Figure 6], and from a wide range of grades, including Foundation Doctors [24%], Medical Trainees [21%], Specialist Registrars [23%], Consultants [18%], and Professors/Lecturers [6%]. In addition, within the UK notifications have not been limited to centres with specific research links to our institution, as has been a major limitation of previous epidemiological research for acute CNS infections (Goenka et al 2013) [Figure 7].

Figure 6. Real-time global disease database generation collected using the ClickClinica © application on 10.3.2013

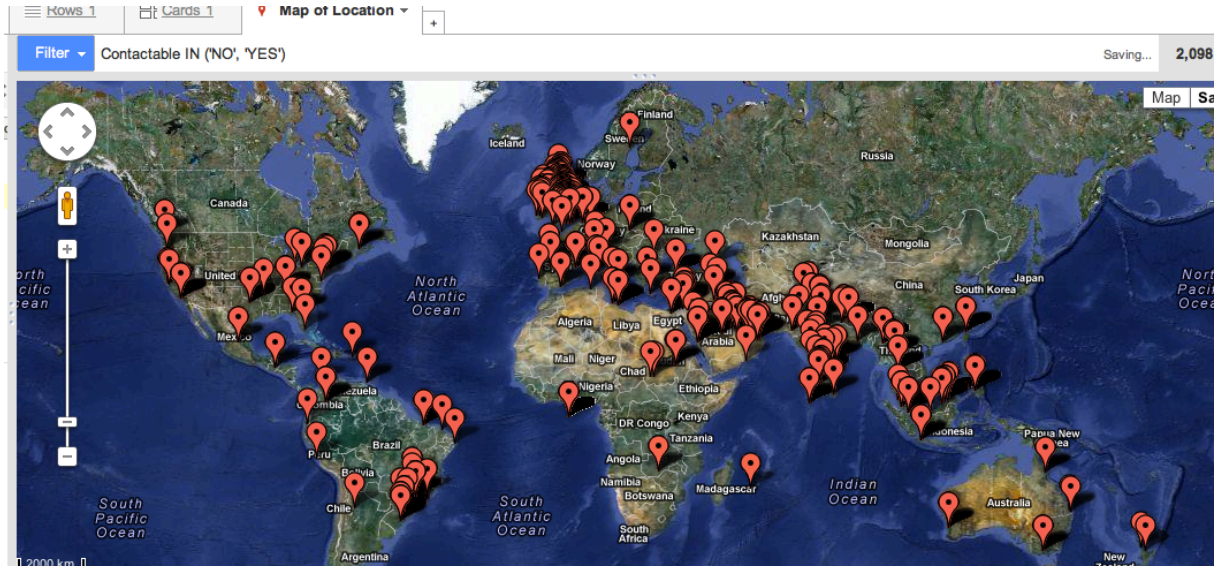


Figure 7. Comparison between geographical location of cases notified by neurologists through national study through BNSU and BPNSU over 2 years [a] and through the application in 3 months [b]

Our data collection approach allows collection of both the denominator data [i.e. which guidelines are being read by whom] and the numerator data [i.e. specific disease notifications]. This allows assessment of data collected as both simple frequencies and also as a proportion of usage for that individual user and also as a

proportion of usage of the specific disease guideline of interest. Furthermore, these data could be visualised as a global or regional heat-map which can incorporate population data, for example disease notifications could be represented as an incidence within a population or even and incidence within a population exposed to a particular environmental risk factor.

During this pilot period, the encephalitis guideline was read on 32 separate occasions and 5 cases of encephalitis were notified as having received aciclovir within the recommended 6 hours of admission. Notifications came from junior trainees, middle-grade doctors and a professor; 2 in the UK, 1 in Spain, 1 in Brazil, and 1 in China. Influenza guidelines were accessed 19 times, with 3 relating to CNS involvement and with two cases notified as H1N1, one of whom is reported as having received antivirals.

The application does not collect any patient-identifiable demographic data, but rather the data is of the doctor's practice, which they have agreed to provide at the point of installing the app. In addition, it also has the potential to increase recruitment into on-going clinical studies. If a doctor is seeing a patient and reading a guideline for a disease in a hospital in which there is an on-going study, then an automated system can generate an email to the doctor and the research nurse for that hospital allowing them to make contact to consent the patient to be recruited into that study. Therefore, no confidential or patient-identifiable information is made available centrally.

During this initial proof-of-concept pilot phase of the application, notifications from the individual user are available for them to review for themselves within the app

itself. However, the wider database was only available to the central research team. Nevertheless, as there has been growing interest in the potential for this rich data source to inform future research, I have developed a number of research collaborations, including with researchers at the Universities of Liverpool, San Diego and Oxford, through which I am making the data relating to the conditions of interest readily accessible.

However, this approach to data collection is reliant on doctors using clinical guidelines. This will vary between disease areas in response to many factors, such as disease complexity or rarity, new guideline availability, individual clinician experience with the disease in question and available time. In addition, doctors do not typically refer to guidelines for every patient they see, particularly in areas where they are able to practice without seeking further information, and in these areas our data collection will be more limited. Nevertheless, clinicians regularly use guidelines and other reference texts to access information that is difficult to remember, such as drug dosages, which specific investigations are most sensitive and specific, or diseases that are not routinely seen, such as is the case for acute viral encephalitis. Furthermore, routine use of clinical guidelines, and indeed audit of their use, has an increasingly important role in day-to-day clinical practice. Moreover, as it does not require any additional time to notify a disease once they are reading the guideline, it is hoped that, if even only for specific disease areas, some useful data will be collected. Nevertheless, as novel technologies start to generate a new type of epidemiological data, new approaches to understanding and automating how best to interpret these data, including algorithm generation, will be required (Vidal et al 2006; Wang et al 2010).

Future versions of the application will need to be developed for other smartphone platforms, such as the Google Android© market, and will need to be monitored to be compatible with software updates. As many doctors use smart phones on a daily basis there is at least the potential to access a wide cohort from whom to collect data. Moreover, future iterations could provide information for, and access data from, allied health care professionals, scientists and the wider public. Already the application has received broad-based interest, such as in the national public press, including the Guardian, and international scientific media, including the Lancet (Sample 2012; Arnold 2013)

4.4 Conclusions

It is unclear at this very early stage if this sort of approach can improve disease surveillance and clinical practice research, or indeed how many doctors would have to use such an app to create the critical mass to collected meaningful data. Further studies are needed to assess the data collected through these approaches in parallel with that collected through on-going studies and routine surveillance approaches.

Nevertheless, current approaches appear to miss many cases, particularly of encephalitis, and novel approaches, such as ClickClinica ©, may provide data to start to address this.

However, specific data of epidemiological interest, such as specific pathogens

identified, is limited not just by disease notification but also by the attending clinician establishing the diagnosis and aetiology of an acute CNS infection. Particularly, a LP and appropriate investigation of the CSF is pivotal to establishing both the diagnosis and the aetiology. Previous pilot work that I conducted in single centres has identified delays in investigation and consequently missed diagnoses in patients with suspected CNS infections (Bell et al 2009; Michael et al 2010). However, this has not been investigated across multiple centres. Therefore, I took this work forward to investigate the aspects of clinical management of patients with suspected acute CNS infection to identify sub-optimal aspects of management and delays that may result in reduced detection rates of acute viral encephalitis.

5. Diagnosis at individual level- Sub-optimal investigation

5.1 Introduction

Central nervous system infections are a neurological emergency requiring urgent investigation and treatment (Van de Beek et al 2006; Solomon et al 2007). Among the most common CNS infections are meningitis, in which the brain meninges are primarily affected, and encephalitis, when the brain parenchyma is primarily involved; however, the distinction between meningitis and encephalitis is not always clear clinically, as many of the symptoms and signs overlap, and the term meningoencephalitis is often used (Kennedy et al 2005; Solomon et al 2007). In the UK, as in other developed countries, *S. pneumoniae* is the most common cause of bacterial meningitis in adults, whilst HSV is the most common cause of viral encephalitis. However, the number of patients with suspected and clinically diagnosed CNS infection is much greater than that of proven aetiology (Bell et al 2009; Michael et al 2010).

Over the last 15 years the management of acute bacterial meningitis in adults has received considerable attention in the UK, with detailed epidemiological studies, vaccination programmes and the introduction of National Guidelines (Heyderman et al 2000; Heyderman et al 2003; Gjini et al 2006). In comparison, CNS infections caused by viruses have been relatively neglected. A recent pilot study from a teaching and secondary care hospital in our region suggested that the management of patients

with suspected viral encephalitis may often be suboptimal, particularly with regard to delays in performing a LP and starting treatment (Bell et al 2009).

To see if these preliminary findings are reproduced more widely, we therefore conducted a detailed, retrospective study of adults with all suspected acute CNS infections at 10 hospitals in the NHS North West region. This allowed us to compare the epidemiology, clinical features, and initial management of patients with suspected viral and bacterial CNS infections, focusing on the key issues of clinical predictors of a CNS infection, the role of neuro-imaging and LP, and the initiation of treatment. This allowed us to assess which aspects of clinical management could results in delays in diagnosis of CNS infection and potentially impact on pathogen identification.

5. 2 Methods

I led a multicentre cross-sectional retrospective cohort study assessing patients managed over three months [1st September to 1st December 2007] of adults [>16 years] with suspected acute CNS infections admitted to 10 hospitals in the NHS North West Region serving a population of more than two million; seven were district general hospitals and three teaching hospitals, but none had tertiary infectious diseases or neurology units. Data were collected between January 1st and September 1st 2008. The study was approved by each of the Clinical Research and Audit Departments of the individual NHS Trusts involved.

To identify patients in whom a CNS infection might have been suspected we screened electronic laboratory records for patients who had had a CSF sample taken, and electronic pharmacy records for patients who had received intravenous aciclovir and/or an intravenous 3rd generation cephalosporin, which are the presumptive treatments for suspected acute viral encephalitis and bacterial meningitis respectively. The case notes were then examined to determine whether there were clinical features suspicious of a CNS infection. These included fever or prodromal flu-like illness, headache, new onset seizures, focal neurological weakness, confusion/altered behaviour, decreased GCS, signs of meningism [photophobia, neck stiffness, Kernig's sign] or rash (Solomon et al 2007; Bell et al 2009). Details, including whether the admitting team had suspected a CNS infection, were recorded on a standardised form by a member of the Northwest Neurological Infections Network team.

Data were collected at the Trusts where the patient had been identified and entered into an anonymised centralised database. Clinical management was compared with the standard of the British Infection Society guidelines for the management of suspected meningitis and with a regional guideline for the management of suspected encephalitis (Heyderman et al 2003); these indicate that patients with suspected CNS infections should have an urgent LP, unless they have one of the following contraindications: GCS<13 or declining [>2 points], seizures, focal neurological signs, strong suspicion of meningococcal septicaemia, systemic shock, coagulation disorder, immune compromise or signs of raised intracranial pressure [Cushing's reflex, papilloedema, altered pupillary response, ocular palsies, decorticate/decerebrate posturing or abnormal respiratory pattern].

5.2.1 Case Definitions

Patients were classified into clinical case definitions on the basis of their presenting features, initial CSF and imaging findings [Table 6] (Solomon et al 2007). Patients that met the definition of purulent or aseptic meningitis or encephalitis were considered to have a clinically diagnosed CNS infection whereas those with meningism only or encephalopathy only were not. After microbiological analysis was completed patients were further classified as microbiologically or virologically confirmed, if a pathogen was identified.

Table 7. Clinical case definitions

Clinical Case Definition	Clinical and initial investigatory criteria	Microbiological and virological confirmation
Encephalopathy Only	Altered consciousness with no evidence of inflammation in the CNS on imaging or CSF analysis (i.e. CSF white cell count <5/ μ L)	
Encephalitis	Altered consciousness with no other cause identified and evidence of CNS inflammation on imaging or CSF analysis (i.e. CSF white cell count ≥5/**µL**)	Defined as 'virologically confirmed' if a pathogen was identified by culture or PCR of the CSF
Meningism Only	Meningism (headache with neck stiffness and/or photophobia) without evidence of altered consciousness and no evidence of inflammation on imaging or CSF analysis (i.e. white cell count <5/**µL**)	
Purulent Meningitis	Meningism (as detailed above) with a CSF white cell count >1000/**µL**or between 100 and 1000/**µL**with a predominance of polymorphonuclear cells and a CSF: plasma glucose ratio <0.5 (or an unpaired CSF glucose <5mmol)	Defined as 'microbioloigcally confirmed' if a pathogen was identified by culture or PCR of the CSF or blood
Aseptic meningitis	Meningism (as detailed above) in a conscious patient, with a normal CSF: plasma glucose ratio (>0.5) and either a CSF white cell count of 5-20/**µL**, or 20-1000/ml with a lymphocyte predominance	Defined as 'virologically confirmed' if a viral pathogen was identified by culture or PCR of the CSF, or 'microbiologically confirmed' TB meningitis if *Mycobacterium tuberculosis* was identified by culture or PCR of the CSF or blood

Where cases met none or more than one of these clinical case definitions the most likely diagnosis as judged clinically was used. Patients who received antibiotics for purulent meningitis or an extra-CNS bacterial infection were considered to have received antibiotics appropriately.

5.2.2 Statistical methods

Data were analysed with the SPSS program [SPSS Inc. 2007], using the chi-squared test to compare categorical data, the Mann Whitney test for non-parametric data and odds ratios with 95% confidence intervals where appropriate, with statistical significance defined as $p<0.05$. Methodology and results are presented in accordance with the STROBE Guidelines [STrengthening the Reporting of OBservational studies in Epidemiology; www.strobe-statement.org, www.annals.org]. Epidemiological incidence data were calculated with the assumption that all patients within the catchment areas would attend the corresponding hospital and that people from outside of the catchment area would not attend the hospital.

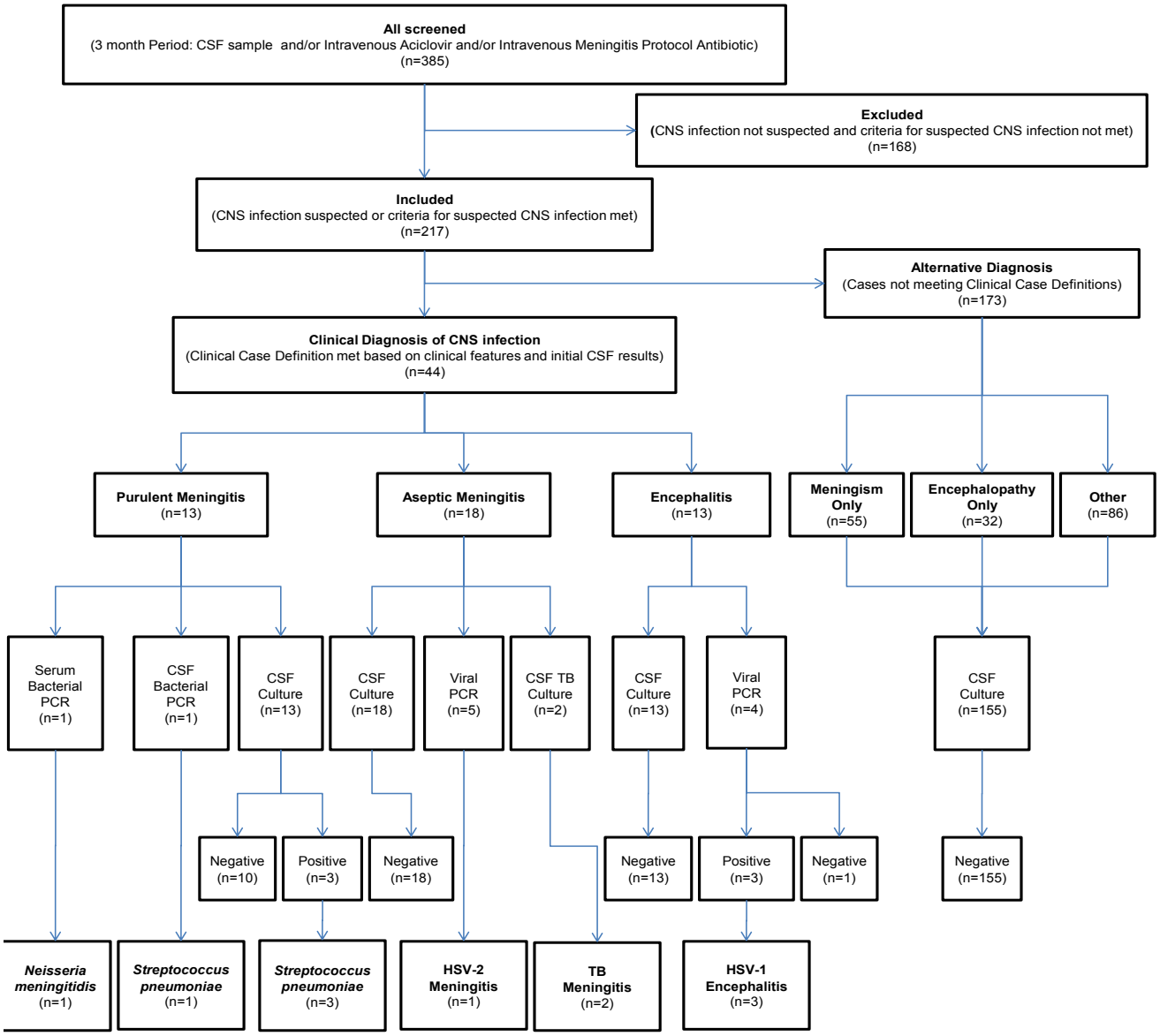
The number of people within the catchment population was identified from the published literature for each of the 10 NHS Trusts involved and the proportion of adults was defined as 79.6% of the catchment population as per the general UK population (Office of National Statistics 2009).

5.3 Results

5.3.1 Identification and clinical diagnosis

Initially 385 patients were identified by CSF and pharmacy screens, of whom 217 [56%] had a suspected CNS infection [Figure 8]. All CSF cultures were bacterial cultures and no viral cultures were performed.

Figure 8. Identification of patients with suspected CNS infection, clinical case definitions and microbiological/virological confirmation



Of these 18 were identified by the pharmacy screen only, 116 by the CSF screen only and 83 by both screens. All patients identified were acute admissions. The median [range] age of these 217 patients was 41 [16-87] years and 99 [46%] were male. Ten of them did not have CNS infection suspected by the clinical team, even though they had relevant clinical features; one of these was ultimately diagnosed with aseptic meningitis, following a LP for presumed subarachnoid haemorrhage. After initial investigations, 44 [20%] met the clinical case definitions for CNS infection [18 aseptic meningitis, 13 purulent meningitis, 13 encephalitis]; the 173 that did not included 32 [18%] with encephalopathy only, 55 [32%] meningism only and 86 [50%] other [Table 8]. Patients with a CNS infection were significantly older than those with other diagnoses [median [range] 44 [17-84] years, versus 37 [19-87] years, p=0.016].

Table 8. Diagnoses of 173 patients with suspected CNS infection who did not meet the clinical case definition of CNS infection

Diagnostic category	Diagnosis	Number
Non CNS infection	Unspecified viral illness	42
	Respiratory tract infection	6
	Urinary tract infection	4
	Gastroenteritis	3
	Otitis media	1
	Tympanomastitis	1
	Vestibulocochlear Varicella Zoster	1
Vascular	Cerebrovascular accident	6
	Transient Ischaemic Attack	2
	Subarachnoid Haemorrhage	2
Neoplastic	CNS Lymphoma	2
	Glioma	1
	Cerebral metastases	1
Immune	Guillain-Barre Syndrome	2
	Graft versus Host Disease	1
Chronic CNS infection	Progressive Multifocal Leukoencephalopathy	3
Metabolic	Metabolic encephalopathy	3
Psychiatric	Acute psychosis	2
Toxic	Alcohol withdrawal	2
Iatrogenic	Medication side effect	2
Idiopathic	No diagnosis reached	34
Other	Migraine	23
	Headache (unspecified)	18
	Idiopathic Intracranial Hypertension	1
	Transient Global Amnesia	1
	Pancreatic pseudocyst	1
	Epilepsy	4
	Epilepsy plus poor drug adherence	2
	First seizure	2
	Total	173

The catchment population was 2,218,000 of whom an estimated 1,765,528 were adults giving an approximate annual incidence for any CNS infection of 9.9 per 100,000; 4.1/100,000 for aseptic meningitis, 2.9/100,000 for purulent meningitis and 2.9/100,000 for encephalitis. On average each hospital admitted approximately 1.5 patients with a CNS infection per month.

5.3.2 Microbiological and virological diagnoses

CSF bacterial culture was performed for all 199 patients who had a LP and was positive for three patients [all with *S. pneumonia*]. Bacterial PCR was performed on the CSF of one patient which identified *S. pneumoniae* and the serum of one patient, positive for *N. meningitidis*; both patients had negative CSF and blood cultures. All patients with microbiologically confirmed acute bacterial meningitis came from the group with a clinical diagnosis of purulent meningitis. Viral PCR was performed for five [31%] of the patients with aseptic meningitis and herpes simplex virus-2 [HSV-2] was identified in one case. CSF Tuberculosis culture was performed for two patients with aseptic meningitis and was positive in both cases. The causative pathogen was not identified in 15 [94%] patients with aseptic meningitis. Only 4 [31%] of the encephalitis cases had a viral PCR performed, which identified herpes simplex virus-1 [HSV-1] in three cases. Thus the estimated annual incidences for pneumococcal meningitis, HSV-2 meningitis, and HSV-1 encephalitis, per 100,000 adults were 0.9, 0.2, and 0.7, respectively.

5.3.3 Clinical features

For the 217 patients in whom a CNS infection was suspected, the median [range] time to suspicion was 1 [0.3-312] hour for patients ultimately diagnosed with a CNS infection and 2 [0.2-720] hours for those with other diagnoses [p=0.259]; within the former group, time to suspicion of a CNS infection was significantly longer for patients with encephalitis than meningitis [4 [0.3-312] versus 0.3 [0.1-12] hours, p<0.001].

In table 9 the clinical features of the patients are compared, according to diagnostic group. Compared with patients with other diagnoses, patients with CNS infections were more likely to have a history of foreign travel, a history of fever, a documented pyrexia on admission, or confusion or behavioural change on examination. However, the sensitivity and negative predictive values of these parameters were not sufficiently high to be clinically useful as predictors of a CNS infection. Both patients with tuberculous meningitis had recently travelled from Africa [Nigeria and Ghana]. Two patients with CNS infections were immunocompromised due to human immunodeficiency virus infection. Overall, patients with CNS infections were not more likely to be immunocompromised than those with other diagnoses, nor to have a low GCS.

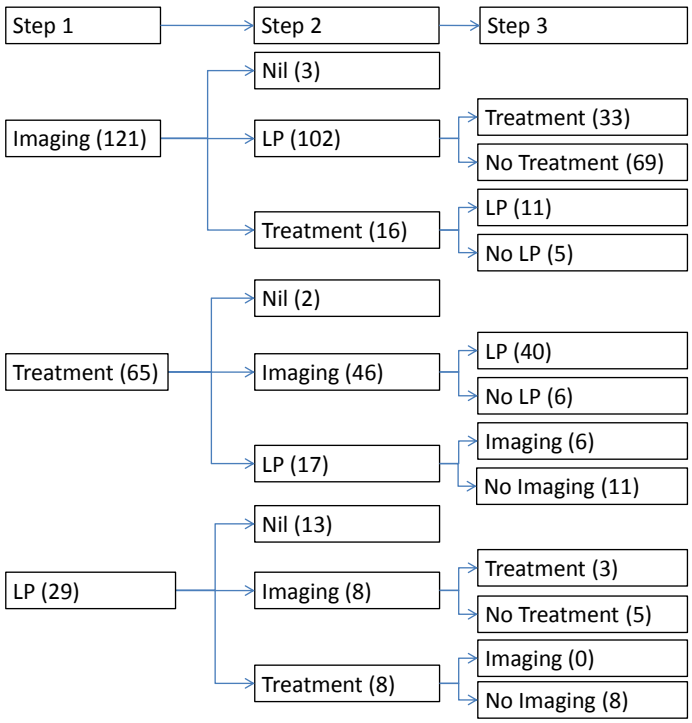
Table 9. The clinical features of patients meeting a clinical case definition of a CNS infection as compared with those who did not

Clinical Features	Clinical Case Definition					Sensitivity	Specificity	PPV	NPV	OR	*p* value
	Aseptic Meningitis (n=18)	Purulent Meningitis (n=13)	Encephalitis (n=13)	All CNS Infections (n=44) (%)	Other Diagnosis (n=173) (%)					(95% CI)	
History											
Fever	10	8	3	21 (48)	42 (24)	44	75	32	83	2.85 (1.36-5.98)	0.002
Febrile Prodrome	8	7	4	19 (43)	63 (36)	43	64	23	81	1.33 (0.64-2.74)	0.409
Headache	17	11	5	33 (75)	118 (68)	75	32	22	83	1.40 (0.62-3.19)	0.382
Nausea	10	8	4	22 (50)	84 (49)	50	51	21	80	1.06 (0.52-2.16	0.864
Vomiting	10	5	4	19 (43)	63 (36)	42	59	21	79	1.33 (0.64-2.74)	0.409
Photophobia	12	6	0	18 (41)	55 (32)	46	67	27	83	1.49 (0.71-3.09)	0.253
Immunocompromised	1	0	1	2 (5)	8 (5)	5	95	20	80	1.88 (0.26-10.54)	0.350
Travel	3	3	0	6 (14)	7 (4)	14	96	46	81	3.74 (1.04-13.35)	0.028
Seizures	2	1	5	8 (18)	12 (7)	10	93	27	79	2.98 (1.02-8.58)	0.021
Speech Disturbance	1	1	2	4 (9)	15 (9)	9	91	21	80	1.05 (0.28-3.65)	0.564
Neck Stiffness	8	7	1	16 (36)	59 (34)	42	67	25	81	1.10 (0.52-2.32)	0.778
Examination											
Drowsiness	3	2	6	11 (25)	25 (14)	25	86	31	82	1.97 (0.82-4.71)	0.093
Confusion/Behaviour change	0	4	12	16 (36)	33 (19)	36	81	33	83	2.42 (1.11-5.29)	0.014
GCS<15	0	5	5	10 (23)	30 (17)	23	83	25	81	1.40 (0.58-3.35)	0.411
Pyrexia	9	10	3	22 (50)	43 (25)	50	75	34	86	3.02 (1.45-6.34)	0.001

5.3.4 Investigation

Data were available for 215 [99%] patients regarding the timing of both initial investigations [imaging or LP] and antibiotic and/or antiviral treatment [figure 9]. Most patients had imaging [n=121, 56%] or treatment [n=65, 30%] first; only 29 [13%] had the LP first. Sixteen [7%] did not have a LP at any point. Treatment was delayed for imaging in 52 [29%] of the 181 who had imaging and it was delayed for a LP in 41 [21%] of the 199 who had a LP.

Figure 9. The order of investigations and treatment in the clinical management of 215 patients with suspected CNS infection



In total, 183 [84%] patients underwent neuro-imaging; 144 had CT only, 5 had MRI only and 34 had both. 153 [71%] patients had imaging before the LP, although in 101 [66%] there was no contraindication to an immediate LP. Of the 52 [24%] with clinical contraindications to an immediate LP these were predominantly focal neurological signs [n=17], seizures [n=15] and a high suspicion of meningococcal septicaemia [n=14].

The CT scan was abnormal in nine [17%] of these patients, but seven still required a LP to make the diagnosis [one with a suspected tuberculoma, two with enhancing cerebellar lesions ultimately diagnosed as progressive multifocal leukoencephalopathy, two suggestive of encephalitis, one hyperdense unilateral parietal lesion and one possible unilateral internal capsule infarct]. Two patients had a definitive diagnosis made by the CT scan, and so did not subsequently need a LP [one with cerebral metastases and one with a cerebrovascular accident]. In no cases was a radiological contraindication to a LP identified in the absence of clinical contraindications to LP. One patient with a CT scan suggestive of encephalitis, ultimately proved to have a glioma diagnosed by MRI, whilst the other had pneumococcal meningitis diagnosed by CSF examination. Only one of the three patients with HSV-1 encephalitis had an abnormal CT, which was thought to show a possible unilateral internal capsule infarct.

Of the 101 patients in whom there was no contraindication to an immediate LP, the neuro-imaging was normal in 88 [87%]. In the 13 cases who had an abnormal CT this typically showed periventricular ischaemia or atrophy, and in none did the CT scan demonstrate mass effect or brain shift precluding a LP and 12 [92%] patients went on

to have a LP, with no complications. The one patient who did not have a LP had progressive multifocal leukoencephalopathy diagnosed by MRI.

Thirty-nine [18%] patients underwent an MRI scan a median [range] 5 days [2 hours-23 days] after admission. MRI was abnormal in 20 [51%] patients, diagnosing generalized ischaemia [n=6], cerebral atrophy [n=3], progressive multifocal leukoencephalopathy [n=3], acute cerebrovascular accidents [n=3], CNS lymphoma [n=2], cystic metastases [n=1], glioma [n=1] and a tuberculoma [n=1].

The median [range] time from admission to LP was significantly longer for patients who had neuro-imaging first than for those who did not [18.5 [2-384] versus 6 [1-72] hours, $p<0.0001$]. There was also a significant delay in performing the LP in those who proved to have encephalitis compared with meningitis [23 [4-360] versus 12 [2-48] hours, $p=0.042$].

CSF analysis

The CSF opening pressure was recorded for only 71 [36 %] of the 199 patients who had a LP; 41 had a pleocytosis and the white blood cell differential was recorded in 29 [70%]; the protein was measured in 180 [90%] and although the CSF glucose was recorded in 176 [88%] a simultaneous plasma glucose was only taken in 74 cases [37%].

Of the 44 patients who met the clinical case definition of a CNS infection, 25 [57%] had antibiotic and/or antiviral treatment commenced before the LP of whom 6 [24%] had CNS infection confirmed microbiologically or virologically. For the 19 patients

with a CNS infection who had the LP prior to treatment, 5 [26%] were confirmed microbiologically or virologically. Six [55%] of the 11 patients with microbiologically or virologically confirmed CNS infection, had the diagnosis made by PCR of the CSF or serum rather than culture. Two of the three patients who had a positive CSF culture had the LP before treatment whilst the third had one dose of a 3rd generation cephalosporin 10 hours before the LP.

5.3.5 Treatment

The median [range] time from admission to starting treatment was longer for aciclovir than cephalosporin [7 [0.5-312] versus 3 [0.3-312] hours, respectively, *p*=0.002] [table 9]. However there was no such delay for aciclovir treatment in patients who had the LP performed before imaging. The median [range] time from suspicion of encephalitis to aciclovir and of suspicion of meningitis to a 3rd generation cephalosporin was similar [2 [1.5-299] versus 2 [0.3-48] hours, respectively, *p*=0.148]. Of the 112 patients who had both a LP and antimicrobial treatment, 68 [61%] had treatment prior to the LP.

Table 10. The time from admission to empirical treatment in patients with suspected CNS infection in relation to the order of investigations

	Time to Aciclovir (n=48)	Time to Cephalosporin (n=88)	*p value*
All patients	7 (0.5-312)	3 (0.3-312)	0.002
Imaging then LP	7.5 (0.5-312)	3 (0.5-312)	0.004
LP then Imaging	5 (2-21)	5 (0.3-76)	0.725

Thirteen patients with purulent meningitis and ten with non-CNS bacterial infections clearly needed antibiotic treatment; 84 additional patients received intravenous 3rd generation cephalosporin for a median [range] duration of 3 [0.25-13] days. 114 patients received antimicrobial therapy [a cephalosporin and/or acyclovir] that proved unnecessary, and had information available about the timing of the LP. Antimicrobial treatment was more likely in those that had treatment started before LP, than those who had treatment started after the LP [52 [74%] of 70, versus 14 [32%] of 44 [p<0.0001].

5.3.6 Outcome

The median [range] in-patient hospital stay was 12.3 [1-163] days for the 44 patients with a CNS infection. Microbiological advice was sought for 18 [38%] of them and neurological advice for 13 [28%]. One patient was transferred to the intensive care unit and one to the regional neurological centre; both had HSV1 encephalitis complicated by seizures and reduced consciousness. Both survived, but with sequelae; one had a residual hemiplegia and speech disturbance and the other had epilepsy. A third patient had impaired hearing following pneumococcal meningitis. One patient,

who presented with suspected encephalitis proved to have a large cerebrovascular accident and subsequently died.

5.4 Discussion

Central nervous system infections remain an important cause of morbidity and mortality in the UK in the twenty-first century. Hospital Episode Statistics show more than 14 million finished consultant episodes in the UK NHS, using nearly 53 million bed-days (Davison et al 2003; Whitley 2006). Although there are few good health economic studies, the burden to the NHS and society can be judged from medical negligence costs, which typically exceed three million pounds for the most serious cases (Schlesinger 2009).

Over the last 15 years acute bacterial meningitis has attracted considerable attention in the UK, with increased awareness among the public and health care workers, national management guidelines, vaccination programmes and improved outcomes (Heyderman et al 2000; Heyderman et al 2003; Snyder 2003). Neurological diseases caused by viruses have received less attention, and are generally considered to be less important, but as far as we are aware, there has been no comparative study, examining the incidence, clinical features and management of acute viral and bacterial CNS infections in a single population.

In our study of adults admitted to ten hospitals across the NHS North West region, we calculated an annual incidence for purulent meningitis of 2.9/100,000, for aseptic

meningitis it was 4.1/100,000 and for encephalitis it was 2.9/100,000. Thus during the study period the incidence of encephalitis was the same as that of purulent meningitis. Our calculated incidence for purulent meningitis was similar to those reported from previous studies of adults in Western Industrialised settings of 0.6-4 per 100,000 (Fitch et al 2007) the incidence of aseptic meningitis was lower than previous studies 5.2-7.6/100,000 (Kupila et al 2006; Logan et al 2008) whilst the incidence of encephalitis was higher than previous reports from similar settings 1.5-2.2/100,000 (Davison et al 2003; Kupila et al 2006). We conducted our study during the winter months only; if anything this might have led to an over-estimate of the annual incidence of purulent meningitis, which is more common in the winter, and an under-estimate of viral meningitis, which is more common in the summer months; in non-tropical countries the incidence of viral encephalitis has no seasonality (Fitch et al 2007; Solomon et al 2007).

In terms of aetiology, we found that HSV was equally as common as *S. pneumoniae*, both accounting for an annual incidence of 0.9 CNS infections per 100,000 adults [0.7 cases of encephalitis and 0.2 of meningitis for HSV]. Although our study was limited in scope, the incidences we found were similar to those reported previously: 1.2-2.3/100,000 for pneumococcal meningitis in all ages (Weisfelt et al 2006), 0.2-0.4/100,000 for HSV encephalitis in all ages (Whitley 2006), and 1.4/100,000 adults for HSV meningitis in adults (Kupila et al 2006). Although the denominator in our study, based on the reported hospital catchment populations, is likely to be an overestimate, the fact that it was the same for bacterial and viral diseases allows for a meaningful comparison. Whereas all patients had bacterial culture of CSF performed,

only a handful had CSF PCR for viruses, so the true incidence of HSV infection may be even higher.

There were also important differences in the clinical management between patients with suspected meningitis and suspected encephalitis in our study. The average time from admission to suspicion of CNS infection was significantly longer for encephalitis [4 hours] than for purulent or aseptic meningitis [0.3 hours]. Moreover, there was a significant delay in performing the LP in patients with encephalitis as opposed to meningitis, and patients with suspected meningitis had antimicrobial treatment commenced sooner after admission than those with suspected encephalitis.

There may be a number of possible explanations for these differences including the lack of awareness of the clinical features of encephalitis, and the broad differential diagnosis of patients with confusion or altered behaviour, focal neurological signs, or seizures; of note less than half the patients with encephalitis had a reduced GCS, but nearly all had altered behaviour or confusion. In addition the management of patients with suspected meningitis appears to have improved following the publication of British Infection Society guidelines, particularly with regard to time to treatment (Heyderman et al 2003; Michael et al 2010).

Compared to patients that ultimately had other diagnoses, those with CNS infections were more likely to have a travel history, altered behaviour or confusion, and pyrexia. However none of these features in isolation, or in combination, could identify such patients with sufficient sensitivity or specificity to reliably diagnose a CNS infection. This can only be done with CSF analysis (Chadwick 2005; Solomon et al 2007). The

management guidelines and reviews for meningitis and encephalitis emphasise the need for an urgent LP in all patients, unless there are clinical contraindications (Heyderman 2005; Solomon et al 2007). The LP gives an immediate indication of whether there is a CNS infection, whether this is more likely to be bacterial or viral, and subsequently, what the pathogen is. This allows rationalisation of treatment and avoidance of unnecessary antimicrobials, thus minimising the chances of resistance developing, and of avoidable iatrogenic events; these include nephrotoxicity with aciclovir, and opportunistic infection with cephalosporins (Hasbun et al 2001; Stone 2007). In our study many patients were exposed to antibiotics they did not require, which might have been avoided with an earlier LP. There was a significantly higher percentage of patients who received antibiotics that proved to be unnecessary if treatment was started before rather than after the LP.

We found, as have others previously, that the majority of patients underwent unnecessary imaging before the LP, which can cause delays in treatment (Chadwick 2005; Proulx et al 2005; Bell et al 2009). Only 13% of the patients in this study had a LP undertaken before any imaging or treatment was started. Patients with encephalitis that did not have imaging before a LP were started on aciclovir sooner. Although some have postulated that a CT scan first may obviate the need for a LP, in our study only two patients who had a CT first did not subsequently need an LP performing. Encouragingly, the median time from admission to starting aciclovir treatment in this study was 7 hours; this is a considerable improvement on the median 48 hours, from our previous pilot study, which was conducted 3 years earlier at one of the hospitals included in the current study (Bell et al 2009).

In summary, this study conducted across 10 hospitals in the NHS North West Region showed that in adults clinically diagnosed encephalitis was as common as purulent meningitis, and that HSV was a more common cause of CNS infection than previously thought. The management of patients with suspected encephalitis was worse than that of suspected meningitis, in terms of time to suspicion of the diagnosis, time to LP, and time to initiation of treatment. National encephalitis guidelines are needed to assist clinicians in the management of these complex patients where early investigation and treatment has a significant effect of morbidity and mortality.

In this study I also identified several aspects of clinical management that may result in reduced diagnosis of viral encephalitis in at least two thirds of cases. At the time of performing the LP only 36% had the opening CSF pressure measured, only 37% had a paired plasma glucose sent, and only 31% of cases meeting the criteria for encephalitis had viral PCR performed. All of these basic investigations are pivotal to establishing both the diagnosis and the aetiology, and therefore directing treatment (Solomon et al 2012). In addition, failure to establish the aetiology for the individual patient concerned has a further impact on disease surveillance. Moreover, only 13% of patients had the LP without waiting for imaging and this practice resulted in statistically significant delays in performing the LP. The impact of these delays on the viral load, and therefore pathogen detection, is poorly understood. Therefore, to assess this I undertook a quantitative study of the viral load in the CSF of patients with acute viral encephalitis; this allowed ask whether the time from presentation to LP had an impact on viral load detected, and also whether the viral load corresponded to disease severity and outcome.

6. Impact of delayed investigation on viral load

6.1 Introduction

The most common cause of sporadic viral encephalitis is infection with the herpes viruses HSV and VZV, with the latter being one of the most common viral CNS infections in Western Europe (Koskiniemi 2001; Minjolle 2002; Persson 2009). However, in approximately 30-40% of cases of suspected viral encephalitis no cause is identified (Granerod et al 2010).

The advent of PCR analysis to detect viral DNA in CSF revolutionised the diagnosis of viral encephalitis, as viral culture of CSF is typically negative despite active CNS infection (Wildemann 1997; Domingues et al 1998). Subsequently, real-time quantitative PCR [qPCR] has been used, both in research and clinical settings, to quantify the viral load in the CSF (Munoz-Almagro et al 2008). Previous studies have identified that, generally across the range of suspected CNS infections, the CSF is most likely to be positive by PCR in those who have the LP between 3-14 days after symptom-onset and that there is a progressive decline in the proportion of CSF samples positive from day 5 after symptom onset (Davies et al 2005). However, it is unclear if the declining rates of pathogen detection reflect declining viral load with time, or whether this is simply that those patients with a non-viral cause tend to have the LP later. This is of importance as several studies have identified delays in performing the LP in patients with suspected viral encephalitis in routine clinical practice (Bell et al 2009; Michael et al 2010).

As the most common viral cause of CNS infection, VZV has a varied presentation and those with encephalitis have a very varied outcome, from recovery with few sequelae, to deterioration and death despite antiviral treatment (Gilden 2004; Kennedy 2005; Easton et al 2007; Solomon et al 2007), there has been interest in whether the viral load might help predict prognosis (Aberle et al 2005; Persson et al 2009). However, previous studies have not included the time from symptom onset to LP in the analysis.

Therefore, I investigated the relationship between the VZV viral load in the CSF of patients with encephalitis relative to the time from symptom onset and also assessed this in relation to markers of clinical disease severity and outcome.

6.2 Subjects, materials and methods

6.2.1 Study participants and procedures

I screened the electronic records of the Liverpool specialist virology laboratory over a five year period [2004-2009] to identify adults [>18 years old] who had a CSF sample that that had been requested as clinically indicated for VZV PCR, to then identify those who were positive. The clinical case notes were assessed to confirm that the clinical features were consistent with encephalitis; i.e. clinical signs of parenchymal brain dysfunction, such as focal neurological deficit; and/or altered consciousness; and/or seizures; and at least two of: fever >38^{o}C; CSF lymphocytic pleocytosis >5x10^{6}/L; electro-encephalography or neuro-imaging abnormality consisted with encephalitis (Jmor et al 2008; Bell et al 2009; Persson et al 2009). Those with clinical features

consistent with meningitis were excluded; i.e. meningism [headache with neck stiffness and/or photophobia] in a conscious patient, without seizures or focal neurological signs, as described previously (Michael et al 2010).

6.2.2 Laboratory methods

I conducted qPCR using a validated commercial assay [Artus VZV LC-PCR, Qiagen Group], with a uniform volume of CSF and processed this using a Light-Cycler analyser [Roche Diagnostics ™ 2007]. Patients were dichotomised into those with high and low viral loads as described previously, with a high viral load defined as $>5.0x10^4$ copies/μL (Domingues et al 1998). All CSF samples also underwent PCR for HSV1 and 2. The time to LP was defined as the duration from the onset of symptoms of acute encephalitis, as defined above, to performing the LP.

Data were collected from the clinical case notes and electronic records in accordance with national guidelines (Government, UK 1998). Outcome was assessed retrospectively from the notes using the GOS score, as described previously (Mailles et al 2012; Poissy et al 2012). A good outcome was defined as those with a GOS of 5 [good recovery] and a poor outcome as a GOS of <5 [moderate disability-death]. The GCS score at admission was recorded from the clinical case notes and used as a marker of clinical disease severity as this has been shown to correlate with outcome in viral encephalitis (Winter et al 2004; Whitley 2006).

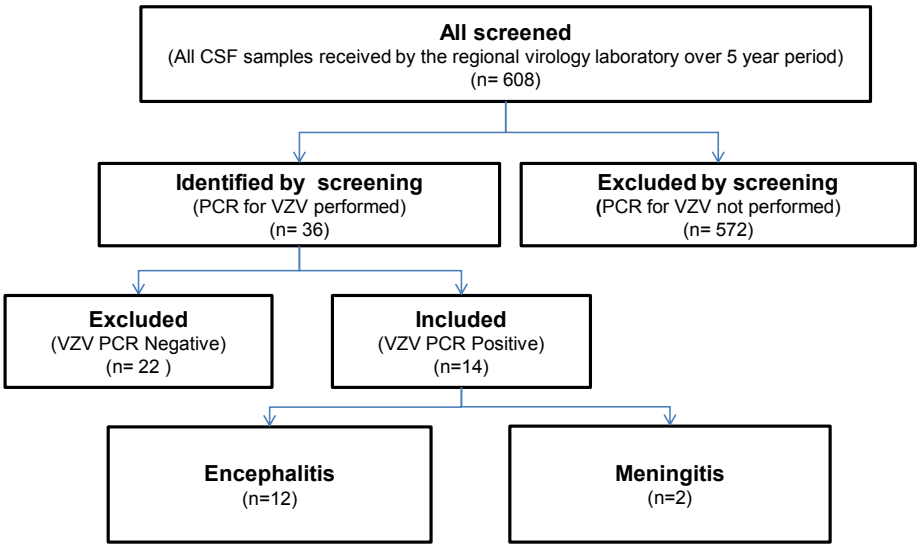
6.2.3 Statistical analysis

Analysis was performed using SPSS [2009]; the Mann-Whitney U test and linear regression performed for continuous nonparametric data; chi-squared test performed for categorical data; and Kendall's tau test performed for nonparametric correlation analysis. A *p value* <0.05 was defined as statistically significant. The incidence was calculated from the specialist virology catchment population [1,515,932] (Government, UK 2001).

6.3 Results

Over five years, the Liverpool Specialist Virology laboratory received 608 CSF samples, of which 36 had a PCR for VZV performed and 14 [39%] were positive [Figure 10]; 12 had clinical features consistent with encephalitis and 2 were consistent with meningitis. The median [range] age was 49 [20-79] years and 8 [67%] were male [Table 11].

Figure 10. Identification and classification of cases of VZV encephalitis



Abbreviations:

CSF: Cerebrospinal Fluid
VZV: Varicella Zoster Virus
PCR: Polymerase Chain Reaction

Table 11. Clinical, microbiological, viral load and outcome data in twelve patients with varicella zoster virus encephalitis

Patient Number	Sex	Age (years)	Immune Compromise	Glasgow Coma Score	Viral Load (copies/µl)	CSF WCC (/mm^3)	CSF Protein (g/l)	Time from symptom onset to LP (hrs)	Aciclovir before LP Duration (hours)	Glasgow Outcome Score (at discharge)
1	M	74	N	15	1720	42	0.70	192	168	3
2	M	64	Cyclophosphamide	14	28.73	9	0.35	106	N	3 *
3	M	76	N	14	56,000	66	0.77	48	N	3
4	M	20	N	15	1840	224	1.82	96	N	5
5	F	29	HIV	14	160,000	48	1.44	8	N	4
6	F	27	N	NA	9860	200	NA	72	NA	5
7	M	27	N	15	1510	158	1.12	120	24	5
8	F	21	N	15	4.57	14	0.81	156	N	5
9	F	65	Metastatic ovarian cancer: Chemotherapy	NA	7,000,000	6	NA	96	NA	1
10	M	73	NA	NA	1.59	39	0.54	NA	NA	NA
11	M	79	N	15	NA	NA	0.66	189	N	4
12	M	34	N	15	NA	78	1.58	96	N	5

Glasgow outcome score: 5 little-minor disability; 4 moderate disability; 3 severe disability; 2 persistent vegetative state; 1 death
* Glasgow Outcome Score 3 on admission and discharge
(N: No; NA: Not available; CSF: Cerebrospinal fluid; HIV: Human Immunodeficiency Virus. LP: Lumbar puncture; WCC: White cell count)

The median duration from onset of symptoms to admission was 3 [1-7] days. Five patients had a good outcome [GOS 5] and six a poor outcome, comprising two with moderate disability [GOS 4], three with severe disability [GOS 3], and one death [GOS 1]. One patient had the same disability on discharge [GOS 3] as on admission due to neurological disability secondary to Parkinson's disease [patient 2]. There was a positive correlation between age and a poor outcome [tau b 0.57, p = 0.02]. Three patients were immunocompromised; one due to HIV [CD4 count and HIV viral load at admission were $261x10^9$/L and 1920 copies/mL, respectively]; one due to cyclophosphamide for a renal transplant; and one had received chemotherapy during the month preceding admission for metastatic ovarian cancer.

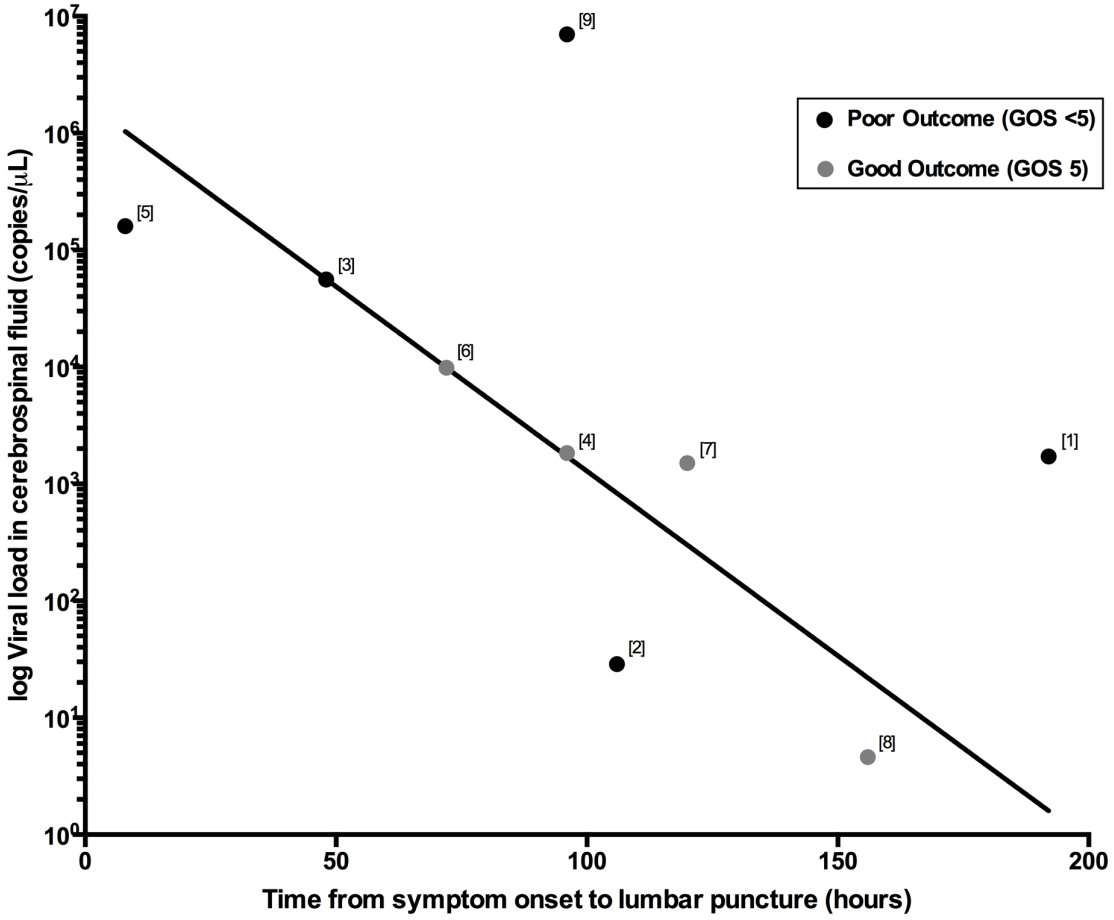
All patients had one LP. The median [range] time from the onset of symptoms to LP was 101 [8-192] hours. Two patients received intravenous aciclovir prior to LP [24-168 hours). The median CSF white cell count and protein level were 66 [6-224] cells/mm^3 and 0.79 [0.35-1.82] g/L, respectively. All patients had a CSF lymphocytosis [85-100%]. There was a significant correlation between the CSF white cell counts and a good outcome [tau b 0.63, p=0.03], and a trend towards a negative correlation between CSF protein and good outcome [-0.58, p=0.055].

The median VZV load was 1780 [1.6 – 7 x 10^6] copies/µL. All CSF samples were negative for HSV1 and 2 by PCR. There was no correlation between age and viral load [p=0.59]. Of the three patients with immune-compromise, 2 had a high viral load and the patient who was immunosuppressed following chemotherapy [patient 9] had the highest viral load in this study, 7.0 x 10^6 copies/µL, and was the only patient to die from encephalitis. Two of 3 patients with a reduced GCS score on admission had a

high viral load, compared with one of 6 with a normal coma score, although this was not significant [p=0.23]. The only patient to suffer acute symptomatic seizures had a high viral load. The two patients who had aciclovir started before the LP had a low viral load [1510 and 1720 copies/μL]. All patients received the same dosing regimen of aciclovir [10mg/kg 8 hourly] for a median duration of 11.5 [5-21] days. There was no significant difference in the duration of treatment with intravenous aciclovir between those patients with a high or low viral load [p=1.0].

Nine patients had data available on viral load, time to LP, and outcome [Table 12]. There was a negative correlation between the time from symptom onset to LP and the viral load [tau b= -0.59, p = 0.036] [Figure 11]. There was no correlation identified between the timing of the LP and outcome [tau b = 0.02, p = 0.93]. There was no significant correlation between the viral load and the outcome overall [tau b= 0.35, p=0.26].

Figure 11. Relationship between timing of lumbar puncture, CSF viral load and outcome in nine patients with VZV encephalitis



CSF: Cerebrospinal fluid; GOS: Glasgow outcome score; VZV: Varicella zoster virus

The median [range] viral load of those with a good outcome was not significantly lower than those with a poor outcome [1675 [4.6-9860] and 56,000 [1720-7.0 x 10^6] copies/μL respectively, p=0.3]. All the three patients with a high viral load had a poor outcome and four of the six patients with a low viral load had a good outcome [p=0.14]. The two patients with low viral load and a poor outcome were patient 2, who had a GOS of 3 on both admission and discharge, and patient 1, who was the only patient to receive aciclovir for >24 hours prior to LP [168 hours], which may have contributed to the low viral load. Interestingly, if these two patients are removed from the analysis, a high viral load was significantly associated with a poor outcome [tau b= -0.73, p=0.04].

Table 12. Clinical, microbiological, management and outcome data for nine patients with high versus low varicella zoster viral load

Viral Load (copies /µl)	Number of cases	Viral Load (copies/µl) Median (range)	Age (years) Median (range)	Duration of symptoms before LP (hours) Median (range)	Aciclovir before LP	Duration of Aciclovir (days) Median (range)	CSF WCC (cells/mm^3) Median (range)	Glasgow Coma Score below 15 (on admission)	Glasgow Outcome Score on discharge	
									Good (GOS = 5)	Moderate/ Severe impairment/ or death (GOS <5)
<50,000	6	1510 (1.59-9860)	34 (20-79)	113 (72-192)	2	11.5 (5-14)	78 (9-224)	1	4	2
>50,000	3	160,000 (5.6-700x10^4)	65 (29-76)	28 (8-48)	0	9.5 (5-14)	27 (6-48)	2	0	3
			(p=0.46)	(p=0.051)			(p=0.42)	(p=0.11)		(p=0.14)

6.4 Discussion

Viral encephalitis is a severe life-threatening infection of the CNS with an annual incidence of 2.9-10/100,000 (Granerod et al 2010). VZV accounts for up to 29% of cases of viral CNS infection, with encephalitis being the most common manifestation (Persson et al 2009). Despite advances in PCR the aetiology remains unknown in 30-40% of patients with suspected viral encephalitis (Granerod et al 2010). Previous studies have identified delays in performing the LP in patients with suspected viral encephalitis (Bell et al 2009; Michael et al 2010). Delays in performing the LP have been shown to be associated with a lower proportion of cases in which a virus is identified (Davies et al 2005). However, it is unclear if this reflects a reduction in viral load over time or whether patients with non-viral causes of encephalitis undergo the LP later. In those patients in whom a virus is identified, there is a varied prognosis ranging from complete recovery to severe cognitive impairment and epilepsy, or death (Domingues et al 1998). However, for the most part the variation in outcome remains poorly understood, and few prognostic markers are available (Raschilas et al 2002; Ooi et al 2007).

PCR analysis of CSF has revolutionised the diagnosis of viral encephalitis and avoids the need for a brain biopsy, the previous gold standard (Wildemann et al 1997). Real-time qPCR can determine the viral load and is used in clinical practice increasingly; for example it can distinguish between a low viral load, which might represent latent infection, from a high viral load, which is more likely to represent active infection (Wildemann et al 1997). More recently there has been interest in whether qPCR might

shed light on pathogenesis and prognosis in viral encephalitis (Munoz-Almagro et al 2008).

In our study of the Liverpool specialist virology laboratory database over 5 years, I identified 12 adults with acute encephalitis due to VZV. In this, the first study to assess the temporal relationship of VZV load, we identified a negative correlation between the time from symptom onset to LP and viral load [tau b = -0.59, p = 0.036]. This may be explained by earlier presentation and investigation of patients with more clinically severe infection, as these patients were also more likely to have a reduced GCS or seizures on admission. However, there was no significant correlation between the time of the LP and outcome overall. We did not identify a significant correlation between viral load and outcome and the difference in outcome between those in the low and high viral load groups was not significant. However, interestingly, if the two potentially confounding patients are removed from the analysis [one with neurological disease on admission and the other, who received prolonged aciclovir treatment prior to the LP], a high CSF viral load was associated with a poor outcome [p=0.04]. There is a potential for the volume of CSF to bias qPCR results therefore standard sample volumes were used for all cases. However, sample storage may also affect qPCR results and we did assess samples collected over several years. Nevertheless, we did not find a significant difference in viral load between samples obtained at different times in this small cohort. One further source of bias is the site of viral reactivation with higher CSF viral loads expected in those with lumbar reactivation than those with reactivation in the cervical region or more superiorly. Therefore, we excluded patients with a zoster rash although this group warrants further investigation in the future.

Previous studies of VZV infection have found that patients with encephalitis had higher CSF viral loads than those with meningitis and that those who required intensive care also had higher loads (Aberle et al 2005; Persson et al 2009). However, neither assessed the viral load in relation to outcome and neither assessed the viral load in VZV encephalitis in relation to the time from symptom onset to LP. In HSV encephalitis, six previous studies found no clear correlation between viral load and outcome overall, (Wildemann et al 1997; Domingues et al 1998; Kamei et al 2004; Ruzek et al 2007; Munoz-Almagro et al 2008; Poissy et al 2012) though two did find that those with either very high viral loads or an initial rise in viral load after treatment had a worse outcome (Kamei et al 2004; Ruzek et al 2007). One additional study in adults found a higher viral load in patients with poor outcome (Domingues et al 1998). However, none of these studies took into account the time, after symptom onset, at which CSF viral load was assessed.

This study confirmed the findings of others that older patients have a worse outcome (Kennedy 2005; Ooi et al 2007). However, there was no correlation between age and viral load. In this small cohort I identified that a low CSF white cell count correlated with a poor outcome. This may reflect the low CSF white cell counts identified in those patients with immune compromise who had a poor outcome, confirming the findings of others that an impaired ability to mount a sufficient host immune response is associated with a poor prognosis (Cinque et al 1998; Solomon et al 2007). On the other hand other studies of viral encephalitis have found a strong inflammatory response is associated with a poor outcome (Winter et al 2004; Kamei et al 2009).

Clearly there is a complex range of factors involved, and a targeted or balanced host response may be the key to a better outcome.

In conclusion, whilst delays in performing the LP may result in a higher proportion of patients for whom no virus is identified, even in those with proven VZV encephalitis, delay in the LP was associated with a significant decline in viral load. Although this may in part reflect those with less severe clinical presentations having the LP performed later, a potential implication is that for patients with suspected viral encephalitis, the greatest likelihood of identifying a pathogen is if the LP is performed in a timely manner and before treatment is started. The prognostic value of the CSF viral load has not been established and this may reflect a rapid reduction in the viral load over time or that a key component of the pathogenesis of viral encephalitis is due to the inflammatory response and related down-stream effects.

Given the identification of delays in investigation in routine practice and the potential impact of this on viral PCR results, along with the lack of parallel CSF investigations in the majority, I wanted to determine whether the introduction of a simple clinical intervention could improve the collection of the appropriate clinical samples and reduce the time to performing the LP.

7. Improving investigation through a lumbar puncture pack

7.1 Background

Although cases of proven CNS infections, such as meningitis and encephalitis, are relatively rare, their recognition is important because rapid diagnosis and treatment significantly reduces morbidity and mortality (British Infection Society 2003; Van de Beek et al 2006; Solomon et al 2007; Tunkel et al 2008; Bell et al 2009).

Investigations on serum and CSF samples obtained at the time of LP, when interpreted in concert, are vital to directing acute treatment towards a viral, bacterial, mycobacterial or fungal pathogen, or a non-infectious diagnosis (Tunkel et al 2008; Solomon et al 2012). Furthermore, advances in molecular techniques, such as PCR, have improved pathogen detection (Chadwick 2002; Granerod et al 2010). Indeed, CSF PCR is the now the gold standard for viruses and also for bacteria, if the culture is negative, as if often the case when antibiotics have been given before the LP (British Infection Society 2003; Michael et al 2010; Solomon et al 2012).

Despite guidelines for CNS infections, research by our group and others has demonstrated typically inadequate samples are taken at the time of the LP and there are often delays in performing the LP; often because there is uncertainty about whether a CT scan of the head is required first (Bell et al 2009; Michael et al 2010; Michael et al 2010). These delays have been shown to reduce the chances of establishing the diagnosis and thus of giving the right treatment (Rasmussen et al 1992; Aronin et al 1998; Michael et al 2010). Notably, for viral encephalitis there is a

progressive decline in the proportion of patients who have a positive PCR of the CSF beyond 5 days from symptom-onset, this may be associated with a lower viral load, and prior acyclovir treatment results in a rapid reduction in viral load (Kamei et al 2004; Davies et al 2005; Michael et al 2011). In addition, failure to obtain the full set of first line investigations, including a paired serum glucose sample, reduces the accuracy of interpreting the CSF findings (Solomon et al 2007; Tunkel et al 2008).

For some conditions, simple clinical interventions can improve management; for example in sepsis (Minton et al 2008). In addition, management of suspected sub-arachnoid haemorrhage [SAH] improved following the publication of guidelines and the introduction in many hospitals of a LP pack for SAH (Bederson et al 2009). However, this improvement in investigation for SAH, may have contributed to inadequate sample collection for patients with suspected CNS infections. As these two diagnoses are the most common reason for a LP in the acute medical setting and these guidelines and packs may have focused sample collection on those required for SAH diagnosis.

Therefore, to address this I modified an existing LP pack for SAH to create a LP pack which would guide clinicians on the appropriate investigation for both a suspected CNS infection and/or a suspected SAH; we evaluated the pack's impact on the quality of investigations for suspected CNS infections.

7.2 Methods

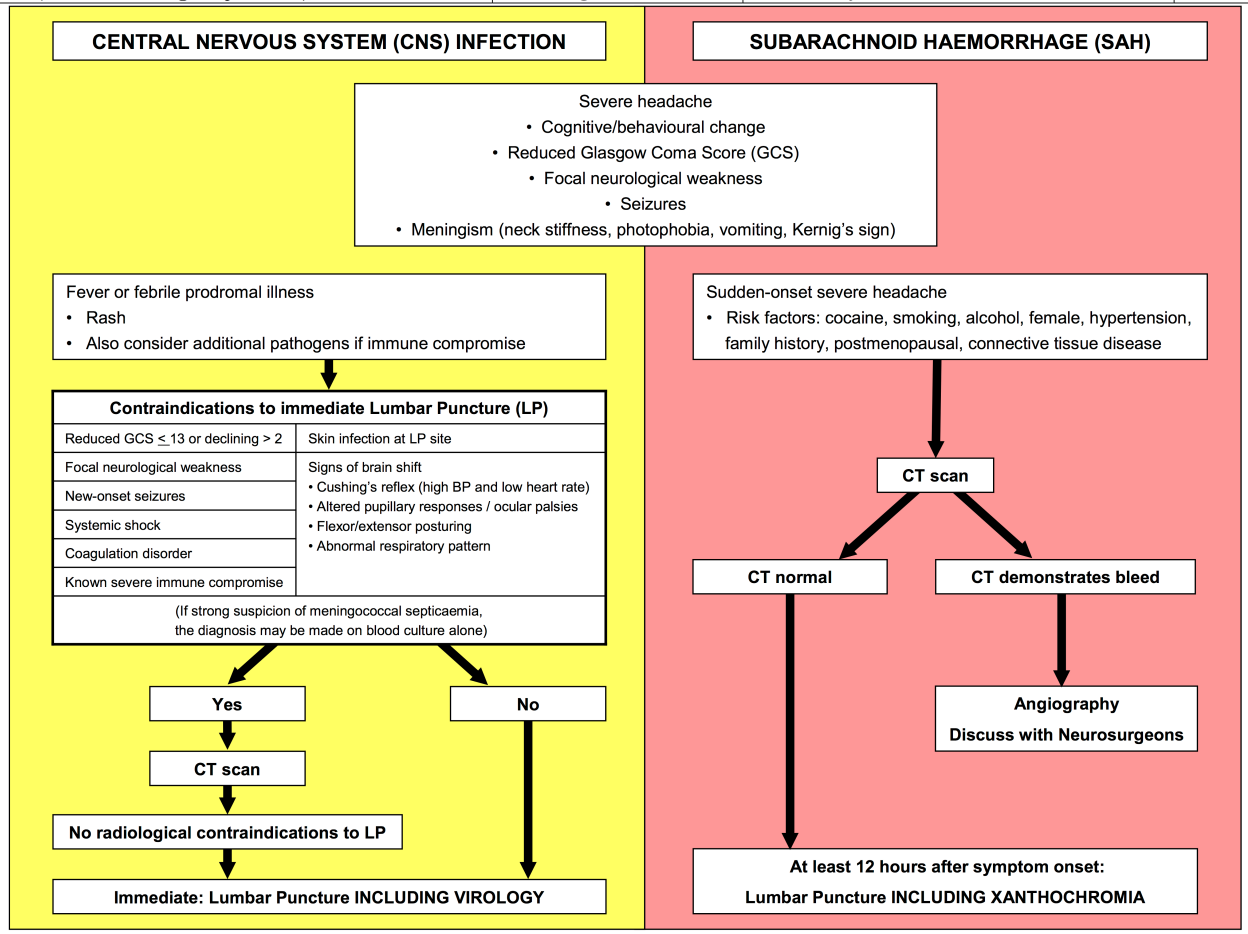
7.2.1 Setting

This study took place in the acute medical admissions unit [MAU] of the Royal Liverpool University Hospital; a large inner city teaching hospital in the NHS North West region of England with a catchment population of 450,000 adults. Based on existing data we would expect approximately 32-65 patients with an acute CNS infection each year; including 2.7-18 cases of bacterial meningitis, 23.4-34.2 cases of aseptic meningitis and 6.75-13 cases of encephalitis (van de Beek et al 2004; Michael et al 2010; Solomon et al 2012).

7.2.2 Lumbar puncture pack

To improve the diagnosis of CNS infections we developed a LP pack [Figure 12]. This involved building on the existing SAH flowchart and considering recommendations from the national meningitis and encephalitis guidelines (Heyderman 2005; Solomon et al 2007). This describes the clinical features directing investigation for a CNS infection or a SAH and guides when to perform a CT or a LP first. The second side indicates which samples should be taken, the volumes required, the bottles to fill, where to send and how to transport. The pack also contains numbered bottles for CSF and blood, which correspond to the flowchart.

Figure 12. Lumbar puncture pack management algorithm


CENTRAL NERVOUS SYSTEM (CNS) INFECTION
SUBARACHNOID HAEMORRHAGE (SAH)
Severe headache
• Cognitive/behavioural change
• Reduced Glasgow Coma Score (GCS)
• Focal neurological weakness
• Seizures
• Meningism (neck stiffness, photophobia, vomiting, Kernig's sign)
Fever or febrile prodromal illness
• Rash
• Also consider additional pathogens if immune compromise
Contraindications to immediate Lumbar Puncture (LP)
Reduced GCS ≤ 13 or declining > 2
Skin infection at LP site
Focal neurological weakness
Signs of brain shift
• Cushing's reflex (high BP and low heart rate)
• Altered pupillary responses / ocular palsies
• Flexor/extensor posturing
• Abnormal respiratory pattern
New-onset seizures
Systemic shock
Coagulation disorder
Known severe immune compromise
(If strong suspicion of meningococcal septicaemia, the diagnosis may be made on blood culture alone)
Yes
No
CT scan
No radiological contraindications to LP
Immediate: Lumbar Puncture INCLUDING VIROLOGY
Sudden-onset severe headache
• Risk factors: cocaine, smoking, alcohol, female, hypertension, family history, postmenopausal, connective tissue disease
CT scan
CT normal
CT demonstrates bleed
Angiography
Discuss with Neurosurgeons
At least 12 hours after symptom onset:
Lumbar Puncture INCLUDING XANTHOCHROMIA

Diagnostic Question	
? CNS Infection only	Immediate lumbar puncture; collect samples 1 to 3 (CSF), 5 (CSF), 6 (blood) and 8 (blood) only
? SAH only	CT normal and > 12 hours after symptom onset; collect samples 1 to 4 (CSF) and 7 (blood) only
? SAH and ? CNS Infection	Collect samples 1 to 5 (CSF) and 6 to 8 (blood)

Order	Sample	Container	Volume	Tests	Specimen Transport
1st	CSF	Universal 	2.5 mL	Microbiology	**MICROBIOLOGY** Write tests required + clinical details on Microbiology form Pneumatic tube
2nd	CSF	Fluoride EDTA 	0.5 mL	Glucose + Protein	
3rd	CSF	Universal 	2.5 mL	Microbiology	
4th	CSF	Minicollect + carrier 	1 mL (Fill to ↑)	Xanthochromia	**BIOCHEMISTRY** Write tests required + clinical details on Biochemistry form Insert labelled minicollect tube into labelled bilirubin carrier tube PROTECT FROM LIGHT (envelope) Transport by hand within 30 minutes DO NOT USE PNEUMATIC TUBE
5th	CSF	Universal 	2 mL	Virology	
6th	Blood	Plain Serum 	4 mL	Virology	
7th	Blood	Serum Gel 	4 mL	Liver Profile	
8th	Blood	Fluoride EDTA 	3 mL	Glucose	

To keep the introduction as simple and inexpensive as possible we aimed for it not to require any additional educational programme. Therefore, in this pilot study it was introduced to the MAU stock room without any educational intervention. Prior to this project there was no mechanism to assist in sample collection for CNS infections.

7.2.3 Outcome measures

Our primary aim was to increase the proportion of patients who had a LP for a suspected CNS infection for whom appropriate CSF investigations were performed based on the guidelines:

- CSF for protein
- CSF and paired plasma for glucose
- CSF for cell count and differential
- CSF for bacteria: MC and S and PCR if culture negative

- CSF for viruses: PCR [or sample stored]

Failure to perform each of the above investigations was defined as a major error. Secondary aims were to increase the proportion of patients who had a LP for a suspected CNS infection for whom a pathogen was identified and the proportion of patients with a CSF pleocytosis who had a pathogen identified, and to reduce the time to LP.

We screened laboratory records to identify any patient who had a CSF sample sent from the MAU for the six-month period before and six-month period after introduction of the pack [December 2009-May 2010 and June-November 2010 respectively]. I then assessed the electronic hospital records to identify those who had the LP for a suspected CNS infection as determined by the admitting team. If a patient had more than one LP, each was assessed. The case records were examined by two authors [BDM and GP], with any disagreements resolved through discussion with the senior author [TS]. A CSF pleocytosis was defined by the laboratory as a white cell count >4/mm^3. None of the investigators performed a LP in the hospital during this time period.

The x^2 and Fischer's exact tests were used for categorical data and Mann Whitney U test for nonparametric continuous data, with statistical significance defined as $p<0.05$.

7.3 Results

The laboratory database screen identified 177 LPs that had been performed on 168 patients in the MAU during the study period, 93 before and 84 after the introduction of the pack; 52 [55%] and 41 [48%], respectively were performed for a suspected CNS infection. The age and gender distribution in the two groups was comparable [Table 13].

Table 13. Samples collected in patients with suspected CNS infections before and after the introduction of a simple lumbar puncture pack

		Pre-LP Pack	Post-LP Pack	P value
Number of Patients		52	41	
Age [Median (range)]		39 (18-78)	37 (17-82)	0.19
Male (%)		30 (58)	29 (71)	0.5
CSF Pleocytosis (%)		18 (35)	14 (34)	1
Number of LPs performed with major errors (%)	**Any major error**	44 (85)	20 (49)	0.0003
	No CSF Glucose	9 (17)	0	0.005
	No Plasma Glucose	28 (54)	0	0.0001
	No CSF Protein	1 (2)	0	1
	No CSF Cell count and differential	0	0	1
	No CSF MC+S	0	0	1
	No CSF Virology	44 (85)	20 (49)	0.0003
Positive Investigations (%)	**CSF Bacterial culture**	2 (4)	2 (5)	1
	CSF Bacterial PCR	1 (2)	2 (5)	0.49
	CSF Viral PCR	2 (4)	5 (12)	0.16
	All CSF PCR	3 (6)	7 (17)	0.09
	All CSF PCR for patients with a CSF pleocytosis	3 (17)	7 (50)	0.059
Time to LP (hours) [Median (range)]		8 (1-71)	8 (1-46)	0.28

Abbreviations: LP Lumbar puncture; CSF Cerebrospinal fluid; MC+S Microscopy, culture and sensitivity; PCR Polymerase chain reaction

Following introduction of the pack, there was an improvement in all parameters assessed. All 41 patients had CSF protein and CSF bacterial studies requested; all patients had CSF glucose and paired blood glucose sent, which were significant improvements compared to the pre-pack period [p=0.005 and p=0.0001 respectively]. The number with major errors in sample collection decreased from 44 [85%] to 20 [49%] [p=0.0003]. The remaining cases were due to failure to take a sample for viral PCR. Prior to introduction of the LP pack 2 [4%] patients had a virus identified in the CSF; after the introduction of the pack 5 [12%] patients had a virus identified in the CSF, this was not statistically significant. Prior to introduction of the pack two patients had a virus identified HSV type 2 [n=1] and VZV [n=1]]; after introduction of the pack five had a virus identified [HSV type 1 [n=1], VZV [n=1] and Enterovirus [n=3]].

Of those patients with a CSF pleocytosis, there was a trend towards an increased proportion with viral or bacterial pathogen being detected by PCR after introduction of the pack, although this did not reach statistical significance [3 [17%] and 7 [50%] respectively, p=0.059]. Bacteria were identified in the CSF by PCR in 1 patient prior to the pack [*N. meningitides*] and 2 patients following the pack [*N. meningitidis* and *S. pneumoniae*]. No patient without a CSF pleocytosis had a pathogen detected by PCR. Prior to the pack one patient had an alpha-haemolytic streptococcus and one a coagulase-negative staphylococcus cultured; after the introduction two patients had coagulase-negative staphylococci cultured. All these cultures were considered to be contaminants as the pathogen is a common skin contaminant, there was no CSF pleocytosis and the patients recovered fully with only symptomatic treatment. There was no significant

difference between the time from admission to LP following introduction of the pack.

7.4 Discussion

Analysis of samples obtained at the time of LP are key to directing treatment for patients with CNS infections; for whom early accurate diagnosis and treatment have a dramatic effect on outcome. Without the full complement of investigation results available it is more difficult for the clinician to direct treatment appropriately. For example, whilst a raised CSF white cell count and neutrophil predominance might direct the clinician to start antibiotics for presumed bacterial meningitis, the additional finding of a very low glucose ratio [<33%] and or very high protein should also direct investigation towards *M. tuberculosis* infection (Heyderman 2003; Solomon et al 2012). In addition, if the LP is performed early in viral encephalitis CSF neutrophils may predominate, which may direct the clinician towards treating for a bacterial infection. Therefore, without the identification of a normal glucose ratio and only slightly raised CSF protein, the clinician would not necessarily be directed towards investigating and treating for a viral pathogen also (Solomon et al 2007). Conversely, when the LP is performed early in patients with bacterial meningitis there may be a lymphocyte predominance, therefore without performing the investigations demonstrating a raised protein and low glucose ratio [<50%], the clinician may fail to start antibiotics appropriately (Heyderman 2000, Heyderman 2005). Moreover, a complete set of normal CSF results can reduce both the duration of

inappropriate antibiotics and the duration of hospital stay for those patients who are found not to have a CNS infection (Chadwick 2002).

Our study suggests that this simple intervention can significantly increase the proportion of patients having a LP who have the correct investigations performed. The proportion not having a CSF glucose sent decreased from 17% to 0% and the proportion not having a paired serum glucose sent was decreased from 54% to 0%. As well as more patients having CSF sent for virological analysis, there were more patients in whom a pathogen was detected. This is pivotal to guiding further treatment and investigation. For example, identification of a virus in patients with meningitis reduces antibiotic use and hospital stay (Durand et al 1993; Chadwick 2002). Moreover, detection of HSV type 2 should direct investigation towards possible genital infection (Solomon et al 2007). In addition, identification of some viruses, such as VZV and HSV type 2, should prompt investigation for HIV infection (Tunkel et al 2008). Detection of bacteria not only guides treatment but also informs important public health measures, such as prophylaxis (The National Institute for Health and Clinical Excellence 2010). However, as the pre-intervention period was between December and May and the post-intervention period was between June and November, more cases of Enterovirus would be expected during this period. Indeed this study identified 3 cases of Enterovirus infection in the post-intervention period in comparison to none in the preceding period. Therefore, this seasonality may account for some of the extra cases identified, and future studies should compare whole years or the same months to account for this potential confounding factor.

Potentially, some of the improvements in practice identified in this study may have been due to the Hawthorne effect if the doctors perceived that the introduction of a new LP pack was being researched and therefore changed their practice. However, we endeavoured to minimise the potential for this by only performing the data collection retrospectively after the study period was complete. Therefore, none of the doctors were overtly aware that any data collection on their practice was going to be performed. Nevertheless, the limitations of this retrospective data collection include the potential for data to be missed, as this approach is dependent on the information documented. Also as this study was conducted to assess the completeness of sample collection at the time of LP by screening laboratory records to identify patients who had had a LP, this study did not include those patients with a suspected CNS infection who did not have a LP. Previous studies have assessed all patients with suspected CNS infections and reported that, whilst the majority ultimately have a LP, there is often sub-optimal sample collection and delays in performing the LP (Bell et al 2009; Michael et al 2010; Michael et al 2010). Whilst sub-optimal sample collection may have an impact of the accuracy of interpretation of the results, the delays may further result in lower viral loads and decreased rates of viral detection (Davies et al 2005; Michael et al 2011).

Despite our intervention, the number of LPs performed with major errors in CSF sample collection was only reduced to 20 [49%]. Whilst this is a significant

improvement from the 44 [85%] without major errors prior to the intervention, many patients still did not have a complete set of CSF investigations sent. The main reason for an error in performing a LP was failure to send a CSF sample for virological investigation. To try to increase awareness and appropriate use of the LP pack I have adapted the electronic ordering so that the clinician has only to click on a tick box for 'suspected CNS infection' and/or 'suspected SAH' and the appropriate investigations are automatically populated and sample bottle labels automatically printed which correlate with the sample bottles in the LP pack. I have also added a patient information leaflet, consent form and an adhesive sticker to allow the easy documentation of the procedure. I have also piloted an educational programme of lectures, demonstrations, video and online tutorials, which now form a key component of an intervention in an NIHR-funded cluster intervention trial to try to improve the management of suspected viral encephalitis.

Following the results of this pilot study, the CSF collection pack has been adopted across the Royal Liverpool University Hospital NHS Foundation Trust and was easily incorporated into clinical practice. The CSF collection pack and the data from this study have also been presented to the National Patient Safety Agency and it has been included in their latest update paper (National Patient Safety Agency 2011).

In summary, this study has shown that the introduction of a simple LP pack to a busy acute medical admissions unit in a teaching hospital results in improvements in the investigation of patients with suspected CNS infection.

Larger studies will be needed, to be sufficiently powered, to determine whether the pack improves patient investigation in a range of different clinical settings, whether it does so in a cost effective manner, and ultimately whether it improves patient management.

Nevertheless, in both the pre- and post-LP pack periods only a low proportion of CSF samples tested were positive by routine PCR for HSV, VZV and enteroviruses, 2 (25%) and 5 (24%), respectively. This is a similar proportion to the 48 (24%) of 203 patients with encephalitis who were positive by PCR for these viruses in the largest UK study (Granerod et al 2010). This low rate of pathogen detection may be because of the limited panel of pathogens assessed, as a secondary panel of PCR, CSF culture and antibody testing in this study identified a causative pathogen in 86 (42%). However, despite this expert panel review of cases with an extended panel of tests for pathogens and further antibody testing for autoimmune encephalitides, the cause remained unknown in 75 [37%]. This is a similar proportion to that in whom no cause is identified across previous studies in both the developed and developing world setting (Granerod et al 2010). It is unclear if this significant proportion are due to novel infectious agents or antibodies, or missed detection of known pathogens or antibodies due to delays in testing, particularly following treatment as is the case in a significant proportion (Michael et al 2010; Kelly et al 2012). It is unknown if determining the host inflammatory profile may be useful in distinguishing between viral and immune-mediated encephalitis, and if so, what implications this may have for those cases in whom no cause is found. The only previous study to attempt to assess this was limited to small numbers of cases comparing

between HSV and a heterogeneous, ill-defined group of HSV-negative cases with radiological evidence of limbic encephalitis (Ichiyama et al 2008). In addition, all published studies of cytokines and associated mediators in encephalitis have assessed a narrow range of potential mediators, have also analysed the role of each mediator in isolation, and have not assessed the impact of these findings on the group in whom no aetiology is identified (Ichiyama et al 2008; Kamei et al 2009). Therefore, I assessed a wide range of mediators, in a large number of prospectively recruited patients and analysed mediator concentrations both in isolation and in concert. I undertook this work both to assess whether the host inflammatory mediator profile differed between those with proven infective and proven immune-mediated pathologies and also whether the inflammatory profile in those in whom no cause was found, despite extensive viral and antibody detection techniques, better reflected that seen in the infective or immune-mediated patients.

8. Augmenting investigation of aetiology with cytokine signatures

8.1 Introduction

Encephalitis is condition in which there is a pathological inflammation of the brain parenchyma, manifest as headache, cognitive and behavioural disturbances, focal neurological signs, seizures and coma (Solomon et al 2012). The most common aetiology is viral infection, with HSV type 1 being the most common sporadic pathogen, accounting for approximately 20% of cases in the UK (Granerod et al 2010). The recent identification of antibody-mediated encephalitis due to antibodies directed against the voltage-gated potassium channel complex and N-methyl-D-aspartate receptor has raised the possibility that the aetiology of encephalitis in those in whom no pathogen is identified may also be primarily antibody-mediated (Dalmau et al 2008; Vincent et al 2010). However, despite including testing for these antibodies the aetiology remains unknown in 37% [95% CI: 30-44%] in the largest UK study (Granerod et al 2010). In addition, whilst this study identified 111(55%) by first-line testing, and a further 17 (8%) by second-line testing, of the remaining 75 unknown cases, 36 underwent next-generation sequencing and no significant pathogen nucleic acid was identified (Ambrose et al 2013). The similarities and differences in the cytokine-mediated inflammatory profiles between viral and antibody-mediated encephalitis are not known. Moreover, the cytokine-mediated inflammatory profile in those of unknown aetiology has not yet been studied. Cytokines and chemokines are key mediators orchestrating leucocyte recruitment and

chemotaxis of leucocytes into the brain parenchyma (Vilela et al 2013). However, few histopathological studies have been conducted and these have been primarily in the pre-PCR and pre-aciclovir era (Love et al 2008). Therefore, little is known about the both the differences in mediator profiles and histopathological changes between aetiologies of acute encephalitis. In addition, even in cases of viral encephalitis, the timing of the LP may impact the likelihood of the PCR being positive, which may in part reflect falling viral load with time (Davies et al 2005, Michael et al 2011). Whilst mass spectroscopy has been used primarily to assess host inflammatory profiles, the potential of this technology to assess for viral peptides has only recently started to be explored in viral CNS infections (Zhang et al 2013; Leveque et al 2014).

Therefore I conducted a study to assess whether the cytokine signature in those in whom no aetiology has been established more closely resembled that of those with a viral or an antibody-mediated aetiology. I also took this forward to assess whether the histopathological changes were different between aetiological groups, and whether viral peptides could be identified in the CSF of patients in whom no cause was identified.

8.2 Aims

1. To determine whether the cytokine/chemokine signatures in the CSF and serum of patients with acute encephalitis due to viral infection differ from immune-mediated disease.

2. To determine if the mediator signature in those of unknown aetiology more closely resemble that in viral or immune-mediated disease.

3. To assess whether these findings are associated with histopathological differences.

4. To assess whether mass spectrometric analysis of CSF can identify viral peptides in those patients in whom no cause has been identified.

8.3 Methods

8.3.1 Pathogen detection

Patient recruitment is described in detail in the general methods section. In addition all patients underwent extensive initial testing [Table 13], if these were negative, they then underwent further second-line testing as determined by a multi-disciplinary panel of virology, microbiology, neurology, infectious diseases and acute medical experts in the HPA [Table 14]. Serum and CSF underwent cytometric bead array as described in detail in the methods section. A second cohort of patients was identified from the Walton Centre Biobank and histopathological tissue examined, as also detailed in the methods section. Control CSF samples were obtained from non-smoking adults (>18 years) recruited in the USA to a commercial biobank who were without clinical or microbiological evidence of neurological disease (Precision Med© 2014).

Table 14. First line testing for all patients with encephalitis recruited through the prospective HPA study of Encephalitis in England

First-line testing for cases of encephalitis*

If immunocompetent
Routine CSF PCR testing
- Herpes simplex virus 1/2
- Varicella zoster virus
- Enterovirus
- Parechovirus
- Adenovirus
- Human herpesvirus-6/7 [<30 years]
- Consider other tests depending on clinical features† *Routine serology*
- If increased activity:
  - Mumps or measles
  - Influenza A or B
- Human herpesvirus-6/7 [<30 years]

If immunocompromised *CSF PCR* As for immunocompetent, and consider:
- Cytomegalovirus
- Epstein-Barr virus
- Human herpesvirus-6/7
- JC virus
- Lymphocytic choriomeningitis virus
- HIV *Serology*
- JC virus

If travelled abroad *CSF PCR* As for immunocompetent, and consider:
- Arboviruses (Japanese encephalitis, dengue, tickborne encephalitis, Nipah virus, Murray Valley encephalitis, St Louis encephalitis)
- Poliomyelitis
- Rabies
- West Nile virus *Serology*
- Arboviruses (Japanese encephalitis, dengue, tickborne encephalitis, Nipah, Murray Valley encephalitis, St Louis encephalitis)
- Rabies

*Algorithm assumes appropriate investigations were also done when clinically indicated to exclude bacterial, fungal, and parasitic infections. †For cervical lymphadenopathy consider cytomegalovirus and Epstein-Barr virus; for respiratory illness consider influenza A and B; for parotitis and orchitis consider mumps.

Table 15. Additional pathogens investigated in patients in whom first line testing was negative as determined by the expert review panel of the HPA study of Encephalitis in England

Rare causes of encephalitis in England (not included in first-line testing)

Viral
Cytomegalovirus, Epstein-Barr virus, flaviviruses, hepatitis viruses, human T-cell lymphotropic virus, lymphocytic choriomeningitis virus, parainfluenza virus, parvovirus B19, poliovirus, rabies virus, respiratory syncytial virus

Bacterial
Bacillus anthracis, *Bartonella henselae*, *Chlamydophila psittaci*, *Chlamydia trachomatis*, *Legionella pneumophila*, *Leptospira* spp, *Listeria monocytogenes*, *Borrelia burgdorferi*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, *Salmonella* spp, *Streptococcus pneumoniae*, *Streptococcus pyogenes*

Rickettsial
Coxiella burnetii, *Rickettsia rickettsia*

Parasitic
Toxoplasma gondii

Fungal
Histoplasma capsulatum

8.3.2 Cytometric bead array

Cytometric bead array techniques and data analysis are described in detail in the methods section.

8.3.3 Mass spectrometry for viral peptides

Liquid chromatography, mass-spectrometry [LC/MS/MS] was performed on samples where sufficient CSF was available for those with viral encephalitis, encephalitis of unknown aetiology, and 5 laboratory control CSF samples

[Precision Med, California, USA]. Samples were stored in low-protein-binding tubes and underwent overnight protein digestions.

Because the CSF samples were of variable and unknown infectivity, all digestion reactions were performed in a category 3 laboratory. CSF was incubated with RapiGest [*Waters Corporation*] at a final concentration of 0.05 % [w/v] for 10 min at 80 °C. Protein samples were then reduced with 3 mM dithiothreitol [DTT] for 10 min at 60 °C, followed by alkylation with 9 mM iodoacetamide for 30 min in the dark at room temperature. Finally, trypsin was added and incubated overnight at 37 °C. To stop the proteolytic reaction and to inactivate and precipitate the detergent, trifluoroascetic acid [TFA] [final concentration 0.5 % [v/v]] was added, followed by incubation for 45 min at 37 °C. To remove all insoluble material, samples were centrifuged at 13,000*g* for 15 min. All peptide separations were carried out using an Ultimate 3000 nano system [Dionex/Thermo Fisher Scientific]. For each analysis, the sample was loaded onto a trap column [Acclaim PepMap 100, 2cm x 75mm inner diameter, C_{18}, 3mm, 100Å] at 5mL/min with an aqueous solution containing 0.1% [v/v] TFA and 2% [v/v] acetonitrile. After 3min, the trap column was set on-line with an analytical column [Easy-Spray PepMap® RSLC 15cm x 75mm inner diameter, C_{18}, 2mm, 100Å] [Dionex]. Peptide elution was performed by applying a mixture of solvents A and B. Solvent A was high-performance liquid chromatography [HPLC] grade water with 0.1% [v/v] formic acid, and solvent B was HPLC grade acetonitrile 80% [v/v] with 0.1% [v/v] formic. Separations were performed by applying a linear gradient of 3.8% to 50% solvent B over 120 mins at 300nL/min followed by a washing step [5min at 99% solvent B] and an equilibration step

[15 min at 3.8% solvent B]. A volume of 2 mL of each sample was injected. The Q Exactive instrument was operated in data dependent positive (ESI+) mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra [*m/z* 300-2000] were acquired in the Orbitrap [Quadrupole-Orbitrap] with 70,000 resolution [*m/*z 200] after accumulation of ions to $1x10^6$ target value based on predictive automatic gain control values from the previous full scan. Dynamic exclusion was set to 20s. The 10 most intense multiply charged ions [$z \geq 2$] were sequentially isolated and fragmented in the octopole collision cell by higher energy collisional dissociation [HCD] with a fixed injection time of 100ms and 35,000 resolution. Typical mass spectrometric conditions were as follows: spray voltage, 1.9kV, no sheath or auxiliary gas flow; heated capillary temperature, 275°C; normalised HCD collision energy 30%. The MS/MS ion selection threshold was set to 1×10^4 counts. A 2Da isolation width was set for MS. Raw data files were uploaded into Proteome Discoverer 1.3 and searched against the human UniProt database using the Mascot search engine [version 2.4.1]. Further searches were performed using the Swissprot database with 'viruses' taxonomy. A precursor ion tolerance of 10ppm and a fragment ion tolerance of 0.01Da were used with carbamidomethyl cysteine set as a fixed modification and oxidation of methionine as a variable modification. The false discovery rate against a decoy database was 1-5%.

8.3.4 Statistics

Cytometric bead array data for cytokines and associated mediators was analysed using the univariate and multivariate techniques described in detail in the methods section.

Protein homologies were interrogated using the European Bioinformatics Institute Database (European Bioinformatics Institute 2014). Data of viral peptides identified by mass-spectrometry were analysed by both the proportion of all samples of each aetiology in which any viral peptide was identified, in addition to the proportion of samples of each aetiology in which a viral peptide known to either cause human disease or be associated in close homology with an associated virus known to cause human disease, as described previously (Leveque et al 2014).

8.4 Results

8.4.1 Patients

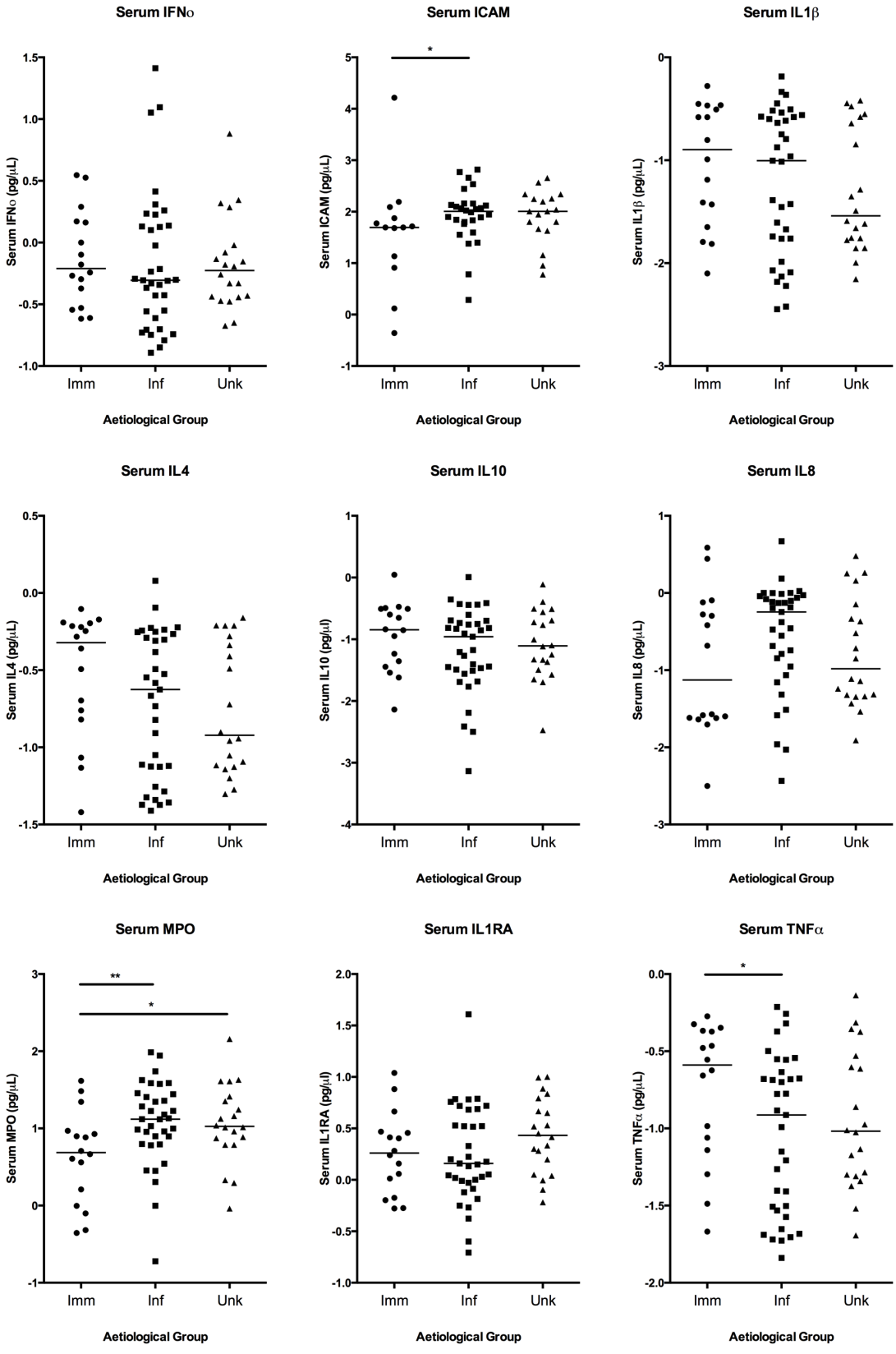
From the HPA cohort, cinical samples were available for 95 patients who presented with acute encephalitis. Serum samples were available for 78 patients, constituting 38 of an infectious aetiology [17 HSV, 7 VZV, 5 TB, 3 bacterial, 2 dual infection, 1 influenza A, 1 measles, 1 HIV, 1 toxoplasmosis], 20 immune-mediated cases [9 antibody-mediated, 8 ADEM, 1 paraneoplastic, 1 vasculitis, 1 multiple sclerosis], and 20 of unknown aetiology. CSF samples were available for 35

patients, constituting 18 of an infectious aetiology [11 HSV, 5 VZV, 1 JC virus, and 1 toxoplasmosis], 9 immune-mediated [5 ADEM, 3 antibody-mediated, 1 paraneoplastic], and 8 of unknown aetiology.

8.4.2 Mediator profiles between aetiological groups

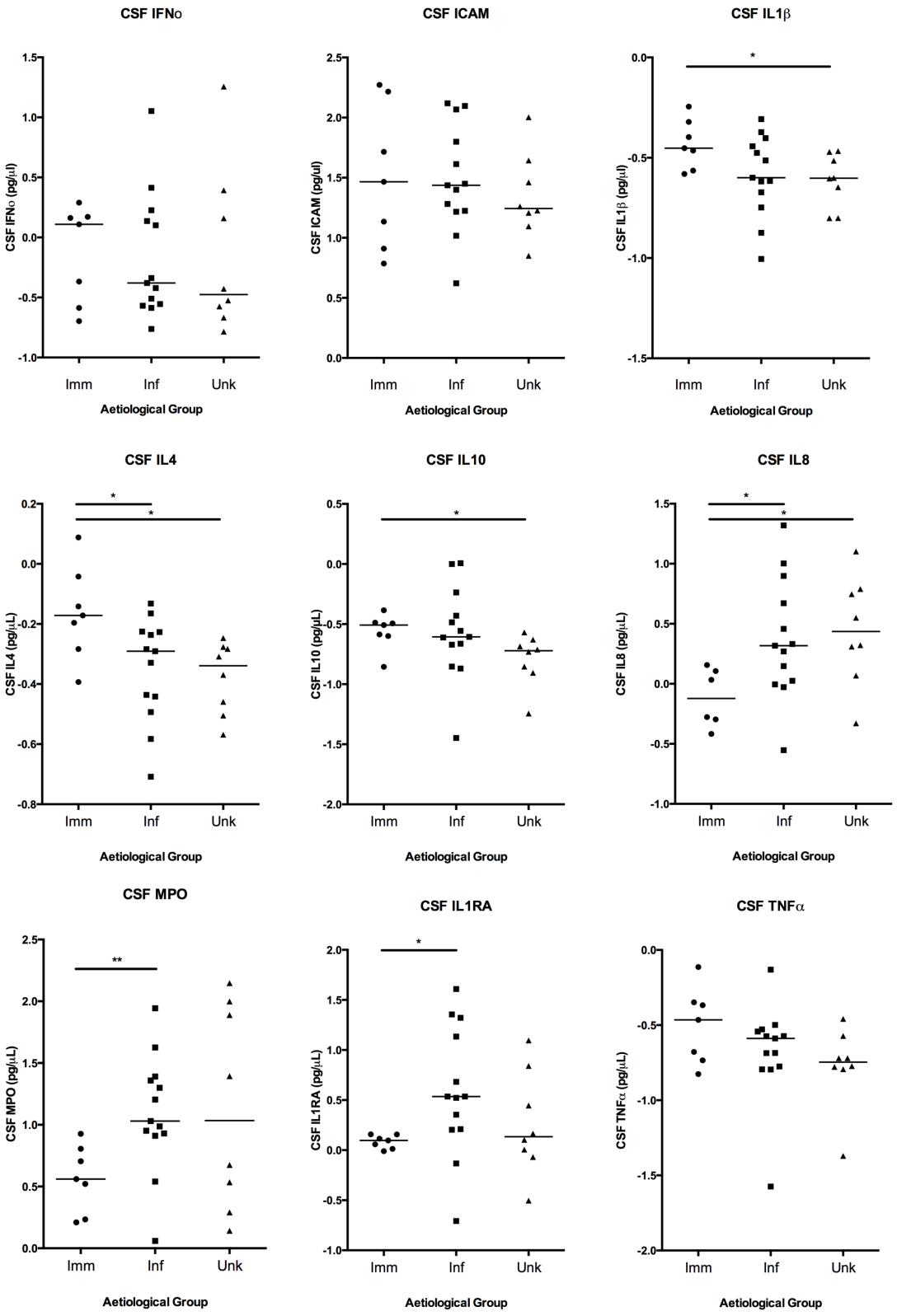
Univariate analysis of mediators in CSF and serum identified differences in concentrations for several mediators between the aetiological groups [Figure 13 and 14]. In both serum and CSF MPO was present at lower concentrations in samples of immune-mediated aetiology. In serum ICAM was highest in patients with an infective aetiology and TNFα in those with immune-mediated aetiology. In CSF concentrations of IL1β and IL10 were higher in immune-mediated than unknown, and IL4 was higher in immune-mediated than both unknown and infection groups. IL8 was present at highest concentration in the CSF of those of unknown aetiology.

Figure 13. Serum concentrations of mediators in patients with encephalitis of immune-mediated, infectious, and unknown aetiology



Log-transformed and median-centred data. * $p<0.05$; ** $p<0.01$

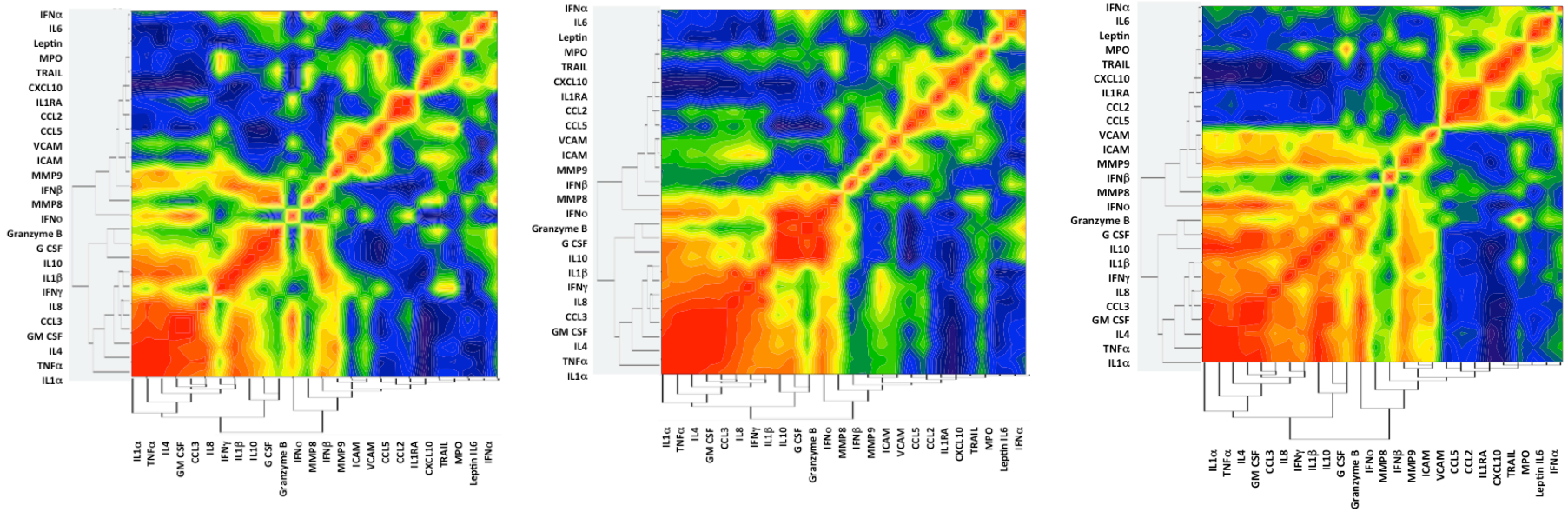
Figure 14. CSF concentrations of mediators in patients with encephalitis of immune-mediated, infectious, and unknown aetiology



Log-transformed and median-centred data. * p<0.05; ** p<0.01

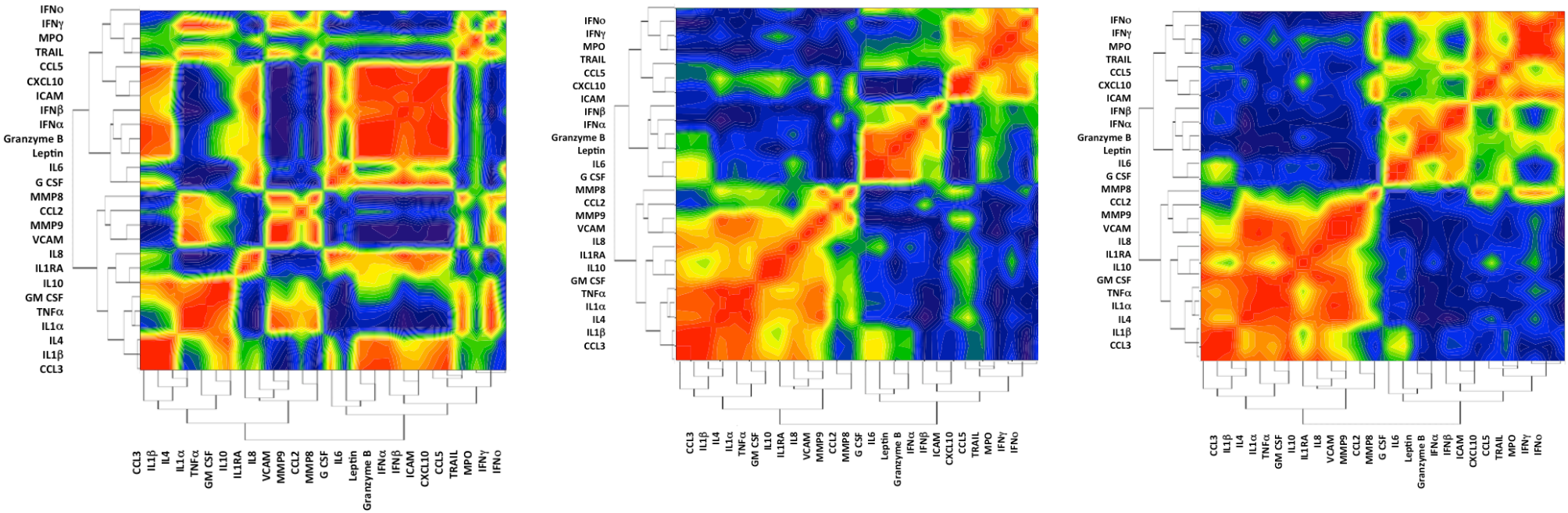
Hierarchical cluster analysis identified two broad clusters of mediators in both serum and CSF. The group A cluster contained IL1α, IL1β and IL10, in addition to other mediators; in CSF these same mediators were identified in group A with the addition of IL1RA. The group B cluster contained IFNα, IFNβ, IFNγ and IFNο, in addition to other mediators. Heatmaps derived from serum mediator concentration correlations identified strong positive correlations between group A mediators in all aetiological groups and this did not appear to be substantially different between groups [Figure 15 a-c].

Figure 15. Heatmaps of mediator correlations in serum from patients with encephalitis of immune-mediated [a], infectious [b], and unknown aetiologies [c]



However, heatmaps derived from CSF mediator concentration correlations appeared to demonstrate strong positive correlations between group A mediators in the infectious and unknown groups that was not present in those with immune-mediated aetiology. Interestingly, the heatmaps of CSF mediators appeared to be similar for the infectious and unknown aetiology groups and both appeared substantially different from those with an immune-mediated aetiology [Figure 16 a-c].

Figure 16. Heatmaps of mediator correlations in cerebrospinal fluid from patients with encephalitis of immune-mediated [a], infectious [b], and unknown aetiologies [c]

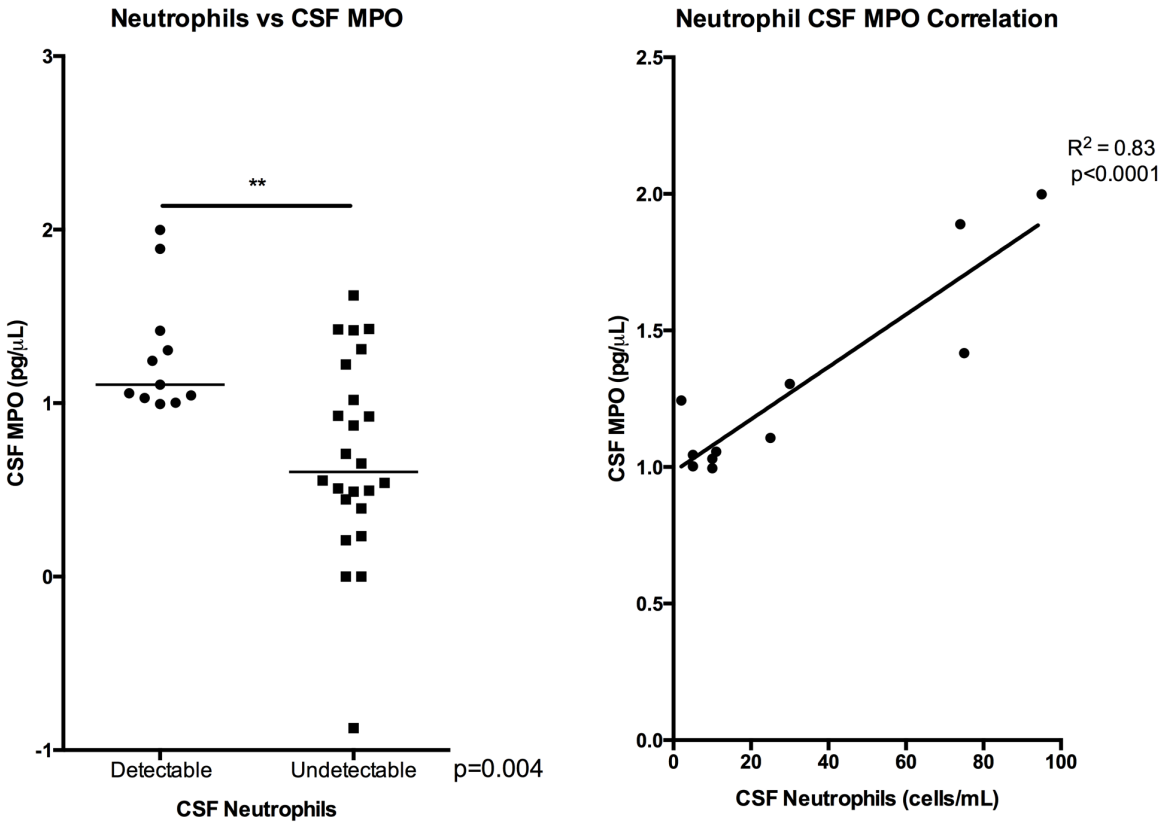


Discriminant function analysis of serum data identified 31 [91%] as infective and 5 [31%] as immune-mediated using MPO alone [Wilks' lambda 0.83, p=0.008]. This technique also identified 17 [85%] samples of unknown aetiology from 6 [38%] immune-mediated samples, again using MPO alone [Wilks' lambda 0.78, p=0.009]; both were confirmed on the leave-one-out cross validation. No serum variables qualified as being capable of distinguishing between samples of infective or unknown aetiology. Discriminant function analysis of CSF data again identified MPO as it correctly identified 11 [85%] of CSF samples as infective and 4 [57%] as immune-mediated, with 15 [75%] correctly classified overall [Wilks' lambda 0.77, p=0.036]; this was confirmed on the leave-one-out cross validation. An MPO concentration cut-off of 1.0pg/μL correctly identified 9 [69%] infective cases and 4 [67%] immune-mediated cases; in the split-reliability analysis 3/5 and 1/3 cases were identified as infective or immune-mediated respectively. The proportion of cases correctly classified was the same when applied to the raw data using a cut-off of 1000pg/μL.

This may reflect relative neutrophil concentration, as 8 [62%] of samples from the infective group had detectable neutrophils in the CSF, as opposed to only 1 [14%] sample from the immune-mediated group [p=0.007] and overall there was a strong positive correlation between CSF neutrophil count and CSF MPO [tau b [95% CI] 0.70 [0.31-1], p=0.005] [Figure 17]. CSF neutrophil count did not correlate with serum MPO concentration. In the split reliability analysis neutrophils were detectable in 3/5 infective samples and 0/2 immune-mediated samples, for whom data were available. There were no significant differences in the proportion of samples with a detectable CSF neutrophil count between the other aetiological groups. Interestingly, examining those with encephalitis due to herpes viruses [HSV and VZV] there was a significantly higher

proportion with a detectable CSF neutrophil count than in the immune-mediated group [15 [58%] vs 3 [14%] respectively, p=0.003].

Figure 17. Cerebrospinal fluid myeloperoxidase concentration and cerebrospinal fluid neutrophil count for all cases of encephalitis



Discriminant function analysis was unable to generate a model that could distinguish between CSF samples of an infective from an unknown aetiology. CSF analysis identified 8 [100%] of unknown and 5 [83%] of immune-mediated samples using IL8 and CCL3 [Wilks' lambda 0.62, p=0.018 and 0.21, p<0.0001 respectively].

8.4.3 Neurohistopathological findings between aetiological groups

Histopathological tissue was available for 11 patients from the Walton Centre Neurohistopathology database (2 HSV, 4 immune-mediated [ADEM, limbic encephalitis, Rassussen's encephalitis], and 5 of unknown aetiology [both HSV and VZV stain and PCR negative]). Of the three vessels assessed per patient (16-90 μm), neutrophils were only identified in 2 cases (1 HSV and 1 of unknown aetiology). No neutrophils were identified in either the perivascular space or immediate parenchyma in any of the immune-mediated cases. Samples from patients with HSV had more CD4 positive cells in both the perivascular space and parenchyma than those of unknown aetiology (24.5 [6-82] vs 3 [0-12], p=0.007 and 14.5 [3-97] vs 0 [0-42], p=0.05, respectively). The number perivascular CD4 positive cells was also identified by DFA (Wilks' lambda 0.602, p=0.012). In the parenchyma CD68 positive cells were also more frequently identified in the HSV patients than those of unknown aetiology (98.5 [29-325] vs 1 [0-89], p=0.004). This was also identified on DFA (Wilks' Lambda 0.634, p=0.002). There were no other statistically significant differences in the proportion of leucocytes identified in the perivascular space or parenchyma of those between the different aetiological groups. The data were median-centred and there were no statistically significant differences in the proportion of each cell subtype between the aetiological groups.

8.4.4 Mass-spectrometry analysis for viral-associated peptides

For mass-spectrometry, CSF from patients in the HPA Study was available for 7 patients with encephalitis due to HSV or VZV, 6 of unknown aetiology and 5 control samples of

CSF from asymptomatic patients [Table 15]. Using protein library software, viral-associated peptides were identified in all 7 viral cases, and all 6 of the samples of unknown aetiology, in comparison to none of the control samples (p=0.001 and p=0.002, respectively). Restricting the results to those patients in whom viral-associated peptides of known human infection there was still a greater proportion of patients in whom peptides were identified in the viral and unknown groups than the controls (5 vs 0, p=0.015 and 5 vs 0, p=0.015, respectively). However, restricting this analysis to viral-associated peptides known to cause encephalitis in humans there were no significant differences in the number in whom viral-associated peptides were identified between the HSV/VZV group and controls (3 vs 0, p=0.2 and 2 vs 0, p=0.45 respectively).

Table 16. Viral peptides identified by mass spectrometry in cerebrospinal fluid from patients with encephalitis due to viral and unknown aetiologies in comparison to asymptomatic controls

	Aetiological Groups		
Viral Peptide	**HSV/VZV Samples**	**Unknown Aetiology Samples**	**Control**
Number (percentage) of samples in which identified	(n=7)	(n=6)	(n=5)
Psittacid herpesvirus 1**	2 (29)	1 (17)	0
Varicella-zoster virus (DNA polymerase catalytic subunit)**	1 (14)	0	0
Human immunodeficiency virus type 2 subtype A (Gag-Pol)**	1 (14)	1 (17)	0
Human papillomavirus type 22 (Regulatory protein E2)*	5 (71)	3 (50)	0
Coronavirus (Replicase polyprotein 1ab)**	1 (14)	1 (17)	0
Feline sarcoma virus (Tyrosine-protein kinase)	6 (86)	4 (67)	0
Aquareovirus C (RNA-directed RNA polymerase)	1 (14)	6 (100)	0
Enterobacteria phage T7 (Gene 4.3)	1 (14)	0	0
Invertebrate iridescent virus 6 (Putative helicase)	1 (14)	0	0
Apple stem grooving virus (Genome polyprotein)	1 (14)	0	0
Lettuce big-vein associated virus (RNA-directed RNA polymerase)	1 (14)	0	0

Although multiple viral peptides were identified in some samples, at least one viral peptide was identified in all HSV/VZV samples and all Unknown samples.
* Viral-associates peptides related to viruses known to cause human infection
** Viral-associates peptides related to viruses known to cause human encephalitis

8.5 Discussion

There are an estimated 0.07 to 12.6 cases per 100,000 cases of encephalitis per year (Granerod et al 2010; Solomon et al 2012). Despite current PCR and antibody analysis the aetiology remains unknown in around 30-40% in most studies [range 15-60%].(Granerod et al 2010; Granerod et al 2010) However, little is known about whether the nature of the cytokine/chemokine mediated inflammatory response differs between those with viral or immune-mediated encephalitis and, if so, does the response in those with encephalitis of unknown aetiology better reflect that in those with viral or immune-mediated disease.

Therefore, I analysed CSF and serum samples from 95 patient recruited prospectively from 24 hospitals in the HPA Encephalitis Study (Granerod et al 2010). Cluster analysis identified a group containing IL1α, IL1β, IL1RA and IL10. CSF heatmaps demonstrated positive correlations between these mediators in infectious and unknown groups but not in immune-mediated. DFA of both CSF and serum identified MPO, distinguishing infective from immune and unknown from immune. Overall CSF concentrations of MPO correlated with CSF neutrophil count suggesting that CSF neutrophils may be the primary source of MPO identified. No mediators could distinguish infectious from unknown. Interestingly, recently in murine models of HSV encephalitis neutrophils have been identified, and may act as a potentially important component of reducing viral load, which may in part be dependant on CCL5 and CCR5 (Melchjorsen et al 2002; Vilela et al 2013). Although the presence of neutrophils has also been identified to be associated with the production of potentially neurotoxic reactive oxygen species and high levels of CCL5 have been associated with worse outcomes in encephalitis due to

Japanese encephalitis virus (Winter et al 2004; Schachtele et al 2012). Nevertheless, this is the first study to identify that CSF MPO may represent a potential biomarker to distinguish viral from immune-mediated encephalitis, and that this may be more sensitive than the CSF neutrophil count alone. MPO concentrations in serum were also associated with an infective aetiology and, although serum neutrophil counts were not available for analysis in this study, this may represent a useful clinical biomarker that warrants further investigation. In addition, that MPO, and broader cytokine signatures in the CSF of those of unknown aetiology better reflected those due to infective rather than immune-mediated disease may shed further light on the potential pathogenesis of these cases and also merits further investigation. Although multiple comparisons were undertaken this represents the largest cohort of patients with encephalitis from whom samples have been analysed for these mediators. A Bonferroni, or other, calculation was not performed following advice from the research group statistician as we did not assess more mediators than patients and previous work in this field has identified and validated significant differences in mediator concentrations for similar acute neurological infections by applying this paradigm (unpublished observations).

Histopathological analysis was limited to only 11 patients, despite a thorough search of both biopsy and post-mortem samples in the regional Neuroscience centre over 10 years this is all the samples that were available. This reflects the impact of modern molecular diagnostic techniques, as the majority of patients will have the diagnosis made by PCR testing of CSF, rather than brain biopsy, as was the previous standard. Therefore, even this small number of patients provides a valuable opportunity to assess histopathological changes in encephalitis. Moreover, this cohort is particularly useful as the majority of histopathological data are prior to the establishment of aciclovir as an

effect antiviral drug and the early initiation of treatment with the advent of PCR. Therefore, this small cohort represents a valuable opportunity to assess the little-studied histopathological changes in encephalitis in the PCR and acyclovir era. Data from the pre-aciclovir/PCR era identified that histopathological changes in HSV encephalitis predominantly affected the mesial temporal lobes and orbitofrontal cortex, and to a lesser extent anterior parahippocampal fusiform and inferior and middle temporal gyri, bilaterally but asymmetrically in the majority. As the olfactory rods are not covered by dura, have close links with these areas and HSV is spread by droplet transmission, this led to the hypothesis that this may represent the pathogenic pathway of this neurotropic virus (Höllinger et al 2000). Histopathological changes are characteristic with widespread necrosis involving the both the cortex and white matter. Macroscopically, post-mortem cases showed generalised cerebral swelling, with consequent compression of the ventricles and midline-shift. Early histopathological changes show a predominance of polymorphonucelar leucocytes around the first 2-3 days (Love et al 2008). Subsequently, after 10-15 days, plasma cells, macrophages and lymphocytes are the predominant leucocyte populations. Nevertheless, there is progressive necrosis, with these necrotic areas tending to be infiltrated by polymorphonuclear cells with surrounding amoeboid microglia, with microglial hyperplasia by 7-14 days. Indeed the perivascular infiltration of infiltrating polymorphonuclear cells may be associated with areas of necrosis around blood vessel walls. Therefore, whilst the presence of neutrophils is historically thought to predominate in the CSF early in infection, there may be on-going influx of neutrophils into the CSF and brain parenchyma later in the disease process, even if they do not represent the predominant leucocyte subset. The increase in the population of microglia and their progression to an 'activated' morphology can be widespread throughout the

brain parenchyma, and may be associated with reactive changes in astrocytes, the co-localisation of which is termed 'glial nodules', and is characteristic of viral encephalitis. However, these studies typically pre-date the RCTs in the 1980s that demonstrated the efficacy of intravenous acyclovir, and no systematic study of HSV encephalitis, and associated encephalitides in the UK has been produced in the era of widespread acyclovir use.

Mass spectroscopy has recently been utilised to augment viral identification in CNS infections (Leveque et al 2014). In this preliminary study I identified viral proteins that share close homology with proteins associated with viral CNS infections in many of the CSF samples from patients with encephalitis due to herpes viruses and, to a lesser extend in those of unknown aetiology, whilst none were detected in the control samples. However, these findings need to be evaluated prospectively in a larger cohort, with paired proteomic and PCR analysis. Moreover, proteomic techniques would need to be optimised before they could form part of routine clinical work-up.

8.6 Conclusions

Cytokine and chemokine profiles identified in the CSF of patients with encephalitis of an infectious aetiology differed from those with an immune-mediated aetiology; those with an unknown aetiology best reflected those of an infectious aetiology. These differences may, at least in part, relate to neutrophils in the CSF.

This analysis of the cytokine/chemokine and associated mediator profiles in the CSF and serum of patients with acute encephalitis has identified that these mediators may

have a role in distinguishing between those of various aetiologies. However, there has been increasing interest as to whether differences in these profiles might predict disease severity and outcome in within a given aetiology of encephalitis. In the most common form of encephalitis, that due to viral infection, a pro-inflammatory profile of these mediators has been identified to correlate with disease severity and poor outcome in those with sporadic viral encephalitides due to Enterovirus and Japanese encephalitis virus (Winter et al 2004; Griffiths et al 2012) However, although some work has been conducted in cellular and animal models, I sought to determine whether the profiles of these mediators may correlate with clinical severity and outcome, and whether these might represent potential targets for adjunctive immunomodulatory therapy in the most commonly identified cause of encephalitis, that due to HSV.

9. Relationship between cytokine signature and disease severity

9.1 Introduction

HSV1 encephalitis has a 10-30% mortality despite current best antiviral treatment, with neurological morbidity in approximately 60% of survivors (Whitley et al 1987; Solomon et al 2012). It is not clear whether this morbidity and mortality is primarily due to the virus directly or the inflammatory response (Wildemann et al 1997; Kamei et al 2009). Aciclovir effectively reduces viral load but does not inhibit immune-mediated pathogenesis (Lokensgard et al 2001). Inflammation of the CNS is evident within the CSF, showing a pleocytosis, and on neuro-imaging and there is mounting evidence that this inflammation, and particularly the contribution of cytokines and associated mediators, may play a key role in pathogenesis (Ichiyama et al 2008; Kamei et al 2009). IL-1 is the prototypical inflammatory cytokine, and has been found to be elevated in murine models and cell culture studies of viral encephalitis resulting in fever, blood-brain barrier permeability, cerebral oedema and release of additional pro-inflammatory cytokines and chemokines (Lokensgard et al 2001; Saxena et al 2008; Saxena et al 2008). Many of these actions are directly opposed by the IL-1 receptor antagonist [IL-1Ra] and IL-10 (Alheim et al 1998; Dietrich et al 1999; Boutin et al 2003). However, these models only provide a limited representation of the pathogenesis of the host inflammation in response to viral re-activation in the human CNS (Vitkovic et al 2001; Winter et al 2004; Ellermann-Eriksen et al 2005; Conrady et al 2010) and a recent review concluded that future studies must focus on human subjects (Vitkovic et al 2001).

However, key inflammatory mediators in viral encephalitis have not been established as previous studies in humans have been limited both by the small numbers of patients and the limited range of mediators assessed (Winter et al 2004; Ichiyama et al 2008; Kamei et al 2009). As these mediators do not act in isolation, but rather in concert, with agonistic and antagonistic common actions, previous work has highlighted the importance of not assessing cytokines in isolation, but instead assessing their relative abundance (Marcotte et al 2013; Michael et al 2013). The ratio of pro- to anti-inflammatory cytokines has been recommended, for example IL6:IL4 and IL6:IL10 ratio have demonstrated correlations with outcome in Japanese encephalitis virus and cerebral malaria, respectively (Day et al 1999; Winter et al 2004). Cytokines and associated mediators have been demonstrated to act in part through increasing BBB permeability with resultant oedema in animal models (Wright et al 1994; Szelenyi et al 2001). To date, no study has compared the cytokines identified in clinical samples with downstream markers of BBB permeability, therefore the pathophysiological mechanisms remain unclear (Tse et al 2009). Nevertheless, clinical studies are typically only able to access a limited number of CSF samples from variable time-points, and often late in the disease process. Therefore, it is unclear to what extent mediators identified at higher concentrations in the CSF reflect intrathecal production or diffusion across an increasingly permeable BBB. Therefore, human BBB models have been developed which allow the ability to determine the relative contribution of mediators produced on the abluminal and luminal sides reflecting intrathecal and intraluminal compartments, and at early time-points (Patabendige et al 2012).

This is a key translational research question as, improved understanding of the pathophysiology of this inflammation, would be pave the way for the use of existing

adjunctive immune-modulatory therapies which have shown efficacy in other inflammatory conditions of the CNS, such as steroids in multiple sclerosis, or targeted therapies, such as IL1RA in stroke-related inflammation (Emsley et al 2005; Burton et al 2012). However, any future potential therapies will be dependent on whether mediators in the CSF of serum are of greatest importance and therefore whether intravascular or intrathecal administration may be required. Mediators identified in the CSF and serum in clinical samples taken at times when there is significant BBB permeability, i.e. later on in the disease, may not necessarily represent intrathecal and intravascular production. Therefore, I also wanted to assess whether the mediators assessed were primarily produced on the intrathecal or intravascular side at early time-points in a BBB model of HSV encephalitis.

9.2 Aims

1. To determine if, in HSV encephalitis and the wider cohort, an elevated pro- to anti-inflammatory balance, as measured by the IL6 to IL4 ratio and the IL-1to IL-1RA and IL10 ratios within the CSF and serum, correlates with
    a. Markers of disease severity
    b. Poor outcome at discharge
2. To determine if this pro- to anti-inflammatory balance correlates with:
    a. The CSF leucocyte count
    b. Markers of BBB permeability
3. To determine if the outcome is more closely associated with CSF viral load in those with HSV encephalitis.

4. To determine if the mediators identified in the clinical samples are produced primarily on the luminal or abluminal side of the BBB, through use of a humanised model

9.3 Methods

9.3.1 Patients

Patients were recruited through the Health Protection Agency study as detailed in section 8.3.

9.3.2 Clinical outcome measures

Clinical severity was determined by the admission GCS as this has been identified to be associated with worse outcome in previous studies (Whitley et al 1987; Winter et al 2004). A normal GCS (15/15) was defined as good and an altered GCS ($\leq$14/15) as poor. Outcome was determined by the GOS score recorded at hospital discharge, with a good outcome defined as a GOS of 5/5 (minor-no disability) and a poor outcome as $\leq$4/5 (moderate disability-death), as described previously (Kamei et al 2009). CSF white cell count and differential were recorded where available and a white cell count of <6 was defined as normal and >5 as elevated.

9.3.3 Blood-brain barrier permeability

I assessed BBB permeability by determining the CSF:serum albumin ratio using radial immunodiffusion, in accordance with the manufacturers' instructions (Binding Site, Birmingham, UK©). Analysis of the relationship between this ratio and the CSF albumin in isolation was determined so that analysis could be undertaken using patients where

only CSF samples were available without paired serum.(Reiber 1995) The nomenclature IL1 was used to denote total IL1α and IL1β concentration.

9.3.4 Viral load

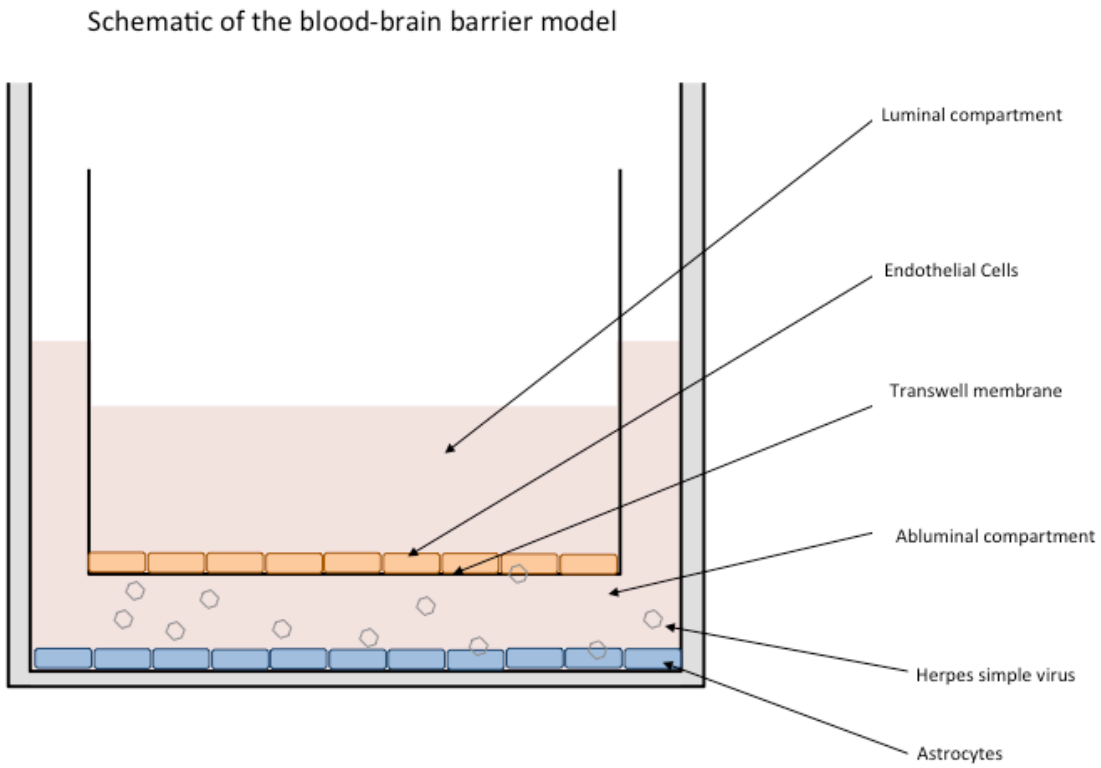
I performed real-time quantitative polymerase chain reaction (qPCR) to qualify the viral load in the CSF of all HSV patients using a validated commercial assay (Artus VZV LC-PCR, Qiagen Group), and processed this using a Light-Cycler analyser (Roche Diagnostics ™ 2007).

9.3.5 Blood brain barrier model

In order to assess the relative contribution of cytokines and associated mediators produced on the luminal and abluminal sides of the BBB, the cytometric bead array was repeated on samples obtained from a BBB model of HSV infection.

The BBB model is described in the main methods section, and in greater detail elsewhere (Patabendige 2012). In brief, the BBB model is a co-culture of confluent primary human astrocytes and immortalised human brain endothelial cells seeded on Transwell inserts [Figure 18]. The model was infected with HSV type 1 from the abluminal 'brain' side at an MOI of 0.01 and virus diluent media [DMEM with 2% FBS] was used for mock-infected controls. TEER measurements and samples were taken immediately post infection and at time points 6, 12, 24 hrs from both the luminal and abluminal sides for cytokine analysis. Mediators were assessed using cytometric bead array as described previously.

Figure 18. Schematic diagram of blood-brain barrier model



9.4 Results

9.4.1 Patients

Full details of the patient characteristics are included in section 8.4.1. In summary, samples were available for 95 patients with acute encephalitis. For 37 patients CSF was available, of whom 12 had HSV infection. For 78 patients serum was available, of whom 17 had HSV infection.

9.4.2 Clinical features associated with poor outcome

There was a significant positive correlation between GCS and GOS (tau b 0.22 [0.08-0.35], p=0.017), and a negative correlation between age and GOS for patients with encephalitis (tau b -0.15 [-0.04 - -0.27], p=0.03). For those with HSV encephalitis, there were no significant differences between those with good and poor GOS with regard to admission GCS, age, or sex. There was no significant difference in the median [range] duration of symptoms before CSF sample collection in those with poor and good outcomes for the cohort overall or the subgroup with HSV encephalitis (10.5 [0-263] vs 4 [0-22], p=0.2 and 2 [1-22] vs 9 [1-22] days, p=0.5, respectively). There was also no significant difference in the duration of symptoms before serum sample in those with poor and good outcomes for the cohort overall or the subgroup with HSV encephalitis (14 [0-263] vs 14 [4-317], p=0.9 and 10 [1-32] vs 10 [4-30] days, p=0.8 respectively).

9.4.3 Mediators in CSF and serum

Samples were only available for the measurement of MMPs 1-3, 7, and 12-13, for 60 patients; therefore these mediators were removed from multivariate analyses. The following mediators were identified in the <80% of the cohort and were therefore removed from further analysis: TNFR1 and 2, eSelectin, CXCL9, IL17a, and VEGFα. In addition, in CSF the following mediators were detected at low concentrations approaching the lower limits of quantification in >20% of the samples: G-CSF, GM-CSF, IFNα2, IFNβ, leptin, IL6. In serum the following mediators were also detected at low concentrations approaching the lower limits of quantification: IFNα2, CCL5, IL6. For all encephalitis cases there was a positive correlation between CSF and serum concentrations for MPO (tau b [95%CI] 0.42 [0.11-0.74], p=0.009), CXCL10 (0.43 [0.18-

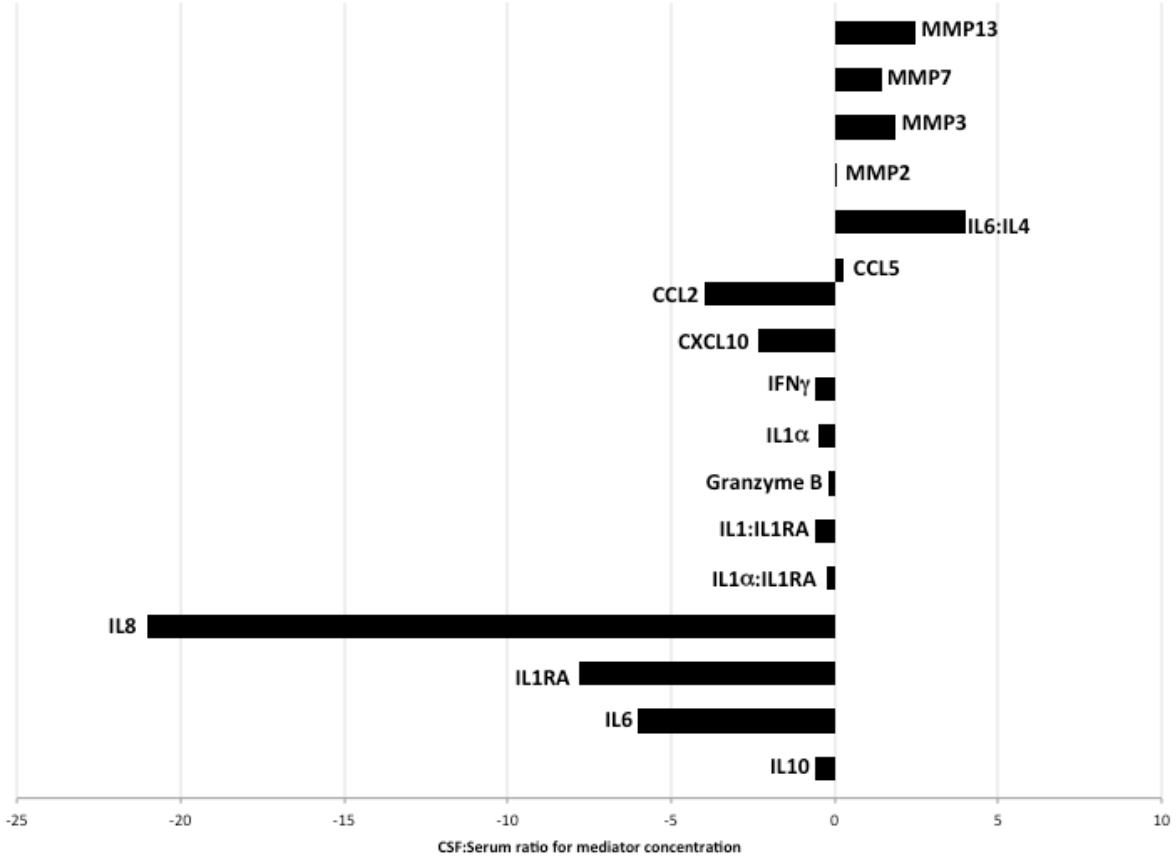
0.68], p=0.01), and IL4 (0.31 [0.01-0.61], p= 0.04). In cases of HSV encephalitis there was a positive correlation between CSF and serum concentrations for GM-CSF only (0.87 [0.5-1], p=002). In cases of HSV encephalitis higher concentrations of several MMPs, CCL5, and the IL6:IL4 ratio were found in the serum than the CSF [Table 17]; higher CSF concentrations than serum were identified for IL1α, IL1RA, IL8, IL10, and several other mediators [Figure 19].

Table 17. Comparison between the relative concentration of mediators in the serum and cerebrospinal fluid in patients with HSV encephalitis

	HSV clinical samples		
	Serum	**CSF**	**p value**
CCL2	0.15 [-0.65-0.41]	0.62 [-0.24-1.93]	0.005
CCL5	1.39 [-0.85-2.79]	-0.37 [-1.04-1.02]	0.058
CXCL10	0.58 [-0.04-1.72]	1.35 [0.37-2.21]	0.04
Granzyme B	-0.25 [-1.15- 0.86]	0.05 [-0.39-0.86]	0.04
ICAM	2.05 [0.40-2.80]	1.46 [1.21-2.43]	0.19
IFNg	-0.77 [-1.56- -0.59]	-0.47 [-0.99-0.37]	0.003
IL10	-0.73 [-2.10- 0.01]	-0.43 [-0.63-0.71]	0.007
IL1a	-0.42 [-0.61- -0.04]	-0.20 [-1.60- 0.10]	0.05
IL1RA	0.16 [-0.11-1.61]	1.23 [0.24-2.06]	0.003
IL6	-0.10 [-0.34-0.25]	0.62 [-0.54-1.17]	0.015
IL8	-0.03 [-2.12-1.19]	0.58 [-0.03-2.27]	0.0004
MMP2	1.31 [-1.77-2.03]	-0.09 [-2.15-0.42]	0.005
MMP3	0.76 [-2.25-1.77]	-1.42 [-2.15-0.38]	0.002
MMP7	0.68 [-1.44-1.77]	-1.01 [-1.87- -0.29]	0.02
MMP13	-1.31 [-2.11- -0.20]	-3.24 [-3.33- -2.45]	0.004
VCAM	1.56 [-0.43-2.30]	1.43 [0.88-2.19]	0.65

Data are log-transformed and median-centred * median [range] concentration [pg/μL]

Figure 19. Relative concentration of mediators between the CSF and serum of patients with HSV encephalitis



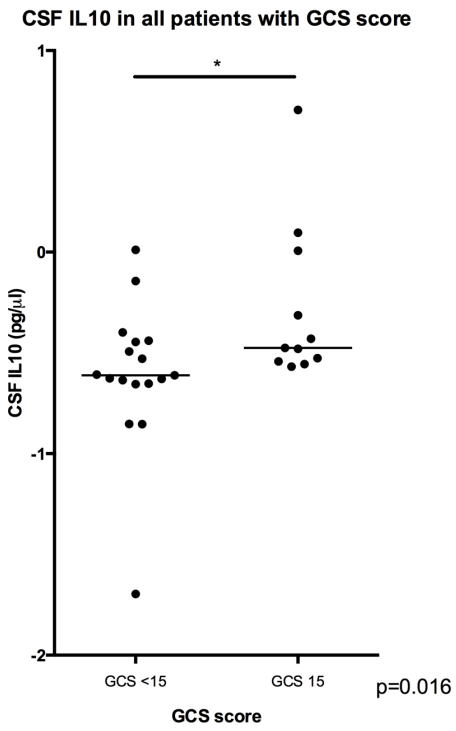
9.4.4 Mediators associated with clinical severity

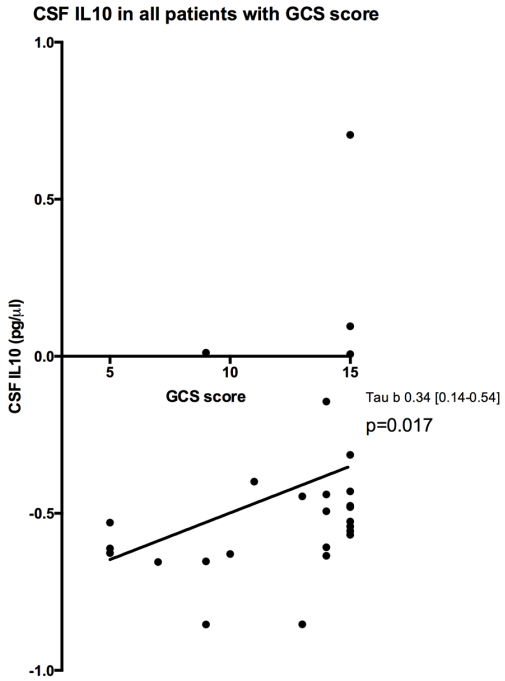
For all patients, the concentration of IL10 in the CSF was significantly higher in those with a normal GCS (p=0.016) and IL10 also had a positive correlation with GCS (p=0.017) (Figure 20 a,b). In serum, both IL1RA and IFNγ were present at significantly higher concentrations in those with a normal GCS (0.68 [0.04-1.61] vs 0.24 [-0.15-1.21] µL, p=0.048 and -0.67 [-1.04- -0.29] vs -0.84 [-1.56- -0.06] µL, p=0.02 respectively).

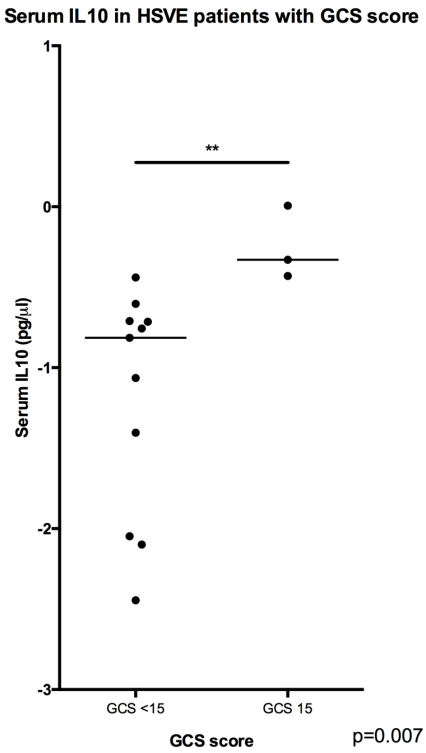
In patients with HSV encephalitis, CSF mediator concentrations did not differ significant according to GCS. In serum the mediator most closely associated with the GCS was also IL10, which was higher in those with normal GCS (p=0.007), and IL10 also had a

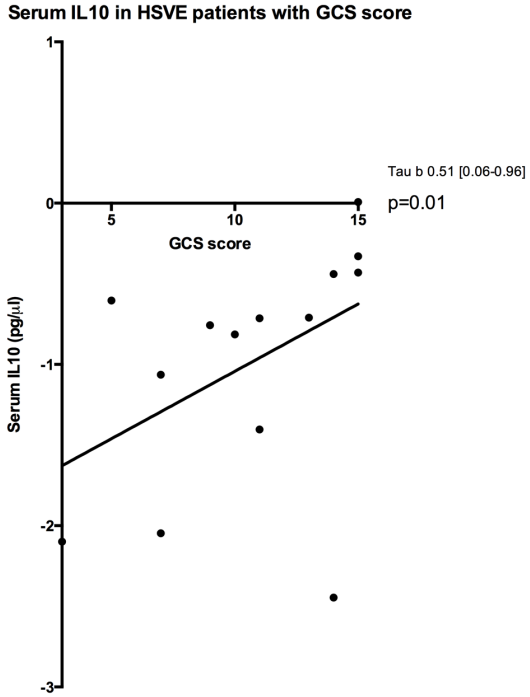
positive correlation with the GCS score (p=0.01) (Figure 20 c,d). Moreover, the serum IL1α:IL10 ratio was lower in those with a normal GCS (0.006 [-37.2-0.24] vs 0.55 [-0.23-1.34] μL, p=0.04). There was also a trend towards a positive correlation between the serum IL1RA concentration and GCS (0.4 [-0.001-0.80], p=0.05).

Figure 20. Cerebrospinal fluid concentration of IL10 and Glasgow coma score in patients with encephalitis [a,b], and serum IL10 concentration and Glasgow coma score in those with HSV encephalitis [c,d]









Data are log-transformed and median-centred.

9.4.5 Mediators associated with poor outcome

Glasgow outcome score at discharge was available for all 95 patients [Table 18]. For all patients with encephalitis, the only individual mediator in the CSF associated with a poor outcome was CCL2. In addition, a high IL1β:IL10 ratio in the CSF was associated with a poor outcome. In serum the mediator associated most significantly a good outcome was IL1RA, which was higher in those with a good outcome (p=0.004) and also had a positive correlation with the GOS score (p=0.01) (Figure 21a,b).

For cases of HSV encephalitis, no single mediator in CSF was significantly associated with the GOS. However, the CSF IL1β:IL1RA ratio was significantly higher in those with a worse outcome score (p=0.009) and this also had an inverse correlation with GOS score (p=0.003)(Figure 21c,d). Discriminant function analysis using the CSF IL1β:IL1RA ratio identified 4/5 and 6/6 patients with good and poor outcomes, respectively, and this was confirmed in the leave-one-out cross validation (Wilks' Lambda 0.528, p=0.02). Applying a CSF IL1β:IL1RA cut-off of >-0.55pg/μL had a high specificity and sensitivity (100% and 83%, respectively, p=0.015). In serum of HSV encephalitis cases, IL1α was weakly associated with GOS, but a high IL1α:IL10 ratio was more significantly higher in those with a poor outcome.

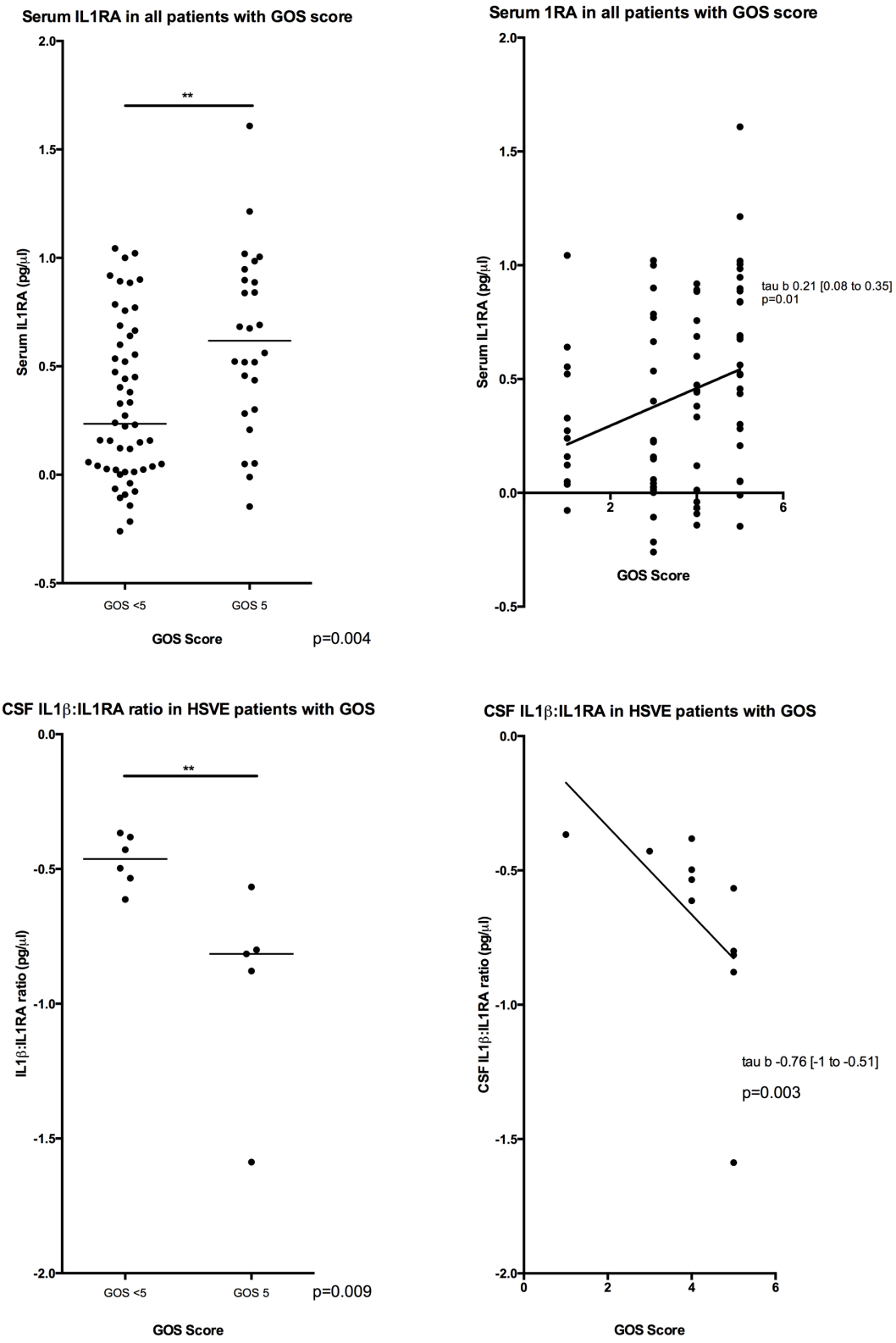
Table 18. Cytokines, chemokines and associated mediators in 95 patients with acute encephalitis and a sub-group of 29 due to herpes simplex virus in relation to Glasgow outcome score at discharge

		All Encephalitis			Herpes Simplex Encephalitis		
	Mediator Median (range)	**GOS 5 (minimal disability)**	**GOS <5 (moderate disability-death)**	**p value**	**GOS 5 (minimal disability)**	**GOS <5 (moderate disability-death)**	**p value**
CSF	IFNo	-0.39 [-0.94 to 0.41]	-0.34 [-0.76 to 0.39]	0.20	-0.33 [-0.52 to 0.41]	-0.46 [-0.56 to 0.08]	0.25
	IFNγ	-0.47 [-0.88 to 0]	-0.47 [-0.99 to 0.61]	0.86	-0.53 [-0.88 to -0.11]	-0.02 [-0.99 to 0.13]	0.13
	CCL5	-0.40 [-1.17 to 0.71]	-0.57 [-1.04 to 1.02]	0.20	-0.44 [-0.71 to 0.48]	-0.22 [-1.04 to 1.02]	0.79
	ICAM	1.48 [1.05 to 2.64]	1.49 [0.79 to 2.69]	0.97	1.46 [1.05 to 2.07]	1.44 [1.21 to 2.43]	0.93
	IL1α	-0.16 [-0.88 to 0.12]	-0.16 [-0.50 to 0.06]	0.96	-0.04 [-0.43 to 0.04]	-0.25 [-0.50 to 0]	0.33
	IL1β	-0.41 [-1.10 to -0.16]	-0.51 [-0.95 to -0.11]	0.56	-0.43 [-1.06 to -0.24]	-0.65 [-0.92 to -0.28]	0.54
	MPO	1.01 [0 to 1.49]	1.02 [-0.87 to 2.00]	0.99	1.03 [0.49 to 1.42]	1.08 [-0.87 to 1.62]	0.93
	CXCL10	1.35 [0 to 2.28]	1.56 [-0.07 to 2.35]	0.33	1.37 [0.37 to 1.92]	1.18 [0.41-1.96]	0.93
	CCL2	**0.36 [0.01 to 0.88]**	**0.7 [-0.24 to 1.93]**	**0.02**	0.49 [0.09 to 0.72]	0.78 [-0.24 to 1.93]	0.33
	CCL3	-0.15 [-0.88 to -0.01]	-0.19 [-1.67 to 0.16]	0.8	-0.27 [-0.45 to -0.11]	-0.13 [-1.67 to -0.08]	0.43
	IL10	-0.48 [-1.70 to 0.10]	-0.53 [-0.75 to 0.01]	0.31	-0.44 [-0.66 to 0.36]	-0.31 [-0.63 to 0]	0.69
	IL1RA	0.54 [0.13 to 1.83]	0.63 [-0.01 to 1.67]	0.73	0.54 [0.24 to 1.84]	1.43 [0.73 to 1.67]	0.13
	IL8	0.58 [-0.03 to 1.47]	0.55 [-0.42 to 2.03]	0.8	0.58 [-0.03 to 1.47]	0.73 [0.17 to 2.03]	0.93
	VCAM	1.2 [-0.37 to 2.19]	1.44 [-0.29 to 2.03]	0.44	1.43 [0.98 to 2.19]	1.31 [0.88 to 2.03]	0.79
	TNFα	-0.58 [-1.52 to -0.37]	-0.68 [-1.26 to -0.09]	0.75	-0.55 [-1.11 to -0.37]	-0.81 [-1.00 to -0.38]	0.33
	IL4	-0.16 [-1.73 to 0.13]	-0.22 [-1.66 to 0.18]	0.9	-0.2 [-0.80 to -0.01]	-0.27 [-0.57 to 0]	1
	TRAIL	0.14 [-0.17 to 0.27]	0.24 [-0.04 to 0.69]	0.07	0.17 [0.09 to 0.24]	0.3 [-0.04 to 0.78]	0.33
	IL1α/IL1RA	-0.3 [-1.18 to 0.25]	-0.19 [-29.57 to 14.83]	0.58	-0.07 [-0.68 to 0.17]	-0.24 [-0.31 to -0.10]	0.31

Serum	G-CSF	-0.36 [-1.11 to 0.05]	-0.49 [-1.29 to 0.77]	0.75	-0.15 [-0.45 to -0.06]	-0.42 [1.2 to 0.45]	0.95
	GM-CSF	-0.07 [-1.26 to 0.40]	-0.37 [-1.39 to 0.54]	0.36	0.03 [-0.70 to 0.33]	-0.06 [1.36 to 0.54]	0.39
	Granzyme B	-0.33 [-1.30 to 0.86]	-0.37 [-1.182 to 0.32]	0.86	-0.24 [-0.34 to 0.86]	-0.81 [-1.15 to 0.32]	0.28
	IFNβ	-0.38 [-2.43 to 0.28]	-1.3 [-2.55 to 0.09]	0.22	-0.21 [-0.91 to 0]	-0.18 [-1.89 to -0.09]	1
	IFNo	-0.28 [-0.77 to 0.88]	-0.19 [-0.71 to 0.60]	0.61	0.17 [-0.56 to 0.41]	-0.31 [-0.70 to 0.26]	0.059
	IFNγ	-0.78 [-1.16 to -0.43]	-0.82 [-1.66 to 0.18]	0.46	-0.70 [-1.00 to -0.59]	-0.83 [-1.56 to -0.29]	0.33
	ICAM	2.06 [0.78 to 2.85]	2.03 [-0.30 to 4.40]	0.90	2.06 [0.78 to 2.39]	1.90 [0.40 to 2.80]	0.85
	IL1α	-0.86 [-1.69 to 0.11]	-0.86 [-1.93 to 0.06]	0.86	**-0.14 [-0.95 to 0.10]**	**-0.55 [-1.60 to -0.08]**	**0.04**
	IL1β	-0.95 [-1.85 to -0.36]	-1.22 [2.34 to -0.07]	0.63	-0.61 [-1.37 to -0.36]	-0.73 [-2.14 to -0.07]	0.96
	MPO	**1.33 [-0.04 to 2.28]**	**1.01 [-0.43 to 2.89]**	**0.02**	1.54 [0.30 to 1.82]	0.92 [0.57 to 2.22]	0.49
	CXCL10	0.48 [-0.32 to 1.72]	0.56 [-0.17 to 1.87]	0.89	1.37 [0.13 to 1.72]	0.58 [-0.04 to 1.30]	0.14
	CCL2	0.08[-0.45 to 0.56]	0.10[-0.79 to 1.04]	0.85	0.21 [-0.28 to 0.41]	0.14 [-0.04 to 1.30]	0.33
	CCL3	-0.33 [-1.49 to -0.01]	-0.48 [-1.34 to -0.05]	0.1	-0.35 [-0.53 to -0.23]	-0.27 [-0.91 to -0.05]	0.87
	IL10	-0.89 [-1.62 to 0.16]	-0.88 [-2.45 to 0.05]	0.52	-0.52 [-1.26 to 0.01]	-0.91 [-2.10 to -0.33]	0.18
	IL1RA	**0.62 [-0.15 to 1.61]**	**0.23 [-0.26 to 1.04]**	**0.004***	0.48 [-0.01 to 1.61]	0.04 [-0.11 to 1.02]	0.37
	IL8	**-0.13 [-1.51 to 2.21]**	**-0.59 [-2.47 to 1.19]**	**0.02**	-0.07 [-0.83 to 0.67]	-0.04 [-2.12 to 1.19]	0.96
	VCAM	1.75 [0.68 to 2.30]	1.77 [-0.48 to 2.55]	0.76	1.9 [1.03 to 2.30]	1.31 [-0.43 to 1.99]	0.12
	TNFα	-0.8 [-0.91 to -0.37]	-0.89 [-1.67 to -0.10]	0.76	-0.61 [-0.91 to -0.26]	-1.02 [-1.67 to -0.10]	0.07
	IL4	-0.46 [-1.27 to -0.03]	-0.56 [-1.39 to 0.19]	0.94	-0.34 [-0.67 to -0.10]	-0.25 [-1.11 to 0.19]	0.79
	TRAIL	0.27 [-0.06 to 1.13]	0.21 [-0.11 to 1.30]	0.53	0.4 [0.16 to 0.49]	0.28 [0.02 to 0.73]	0.57
	IL1α/IL1RA	-0.95 [-5.06 to 10.75]	-1.22 [-243.56 to 34.72]	0.41	-0.47 [-2.18 to 1.97]	-0.62 [-56.12 to 24.51]	0.56
	IL1β/IL1RA	-1.39 [-9.66 to 59.58]	-2.02 [-239.23 to 59.24]	0.23	-0.99 [-6.99 to 59.58]	-0.79 [-84.69 to 32.78]	0.79
	IL1α/IL10	0.78 [-37.20 to 8.61]	0.72 [-13.43 to 8.25]	0.82	**0.03 [-37.20 to 0.76]**	**0.55 [0.24 to 1.34]**	**0.03**
	IL1β/IL10	0.99 -112/50 to 7.70]	1.02 [-25.61 to 9.29]	0.69	0.93 [-112.50 to 1.39]	0.85 [0.22 to 1.25]	0.64

Data are log-transformed and median-centred. * Statistically significant correlation also.

Figure 21. The relationship between CSF concentrations of IL1RA and outcome score in patients with encephalitis [a,b], and the cerebrospinal fluid IL1β:IL1RA ratio with outcome score in patients with HSV encephalitis [c,d]



Data are log-transformed and median-centred.

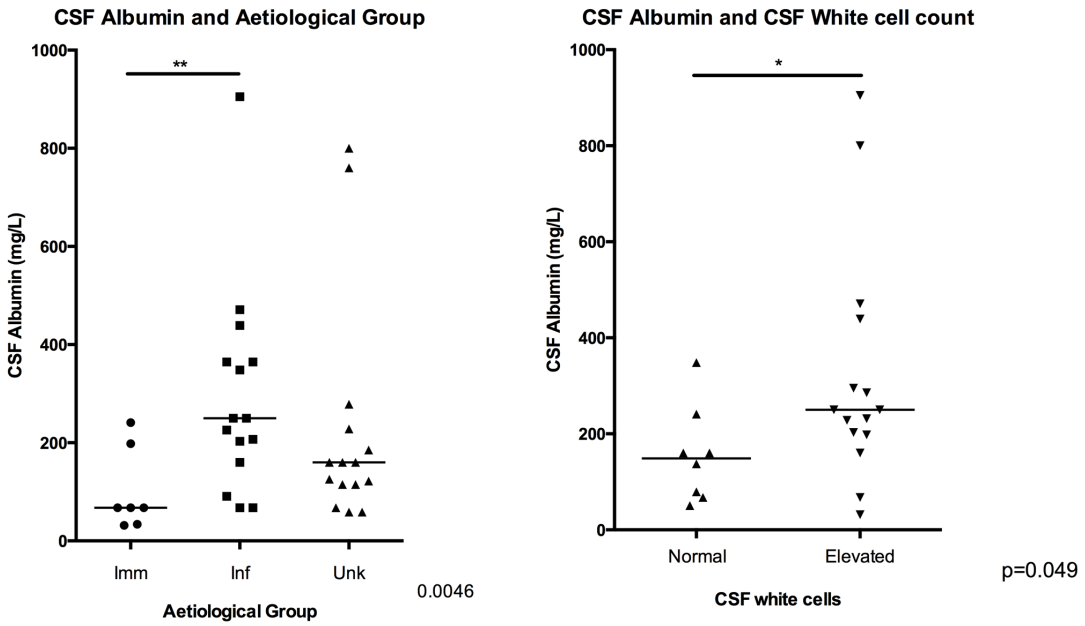
9.4.6 CSF White cell count

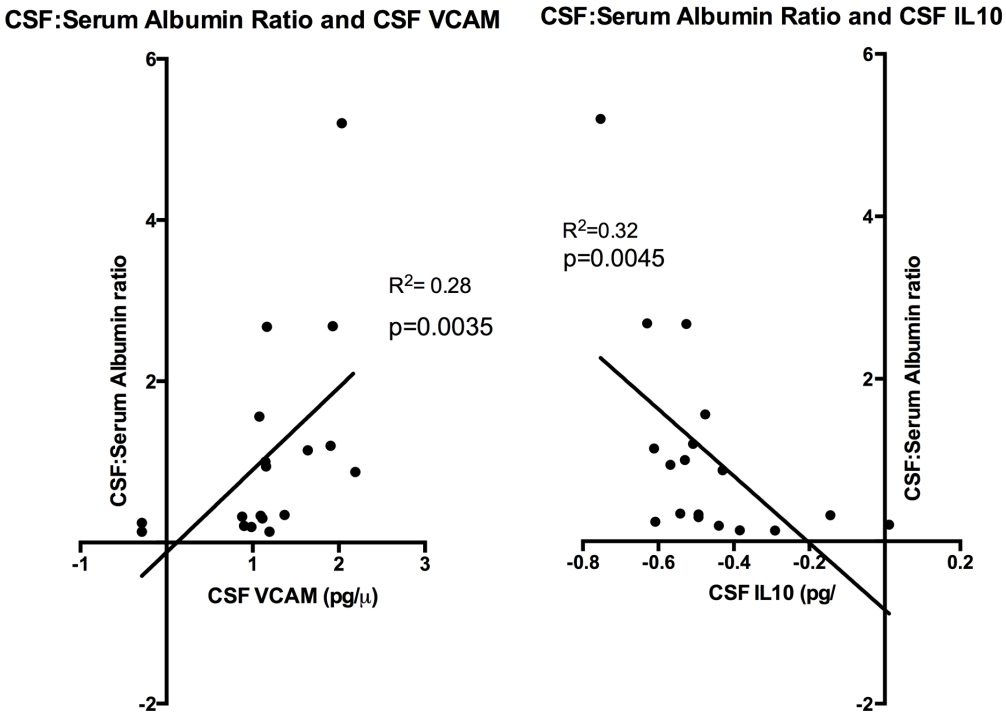
In patients with HSV encephalitis CSF white cell count was available for 26 patients. There was no significant difference in the CSF white cell count in those with a poor outcome compared with a good outcome (48 [4-455] vs 12 [4-542], p=0.09). No mediators in the CSF or serum correlated with CSF white cell count.

9.4.7 CSF:Serum albumin ratio

CSF samples to determine the albumin concentration were available for 22 patients (10 infective, 6 immune-mediated, and 6 unknown aetiology), and paired CSF and serum samples were available for 20 (8 infective, 6 immune-mediated, and 6 unknown aetiology). Overall there was a positive correlation between the CSF albumin and the CSF:serum albumin ratio (tau b [95%CI] 0.55 [0.26-0.83], p=0.002). Overall there was a higher CSF albumin concentration in those with infective than immune-mediate aetiology (364.5 [67.5-905] vs 67.5 [67.5-241] p<0.005). There were no significant differences in CSF albumin concentration between other groups [Figure 22a].

Figure 22. Cerebrospinal fluid concentration of albumin in patients with encephalitis due to immune-mediated, infectious, and unknown aetiologies [a]. Relationship between CSF albumin concentration in those with elevated and normal white cell counts in the CSF [b]. CSF:serum albumin ratio in patients with encephalitis in relation to CSF VCAM [c] and IL10 [d]





For all patients there was no association between the CSF:serum albumin ratio and GOS score (tau b -0.16 [-0.50-0.19], p=0.4). Nevertheless, those with an elevated CSF WCC had a higher albumin concentration (250 [67.5-905] vs 149 [50.65-348.5], p=0.049) [Figure 22b]; although there was no significant association with the CSF:serum albumin ratio. For all patients the only mediator in the CSF with a positive correlation with the CSF:serum albumin ratio was VCAM (0.50 [0.20-0.81], p=0.004) [Figure 22c], and the concentration of CCL2 in the CSF approached significance (0.33 [-0.04-0.71], p=0.057). The mediator in the CSF with the strongest negative correlation with the CSF:serum albumin ratio was IL10 (-0.49 [-0.79- -0.20], p=0.0045) [Figure 22d]

In those with HSV there was no difference between the CSF:serum albumin ratio and outcome or CSF WCC. Of those with HSV encephalitis the only CSF mediator that correlated with the CSF albumin concentration was VCAM (tau b 0.93 [0.70-1], p=0.017).

9.4.8 CSF viral load in HSV encephalitis

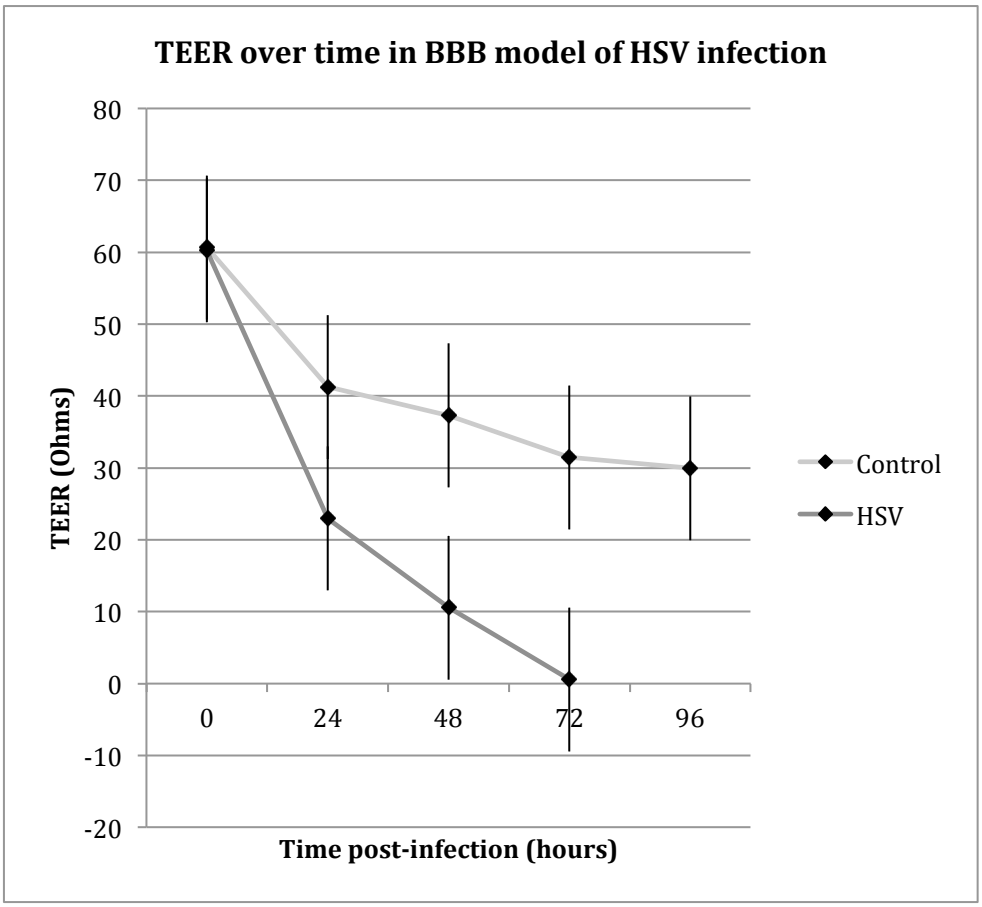
All 12 cases of HSV encephalitis underwent RT-PCR. HSV DNA was only detected in two cases (187,680 log 5.3 and 664,400 log 5.8 copies/uL). Of the ten cases for whom HSV DNA was not detectable, 2 were negative by PCR at recruitment and the diagnosis was made by HSV antibody, and 1 further case had been border line low-positive by PCR at recruitment. The difference between the median [range] time from admission to CSF sample was not statistically significant for

those for whom there was a detectable versus undetectable viral load (2 [1-3] vs 11 [1-22] days, p=0.5). The cases with a detectable HSV viral load had a lower median [range] GOS than those in whom HSV DNA was not detected (2 [1-3] vs 5 [4-5], p=0.04). However, although retrospectively applying the criteria of detectable HSV DNA to predict poor outcome had a high specificity (100%) the sensitivity was low (33%), p=0.5.

9.4.9 Comparison with blood-brain barrier model

In the HSV infected BBB model there was a progressive decline in the TEER greater than that seen in the control sham-infected model, reflecting increasing BBB permeability, and at 72 hours the TEER in the HSV infected model was effectively 0 [Figure 23].

Figure 23. Transendothelial electrical resistance over time in a human blood-brain barrier model following infection with HSV and control sham infection

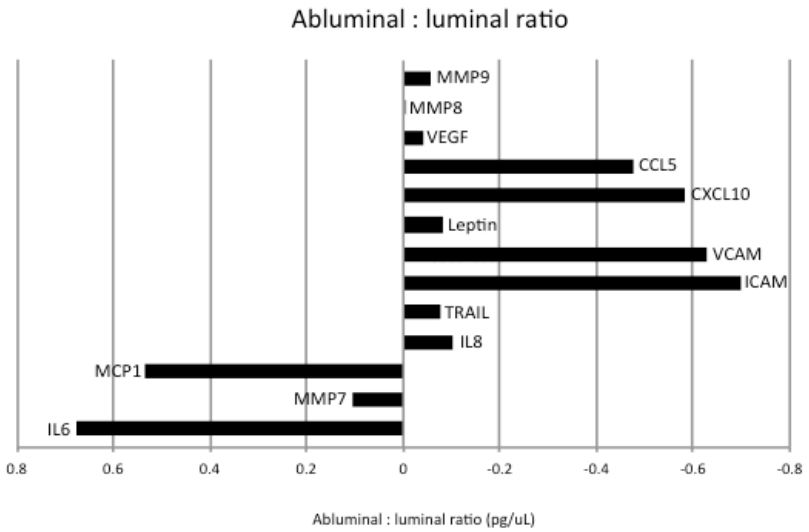


In the HSV infected BBB model several of the mediators assessed were present at levels at the lowest limit end of detection and therefore were excluded from further analysis as the could not be accurately quantified: G-CSF, IFNβ, IFNγ, IFNo, IL1α, IL1β, IL1RA, IL4, IL10, CCL3, CXCL9, TNFα, MPO, eSelectin, MMP8, MMP9, and IL17a.

In the HSV infected BBB model higher concentrations of ICAM (496.81 [277.6-1310.3] vs 185.62 [14.0-369.6], p=0.007) and VCAM (76.21 [51.1-136.9] vs 32.15 [12.2-47.7], p=0.0002) were identified on the luminal as opposed to the

abluminal samples [Figure 24]. The median concentrations of the other mediators did not differ significantly between luminal and abluminal samples. Discriminant function analysis identified three mediators (VCAM [Wilks' lambda] [0.448], p=0.035; ICAM [0.089], p=0.002; MMP7 [0.018], p=0.001) as identifying all 8 samples as having been taken from the luminal or abluminal sides. In addition, the rate of change was greater in samples obtained from the luminal as opposed to abluminal samples for ICAM and CCL2 (16.1 vs 8.7 [pg/µL/hr], p=0.04 and 184.6 vs -9.7 [pg/µL/hr], p=0.06, respectively).

Figure 24. Comparison between relative mediator concentrations in samples from the luminal as opposed to abluminal side of a human blood-brain barrier model of HSV infection



In comparison between the early and late time points there were statistically significant increases in the total concentration for several mediators in the HSV infected BBB model; the only mediator that increased significantly in the control model was MMP7 [Table 19].

Table 19. Comparison between mediator concentrations at early and late time-points in a BBB model of HSV infection and control

	HSV			Control		
Total mediator concentration	**Early** median (range) [pg/μL]	**Late** median (range) [pg/μL]	**p value**	**Early** median (range) [pg/μL]	**Late** median (range) [pg/μL]	**p value**
MMP7	51.2 (40.0-62.7)	67.6 (59.0-77.4)	0.0003	56.5 (39.0-54.0)	84.8 (64.9-87.4)	0.03
CCL2	2970.5 (1604.5-3324.4)	3252 (2436.8-9180.0)	N/S			N/S
CCL5	136.0 (21.1-451.7)	695.6 (331.7-1116.9)	0.003			N/S
CXCL 10	9.5 (5.0-37.0)	80.3 (18.6-125.3)	0.005			N/S
ICAM	196.5 (14.0-364.5)	499.4 (254.5-1310.3)	0.02			N/S
IL6	355.0 (11.9-1317.9)	1280.1 (837.1-1512.6)	0.005			N/S
IL8	697.6 (262.0-808.8)	1317.7 (856.2-1484.5)	0.0006			N/S
TRAIL	60.6 (31.7-87.0)	85.5 (64.5-104.1)	0.002			N/S
VCAM	38.2 (12.2-64.7)	67.7 (39.1-136.9)	N/S			N/S
VEGF	170.0 (34.1-289.0)	443.8 (349.8-539.2)	0.0002			N/S

The total concentration of all mediators identified had a negative correlation with the TEER reflecting increasing BBB permeability [Table 19]. In the control model, the total concentration of none of the mediators correlated with the TEER.

Table 20. Correlations between mediators and transendothelial electrical resistance in a BBB model of HSV infection

Total concentration	Pearson's	p value
MMP7	-0.86	0.006
CCL2	-0.76	0.03
CCL5	-0.88	0.004
CXCL10	-0.83	0.01
ICAM	-0.83	0.01
IL6	-0.97	<0.0001
IL8	-0.92	0.001
TRAIL	-0.92	0.001
VCAM	-0.85	0.008
VEGF	-0.99	<0.0001

9.5 Discussion

The most common cause of encephalitis is infection, with HSV accounting for approximately 40% of cases in whom an infectious aetiology is established (Kennedy 2005; Granerod et al 2010). Despite antiviral therapy with aciclovir, approximately 10-30% patients die, and moderate-severe neurological and neuropsychiatric sequelae are common, with less than 20% able to return to

work (Whitley et al 1987; Raschilas et al 2002). There is mounting evidence from both animal models and clinical studies that cytokine-mediated inflammation may play an important role in the pathogenesis of viral encephalitis (Asaoka et al 2004; Winter et al 2004; Kamei et al 2009). These mediators modulate both the innate and adaptive inflammatory responses and mediate BBB permeability, which is vital for leucocyte infiltration and oedema (Ellermann-Eriksen et al 2005; Conrady et al 2010).

To date, studies of humans with HSV encephalitis have been limited and little is known about whether this inflammatory response is associated with greater clinical disease severity and outcome. Although a wide range of cytokines/chemokines have been implicated, a potentially useful indicator is the ratio of pro- to anti-inflammatory mediators, for example the ratio of IL-6 to IL-4 and to IL10 has been found to correlate with outcome in encephalitis due to Japanese encephalitis virus and in severe malaria, respectively (Day et al 1999; Winter et al 2004). Indeed the significance of the concentration of many mediators is dependent on the relative concentration of natural endogenous antagonists (Paludan et al 2001; Szelenyi et al 2001; Boutin et al 2003) Unfortunately, previous clinical studies of HSV encephalitis have not addressed this (Rosler et al 1998; Ichiyama et al 2008; Kamei et al 2009). Research is needed to delineate the pathophysiological mechanisms of the inflammatory response in encephalitis. This could pave the way for the utilisation of novel or existing adjunctive immunomodulatory therapies to improve the outcome (Emsley et al 2005; Burton et al 2012). Therefore, I analysed CSF and serum

samples from 95 patients recruited prospectively from 24 hospitals in the Health Protection Agency Encephalitis Study to determine whether the cytokine/chemokine profiles correlated with markers of clinical severity and outcome, and whether these were associated with increased BBB permeability and leucocyte infiltration. I also sought to assess whether the viral load was higher in those with a worse outcome.

As identified in previous clinical studies of viral encephalitis, for both encephalitis due to HSV and overall in this study, a lower GCS score on admission was associated with worse GOS score (Skoldenberg et al 1984; Whitley et al 1987). Several MMPs and CCL5 were higher in serum, which is presumed to reflect endothelial production, whereas IL1α, IL1RA, and IL10 were at higher in CSF.

A lower anti-inflammatory response was associated with a worse level of consciousness; as the GCS correlated with CSF IL10 in the cohort overall and with serum IL10 in those with HSV encephalitis. Moreover, a lower anti-inflammatory response was associated with a worse outcome both in the cohort overall and in those with HSV encephalitis. Overall the serum IL1RA had the strongest positive correlation with a good GOS score and a high CSF ILβ:IL10 ratio was associated with a poor GOS score. In HSV cases, a high IL1β:IL1RA ratio in the CSF was most strongly associated with poor GOS score. There was no association between the CSF:serum albumin ratio and the GOS score either overall or in the HSV group. Although, the CSF albumin concentration was higher

in those with a raised CSF white cell count. Overall the mediator with the strongest negative correlation with the CSF:serum albumin ratio was CSF IL10.

The IL1 family, particularly IL-1β, are the prototypical pro-inflammatory cytokines. IL1α and IL1β act through interaction with the IL1-receptor 1, however in addition to the membrane-bound and soluble decoy receptor, IL1-R2, the actions are antagonised by IL1RA, which blocks the active receptor, IL1R1 (Alheim et al 1998; Boutin et al 2003). IL1α, IL1β and IL1RA have been identified in murine models of HSV encephalitis in association with increased cerebral oedema (Tse et al 2009; Pasieka et al 2011). IL-1β has been identified consistently to be up-regulated to robust levels in human microglial cells infected with HSV, without productive infection (Lokensgard et al 2001). Although productive infection with HSV has been demonstrated in both human astrocytes and neurones, cytokine production may be lesser (Lokensgard et al 2001). Nevertheless, although microglia are the main source of IL1β, particularly at early time-points, expression has also been demonstrated in astrocytes, oligodendrocytes, neurons and endothelial cells (Boutin et al 2003; Gibson et al 2004; Pinteaux et al 2009). IL1 acts through IL1-receptors present on every neuroglial cell type, excluding microglia, and is important in up-regulating many pro-inflammatory mediators including MMPs, adhesion molecules, IL6, chemokines and may further mediate endothelial permeability through induction of nitric oxide and phospholipase A2 in astrocytes (Alheim et al 1998; Boutin et al 2003; Gibson et al 2004; John et al 2005; Jensen et al 2009; Pinteaux et al 2009). However, only two previous studies have attempted to assess IL1 in the CSF of patients with HSV encephalitis, and both failed to identify

concentrations above the limit of detection in the majority of the 20 and 9 adults in each study (Aurelius et al 1994; Kamei et al 2009). Although, in both studies at least 50% of patients received steroids and the timing of the administration of these relative to the samples being obtained is not disclosed in one. In addition, the time from symptom onset or admission to sample is not described and the failure to identify IL1 in the majority may reflect delayed presentation and/or investigation. Neither study assessed IL1 relative to IL1RA or IL10. However, elevated concentrations of IL10 were identified in the majority of patients, and this did not differ between those with moderate sequelae/death and those with mild/no disability (Aurelius et al 1994; Kamei et al 2009). The largest study of IL1 in encephalitis assessed patients with Enterovirus 71 infection and identified elevated levels of both IL1β and IL1RA in those with severe disease and elevated IL1RA in those who died (Griffiths et al 2012). However, the IL1β:ILRA ratio was not reported. A degree of IL1β production may be required in HSV encephalitis as IL1β knock-out mice succumb to fatal HSV encephalitis (Sergerie et al 2007). Nevertheless, IL1 is primarily pro-inflammatory and neurotoxic in mixed neuroglial cultures and *in vivo*, and injection of IL1RA has been demonstrated to significantly reduce inflammation and neuroglial injury following ischaemia and in a phase 2 clinical trial demonstrated improved outcomes in patients with cortical infarcts (Emsley et al 2005; Pinteaux et al 2009). In addition, Glatiramer acetate, which is an effective immunosuppressant used in multiple sclerosis, exerts its action, at least in part, through induction of IL1RA expression and inhibition of IL1β production (Burger et al 2009).

IL1β is required to control some viral infections and therefore antagonists may potentially increase the severity of infection (Sergerie et al 2007). However, IL1β has not been demonstrated to suppress HSV infection in human astrocytes and IL1α administration was not required to reduce viral replication in JEV infection of human monocytes (Hasegawa et al 1990: Lokensgard et al 2001). In a recent meta-analysis of 4 controlled trials involving 2771 patients followed-up for 24 weeks whilst receiving IL1RA, Anakinra ©, for rheumatoid arthritis only 30 [1.4%] on treatment developed a serious infection and the odds ratio was not significant in comparison to placebo (2.75 [95%CI: 0.90-8.35], p=0.1); only one case was viral, due to herpes zoster (Salliot et al 2009). In addition, excluding patients with co-morbidities, there was also no significantly increased risk with high-dose Anakinra ©. Nevertheless, Anakinra © is currently contra-indicated during infection (British National Formulary 2014). Interestingly, HSV has been used as a vector in gene therapy to induce IL1RA production and effectively ameliorate experimental autoimmune encephalomyelitis (Furlan et al 2007). Glucocorticoids have been demonstrated to inhibit IL1 expression (Gibson et al 2004). Indeed, steroids were routinely used to treat HSV encephalitis before aciclovir was established as an effective antiviral (Upton et al 1971; Habel et al 1972). Moreover, administration of steroids in addition to aciclovir in a murine model of HSV encephalitis has been demonstrated to reduce neuroimaging changes without increasing in viral load (Meyding-Lamade et al 2003). A phase 2 clinical trial is underway to assess the role of adjunctive dexamethasone in HSV encephalitis (Defres et al 2014).

This study also identified a potentially important role for IL10, another key antagonist of IL1. A previous study identified higher CSF concentrations of IL10 in patients with HSV encephalitis than in non-infectious controls (Ichiyama et al 2008). This potent anti-inflammatory cytokine is produced by astrocytes, cells of macrophage/microglial lineage, and T and B lymphocytes and is known to have a protective role for both neurones and glial cells by blocking pro-inflammatory cytokines, particularly IL1, and promoting survival signalling (Vitkovic et al 2001; Swarup et al 2007). IL10 has been demonstrated to be elevated in murine models of encephalitis due to JEV, and to correlate inversely with the levels of pro-inflammatory cytokines, particularly IL1β and IL6, and histopathological changes (Swarup et al 2007; Saxena et al 2008; Saxena et al 2008). In addition, recently the relative ratio of pro-inflammatory mediators to IL10 has been identified to be of importance in *M. tuberculosis* infection (Skolimowska et al 2012). Moreover, *in vitro* treatment of murine BV2 microglial cells with IL10 has been shown to inhibit IL1β and TNFα mediated cyclooxygenase (COX)-2 production and also to reduce neuronal death in murine models of encephalitis due to both JEV and Theiler's virus (Molina-Holgado et al 2002; Swarup et al 2007). Interestingly, inhibition of COX-2 with meloxicam has been demonstrated to reduce both cerebral oedema and BBB permeability in a rat model of traumatic brain injury (Hakan et al 2010). In addition, in HSV infection, the exogenous administration of IL10 has been demonstrated to reduce the production of pro-inflammatory cytokines, particularly IL1β, in human microglial cultures (Marques et al 2004). Recently it has been identified that instillation of IL1β in a BBB model resulted in a rapid and significant reduction in the TEER, reflecting increasing BBB permeability, which was in part due to

increased expression of adhesion molecules (Labus et al 2014). Therefore, this supports the findings of this study that the mediator with the strongest negative correlation with BBB permeability was IL10 and that the mediator with the strongest positive correlation with BBB permeability was VCAM. In addition, low CSF levels of IL-10 have been implicated in several patients with pro-inflammatory relapses of HSV encephalitis despite clearance of viral DNA (Skoldenberg et al 2006).

However, as IL-10 interferes with the efficient effector process, high levels may potentially increase susceptibility to intracellular pathogens (Wilson et al 2005). Nevertheless, treatment with IL10 has been demonstrated to reduce pro-inflammatory cytokines and inflammatory infiltrates in murine HSV keratitis without impairing viral clearance (Tumpey et al 1994). Indeed IL10 knockout mice develop universally lethal encephalitis with CMV infection (Cheeran et al 2007). IL10 administration has also been demonstrated to reduce neuronal death in glutamate-induced and hypoxic ischaemic injury, in rat and murine models, in part through, reductions in the level of IL1β (Dietrich et al 1999; Bachis et al 2001; de Bilbao et al 2009). Interestingly in a recent study administration of intravenous immunoglobulin protected 129 mice from fatal HSV encephalitis, and this benefit was not achieved in IL10 knock-out mice (Ramakrishna et al 2011).

In this study I did not identify significantly elevated concentrations of IL-6 or the IL6:IL4 ratio in HSV encephalitis patients with a worse outcome. IL6 is produced by all CNS cell types in response to viral proteins and other pro-inflammatory

cytokines, and promotes T and B cell activation and differentiation and increases BBB permeability (Szelenyi et al 2001; Wang et al 2008). Although this has been identified in previous studies of severe infection, these have been of conditions in which there is a viraemia, with JEV infection, or a parasitaemia, with malaria (Day et al 1999; Winter et al 2004). This is pathophysiologicaly different from HSV encephalitis in which brain parenchymal dysfunction follows neurotropic migration.

Infection with HSV on the abluminal side of the BBB model produced a progressive decline in the TEER, reflecting increasing BBB permeability, and by 72 hours the TEER was effectively 0. Although several mediators differed in average concentrations between the abluminal and luminal sides, the only mediators that were statistically significantly different were ICAM and VCAM, which were detected at a greater concentration on the luminal side, reflecting endothelial production, as would be expected (Labus et al 2014). In addition, although the total concentration of several mediators increased over time, the rate of change of mediator concentrations over the time-points, was only significantly greater for luminal as opposed to abluminal samples for ICAM and CCL2. Interestingly, the only mediators with a positive correlation with BBB permeability in the clinical samples were VCAM and, to a lesser extent, CCL2. To what extent these correlations reflect a consequence or cause of increasing permeability requires further study. Nevertheless, in the model, in addition to other mediators, VCAM, ICAM, and CCL2 had a positive correlation with increasing permeability of the BBB.

Although identified as the mediator with the strongest negative correlation with BBB permeability in the clinical samples, IL10 was only detected at very low levels in samples from the BBB model. Therefore, this may imply that astrocytes and endothelial cells do not represent important cells in the production of IL10 in HSV infection. Moreover, IL1α, IL1β, and IL1RA, in addition to other mediators, were also not detected at significant concentrations in the BBB model. This illustrates one of the major limitations of the model studied, notably that both microglia and neurones are not present on the astrocyte side. This is of particular importance when considering the potential role of cytokines and associated mediators, as microglia have been demonstrated to be the major producers of these mediators, and neuronal production may also have an important role in the appropriate circumstances, such as following excitation (Vitkovic et al 2001; Gibson et al 2004). In addition the model does not account for the range of leucocytes present on both the endothelial and brain parenchymal sides (Love et al 2008). Nevertheless, the model does allow the assessment of the changes at the astrocyte-endothelial interface, which is pivotal for the integrity of the BBB, and also allows for the assessment of changes early in infection and at multiple time points, which is not routinely possible with clinical samples.

9.6 Conclusions

During acute HSV encephalitis an excessive pro-inflammatory response may be deleterious. Particularly, the balance between the IL1 family and antagonists, IL1RA and IL10, was associated with coma and a worse outcome. This IL1 balance was also associated with coma and outcome in the cohort of patients with encephalitis overall. IL10 was also inversely associated with BBB permeability. In addition, cellular adhesion molecules and CCL2 demonstrated a positive correlation with BBB permeability in clinical samples, and, along with other mediators, in the HSV infected BBB model also. These may represent modifiable mediators with the potential to improve outcomes in HSV encephalitis and warrant further investigation.

10. Discussion

The aims of the thesis were to assess the detection and diagnosis of acute viral encephalitis at both a population and individual level, and explore the development of novel technological approaches to augment this; this has been successfully met. Detection data at a population level were investigated with the use of clinical disease surveillance networks and through development of a smartphone application. At an individual level, these aims were investigated by assessing current practice across ten centres and using PCR to determine the viral load in relation to timing and disease severity. I then developed a LP pack to improve sample collection, and used cytometric bead array to assess cytokines, chemokines, and associated mediators in the CSF and serum of patients in relation to both aetiological classification and also to markers of clinical severity and outcome. In the clinical cohorts the aims were also addressed though immunohistochemistry to assess leucocyte populations between aetiological classifications, and MS/MS to assess for viral peptides in the CSF of patients with viral and unknown aetiologies; in addition, radial immunodiffusion was used to measure CSF and serum albumin ratios as marker of BBB permeability, and assessment of permeability and mediators was also undertaken in a human BBB model. The results of each chapter build on the experience of the preceding work and demonstrate that current methods to identify viral encephalitis at a population level have several limitations and that novel technological approaches utilising smartphone technology can be developed to allow bi-directional data transfer which has the potential to augment existing approaches.

The results also demonstrate that diagnosis of viral encephalitis is limited by detection at an individual level, in part through delays in performing the LP, which may be associated with a falling viral load, and inadequate sample collection, and that the latter can be improved with the introduction of a simple LP pack. Moreover, these studies found that cytokine signatures may improve identification of viral encephalitis, particularly relating to MPO, and that this may reflect leucocyte populations in the CSF and parenchyma. In addition, cytokine profiles, particularly relating to antagonism of IL1, may improve the identification of those with most severe disease, which may in part reflect BBB permeability. The relationship between the study results and there implications for future research will be discussed here.

10.1 Interaction of study results

The initial study showed that, despite multicentre data collection through the two largest neurological networks across adult and paediatric practice over two years, there were low rates of response and that of the four adult cases for whom data were available only two were identified through the networks and of the 21 paediatric cases, only 10 were identified through the networks. Whilst this study was able to demonstrate several radiologically-defined acute encephalopathy syndrome sub-types due to influenza infection, it is likely that this study methodology was biased towards including patients with more severe manifestations, as 20 (80%) required ITU care and 17 (68%) had a poor outcome score or died. This is reflected in that the study did not identify any patients with acute encephalopathy syndromes at the milder end of the spectrum , and identified only one case of GBS. Interestingly, 10 who had CSF PCR for influenza were negative, despite positive respiratory secretions in all. It is unclear if this reflects delayed presentation with CNS inflammation due to post-infectious phenomena, or whether this is due to the CSF being a poor proxy for CNS parenchymal infection, or a para-infectious process. In the subsequent study of CSF viral load in patients with encephalitis due to VZV infection, a virus also capable of causing clinical presentations of both acute CNS infection and post-infectious immune-mediated disease, the longer the time from symptom-onset to the LP the lower the CSF viral load. Importantly, none of the patients in the influenza cohort had received the efficacious vaccine, despite 8 having clear indications for this, 3 of whom died and the remaining 5 had poor outcomes. Epidemiological methods of disease surveillance that incorporate vaccine usage

in at risk groups are vital to effective public health planning. Current methods of disease surveillance, such as that employed in this study clearly have the potential to miss many cases, in addition to collecting data from a biased cohort. Therefore, novel technological approaches which access data from clinicians across the globe in real-time, including the denominator data of suspected or at-risk cases, in addition to the numerator data of proven or vaccinated cases are needed.

I undertook research to determine whether a novel smartphone based technology could be developed with the potential to collect these data. Previously identified studies have attempted to use crowd-sourcing technology to access Google © search data. However, the data collected are from searches conducted by any individual, with a spectrum of motives, and data from clinicians would be of greater veracity. I was able to develop a smartphone application with the potential to generate a real-time global disease surveillance database based on the usage of clinical guidelines by doctors. Within the initial 4 weeks of release and without any commercial advertising, the application was downloaded by >1000 clinicians; demonstrating the interest and engagement of clinicians to these sort of technologies. However, concerns remain around the applicability of data collected. Nevertheless, these data are potentially of greater utility than that collected through other crowd-sourcing technologies, such as Google Flu. In addition, I endeavoured that the data collected be from the broadest base possible, but with degrees of veracity with which it is considered. For example, it is considered the strongest evidence if the notification includes the user's name, NHS email address and GMC number, all of which can be

assessed on national databases; diminishing user data is therefore viewed with diminishing veracity. Nevertheless, methods to interrogate these data need further exploration, particularly with regards to the generation of automatic data verification and modelling algorithms that can function in real-time. Parallel studies are needed to compare the quality and quantity of data collected through novel approaches, such as ClickClinica, and routine disease surveillance programmes.

Importantly, any attempts to improve the identification of cases of encephalitis at a population level are dependent on the diagnosis of cases at the individual level. A previous study assessed some aspects of clinical practice, which may result in delayed or missed diagnosis of encephalitis in a single centre (Bell et al 2009). In this thesis I describe the largest published multicentre study in the UK to assess this, which I led. In this I identified that encephalitis maybe as common as bacterial meningitis, and HSV infection of the CNS as common as streptococcal infection. Despite this there were delays in the suspicion of encephalitis as a potential diagnosis, delays in undertaking a LP, and delays in commencing treatment, in comparison to meningitis. Whilst this was in part due to the low positive and negative predictive values of the clinical features for distinguishing any CNS infection from the alternative final diagnoses, all these delays were greater for suspected encephalitis as opposed to suspected meningitis. Importantly, 186 (86%) had the LP delayed for either imaging and/or treatment, and when the LP was performed the opening pressure was only measured in 36%, a paired serum glucose sample was only collected in 37%, and viral PCR was only performed in 31% of those meeting the clinical case definition of

encephalitis. Importantly, even of those who were found to meet the clinical case definitions for CNS infection 25 (57%) had received antibiotic and/or antiviral treatment before the LP, which may impact pathogen identification (Michael BD 2010). Whilst this study was limited by retrospective data collection, it avoided the Hawthorne effect, confirmed the findings of others, and may be reflective of the wider population. For example, similar findings have subsequently been identified in a multicentre study of paediatric practice (Kelly C 2012).

I then went on to assess the impact of these delays in performing the LP on viral detection by PCR of the CSF. Whilst previous studies had identified a lower proportion as PCR positive with delays in the LP, it is uncertain if this was due to those with non-viral causes of encephalitis having the LP performed later or a reduction in the viral load with time (Davies et al 2005). Therefore, having screened 608 patients I identified 12 patients with PCR-proven VZV encephalitis and found that the two who had treatment before the LP was performed had low viral loads, and that overall there was a strong negative correlation between the time from symptom-onset to performing the LP. Therefore, early collection of a CSF sample specifically for PCR for viruses may be important in increasing the likelihood of detection of viral nucleic acid. Interestingly, excluding two potential outliers, there was an inverse correlation between the viral load outcome score, suggesting that possibly those with a higher viral load may have a worse outcome. However, previous studies have found conflicting evidence on the role of the viral load in predicting outcome, and several have found evidence to suggest that the cytokine-mediated inflammatory response may be important too (Winter et al 2004; Kamei et al 2009; Zhang et al 2013).

Given the sub-optimal investigation around LPs identified and the potential impact of this on the detection of the load of viral nucleic acid, I undertook further work to determine whether the introduction of a simple LP pack could improve practice. In this study, I identified that, in comparison to the 6 month period prior to the introduction of the LP pack, there was an increase in the proportion of patients who had the appropriate samples collected, including an increase from 15% to 51% having samples collected for viral PCR. This was associated with a trend towards a greater proportion of patients with a CSF pleocytosis having a pathogen identified. However, this may have reflected the seasonality of Enterovirus infection as no cases were identified in the pre-intervention period and 3 were found in the intervention period which spanned periods of low and high Enterovirus infection respectively. In this study, despite improvements in sample collection, as in prospective studies conducted in multiple countries, the proportion in whom no cause was identified remains high; despite using routine methods to detect both pathogens and recognised antibodies responsible for autoimmune encephalitis (Granerod et al 2010; Granerod et al 2010). Therefore, I went on to assess a prospectively recruited cohort of patients with encephalitis who had been thoroughly investigated, to determine whether the cytokine and associated mediator response in those with encephalitis due to infectious aetiologies differed from that in immune-mediated cases, and whether that identified in those of unknown aetiology best reflected the infectious or immune-mediated cases.

In this study I demonstrated that in a cohort of 95 patients with encephalitis prospectively recruited in the UK, that the CSF cytokine signature differed between those of infectious and immune-mediated aetiologies, and interestingly that those of unknown aetiology seemed to best reflect infectious cases. In both the CSF and serum the mediator that was identified as most effective at distinguishing cases of infectious and unknown aetiologies from immune-mediated cases was MPO, which also correlated with the CSF neutrophil count. Therefore, MPO may represent a useful biomarker to determine disease aetiology. CSF and serum biomarkers have been explored in other diseases of the CNS. For example, I have previously identified that the serum concentrations of G-CSF and eosinophil chemotactants could distinguish, within a cohort of patients with CNS inflammation and demyelination, the sub-group of patients in whom this was due to aquaporin 4 antibodies (Michael et al 2013).

Histopathological studies, although predominantly in samples from the pre-aciclovir era, demonstrated a severe necrotising encephalitis in HSV infection with the early infiltration of neutrophils cells (Love et al 2008). This was demonstrated in the analysis of the 11 samples of histopathological tissue in this study, in which neutrophils were only identified in one of the two cases due to HSV and one of the five of unknown aetiology, but none of the four immune-mediated cases. Although this is a small number of patients it is similar to that previously reported as, for obvious reasons, few patients undergo a biopsy or post-mortem examination; therefore despite the small numbers this represents valuable cohort to study. It is hoped that national projects such as the Medical Research Council Brain Bank will make it possible to analyse sufficient samples

of biopsy and post-mortem tissue to better understand the histopathological changes in patients with encephalitis of unknown cause in the future (Medical Research Council 2014). Future studies of leucocyte subsets in HSV encephalitis should include assessment of neutrophils. More recently RNA has been successfully extracted from historical formalin-fixed tissue and assessed by reverse-transcription PCR to determine host transcriptomic profiles (Griffiths et al 2014-unpublished observations). This may also be of use in assessing samples from patients with encephalitis of unknown aetiology, both to assess host patterns, including MPO, but also to assess for pathogen DNA and RNA.

As histopathological tissue is not routinely available, the development of CSF biomarkers has the potential to aid early diagnosis and therefore allow the clinician to determine which patients require acyclovir or not. This is of particular importance in resource poor settings where there is limited access to PCR and such analysis may be further limited by sample transfer and storage. Moreover, biomarkers in serum that represent proxy markers of CNS inflammation could potentially negate the need to perform a LP, and therefore avoid any potential associated risks. Future studies should focus on the development of point-of-care tests for protein biomarkers with the aim of determining three key clinical questions: Firstly, does this patient have CNS inflammation and therefore need transfer to hospital? Secondly, does this patient with CNS inflammation have a CNS infection? And thirdly, does this patient with CNS infection have a viral or bacterial cause? Similar point-of-care testing has been developed for other pathogens in resource-poor settings, including the use of lateral flow assays for lactate to distinguish bacterial from viral meningitis;

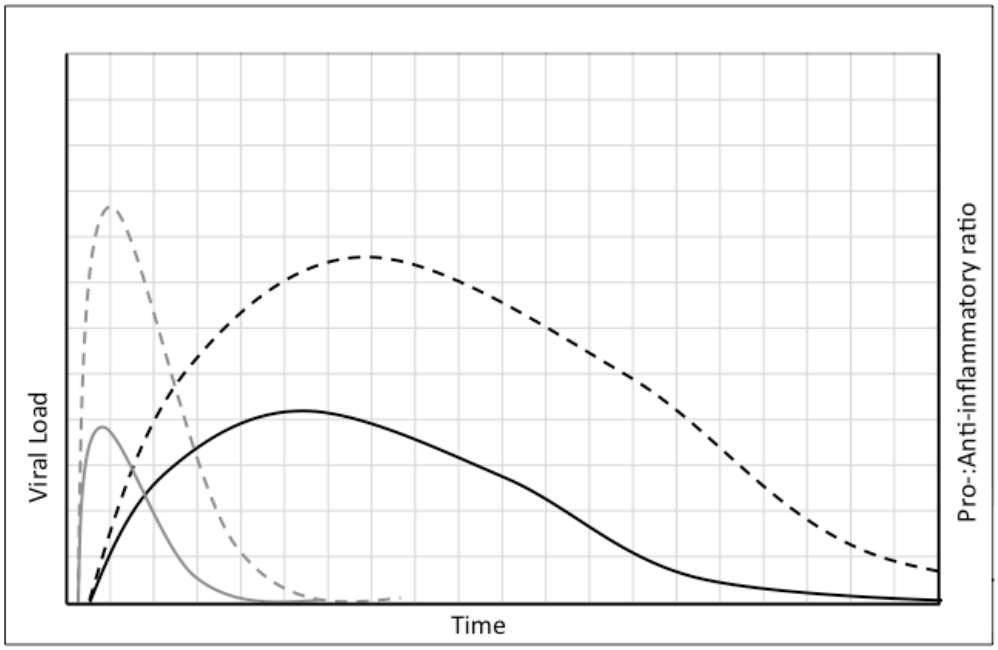
due to the clinical overlap these may need to incorporated into any future test for suspected encephalitis (Majwala et al 2013; Rugemalila et al 2013). However, as this study identified that MPO, a product of activated neutrophils, was the best biomarker at determining infectious from immune-mediated encephalitis, this biomarker could, even at best, only attempt to be used to assess the second of these questions. Nevertheless, even in the UK the average time from collection of sample to receiving the PCR result is 48-72hours and for antibody results is two weeks (National Health Service 2014). Therefore, a simple point-of-care test that could determine which patients should receive antibiotics and antivirals and which should not, pending the results of these further investigations, would be useful. Indeed delays in diagnosis may be made worse in the UK with the further planned centralisation of testing (Public Health England 2014).

Whilst cytokine profiles in the CSF and serum may be useful as biomarkers of aetiology, some have attempted to determine whether they might be associated with the clinical severity and outcome in viral encephalitis (Winter et al 2004; Kamei et al 2004; Griffiths et al 2012). I found that the relative concentration of IL1 and its associated antagonists, IL1RA and IL10, was associated with markers of clinical disease severity and outcome. Moreover, this study found that the mediator with the strongest negative correlation with the BBB permeability was IL10 and that the adhesion and chemotactic molecules, VCAM and CCL2 respectively, were associated with increasing permeability. I took these findings forward to examine these and associated mediators in a BBB model of HSV infection, identifying that, HSV infection resulted in a significant increase in BBB

permeability and that, in addition to other mediators, adhesion molecules VCAM, ICAM and CCL2 were associated with this increasing BBB permeability. Moreover, higher concentrations of both adhesion molecules were identified on the luminal side and the rate of the increase in production of ICAM and CCL2 was also greater on the luminal side. This work found that antagonism of IL1 may represent a potential therapeutic pathway by which to reduce the CNS inflammation associated with HSV encephalitis, in part through the association between IL10 and BBB permeability; and this permeability may also be mediated through adhesion molecules and CCL2. This study also found that a detectable viral load was associated with a lower GOS, but no association with viral load could be assessed. In addition, the previous study of viral load in VZV did not find a significant association between viral load and outcome, except for a post-hoc analysis of a sub-group. This is comparable with previous studies, which have failed to identify a significant correlation between viral load and outcome in viral encephalitis (Kamei et al 2004; Ruzek et al 2007; Munoz-Almagro et al 2008). Conversely, published research assessing the inflammatory response in viral encephalitis has typically identified a significant association between inflammatory profiles and outcome, albeit through different, but related, pro-inflammatory pathways (Winter et al 2004; Kamei et al 2009; Griffiths et al 2012). Whilst this may in part reflect publication bias, taken together I hypothesise from the studies in this thesis, and supporting evidence in the literature, that there may be a relatively rapid reduction in the viral load in the CSF of patients with viral encephalitis, particularly once treatment has been started, as is the case in a significant proportion of patients (Kamei et al 2004; Michael et al 2010; Michael et al 2011). I further hypothesise that the

inflammatory cascade initiated by active viral replication in the CNS may have a more gradual reduction [Figure 25]. Therefore, these hypotheses imply that there is a longer window in which samples are likely to demonstrate a higher pro-inflammatory response in those with a poor outcome than the shorter window within which a higher viral load may be associated with a poor outcome.

Figure 25. Hypothesis generated regarding the sampling window within which a higher viral load or a higher pro- to anti-inflammatory response may be predicative of good [solid line] or poor [dashed line] outcome in HSV encephalitis



10.2 Concluding remarks

Acute encephalitis represents an often severe disease of diverse aetiologies for which detection is sub-optimal at the population level, and diagnosis is sub-optimal at the individual level. Current limitations of epidemiological surveillance practices, reflect user uptake and have associated selection biases. Novel real-time technologies can be developed that have the potential to facilitate enhanced reporting of cases. Nevertheless, epidemiological data are limited by diagnosis at the individual level. The majority of patients with suspected viral encephalitis have the diagnostic LP delayed for other investigations and treatments and this may result in lower viral loads and rates of detection. Nevertheless, simple interventions can increase the proportion of patients having the appropriate samples taken and may increase the proportion in whom a pathogen can be identified. However, despite this the aetiology remains unknown in a significant proportion of patients. Cytokine profiles differ between patients with encephalitis due to infectious and immune-mediated aetiologies, and those with no known aetiology may best reflect those in whom an infection is identified. This may in part reflect neutrophils in the CSF and the perivascular brain parenchyma, and mass spectrometric techniques have the potential to increase pathogen identification in this subgroup of patients. Cytokine signatures, particularly those relating to IL1 and its associated antagonists, IL1RA and IL10, correlate with clinical disease severity and outcome, in part through permeability of the BBB. This permeability appears to be particularly related to IL10 and VCAM in clinical samples and this can be explored in a BBB model. These inflammatory responses are more consistently

associated with outcome than the viral load, although this may relate to the window of sampling. Viral encephalitis, even when detected early, is still associated with significant morbidity and mortality despite current antiviral treatment, therefore these findings should direct future research targeted to address these inflammatory cytokine profiles.

10.3 What this thesis has added to the literature

This study identified, in relation to a pandemic viral infection in the UK, that current disease surveillance mechanisms detected a limited number of cases of CNS manifestations, and may reflect selection bias. This study went on to show that novel technological approaches using real-time user data collection can be created and that these have the potential to augment current surveillance mechanisms. This study also showed that the diagnosis of viral encephalitis on an individual level is limited by sub-optimal CSF collection and that this may have an impact on viral load and pathogen detection. This study found that the cytokines in the CSF , particularly MPO, may augment the distinction of viral encephalitis from immune-mediated disease, and that this may have implications for histopathological features and pathogenesis in those in whom no aetiology is identified. Moreover these cytokine profiles, in particular those relating to antagonism of IL1, were found to correlate, for the first time in clinical samples, with markers of disease severity and outcome. In addition IL10 was identified as having a potential role in reduced BBB permeability, and adhesion molecules and CCL2 with increased permeability, in both clinical samples and a BBB model of HSV infection.

10.4 Future directions

Improved understanding of the epidemiology of acute viral encephalitis is paramount to conducting future research, to developing interventions to improve diagnostic methods and assessing access to therapeutic options. Novel approaches which access user data that may better reflect the wider practice of clinicians may address some of these concerns and have the advantages of real-time capability. However, these need to be validated in parallel prospective population studies, with direct comparisons between these novel technologies, routine surveillance systems, and targeted case identification for prospective studies.

Simple interventions such as a LP pack, can increase the proportion of patients having the appropriate samples taken and may increase the proportion in whom a pathogen can be identified. However, this study was only conducted at one centre and these interventions need to be evaluated in multi-centre studies. Indeed both ClickClinica and the LP pack are currently being trialled in a NIHR-funded cluster intervention trial (Enceph-UK 2014). This is assessing these interventions as part of a clinical package in terms of the impact on the proportion of patients managed appropriately in accordance with the guidelines. The primary outcome measures being assessed are the proportion receiving intravenous acyclovir within 6 hours and the proportion having a LP within 12 hours; the secondary outcome measures are the proportion having a paired plasma glucose collected with the CSF and the proportion having CSF PCR for HSV. In addition this provides a valuable opportunity to assess the cases

identified through ClickClinica in comparison to those identified through routine disease notifications to Public Health England, and those identified through the standard approaches of laboratory-directed screening of clinical notes as employed previously (Kelly et al 2012).

This study identified a potential role for MPO as identifying patients with an infectious aetiology. Nevertheless, the routine CSF parameters of white cell count and differential, when used in combination with the CSF protein and glucose ratio results can go a long way to determining the likely aetiological category (Tunkel et al 2008; Kneen et al 2012; Solomon et al 2012). Therefore, prospective blinded studies of patients with suspected encephalitis are needed comparing the CSF and serum neutrophil counts and MPO concentrations with the routine CSF parameters to determine the relative sensitivity, specificity, and positive and negative predictive values of these approaches. In addition, when considering pathogen-identification studies, such as using next-generation sequencing or proteomic approaches, the CSF cytokine profiles may represent a method by which patients could be selected as 'likely infectious', for example those with higher MPO concentrations or detectable neutrophils within the CSF.

This study also identified a potential role for antagonism of IL1 in improving disease severity and outcome in HSV encephalitis, in part through BBB permeability. The IL1 antagonists, IL1RA and IL10, should be applied in a BBB model of HSV infection; and these should be assessed with both luminal and abluminal administration as a proxy for whether intrathecal or intravenous administration would be required. In addition, cytometric bead array should be

performed on samples within this model to assess the down-stream effects of IL1 antagonism. Also the HSV viral load should be assessed by real-time PCR to assess whether IL1 antagonism results in greater viral loads. The impact should be assessed as an adjunct to acyclovir therapy. There, is also the potential that the adhesion molecules VCAM and ICAM, and the chemokine CCL2 may be associated with increasing permeability and antagonism of these mediators should also be investigated. Targeted antagonism of these mediators has the potential to decrease BBB permeability with a lesser risk of increasing the viral load, as compared to broader spectrum immunosuppression with direct antagonism of IL1, or even more broadly through immunosuppression with steroids. This work will need to be taken forward in a murine model of HSV encephalitis to assess the impact of intra-peritoneal injection on markers of disease severity, pro-inflammatory downstream cytokine profiles, and cerebral oedema. These studies will also need to assess whether the administration of these adjuncts results in an increase in the viral load. A randomised controlled trial of adjunctive dexamethasone is being undertaken in the UK with the primary outcome being language function after discharge (Defres et al 2014). Future studies will need to assess whether there is any additional advantage for targeted pro-inflammatory blockade with IL1 antagonists and/or targeted BBB permeability therapy with VCAM/ICAM/CCL2 blockade, over simply the adjunctive use of dexamethasone.

11. References

Abbott NJ, Patabendige A, Dolman DEM, Yusof SR, Begley DJ (2010). "Structure and function of the blood–brain barrier." Neurobiol Dis **37**: 13-25.

Aberle S, Aberle J, Steininger C, Puchhammer-Stockl E (2005). "Quantitative real time PCR detection of varicella zoster virus DNA in cerebrospinal fluid in patients with neurological disease." Med Microbiol Immunol **194**: 12-17.

Abbott J, Ronnback L, Hansson E (2006). "Astrocyte-endothelial interactions at the blood-brain barrier." Nature Rev Neurosci **7**: 41-53

Akins PT, Belko J, Uyeki TM, Axelrod Y, Lee KK, Silverthorn J (2010). "H1N1 encephalitis with malignant edema and review of neurologic complications from influenza." Neurocrit Care **13**(3): 396-406.

Alemdar M, Selekler HM, Iseri P, Demirci A, Komsuoglu SS (2006). "The importance of EEG and variability of MRI findings in acute hemorrhagic leukoencephalitis." Eur J Neurol **13**(11): e1-e3.

Alheim K, Bartfai T (1998). "The interleukin-1 system: receptors, ligands, and ICE in the brain and their involvement in the fever response." Ann N Y Acad Sci **840**: 51-58.

Ambrose HE, Granerod J, Clewley JP, Davies NW, Keir G, Cunningham R, Zuckerman M, Mutton KJ, Ward KN, Ijaz S, Crowcroft NS, Brown DW; UK Aetiology of Encephalitis Study Group (2013). "Diagnostic strategy used to establish etiologies of encephalitis in a prospoective cohort of patients in England." J Clin Microbiol **49**(10): 3576-83

Antinori A, Arendt G, Becker JT, Brew BJ, Byrd DA, Cherner M, Clifford DB, Cinque P, Epstein LG, Goodkin K, Gisslen M, Grant I, Heaton RK, Joseph J, Marder K, Marra CM, McArthur JC, Nunn M, Price RW, Pulliam L, Robertson KR, Sacktor N, Valcour V, Wojna VE (2007). "Updated research nosology for HIV-associated neurocognitive disorders." Neurology **69**(18): 1789-1799.

Arnold C (2013). "Cloudy with a chance of influenza." Lancet Infect Dis **13**(2): 116-117.

Aronin SI, Peduzzi P, Quagliarello VJ (1998). "Community-acquired bacterial meningitis: risk stratification for adverse clinical outcome and effect of antibiotic timing." Ann Int Med **129**: 862-869.

Asaoka K, Shoji H, Nishizaka S, Ayabe M, Abe T, Ohori N, Ichiyama T, Eizuru Y (2004). "Non-herpetic acute limbic encephalitis: cerebrospinal fluid cytokines and magnetic resonance imaging findings." Intern Med **43**(1): 42-48.

Aurelius E, Andersson B, Forsgren M, Skoldenberg B, Strannegard O (1994). "Cytokines and other markers of intrathecal immune response in patients with herpes simplex encephalitis." J Infect Dis **170**(3): 678-681.

Bachis A, Colangelo A, Vicini S, Doe PP, De Bernardi MA, Brooker G, Mocchetti I (2001). "Interleukin-10 prevents glutamate-mediated cerebellar granule cell death by blocking caspase-3-like activity." J Neurosci **21**(9): 3104-3112.

Bartynski WS, Upadhyaya AR, Petropoulou KA, Boardman JF (2010). "Influenza A encephalopathy, cerebral vasculopathy, and posterior reversible encephalopathy

syndrome: combined occurrence in a 3-year old child." Am J Neuroradiol **31**(8): 1443-1446.

Bederson JB, Sander C, Batjer HH, Dacey RG, Dion JE, Diringer MN, Duldner JE, Harbaugh RE, Patel AB, Rosenwasser RH (2009). "Guidelines for the Management of Aneurysmal Subarachnoid Hemorrhage: a Statement for Healthcare Professionals From a Special Writing Group of the Stroke Council, American Heart Association." Stroke **40:** 994-1025.

Befort P, Gaillard N, Roubille C, Le Quellec A (2010). "Hemorrhagic leukoencephalitis linked to Epstein Barr virus in an adult patient." Clin Neurol Neurosurg **112**(9): 829-831.

Begum F, Pebody R (2013). "Influenza Vaccine Uptake amongst GP Patient Groups in England. Winter Season 2012/2013." Public Health England: PHE gateway number: 2013075. Accessed 28th June 2014.

Behzad-Behbahani A, Abdolvahab A, Gholamali YP, Roshanak B, Mahmood R (2003). "Clinical signs as a guide for performing HSV PCR in correct diagnosis of herpes simplex virus encephalitis." Neurol India **51**: 341-344.

Bell DJ, Suckling R, Rothburn MM, Blanchard T, Stoeter D, Michael BD, Cooke RPD, Kneen R, Solomon T (2009). "Management of suspected herpes simplex virus encephalitis in adults in a UK teaching hospital." Clin Med **9**: 231-235.

British Medical Journal Publising Group (2012). http://group.bmj.com/products/mobile-apps. Accessed 20th November 2012

British Medical Journal Publishing Group and Pharmaceutical Press (2014). British National Formulary (online) London. Accessed 5th June 2014.

Boulos MN, Wheeler S, Tavares C, Jones R (2011). "How smartphones are changing the face of mobile and participatory healthcare: an overview, with example from eCAALYX." Biomed End Online **10**(24): doi:10.1186/1475-1925X-1110-1124.

Boutin H, Kimber I, Rothwell NJ, Pinteaux E (2003). "The expanding interleukin-1 family and its receptors: do alternative IL-1 receptor/signaling pathways exist in the brain?" Mol Neurobiol **27**(3): 239-248.

Bradner S, Thaler C, Lelental N, Buchfelder M, Kleindienst A, Maler JM, Kornhuber J, Lewczuk P (2014). "Ventricular and lumbar cerebrospinal fluid concentrations of Alzheimer's disease biomarkers in patients with normal pressure hydrocephalus and post-traumatic hydrocephalus." J Alzheimers Dis **41**(4): 1057-62

British Infection Society (2003). "Early Management of Suspected Bacterial Meningitis and Meningococcal Septicaemia in Adults Algorithm." http://www.britishinfectionsociety.org/drupal/sites/default/files/MeningitisAlgorithm03.pdf. Accessed 5th February 2010.

Buckley C, Vincent A (2005). "Autoimmune channelopathies." Nat Clin Pract Neurol **1**: 22-33.

Burger D, Molnarfi N, Weber MS, Brandt KJ, Benkhoucha M, Gruaz L, Chofflon M, Zamvil SS, Lalive PH (2009). "Glatiramer acetate increases IL-1 receptor

antagonist but decreases T cell-induced IL-1beta in human monocytes and multiple sclerosis." Proc Natl Acad Sci USA **106**(11): 4355-4359.

Burton JM, O'Connor PW, Hohol M, Beyene J (2012). "Oral versus intravenous steroids for treatment of relapses in multiple sclerosis." Cochrane Database Syst Rev **12**: CD006921.

Catalan M, Naccarato M, Grandi FC, Capozzoli F, Koscica N, Pizzolato G (2009). "Acute hemorrhagic leukoencephalitis with atypical features." Neurol Sci **30**(1): 55-57.

Centers for Disease Control and Prevention (2009). Neurologic complications associated with novel influenza A (H1N1) virus infection in children - Dallas, Texas, May 2009. Morbidity and Mortality Weekly Report **58**(28): 773-778.

Centers for Disease Control and Prevention (2013). [http://wwwn.cdc.gov/nndss/script/history.aspx] Accessed 25th March 2013.

Chadwick DR (2005). "Viral meningitis." Br Med Bull **75-76**: 1-14.

Chadwick DR, Lever AM (2002). "The impact of new diagnostic methodologies in the management of meningitis in adults at a teaching hospital." Q J Med **95**: 663-670.

Chataway J, Davies NWS, Farmer S, Howard R S, Thompson E J, Ward K N (2004). "Herpes simplex encephalitis: an audit of the use of laboratory diagnostic tests." Q J Med **97**: 325-330.

Cheeran MC, Hu S, Palmquist JM, Bakken T, Gekker G, Lokensgard JR (2007). "Dysregulated interferon-gamma responses during lethal cytomegalovirus brain infection of IL-10-deficient mice." Virus Res **130**(1-2): 96-102.

Chen SF, Hu C, Wu HM, Chen SH, Liang YC, Hsu KS (2004). "Seizure, neuron loss, and mossy fiber sprouting in herpes simplex virus type 1infected organotypic hippocampal cultures." Epilepsia **45**: 322-332.

Chen Y, Mizuguchi H, Yao D, Ide M, Kuroda Y, Shigematsu Y, Yamaguchi S, Yamaguchi M, Kinoshita M, Kido H (2005). "Thermolabile phenotype of carnitine palmitoyltransferase II variations as a predisposing factor for influenza-associated encephalopathy." FEBS Lett **579**(10): 2040-2044.

Cinque P, Cleator GM, Weber T, Monteyne P, Sindic CJ, van Loon AM (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.

Cinque P, Lea V (1998). "Herpes simplex virus infections of the central nervous system in human immunodeficiency virus-infected patients: Clinical management by polymerase chain reaction assay of cerebrospinal fluid." Clin Infect Dis **27**: 303-9.

Cisse FA, Antoine JC, Pillet S, Jousserand G, Reynaud-Salard M, Camdessanche J-P (2010). "Acute hemorrhagic leukoencephalopathy associated with influenza A (H1N1) virus." J Neurol **258**(3): 513-514.

Conrady CD, Drevets DA, Carr DJ (2010). "Herpes simplex type 1 infection of the nervous system: is an immune response a good thing?" J Neuroimmunol **220**(1-2): 1-9.

Costiniuk CT, Le Saux N, Sell E, Doja A, Karnauchow T, Jacob P, Hui C (2011). "Miller Fisher Syndrome in a Toddler With Influenza A (H1N1) Infection." J Child Neurol **26**(3): 385-388.

Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, Dessain SK, Rosenfeld MR, Balice-Gordon R, Lynch DR (2008). " Anti-NMDA-receptor encephalitis: case series and analysis of the effects of the antibodies. ." Lancet Neurol **7**: 1091-1098.

Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld M R, Balice-Gordon R (2011). "Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis." Lancet Neurol **10**: 63-74.

Davies N, Brown L, Gonde J, Irish D, Robinsonson R, Swan A, Banatvala J, Howard R, Sharief M, Muir P (2005). "Factors influencing PCR detection of viruses in cerebrospinal fluid of patients with suspected CNS infections." J Neurol Neurosurg Psychiatry **76**: 82-87.

Davies NW, Sharief MK, Howard RS (2006). "Infection-associated encephalopathies: their investigation, diagnosis, and treatment." J Neurol **253**: 833-845.

Davison KL, Crowcroft NS, Ramsay ME, Brown DW, Andrews NJ (2003). "Viral encephalitis in England, 1989-1998: What did we miss?" Emerg Infect Dis **9**: 234-240.

Day NP, Hien TT, Schollaardt T, Loc PP, Chuong LV, Chau TT, Mai NT, Phu NH, Sinh DX, White NJ, Ho M (1999). "The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria." J Infect Dis **180**(4): 1288-1297.

de Bilbao F, Aresenijevic D, Moll T, Garcia-Gabay I, Vallet P, Langhans W, Giannakopoulos P (2009). "In vivo over-expression of interleukin-10 increases resistance to focal brain ischemia in mice." J Neurochem **110**(1): 12-22.

De La Blanchardiere A, Rozenberg F, Caumes E, Picard O, Lionnet F, Livartowski J, Coste J, Sicard D, Lebon P, Salmon-Ceron D (2000). "Neurological complications of varicella zoster virus infection in adults with human immunodeficiency virus infection." Scand J Infect Dis **32**: 263-269.

Defres S, Keller S, Rishma V, Parkes L, Roberts N, Solomon T (2014). "ENCEPH-UK Programme - A pilot Study of the change in temporal lobe volumes in Herpes simplex virus encephalitis using stereological techniques." ESCMID.org. Accessed 1st June 2014

Dennett C, Klapper PE, Cleator GM (1996). "Polymerase chain reaction in the investigation of "relapse" following herpes simplex encephalitis." J Med Virol **48**: 129-132.

Desmond RA, Accortt NA, Talley L, Villano SA, Soong SJ, Whitley RJ (2006). "Enteroviral meningitis: natural history and outcome of pleconaril therapy." Antimicrob Agents Chemother **50**: 2409-2414.

Dietrich WD, Busto R, Bethea JR (1999). "Post-ischemic hypothermia and IL-10 treatment provide long-lasting neuroprotection of CA1 hippocampus following transient global ischemia in rats." Exp Neurol **158**(2): 444-450.

Domingues RB, Lakeman F, Mayo MS, Whitley RJ (1998). "Application of competitive PCR to cerebrospinal fluid samples from patients with herpes simplex encephalitis." J Clin Microbiol Methods **36**: 2229-2234.

Donoso Mantke O, Vaheri A, Ambrose H, Koopmans M, de Ory F, Zeller H, Beyrer K, Windorfer A, Niedrig M; European Network for Diagnostics of Imported Viral Diseases (ENIVD) Working Group for Viral CNS Diseases (2006). "Analysis of the surveillance situation for viral encephalitis and meningitis in Europe." Euro Surveill **13** (3): pii:8017.

Durand ML, Calderwood SB, Weber DJ, Miller SI, Southwick FS, Caviness VS, Swartz MN (1993). "Acute bacterial meningitis in adults: a review of 493 episodes." N Eng J Med **328**: 21-28.

Dworkin RH, Johnson RW, Breuer J, Gnann J W, Levin M J, Backonja M, Betts RF, Gershon AA, Haanpaa ML, McKendrick MW, Nurmikko TJ, Oaklander AL, Oxman MN, Pavan-Langston D, Petersen KL, Rowbotham MC, Schmader KE, Stacey BR, Tyring SK, van Wijck AJ, Wallace MS, Wassilew SW, Whitley RJ (2007). "Recommendations for the management of herpes zoster." Clin Infect Dis **44**(Suppl 1): S1-26.

Easton A, Atkin K, Hare P (2007). "'a light in a very dark place': The role of a voluntary organisation providing support for those affected by encephalitis." Neuropsychol Rehabil **17**: 638-647.

Ekstrand JJ (2012). "Neurologic complications of influenza." Semin Pediatr Neurol **19**(3): 96-100.

Ekstrand JJ, Herbener A, Rawlings J, Turney B, Ampofo K, Korgenski EK, Bonkowsky JL (2010). "Heightened neurologic complications in children with pandemic H1N1 influenza." Ann Neurol **68**(5): 762-766.

Ellermann-Eriksen S (2005). "Macrophages and cytokines in the early defence against herpes simplex virus." Virol J **2**: 59.

Emsley HC, Smith C, Georgiou RF, Vail A, Hopkins SJ, Rothwell NJ, Tyrrell PJ (2005). "A randomised phase II study of interleukin-1 receptor antagonist in acute stroke patients." J Neurol Neurosurg Psychiatry **76**(10): 1366-1372.

Enceph-UK (2014) http://www.encephuk.org/studies/intervention.aspx. Accessed 3rd May 2014.

European Bioinformatics Institute (2014). http://www.ebi.ac.uk/pride/archive/ Accessed 14th June 2014.

Farooq O, Faden HS, Cohen ME, Ramanathan M, Barrett H, Farkas MK, Langan TJ, Yeh EA (2012). "Neurologic complications of 2009 influenza-A H1N1 infection in children." J Child Neurol **27**(4): 431-438.

Fitch MT, van de Beek D (2007). "Emergency diagnosis and treatment of adult meningitis." Lancet Infect Dis **7**: 191-200.

Fodor P A, Levin MJ, Weinberg A, Sandberg E, Sylman J, Tyler KL (1998). "Atypical herpes simplex virus encephalitis diagnosed by PCR amplification of viral DNA from CSF." Neurol **51**: 554-559.

Fujimoto S, Kobayashi M, Uemura O, Iwasa M, Ando T, Katoh T, Nakamura C, Maki N, Togari H, Wada Y (1998). "PCR on cerebrospinal fluid to show influenza-associated acute encephalopathy or encephalitis." Lancet **352**(9131): 873-875.

King's Fund (2010). http://www.kingsfund.org.uk/projects/general-election-2010/faqs - hospital]. Accessed 25th March 2013.

Furlan R, Bergami A, Brambilla E, Butti E, De Simoni MG, Campagnoli M, Marconi P, Comi G, Martino G (2007). "HSV-1-mediated IL-1 receptor antagonist gene therapy ameliorates MOG(35-55)-induced experimental autoimmune encephalomyelitis in C57BL/6 mice." Gene Ther **14**(1): 93-98.

Gibson RM, Rothwell N, Le Feuvre RA (2004). "CNS injury: the role of the cytokine IL-1." Vet J **168**(3): 230-237.

Gilca R, Deceuninck G, De Serres G, Boulianne N, Sauvageau C, Quach C, Boucher FD, Skowronski DM (2011). "Effectiveness of pandemic H1N1 vaccine against influenza-related hospitalization in children." Pediatrics **128**(5): e1084-e1091.

Gilden D (2004). "Varicella zoster virus and central neurological system syndromes." Herpes **11**: 89A-94A.

Ginsberg J, Mohebbi MH, Patel RS, Brammer L, Smolinski MS, Brilliant L (2009). "Detecting influenza epidemics using search engine query data." Nature **457**(7232): 1012-1014.

Gjini AB, Stuart J, Cartwright K, Cohen J, Jacobs M, Nichols T, Ninis N, Prempeh H, Whitehouse A, Heyderman RS (2006). "Quality of in-hospital care for adults with acute bacterial meningitis: a national retrospective survey." Q J Med **99**: 761-769.

Glaser CA, Honarmand S, Anderson LJ, Schnurr DP, Forghani B, Cossen CK, Schuster FL, Christie LJ, Tureen JH (2006). "Beyond viruses: clinical profiles and etiologies associated with encephalitis." Clin Infect Dis **43**: 1565-1577.

Glaser CA, Winter K. DuBray K, Harriman K, Uyeki TM, Sejvar J, Gilliam S, Louie K (2012). "A Population-Based Study of Neurologic Manifestations of Severe Influenza A(H1N1)pdm09 in California." Clin Infect Dis **55**(4): 514-520.

Goenka A, Michael BD, Ledger E, Hart I, Absoud M, Chow G, Lilleker J, Lim M, Lunn M, Peake D, Pysden K, Roberts M, Carrol E, Avula S, Solomon T, Kneen R (2013). "Neurological manifestations of influenza in adults and children: Results of a national British surveillance study." Clin Infect Dis **58** (6): 775-84.

Government, UK (1998). "Data protection act." http://www.opsi.gov.uk/Acts/acts1998/ukpga_19980029_en_1. Accessed 2nd June 2014.

Government, UK (2001). http://www.statistics.gov.uk/census2001/pop2001/Merseyside.asp. Accessed 2nd June 2014.

Granerod J, Ambrose HE, Davies NW, Clewley JP, Walsh AL, Morgan D, Cunningham R, Zuckerman M, Mutton KJ, Solomon T, Ward KN, Lunn MPT, Irani

SR, Vincent A, Brown DWG, Crowcroft NS (2010). "Causes of encephalitis and differences in their clinical presentations in England: a multicentre, population-based prospective study." Lancet Infect Dis **10**: 835-844.

Granerod J, Cousens S, Davies NW, Crowcroft NS, Thomas SL (2013). "New estimates of incidence of encephalitis in England." Emerg Infect Dis **19**(9): doi:10.3201/eid1909.130064

Granerod J, Tam CC, Crowcroft NS, Davies NWS, Borchert M, Thomas SL (2010). "Challenge of the unknown: A systematic review of acute encephalitis in non-outbreak situations." Neurology **75**: 924-932.

Griffith JW, Sokol CL, Luster AD (2014). "Chemokines and chemokine receptors: positioning cells for host defense and immunity." Ann Rev Immunol **32**: 659-702.

Griffiths MJ, Ooi MH, Wong SC, Mohan A, Podin Y, Perera D, Chieng CH, Tio PH, Cardosa MJ, 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**(6): 881-892.

Habel AH, Brown JK (1972). "Dexamethasone in herpes-simplex encephalitis." Lancet **1**(7752): 695.

Hakan T, Toklu H, Biber N, Ozevren H, Solakoglu S, Demirturk P, Aker FV (2010). "Effect of COX-2 inhibitor meloxicam against traumatic brain injury-induced biochemical, histopathological changes and blood-brain barrier permeability." Neurol Res **32**(6): 629-635.

Harvala H, Bremner J, Kealey S, Weller B, McLellan S, Lloyd G, Staples E, Faggian F, Solomon T (2009). "Case report: Eastern equine encephalitis virus imported to the UK." J Med Virol **81**: 305-308.

Hasbun R, Abrahams J, Jekel J, Quagliarello V J (2001). "Computed tomography of the head before lumbar puncture in adults with suspected meningitis." N Eng J Med **345**: 1727-1733.

Hasegawa H, Satake Y, Kobayashi Y (1990). "Effect of cytokines on Japanese encephalitis virus production by human monocytes." Microbiol Immunol **34**(5): 459-466.

Health Protection Agency (2007). http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1251473364307%5D. Accessed 25th March 2013.

Health Protection Agency (2011). [http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1251473364307%5D,. Accessed 25th March 2013.

Health Protection Agency (2011). “Surveillance of influenza and other respiratory viruses in the UK 2010-2011 Report.” Health Protection Agency, London. Accessed 25th March 2013.

Heyderman RS, Klein NJ (2000). " Emergency management of meningitis." J R Soc Med **93**: 225-229.

Heyderman RS, Lambert HP, O'Sullivan I, Stuart JM, Taylor BL, Wall RA (2003). "Early management of suspected bacterial meningitis and meningococcal septicaemia in adults." J Infect **46**: 75-77.

Höllinger P, Matter L, Sturzenegger M (2000). "Normal MRI findings in herpes simplex virus encephalitis." J Neurol **247**: 799-801.

Hoshino A, Saitoh M, Oka A, Okumura A, Kubota M, Saito Y, Takanashi J, Hirose S, Yamagata T, Yamanouchi H, Mizuguchi M (2012). "Epidemiology of acute encephalopathy in Japan, with emphasis on the association of viruses and syndromes." Brain Dev **34**(5): 337-343.

Ichiyama T, Morishima T, Isumi H, Matsufuji H, Matsubara T, Furukawa S (2004). "Analysis of cytokine levels and NF-κB activation in peripheral blood mononuclear cells in influenza virus-associated encephalopathy." Cytokine **27**(1): 31-37.

Ichiyama T, Shoji H, Takahashi Y, Matsushige T, Kajimoto M, Inuzuka T, Furukawa S (2008). "Cerebrospinal fluid levels of cytokines in non-herpetic acute limbic encephalitis: comparison with herpes simplex encephalitis." Cytokine **44**(1): 149-153.

Irani SR, Alexander S, Waters P, Kleopa K A, Pettingill P, Zuliani L, Peles E, Buckley C, Lang B, Vincent A (2010). "Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia." Brain **133**: 2734-2748.

Irani SR, Bera K, Waters P, Zuliani L, Maxwell S, Zandi MS, Friese MA, Galea I, Kullmann DM, Beeson D, Lang B, Bien CG, Vincent A (2010). " N-methyl-D-aspartate antibody encephalitis: temporal progression of clinical and paraclinical observations in a predominantly non-paraneoplastic disorder of both sexes." Brain **133**: 1655-1667.

Irani SR, Michell AW, Lang B, Pettingill P, Waters P, Johnson MR, Schott JM, Armstrong RJ, Zagami A, Bleasel A, Somerville ER< Smith SM, Vincent A (2010). " Faciobrachial dystonic seizures precede Lgi1-antibody limbic encephalitis." Ann Neurol **69**: 892-900.

Ito Y, Ichiyama T, Kimura H, Shibata M, Ishiwada N, Kuroki H, Furukawa S, Morishima T (1999). "Detection of influenza virus RNA by reverse transcription-PCR and proinflammatory cytokines in influenza-virus-associated encephalopathy." J Med Virol **58**(4): 420-425.

Ivers N, Jamtvedt G, Flottorp S, Young JM, Odgaard-Jensen J, French SD, O'Brien MA, Johansen M, Grimshaw J, Oxman AD (2012). "Audit and feedback: effects on professional practice and healthcare outcomes." Cochrane Database Syst Rev **13**(6): CD000259.

Jefferson T, Rivetti A, Harnden A, Di Pietrantonj C, Demicheli V (2008). "Vaccines for preventing influenza in healthy children." Cochrane Database Syst Rev (2): CD004879.

Jeliffe S (1918). "Nervous and mental disturbances of influenza." N Y Med J **108**: 725-728.

Jemsek J, Greenberg SB, Taber L, Harvey D, Gershon A, Couch R B (1983). "Herpes zoster-associated encephalitis: clinicopathologic report of 12 cases and review of the literature. ." Medicine (Baltimore) **62**: 81-97.

Jennett B, Bond M (1975). "Assessment of outcome after severe brain damage." Lancet **1**(7905): 480-484.

Jensen MD, Sheng W, Simonyi A, Johnson GS, Sun AY, Sun GY (2009). "Involvement of oxidative pathways in cytokine-induced secretory phospholipase A2-IIA in astrocytes." Neurochem Int **55**(6): 362-368.

Jmor F, Emsley HCA, Fisher M, Solomon T, Lewthwaite P (2008). "The incidence of acute encephalitis syndrome in Western Industrialised and Tropical Countries." Virology J **5**(134).

John GR, Lee S, Song X, Rivieccio M, Brosnan CF (2005). "IL-1-regulated responses in astrocytes: relevance to injury and recovery." Glia **49**(2): 161-176.

Kamei S, Sekizawa T, Shiota H, Mizutani T, Itoyama Y, Takasu T, Morishima T, Hirayanagi K (2005). "Evaluation of combination therapy using aciclovir and corticosteroid in adult patients with herpes simplex virus encephalitis." J Neurol Neurosurg Psychiatry **76**: 1544-1549.

Kamei S, Taira N, Ishihara M, Sekizawa T, Morita A, Miki K, Shiota H, Kanno A, Suzuki Y, Mizutani T, Itoyama Y, Morishima T, Hirayanagi K (2009). "Prognostic value of cerebrospinal fluid cytokine changes in herpes simplex virus encephalitis." Cytokine **46**(2): 187-193.

Kamei S, Toshiaki T, Morishima T, Mizutani T (2004). "Serial changes of intrathecal viral loads evaluated by chemiluminescence assay and nested PCR with aciclovir treatment in herpes simplex encephalitis." Intern Med **43**: 796-801.

Kawashima H, Morichi S, Okumara A, Nakagawa S, Morishima T (2012). "National survey of pandemic influenza A (H1N1) 2009-associated encephalopathy in Japanese children." J Med Virol **84**(8): 1151-1156.

Kelly C, Sidhu A, Michael BD, Beeching NJ, Riordan A, Solomon T, Kneen R (2012). "The Management of Paediatric suspected CNS infections across Merseyside: A multicentre retrospective audit." BMC Paediatr (7): 145-149.

Kelly C, Sohal A, Michael BD, Riordan A, Solomon T, Kneen R (2012). "Suboptimal management of central nervous system infections in children: a multi-centre retrospective study." BMC Pediatr **12**(1): 12-145.

Kennedy PG (2005). "Viral encephalitis." J Neurol **252**(3): 268-272.

Khan SS, Smith MS, Reda D, Suffredini AF, McCoy Jr JP (2004). "Multiplex bead array assays for detection of soluble cytokines: comparisons of sensitivity and quantitative values among kits from multiple manufacturers." Cytometry B Clin Cytom **61**(1): 35-39.

Khandaker G, Zurynski Y, Buttery J, Marshall H, Richmond PC, Dale RC, Royle J, Gold M, Snelling T, Whitehead B, Jones C, Heron L, McCaskill M, Macartney K, Elliott EJ, Booy R (2012). "Neurologic complications of influenza A(H1N1)pdm09: Surveillance in 6 pediatric hospitals." Neurology **79**(14): 1474-1481.

Kneen R, Jakka S, Mithyantha R, Riordan A, Solomon T (2010). "The management of infants and children treated with aciclovir for suspected viral encephalitis." Arch Dis Child **95**: 100-106.

Kneen R, Michael BD, Menson E, Mehta B, Easton A, Hemingway C, Klapper PE, Vincent A, M Lim, Carrol E, Solomon T (2012). "Management of suspected viral encephalitis in children- Association of British Neurologists and British Paediatric Allergy Immunology and Infection Group National Guidelines." Journal of Infection **64**: 449-477.

Kneen R, Solomon T, Appleton R (2002). "The role of lumbar puncture in children with suspected central nervous system infection." BMC Paediatrics **2**: 8.

Koskiniemi M, Rantalaiho T, Piiparinen H, von Bonsdorff CH, Färkkilä M, Järvinen A, Kinnunen E, Koskiniemi S, Mannonen L, Muttilainen M, Linnavuori K, Porras J, Puolakkainen M, Räihä K, Salonen EM, Ukkonen P, Vaheri A, Valtonen V (2001). "Infections of the central nervous system of suspected viral origin: A collaborative study from finland." J Neurovirol **7**: 400-408.

Koskiniemi M, Vaheri A, Taskinen E (1984). "Cerebrospinal fluid alterations in herpes simplex virus encephalitis." Clin Infect Dis **63:** 608-18.

Kupila L, Vourinen T, Vainionpa R, Hukkanen V, Marttila RJ, Kotilainen P (2006). "Etiology of aseptic meningitis and encephalitis in an adult population." Neurology **66**: 75-80.

Labus J, Hackel S, Lucka L, Danker K (2014). "Interleukin-1beta induces an inflammatory response and the breakdown of the endothelial cell layer in an

improved human THBMEC-based in vitro blood-brain model." J Neurosci Methods **228**: 35-45.

Lann MA, Lovell MA, Kleinschmidt-DeMasters BK (2010). "Acute Hemorrhagic Leukoencephalitis." Am J Forensic Med Pathol **31**(1): 7-11.

Launes C, de-Sevilla MF, Selva L, Garcia-Garcia JJ, Pallares R, Muñoz-Almagro C (2012). "Viral coinfection in children less than five years old with invasive pneumococcal disease." Pediatr Infect Dis J **31**(6): 650-653.

Leveque N, Legoff J, Mengelle C, Mercier-Delarue S, N'Guyen Y, Renois F, Tissier F, Simon F, Izopet J, Andreoletti L (2014). "Virological diagnosis of central nervous system infections by use of PCR coupled with mass spectrometry analysis of cerebrospinal fluid samples." J Clin Microbiol **52**(1): 212-217.

Levin M, Hjelm M, Kay JD, Pincott JR, Gould JD, Dinwiddie R, Matthew DJ (1983). "Haemorrhagic shock and encephalopathy: a new syndrome with a high mortality in young children." Lancet **2**(8341): 64-67.

Logan SAE, MacMahon E (2008). "Viral Meningitis." Br Med J **336**: 36-40.

Lokensgard JR, Hu S, Sheng W, van Oijen M, Cox D, Cheeran MC, Peterson PK (2001). "Robust expression of TNF-alpha, IL-1beta, RANTES, and IP-10 by human microglial cells during nonproductive infection with herpes simplex virus." J Neurovirol **7**(3): 208-219.

Love S, Louis DN, Ellison DW (2008). Greenfield's Neuropathology, Hodder Arnold. ISBN 0340906812: 1293-8.

London School of Hygiene and Tropcial Medicine (2014). "Flusurvey." 2014, from https://flusurvey.org.uk/en/the-project/. Accessed 20th May 2014.

Magazzini S, Nazerian P, Vanni S, Paladini B, Pep G, Casanova B, Crugnola C, Grifoni S (2012). "Clinical picture of meningitis in the adult patient and its relationship with age." Int Emerg Med **7**(4): 359-364.

Mailles A, De Broucker T, Costanzo P, Martinez-Almoyna L, Vaillant V, Stahl JP (2012). "Long-term outcome of patients presenting with acute infectious encephalitis of various causes in France." Clin Infect Dis **54**(10): 1455-1464.

Majwala A, Burke R, Patterson W, Pinkerton R, Muzoora C, Wilson LA, Moore CC (2013). "Handheld point-of-care cerebrospinal fluid lactate testing predicts bacterial meningitis in Uganda." Am J Trop Med Hyg **88**(1): 127-131.

Marcotte TD, Deutsch R, Michael BD, Franklin D, Cookson DR, Bharti AR, Grant I, Letendre SL (2013). "A concise panel of biomarkers identifies neurocognitive functioning changes in HIV-infected individuals." J Neuroimmune Pharmacol **8**(5): 1123-1135.

Marques CP, Hu S, Sheng W, Cheeran MC, Cox D, Lokensgard JR (2004). "Interleukin-10 attenuates production of HSV-induced inflammatory mediators by human microglia." Glia **47**(4): 358-366.

McCoy MK, Tansey MG (2008). "TNF signaling inhibitionin the CNS: implications for normal brain function and neurodegenerative disease." J Neuroinflamm **5**(45): 1-13.

McCullers JA (2006). "Insights into the interaction between influenza virus and pneumococcus." Clin Microbiol Rev **19**(3): 571-582.

McDonald WI, Compston A, Edan G , Goodkin D, Hartung HP, Lublin FD, McFarland HF, Paty DW, Polman CH, Reingold SC, Sandberg-Wollheim M, Sibley W, Thompson A, van den Noort S, Weishenker BY, Wolinsky JS (2001). "Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis." Ann Neurol **50**: 121-127.

McGrath N, Anderson NE, Croxson M C, Powell K F. (1997). "Herpes simplex encephalitis treated with acyclovir: Diagnosis and long term outcome." J Neurol Neurosurg Psychiatry **63:** 321-6.

Medical Research Council (2014). "UK Brain Bank." 2014, from http://www.mrc.ac.uk/resourcesandservices/ukbrainbanksnetwork. Accessed 6th june 2014.

Melchjorsen J, Pedersen FS, Mogensen SC, Paludan SR (2002). "Herpes simplex virus selectively induces expression of the CC chemokine RANTES/CCL5 in macrophages through a mechanism dependent on PKR and ICP0." J Virol **76**(6): 2780-2788.

Meyding-Lamade UK, Oberlinner C, Rau PR, Seyfer S, Heiland S, Sellner J, Wildemann BT, Lamade WR (2003). "Experimental herpes simplex virus encephalitis: a combination therapy of acyclovir and glucocorticoids reduces long-term magnetic resonance imaging abnormalities." J Neurovirol **9**(1): 118-125.

Michael BD, Elsone L, Griffiths MJ, Faragher B, Borrow R, Solomon T, Jacob A (2013). "Post-acute serum eosinophil and neutrophil-associated cytokine/chemokine profile can distinguish between patients with neuromyelitis optica and multiple sclerosis; and identifies potential pathophysiological mechanisms - a pilot study." Cytokine **64**(1): 90-96.

Michael BD, Meneezes BF, Cunniffe J, Miller A, Kneen R, Francis G, Beeching NJ, Solomon T (2010). "The effect of delayed lumbar punctures on the diagnosis of acute bacterial meningitis in adults. ." Emerg Med J **27**(6): 433-438.

Michael BD, Stewart A, Buckley C, Galbraith S, Hopkins M, Hart IJ, Solomon T. (2011). "Varicella zoster virus encephalitis: The relationship between viral load, time, clinical features and outcome." European Journal of Neurology **18**(s2): 66-67.

Michael BD, Stoeter DJ, Manji H (2014). "Encephalitis." Challenging concepts in Neurology, Oxford University Press: In Press.

Michael BD, Sidhu M, Stoeter DJ, Roberts M, Beeching NJ, Bonington A, Hart IJ, Kneen R, Miller A, Solomon T and the North West Neurological Infections Network (2010). "Acute central nervous system infections in adults-a retrospective cohort study in the NHS North West region." Q J Med **103**(10): 749-758.

Michael BD, Solomon T (2012). "Seizures and encephalitis: clinical features, management, and potential pathophysiologic mechanisms." Epilepsia **53 Suppl 4**: 63-71.

Minjolle S, Arvieux C, Gautier A L, Jusselin I, Thomas R, Michelet C, Colimon R (2002). " Detection of herpes virus genomes by polymerase chain reaction in cerebrospinal fluid and clinical findings." J Clin Virol **Suppl 1**: S59-70.

Minton J, Clayton J, Sandoe J, McGann H, Wilcox M (2008). "Improving early management of bloodstream infection: a quality improvement project." BMJ **336**: 440-3.

Misra UK, Kalita J, Nair PP (2008). "Status epilepticus in central nervous system infections: an experience from a developing country." Am J Med **121**: 816-823.

Mizuguchi M, Abe J, Mikkaichi K, Noma S, Yoshida K, Yamanaka T, Kamoshita S (1995). "Acute necrotising encephalopathy of childhood: a new syndrome presenting with multifocal, symmetric brain lesions." J Neurol Neurosurg Psychiatry **58**(5): 555-561.

Mizuguchi M, Yamanouchi H, Ichiyama T, Shiomi M (2007). "Acute encephalopathy associated with influenza and other viral infections." Acta Neurol Scand **115**(Suppl. 186): 45-56.

Mofenson LM, Brady MT, Danner S P, Dominguez K L, Hazra R, Handelsman E, Havens P, Nesheim S, Read JS, Serchuck L, Van Dyke R; Centers for Disease Control and Prevention; National Institutes of Health; HIV Medicine Association of the Infectious Diseases Society of America; Pediatric Infectious Diseases Society; American Academy of Pediatrics (2009). "Guidelines for the Prevention and Treatment of Opportunistic Infections among HIV-exposed and HIV-infected children: recommendations from CDC, the National Institutes of Health, the HIV Medicine Association of the Infectious Diseases Society of America, the Pediatric Infectious Diseases Society, and the American Academy of Pediatrics." MMMWR Recomm Rep **58**(RR-11): 1-166.

Molina-Holgado E, Arevalo-Martin A, Ortiz S, Vela JM, Guaza C (2002). "Theiler's virus infection induces the expression of cyclooxygenase-2 in murine astrocytes: inhibition by the anti-inflammatory cytokines interleukin-4 and interleukin-10." Neurosci Lett **324**(3): 237-241.

Munoz-Almagro C, Jordan I, Cambra FJ, Esteban E, Urrea M, Garcia-Garcia JJ, Palomeque A (2008). "Quantitative real-time PCR in peadiatric patients with herpes simplex infections of the central nervous system." J Virol Methods **147**: 297-300.

Neilson DE, Adams MD, Orr CM, Schelling DK, Eiben RM, Kerr DS, Anderson J, Bassuk AG, Bye AM, Childs AM, Clarke A, Crow YJ, Di Rocco M, Dohna-Schwake C, Dueckers G, Fasano AE, Gika AD, Gionnis D, Gorman MP, Grattan-Smith PJ, Hackenberg A, Kuster A, Lentschig MG, Lopez-Laso E, Marco EJ, Mastroyianni S, Perrier J, Schmitt-Mechelke T, Servidei S, Skardoutsou A, Uldall P, van der Knaap MS, Goglin KC, Tefft DL, Aubin C, de Jager P, Hafler D, Warman ML (2009). "Infection-triggered familial or recurrent cases of acute necrotizing encephalopathy caused by mutations in a component of the nuclear pore, RANBP2." Am J Hum Genet **84**(1): 44-51.

National Health Service (2014). "Southtees Pathology Turnaround times." 2014, from http://southtees.nhs.uk/services/pathology/tests/turnaround-times/. Accessed 6th June 2014.

National Patient Safety Agency (2011). "Neuraxial Update October 2011." from http://www.
patientsafetyfirst.nhs.uk/ashx/Asset.ashx?path1/4/Medicationsafety/NPSA-Neuraxial %20Update%20Newsletter-October%202011-final.pdf.

Okabe N, Yamashita K, Taniguchi K, Inouye S (2000). "Influenza surveillance system of Japan and acute encephalitis and encephalopathy in the influenza season." Pediatr Int **42**(2): 187-191.

Okumura A, Mizuguchi M, Kidokoro H, Tanaka M, Abe S, Hosoya M, Aiba H, Maegaki Y, Yamamoto H, Tanabe T, Noda E, Imataka G, Kurahashi H (2009). "Outcome of acute necrotizing encephalopathy in relation to treatment with corticosteroids and gammaglobulin." Brain Dev **31**(3): 221-227.

Okumura A, Nakagawa S, Kawashima H, Morichi S, Muguruma T, Saito O, Fujimoto J, Toida C, Kuga S, Imamura T, Shimizu T, Kondo N, Morishima T (2013). "Severe form of encephalopathy associated with 2009 pandemic influenza A (H1N1) in Japan." J Clin Virol **56**(1): 25-30.

Ooi MH, Solomon T, Podin Y, Mohan A, Akin W, Yusuf M A, del Sel S, Kontol KM, Lai BF, Clear D, Chien CH, Blake E, Perea D, Wong SC, Cardosa J (2007). "Evaluation of Different Clinical Sample Types in the Diagnosis of Human Enterovirus 71 Associated Hand-Foot-and-Mouth Disease." J Clin Microbiol **45**: 1858-1868.

Openshaw H, Cantin E (2005). "Corticosteroids in herpes simplex virus encephalitis. ." J Neurol Neurosurg Psychiatry **76**: 1469.

Pacheco LR, Tavares HM, Moyses Neto M, Dantas M, Rocha L S, Ribeiro KM, Figueiredo JF (2005). "Acute renal failure related to intravenous acyclovir." Rev Assoc Med Bras **51**: 275-278.

Pandey S, Rathore C, Michael BD (2014). “Antiepileptic drugs for the primary and secondary prevention of seizures in viral encephalitis.” Cochrane Dat Sys Rev : CD010247

Palacios G, Hornig M, Cisterna D, Savji N, Bussetti AV, Kapoor V, Hui J, Tokarz R, Briese T, Baumeister E, Lipkin WI (2009). "Streptococcus pneumoniae coinfection is correlated with the severity of H1N1 pandemic influenza." PLoS One **4**(12): 8540.

Paludan SR (2001). "Requirements for the induction of interleukin-6 by herpes simplex virus-infected leucocytes." J Virol **75**(17): 8008-8015.

Pasieka TJ, Cilloniz C, Carter VS, Rosato P, Katze MG, Leib DA (2011). "Functional genomics reveals an essential and specific role for Stat1 in protection of the central nervous system following herpes simplex virus corneal infection." J Virol **85**(24): 12972-12981.

Patabendige A (2012). "Toward a humanised alternative to the use of laboratory animals for blood-brain barrier research." Altern Lab Anim **40**(5): 12-13.

Persidsky Y, Ramirez S, Haorah J, Kanmogne G (2006). "Blood-brain barrier: structural components and function under physiologic and pathologic conditions." J Neuroimmune Pharmacol **1**: 223-236.

Persson A, Bergstrom T, Lindh M, Namvar L, Studahl M (2009). "Varicella-zoster virus CNS disease - viral load, clinical manifestations and sequels." J Clin Virol **46**: 249-253.

Pinteaux E, Trotter P, Simi A (2009). "Cell-specific and concentration-dependent actions of interleukin-1 in acute brain inflammation." Cytokine **45**(1): 1-7.

Poissy J, Champenois K, Dewilde A, Melliez H, Georges H, Senneville E, Yazdanpanah Y (2012). "Impact of Herpes simplex virus load and red blood cells in cerebrospinal fluid upon herpes simplex meningo-encephalitis outcome." BMC Infect Dis **12**: 356.

Poissy J, Wolff M, Dewilde A, Rozenberg F, Raschilas F, Blas M, Georges H, Chaffaut C, Yazdanpanah Y (2009). "Factors associated with delay to acyclovir administration in 184 patients with herpes simplex virus encephalitis." Clin Microbiol Infect **15**: 560-564.

Proulx N, Frechette D, Toye B, Chan J, Kravcik S (2005). "Delays in the administration of antibiotics are associated with mortality from adult acute bacterial meningitis." Q J Med **98**: 291-298.

Public Health England (2014). "Towards a public health surveillance strategy for England."https://http://www.gov.uk/government/uploads/system/uploads/attachment_data/file/213339/Towards-a-Public-Health-Surveillance-Strategy.pdf. Accessed 29th June 2014.

Ramakrishna C, Newo AN, Shen YW, Cantin E (2011). "Passively administered pooled human immunoglobulins exert IL-10 dependent anti-inflammatory effects that protect against fatal HSV encephalitis." PLoS Pathog **7**(6): e1002071.

Raschilas F, Wolffe M, Delatour F, Chaffaut C, De Broucker T, Chevret S, Lebon P, Canton P, Rozenberg F (2002). "Outcome of and prognostic factors for herpes

simplex encephalitis in adult patients: results of a multicenter study." Clin Infect Dis **35**(3): 254-260.

Rasmussen HH, Sorensen H, Moller-Petersen J, Mortensen FV, Nielsen B (1992). "Bacterial meningitis in elderly patients: clinical picture and course." Age Ageing **21**: 216-220.

Read SJ, Jeffery KJM, Bangham CRM (1997). "Aseptic Meningitis and Encephalitis: the Role of PCR in the Diagnostic Laboratory, ." J Clin Microbiol **35**: 691-696.

Reiber H (1995). "External quality assessment in clinical neurochemistry: survey of analysis for cerebrospinal fluid (CSF) proteins based on CSF/serum quotients." Clin Chem **41**(2): 256-263.

Rinka H, Yoshida T, Kubota T, Tsuruwa M, Fuke A, Yoshimoto A, Kan M, Miyazaki D, Arimoto H, Miyaichi T, Kaji A, Miyamoto S, Kuki I, Shiomi M (2008). "Hemorrhagic shock and encephalopathy syndrome-the markers for an early HSES diagnosis." BMC Pediatr **8**(1): 43.

Rodrigues M, Zoecklein L, Papke L, Gamez J, Denie A, Macura S, Howe C (2009). "Tumour necrosis factor alpha is reparative via TNFR1 in the hippocampus and via TNFR2 in the striatum after virus-induced encephalitis." Brain Pathol **19**: 12-26.

Rosler A, Pohl M, Braune HJ, Oertel WH, Gemsa D, Sprenger H (1998). "Time course of chemokines in the cerebrospinal fluid and serum during herpes simplex type 1 encephalitis." J Neurol Sci **157**(1): 82-89.

Rothwell NJ, Loddick S (2002). Immune and inflammatory responses in the nervous system (2nd Edition) Oxford University Press: ISBN 9780198509806: 36-50.

Rugemalila J, Maro V, Kapanda G, Ndaro AJ, Jarvis JN (2013). "Cryptococcal antigen prevalence in HIV-infected Tanzanians: a cross-sectional study and evaluation of a point-of-care lateral flow assay." Trop Med Int Health **18**(9): 1075-1079.

Ruzek D, Piskunova N, Zampachova E. (2007). " High variability in viral load in cerebrospinal fluid from patients with herpes simplex and varicella zoster infections of the central nervous system." Clin Microbiol Infect **13**: 1217-1219.

Salliot C, Dougados M, Gossec L (2009). "Risk of serious infections during rituximab, abatacept and anakinra treatments for rheumatoid arthritis: meta-analyses of randomised placebo-controlled trials." Ann Rheum Dis **68**(1): 25-32.

Sammon CJ, McGrogan A, Snowball J, de Vries CS (2012). "Factors associated with uptake of seasonal and pandemic influenza vaccine among clinical risk groups in the UK: an analysis using the General Practice Research Database." Vaccine **30**: 2483-2489.

Sawyer MH, Webb DE, Balow JE, Straus S E (1988). " Acyclovir-induced renal failure. Clinical course and histology." Am J Med **84**: 1067-1071.

Saxena V, Mathur A, Krishnani N, Dhole TN (2008). "An insufficient anti-inflammatory cytokine response in mouse brain is associated with increased tissue pathology and viral load during Japanese encephalitis virus infection." Arch Virol **153**(2): 283-292.

Saxena V, Mathur A, Krishnani N, Dhole TN (2008). "Kinetics of cytokine profile during intraperitoneal inoculation of Japanese encephalitis virus in BALB/c mice model." Microbes Infect **10**(10-11): 1210-1217.

Schachtele SJ, Hu S, Lokensgard JR (2012). "Modulation of experimental herpes encephalitis-associated neurotoxicity through sulforaphane treatment." PLoS One **7**(4): e36216.

Sejvar JJ (2006). "The evolving epidemiology of viral encephalitis." Curr Opin Neurol **19**: 350-357.

Sener RN (2001). "Herpes simplex encephalitis: diffusion MR imaging findings." Comput Med Imaging Graph **25**: 391-397.

Sergerie Y, Rivest S, Boivin G (2007). "Tumor necrosis factor-alpha and interleukin-1 beta play a critical role in the resistance against lethal herpes simplex virus encephalitis." J Infect Dis **196**(6): 853-860.

Shaman J, Karspeck A (2012). "Forecasting seasonal outbreaks of influenza." Proc Natl Acad Sci USA **109**(50): 20425-20430.

Sintchenko V, Gallego B (2009). "Laboratory-guided detection of disease outbreaks: three generations of surveillance systems." Arch Path Lab Med **133**(6): 916-925.

Sivadon-Tardy V, Orlikowski D, Porcher R, Sharshar T, Durand MC, Enouf V, Rozenberg F, Caudie C, Annane D, van der Werf S, Lebon P, Raphaël JC, Gaillard

JL, Gault E (2009). "Guillain‐Barré Syndrome and Influenza Virus Infection." Clin Infect Dis **48**(1): 48-56.

Skoldenberg B, Aurelius E, Hjalmarsson A, Sabri F, Forsgren M, Andersson B, Linde A, Strannegard O, Studahl M, Hagberg L, Rosengren L (2006). "Incidence and pathogenesis of clinical relapse after herpes simplex encephalitis in adults." J Neurol **253**(2): 163-170.

Skoldenberg B, Forsgren M, Alestig K, Bergstrom T, Burman L, Dahlqvist E, Forkman A, Fryden A, Lovgren K, Norlin K (1984). "Acyclovir versus vidarabine in herpes simplex encephalitis. Randomised multicentre study in consecutive Swedish patients." Lancet **2**(8405): 707-711.

Skolimowska KH, Rangaka M, Meintjes G, Pepper DJ, Seldon R, Mathews K, Wilkinson RJ, Wilkinson KA (2012). "Altered ratio of IFN-y/IL-10 in patients with drug resistant Mycobacterium tuberculosis and HIV-Tuberculosis immune reconstitution inflammatory syndrome." PLoS One **7**(10): e46481.

Snyder RD (2003). "Bacterial meningitis: diagnosis and treatment." Curr Opin Neurosci Rep **3**: 461-469.

Solomon T, Hart IJ, Beeching NJ (2007). "Viral encephalitis: a clinician's guide." Pract Neurol **7**(5): 288-305.

Solomon T, Michael BD, Smith PE, Sanderson F, Davies NWS, Hart IJ, Buckley C, Holland M, Easton A, Kneen R, Beeching NJ (2012). "Management of suspected viral encephalitis in adults: Association of British Neurologists and British Infection Association National Guideline." Journal of Infection **64**(4): 374-373.

Solomon T, Whitley RJ (2004). "Arthropod-borne viral encephalitides. Infections of the Central Nervous system." Ed Scheld, Whitley RJ, Marra C. Philidelphia, PA, Lippincott Williams and Wilkins. NLM IDL101625260.

Sommer JB, Gaul C, Heckmann J, Neundorfer B, Erbguth FJ (2002). "Does lumbar cerebrospinal fluid reflect ventricular cerebrospinal fluid? A prospective study in patients with external ventricular drainage." Eur Neurol **47**(4): 224-32

Steiner I, Budka H, Chaudhuri A, Koskiniemi M, Sainio K, Salonen O, Kennedy PG (2005). "Viral encephalitis: a review of diagnostic methods and guidelines for management." Eur J Neurol **12**: 331-343.

Stone MJ (2007). "A medical overview of encephalitis. ." Neurpsychol Rehab **17**: 429-449.

Studahl M, Hagberg L, Rekabdar E, Bergstrom T (2000). "Herpesvirus DNA detection in cerebral spinal fluid: differences in clinical presentation between alpha-, beta-, and gamma-herpesviruses. ." Scand J Infect Dis **32**: 237-248.

Surana P, Tang S, McDougall M, Tong CYW, Menson E, Lim M (2011). "Neurological complications of pandemic influenza A H1N1 2009 infection: European case series and review." Eur J Pediatr **170**: 1007-1015.

Swarup V, Ghosh J, Duseja R, Ghosh S, Basu A (2007). "Japanese encephalitis virus infection decrease endogenous IL-10 production: correlation with microglial activation and neuronal death." Neurosci Lett **420**(2): 144-149.

Szelenyi J (2001). "Cytokines and the central nervous system." Brain Res Bull **54**(4): 329-338.

Szodoray P, Alex P, Chappell-Woodward CM, Madland TM, Knowlton N, Dozmorov I, Zeher M, Jarvis JN, Nakken B, Brun JG, Centola M (2007). "Circulating cytokines in Norwegian patients with psoriatic arthritis determined by a multiplex cytokine array system." Rheumatology **46**: 417-425.

Takanashi J (2009). "Two newly proposed infectious encephalitis/encephalopathy syndromes." Brain Dev **31**(7): 521-528.

The National Institute for Health and Clinical Excellence (2010). "The Management of Bacterial Meningitis and Meningococcal Septicaemia in Children and Young People Younger Than 16 Years in Primary and Secondary Care. Clinical Guidelines, CG102." http://guidance.nice.org.uk/CG102 Accessed June 2010.

Thigpen MC, Whitney C, Messonnier NE, Zell ER, Lynfield R, Hadler JL, Harrison LH, Farley MM, Reingold A, Bennett NM, Craig AS, Schaffner W, Thomas A, Lewis MM, Scallan E, Schuchat A (2011). "Emerging infections programs network. Bacterial meningitis in the United States." N Eng J Med **362**(21): 2004-2016.

Thompson KA, Blessing WW, Wesselingh SL (2000). Herpes simplex replication and dissemination is not increased by corticosteroid in a rat model of focal Herpes encephalitis". J Neurovirol **6**(1): 25-32

Tse MC, L. C., Mot K, Onlamoon N, Hsiao HM, Perng GC. (2009). "ICAM-5 modulates cytokine/chemokine production in the CNS during the course of herpes simplex virus type 1 infection." J Neuroimmunol **213**(1-2): 12-19.

Tumpey TM, Elner V, Chen SH, Oakes JE, Lausch RN (1994). "Interleukin-10 treatment can suppress stromal keratitis induced by herpes simplex virus type 1." J Immunol **153**(5): 2258-2265.

Tunkel AR, Glaser CA., Bloch K C, Sejvar JJ, Marra C M, Roos KL, Hartman BJ, Kaplan SL, Scheld WM, Whitley RJ; Infectious Diseases Society of America (2008). "The management of encephalitis: clinical practice guidelines by the Infectious Diseases Society of America." Clin Infect Dis **47**: 303-327.

Tyler KL (2004). "Update on herpes simplex simplex encephalitis." Rev Neurol Dis **1**: 169-178.

University College London (2014). "Flu Watch." http://www.ucl.ac.uk/iph/research/cide/fluwatch. Accessed 16th May 2014.

Upton AR, Foster JB, Barwick DD (1971). "Dexamethasone treatment in herpes-simplex encephalitis." Lancet **1** (7643): 411-2.

van Crevel H, Hijdra A, de Gans J (2002). "Lumbar puncture and the risk of herniation: when should we first perform CT? ." J Neurol **249**: 129-137.

van de Beek D, de Gans J, Spanjaard L, Weisfelt M, Reitsma J B, Vermeulen M (2004). "Clinical features and prognostic factors in adults with bacterial meningitis. ." N Eng J Med **351**: 1849-1859.

Van de Beek D, de Gans J, Tunkle AR, Wijdicks FM (2006). "Community-acquired bacterial meningitis in adults." N Eng J Med **354**: 44-53.

VanLandingham KE, Marsteller HB, Ross GW, Hayden FG (1988). "Relapse of herpes simplex encephalitis after conventional acyclovir therapy. ." JAMA **259**: 1051-1053.

Vidal Rodeiro CL, Lawson AB (2006). "Online updating of space-time disease surveillance models via particle filters." Stat Methods Med Res **15**(5): 423-444.

Vilela MC, Lima G, Rodrigues DH, Lacerda-Queiroz N, Pedroso VS, Miranda AS, Rachid MA, Kroon EG, Campos MA, Teixeira MM, Sellner J, Teixeira AL (2013). "Absence of CCR5 increases neutrophil recruitment in severe herpetic encephalitis." BMC Neurosci **14**: 19.

Vincent A, Buckley C, Schott J M, Baker I, Dewar B K, Detert N, Clover L, Parkinson A, Bien CG, Omer S, Lang B, Rossor MN, Palace J (2004). "Potassium channel antibody-associated encephalopathy: a potentially immunotherapy-responsive form of limbic encephalitis." Brain **127**: 701-712.

Vincent A, Irani SR, Lang B (2010). "The growing recognition of immunotherapy-responsive seizure disorders with autoantibodies to specific neuronal proteins." Curr Opin Neurol **23**: 144-150.

Vitkovic L, Maeda S, Sternberg E (2001). "Anti-inflammatory cytokines: expression and action in the brain." Neuroimmunomodulation **9**(6): 295-312.

Wang G, Zhang J, Weizhong L, Xin G, Su Y, Gao Y, Zhang H, Lin G, Jiao X, Li K (2008). "Apoptosis and proinflammatory cytokine responses of primary mose microglia and astrocytes induced by human H1N1 and avian H5N1 influenza viruses." Cell and Mol Immunol **5**(2): 113-120.

Wang X, Zeng D, Seale H, Li S, Cheng H, Luan R, He X, Pang X, Dou X, Wang Q (2010). "Comparing early outbreak detection algorithms based on their optimized parameter values." J Biomed Inform **43**(1): 97-103.

Weil AA, Glaser CA, Amad Z, Forghani B (2002). "Patients with suspected herpes simplex encephalitis: rethinking an initial negative polymerase chain reaction result." Clin Infect Dis **34**: 1154-1157.

Weisfelt M, van de Beek D, Spanjaard L, Reitsma JB, de Gans J (2006). "Attenuated cerebrospinal fluid leucocyte count and sepsis in adults with pneumococcal meningitis: a prospective cohort study." BMC Infect Dis **6**: 149.

Whitley RJ (2006). "Herpes simplex encephalitis: adolescents and adults." Antiviral Res **71** (2-3): 141-8.

Whitley RJ, Alford CA, Hirsch MS, Schooley RT, Luby JP, Aoki FY, Hanley D, Nahmias AJ, Soong SJ (1986). "Vidarabine versus acyclovir therapy in herpes simplex encephalitis." N Eng J Med **314**: 144-149.

Whitley RJ, Cobbs CG, Alford CA Jr, Soong SJ, Hirsch MS, Connor JD, Corey L, Hanley DF, Levin M, Powell DA; NIAD Collaborative Antiviral Study Group. (1989). "Diseases that mimic herpes simplex encephalitis. Diagnosis, presentation, and outcome." JAMA **262**: 234-239.

Whitley RJ, Gnann JW (2002). "Viral encephalitis: familiar infections and emerging pathogens." Lancet **359**: 507-513.

Whitley RJ (1987). "Factors indicative of outcome in a comparative trial of acyclovir and vidarabine for biopsy-proven herpes simplex encephalitis." Infection **15**(Suppl 1): S3-8.

Wildemann B, Ehart K, Storch-Hagenlocher B, Meydig-Lamade U, Steinvorth S, Hacke W, Haas J (1997). "Quantitation of herpes simplex virus type 1 DNA in cells of cerebrospinal fluid of patients with herpes simplex virus encephalitis." Neurology **48**(5): 1341-1346.

Wilson EH, Wille-Reece U, Dzierszinski F, Hunter CA (2005). "A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis." J Neuroimmunol **165**(1-2): 63-74.

Wingerchuk DM, Lennon V, Pittock SJ, Weinshenker BG (2006). "Revised diagnostic criteria for neuromyelitis optica." Neurology **66**: 1485-1489.

Winter PM, Dung N, Loan HT, Kneen R, Wills B, Thu le T, House D, White NJ, Farrar JJ, Hart CA, Solomon T (2004). "Proinflammatory cytokines and chemokines in humans with Japanese encephalitis." J Infect Dis **190**(9): 1618-1626.

Wong AM, Simon EM, Zimmerman RA, Wang H-S, Toh C-H, Ng S-H (2006). "Acute necrotizing encephalopathy of childhood: correlation of MR findings and clinical outcome." AJNR Am J Neuroradiol **27**(9): 1919-1923.

Wozniak MA, Shipley SJ, Combrinck M, Wilcock GK, Itzhaki RF (2005). "Productive herpes simplex virus in brain of elderly normal subjects and Alzheimer's disease patients." J Med Virol **75**: 300-306.

Wright JL, Merchant RE (1994). "Blood-brain barrier changes following intracerebral injection of human recombinant tumor necrosis factor-alpha in the rat." J Neurooncol **20**(1): 17-25.

Yamada S, Kameyama T, Nagaya S, Hashizume Y, Yoshida M (2003). " Relapsing herpes simplex encephalitis: pathological confirmation of viral reactivation." J Neurol Neurosurg Psychiatry **74**: 262-264.

Yamanouchi H, Mizuguchi M (2006). "Acute infantile encephalopathy predominantly affecting the frontal lobes (AIEF): A novel clinical category and its tentative diagnostic criteria." Epilepsy Res **70**: 263-268.

Yildizdas D, Kendirli T, Arslankoylu AE, Horoz OO, Incecik F, Ince E, Ciftci E (2010). "Neurological complications of pandemic influenza (H1N1) in children." Eur J Pediatr **170**(6): 779-788.

Zhang LK, Chai F, Li HY, Xiao G, Guo L (2013). "Identification of host proteins involved in Japanese encephalitis virus infection by quantitative proteomics analysis." J Proteome Res **12**(6): 2666-2678.

Zhang S, Jouanguy E, Ugolini S, Smahi A, Elain G, Romero P, Segal D, Sancho-Shimizu V, Lorenzo L, Puel A, Picard C, Chapgier A, Plancoulaine S, Titeux M, Cognet C, vo Bernuth H, Ku CL, Casrouge A, Zhang XX, Barreiro L, Loenard J, Hamilton C, Lebon P, Heron B, Vallee L, Quintana-Murci L, Hovnanian A, Rozenberg F, Vivier E, Geissmann F, Tardieu M, Abel, Casanova JL (2007). "TLR3 deficiency in patients with HSV encephalitis." Science **317**(5844): 1522-1527.

12. Appendices

12.1 Publications arising from this thesis

12.1.1 Management of suspected viral encephalitis in adults: Association of British Neurologists and British Infection Association National Guideline.

Solomon T, MICHAEL BD (joint first), Smith PE, Sanderson F, Davies NWS, Hart IJ, Buckley C, Holland M, Easton A, Kneen R, Beeching NJ. On behalf of the National Encephalitis Guidelines Development Group. ***J Infect*** *2012;* 64(4): 347-73

12.1.2 Management of suspected viral encephalitis in children: Association of British Neurologists and British Paediatric Allergy, Immunology and Infection Group National Guideline.

Kneen R, MICHAEL BD (joint first), Menson E, Hemmingway C, Vincent A, Davies NWS, Wilkins E, Holland M, Easton A, Solomon T. On behalf of the National Encephalitis Guidelines Development Group. ***J Infect*** *2012*; 64(5): 449-77

12.1.3 The clinical management of acute central nervous system infections in immunocompetent adults.

MICHAEL BD, Solomon T. ***Oxford Textbook of Diagnosis and Treatment in Internal Medicine*** *2014; Commissioned: Ahead of press*

12.1.4 Clinical management of acute viral encephalitis

MICHAEL BD, Stoeter DJ, Manji H. **Challenging Concepts in Neurology. *Oxford University Press*** *2014; Commissioned: Ahead of press*

12.1.5 Seizures and Encephalitis: Clinical features, management and potential pathophysiological mechanisms.

MICHAEL BD, Solomon T. ***Epilepsia*** *2012;* 53(S4): 63-71

12.1.6 Neurological manifestations of influenza in adults and children: Results of a national British surveillance study.

Goenka A, MICHAEL BD, Ledger E, Hart I, Absoud M, Chow G, Lilleker J, Lim M, Lunn M, Peake D, Pysden K, Roberts M, Carrol E, Avula S, Solomon T, Kneen R. ***Clin Infect Dis*** *2014;* 58 (6):775-84.

12.1.7 Development of ClickClinica: A novel smartphone application to generate real-time global disease surveillance and clinical practice database.

MICHAEL BD, Geleta D. ***BMC Med Inform Decis Mak*** *2013;* 13:70. doi: 10.1186/1472-6947-13-70

12.1.8 The epidemiology and management of adult suspected central nervous system infections - a retrospective cohort study in the NHS Northwest Region.

MICHAEL BD, Sidhu M, Stoeter D, Roberts M, Beeching NJ, Wilkins E, Hart I, Kneen R, Miller A, Solomon T. ***Q J Med*** *2010;* 103(10): 749-5

12.1.9 Varicella zoster virus encephalitis: The relationship between viral load, time, clinical features and outcome

MICHAEL BD, Stewart A, Buckley C, Galbraith S, Hopkins M, Hart IJ, Borrow R, Solomon T. Abstract ***Eur J Neurol*** *2011*

12.1.10 Improving the diagnosis of central nervous system infections in adults through introduction of a simple lumbar puncture pack: A quality improvement project

MICHAEL BD, Powell GA, Hatch S, Bailey L, Almond S, McGill F, Cousins D, Hart IJ, Griffiths M, Kneen R, Solomon T. ***Emerg Med J*** *2013;* 30(5): 402-5

12.2 Related publications

12.2.1 Suboptimal management of central nervous system infections in children: a multi-centre retrospective study.

Kelly C, Sohal A, MICHAEL BD, Beeching NJ, Riordan A, Solomon T, Kneen R. *BMC Paediatr 2012*;12: 145

12.2.2 . Lumbar puncture: diagnosing acute central nervous system infections.

Matata C, MICHAEL BD, Garner V, Solomon T ***Nurs Stand*** *2012*; 27(8); 49-56

12.2.3 Post-acute serum eosinophil and neutrophil-associated cytokine profile can distinguish between patients with neuromyelitis optica and multiple sclerosis and identifies potential pathophysiological mechanisms and therapeutic targets- A pilot study

MICHAEL BD, Elsone L, Griffiths M, Faragher B, Borrow R, Solomon T, Jacob A.. ***Cytokine*** *2013;* 64(1): 90-6. Cited as key paper for 2013 by ***Royal Society of Medicine*** *for* ***Current Medical Literature*** *2013. 5(4): 103-4*

12.2.4 A concise panel of biomarkers identifies neurocognitive functioning changes in HIV-infected individuals.

Marcotte T, Deutesch R, MICHAEL BD, Franklin D, Grant I, Letendre S. ***J Neuroimmune Pharmacol*** *2013;* 8(5): 1123-35.

12.2.5 Lumbar puncture: Diagnosing CNS infections and spinal devices with safer connectors.

National Patient Safety Agency (NPSA) ***Neuroaxial Update*** November 2012.

12.2.6 Antiepileptic drugs for the primary and secondary prevention of seizures in viral encephalitis (Review).

Pandey S, Rathore C, Michael BD. ***Cochrane Database of Systematic Reviews*** *2014;* CD010247