PROTEIN PROMISE? APPLICATION OF MASS SPECTROMETRY-BASED PROTEOMICS TECHNIQUES TO IDENTIFY CEREBROSPINAL FLUID BIOMARKERS FOR DIAGNOSING SUSPECTED CENTRAL NERVOUS SYSTEM INFECTIONS - A FOCUS ON JAPANESE ENCEPHALITIS VIRUS INFECTION

by

Tehmina Manek Bharucha

Merton College

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For the degree of Doctor of Philosophy in the field of Biochemistry

Department of Biochemistry, University of Oxford,

South Parks Road, Oxford OX1 3QU and

the Lao-Oxford-Mahosot Hospital-Wellcome Trust-Research Unit,

Mahosot Hospital, Vientiane, Laos

Statement of contributions

My thesis involved research that would not have been possible without collaboration. I certify that the work detailed here is my own unless explicitly stated below.

Most importantly, I acknowledge the receipt of precious patient samples collected and characterised as part of the Laos central nervous system infection study that represents an immense body of work that started in 1999, detailed in numerous publications, and is still ongoing. My analysis of these samples is reported in Chapter 3, Chapter 6 and Chapter 7.

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[1] https://www.ipa.world/IPA/en/Members/MANEK_PHIROZE_BHARUCHA.aspx
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Abbreviations

A1BG	Alpha-1-B glycoprotein
Aa	Amino acids
ACN	Acetonitrile
AD	Alzhiemer's disease
AFAM	Afamin
ALDOC	Aldolase, fructose-bisphosphate C
AmBic	Ammonium bicarbonate
AUROC	Area under the receiver operator curve
AXL	AXL receptor tyrosine kinase
AZU1	Azurocidin 1
Aβ42/40	Amyloid-β 1–42/1–40 ratio
B2M	Beta-2-microglobulin
BASP1	Brain abundant membrane attached signal protein 1
BBB	Blood-brain-barrier
BBBI	Blood-brain-barrier impairment or dysfunction
BCB	Blood-CSF-barrier
BPI	Bactericidal permeability increasing protein
C	Capsid
CBLN1	Cerebellin 1 precursor
CBPB2	Carboxypeptidase B2
CCT	Collaborations to Catalyse Translation
CDA	Cytidine deaminase
CDC	Centers for Disease Control and Prevention
CEACAM8	CEA cell adhesion molecule 8
CEND1	Cell cycle exit and neuronal differentiation 1
CF	Complement fixation
CHCA/HCCA	α-cyano-4-hydroxycinnamic acid
CHI3L1	Chitinase 3 like 1
CI	Confidence interval
CJD	Creutzfeldt–Jakob disease
CMPK2	Cytidine/uridine monophosphate kinase 2
CNS	Central nervous system
CPE	Cytopathic effect
CRP	C-reactive protein
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CTSL	Cathepsin L
CV	Cross-validation
Cx.	Culex
DABA	Double-antigen binding assay
DALY	Disability-adjusted life year
DBS	Dried blood spots
DCD	Dermcidin

DCS	Dried CSF spot
DDA	Data dependent acquisition
DENV	Dengue virus
DIA	Data independent acquisition
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
ENO2	Gamma-enolase/neuron-specific enolase
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
EV	*Enterovirus* spp.
FC	Fold change
FDA	U.S. Food and Drug Administration
FDR	False discovery rate
FETUA	Alpha-2-HS-glycoprotein
GFAP	Glial fibrillary acidic protein
GO	Gene Ontology
GOLM1	Golgi membrane protein 1
HCMV	Human cytomegalovirus
HDA	Helicase-dependent amplification
HELZ	Helicase with zinc finger domain 2
HI	Inhibition of haemagglutination
HIV	Human immunodeficiency virus
HPA	Human Protein Atlas
HSV	Herpes simplex virus
IAA	Iodoacetamide
IFA	Immunofluorescence
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IGHV3-74	Immunoglobulin heavy variable 3-74
IGLV3-9	Immunoglobulin lambda variable 3-9
IgM	Immunoglobulin M
IL18BP	Interleukin 18 binding protein
IBP7	Insulin-like growth factor-binding protein 7
IQR	Interquartile range
ISG15	ISG15 ubiquitin like modifier
ISGs	Interferon stimulating genes
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
kDa	Kilodalton
kNN	k-Nearest Neighbours
LAMP	Loop-mediated isothermal amplification
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled to mass spectrometry

LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry, also referred to as MS2 or MS/MS
LMICs	Low- and Middle-Income Countries
LOMWRU	Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit
LP	Lumbar puncture
M	Membrane
MAC	IgM antibody-capture
Malaria	Plasmodium falciparum
MALDI-ToF	Matrix assisted desorption ionisation – time of flight
MAPK1	Mitogen-activated protein kinase 1
MAPT	Microtubule associated protein tau (commonly referred to as tau)
MARCKSL1	MARCKS like 1
MBR	Match between runs
MEM	Minimum essential medium
MeSH	Medical Subject Headings
MMP8	Matrix metallopeptidase 8
MMP9	Matrix metallopeptidase 9
mNGS	Metagenomic next-generation sequencing
MORU	Mahidol Oxford Tropical Medicine Research Unit
mRNA	Messenger RNA
MS	Mass spectrometry
MS/MS or MS2	Tandem mass spectrometry
MTX3	Metallothionein 3
MWCO	Molecular weight cut-off
mz	Mass-to-charge ratio
NAb	Neutralising antibodies
NASBA	Nucleic acid sequence-based amplification
NEAR	Nicking and extension enzyme amplification reaction
NIH	National Institutes of Health
Non-JE	Confirmed CNS infections other than Japanese encephalitis
NPA	Negative percentage agreement
NPPC	Natriuretic peptide C
NPV	Negative predictive value
NS	Non-structural protein
NSE	Gamma-enolase/neuron-specific enolase
OLFM4	Olfactomedin 4
OPA	Overall percentage agreement
Ot	Orientia tsutsugamushi
OXTREC	Oxford University Tropical Ethics Research Committee
p-tau	Hyperphosphorylated tau
PALM	Paralemmin
PCP4	Purkinje cell protein 4
PCR	Polymerase chain reaction
PRDX2	Peroxiredoxin-2
PLBD1	Phospholipase B domain containing 1
PLS-DA	Partial least squares-discriminant analysis

PPA	Positive percentage agreement
PPV	Positive predictive value
PRIDE	Proteomics Identification Database
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analyses
prM	Pre-membrane
PRM	Parallel reaction monitoring
PRNT	Plaque-reduction neutralisation test
PSMB1	Proteasome 20S subunit beta 1
PTX3	Pentraxin 3
QC	Quality control sample
QE classic	Q Exactive benchtop hybrid quadrupole-Orbitrap MS
RBC	Red blood cell
RDT	Cerebrospinal fluid
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROC	Receiver operating characteristics
RP	Reverse phase
RPA	Recombinase polymerase amplification
RT	Reverse-transcription
RTN1	Reticulon 1
RT-PCR	Reverse-transcription polymerase chain reaction
RT-qPCR	Real-time quantitative reverse-transcription polymerase chain reaction
RU	Relative units
S100A12	S100 calcium binding protein A12
S100B	S100 calcium-binding protein B
SAMP	Serum amyloid-p component
SCRN1	Secernin 1
SEAe	South-East-Asia encephalitis
SF	Substandard and falsified
SORS	Spatially offset Raman spectroscopy
SPE	Solid phase extraction
spp.	Species
SPP1	Secreted phosphoprotein 1
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
SVM	Support vector machine
tau	Microtubule associated protein tau (MAPT is the gene name)
TB	Mycobacterium tuberculosis
TCID	Median tissue culture infective dose
TEAB	Triethylammonium bicarbonate
TFA	Trifluoroacetic acid
TMA	Transcription-mediated amplification
TMT	Tandem mass tag
TNFSF13B	TNF superfamily member 13b
TNFSF13B	TNF superfamily member 13b
TTHY	Transthyretin
TXNRD1	Thioredoxin reductase 1

UHPLC	Ultra-high-performance liquid chromatography
UNICEF	United Nations Children's Fund
UTR	Untranslated region
VIE	Vaccine Identity and Evaluation
VNT	Virus neutralisation test
VTDB	Vitamin D-binding protein
VZV	varicella zoster virus
WBC	White blood cell
WGCNA	Weighted correlation network analysis
WHO	World Health Organization
WNV	West Nile virus
YWHAG	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma
YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein eta
ZIKV	Zika virus
2D	Two-dimensional
3D	Three-dimensional

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Publications arising from this thesis

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3. Bharucha T, Shearer FM, Vongsouvath M, Mayxay M, de Lamballerie X, Newton PN et al. A need to raise the bar - A systematic review of temporal trends in diagnostics for Japanese encephalitis virus infection, and perspectives for future research. Int J Infect Dis. 2020 Jun;95:444-456. doi:10.1016/j.ijid.2020.03.0392.1

4. Bharucha T, Gangadharan B, Kumar A, de Lamballerie X, Newton PN, Winterberg M et al. Mass spectrometry-based proteomic techniques to identify cerebrospinal fluid biomarkers for diagnosing suspected central nervous system infections. A systematic review. J Infect. 2019 Nov;79(5):407-418. doi:10.1016/j.jinf.2019.08.005

Abstract

Neurological infections account for considerable death and disability worldwide. Even in the best-resourced settings, existing diagnostic tests are inadequate and adversely affect patient outcomes. In this thesis, I propose that the identification of host biomarkers of neurological infection could fill the gap in case ascertainment.

In Laos, as is the case in other countries in Asia, the leading cause of neurological infection is Japanese encephalitis virus (JEV). Specifically for Japanese encephalitis (JE), the pathogen is present at very low levels such that it is rarely detected in any body fluid, and the mainstay of diagnosis is anti-JEV IgM antibody capture enzyme-linked immunosorbent assay. This has poor specificity. Furthermore, there is no rapid diagnostic test to be used in the rural areas that JEV exists. JE represents an important neurological infection to illustrate the potential role of host protein biomarkers for diagnosis.

I retrospectively tested a cohort of 163 patients recruited as part of the Laos central nervous system (CNS) infection study. Application of liquid chromatography and tandem mass spectrometry (LC-MS/MS), using extensive offline fractionation and tandem mass tag labelling, enabled a comparison of the CSF proteome in 68 JE patient vs 95 non-JE neurological infections. 5,069 proteins were identified, including 4,805 human proteins and 265 pathogen proteins. I incorporated univariate analysis of differential protein expression, network analysis and machine learning techniques to build a ten-protein diagnostic signature of JE.

Pathways related to JE infection included neuronal damage, anti-apoptosis, heat shock and unfolded protein responses, cell adhesion, macrophage and dendritic cell activation as well as a reduced acute inflammatory response, hepatotoxicity, activation of coagulation, extracellular matrix and actin regulation. I verified the results by performing DIA LC-MS/MS in 16 (10%) of the samples, demonstrating 82% accuracy using the same model. Ultimately, antibody-based validation will be required, in a larger group of patients, in different locations and in field settings, to refine the list to 2-3 proteins that could be harnessed in a rapid diagnostic test.

COVID-related mitigating circumstances

It would be short-sighted not to write a thesis in the field of infectious diseases without stating from the outset the extensive impact of the SARS-CoV-2 pandemic. Aside from the fact that my DPhil was suspended for a period so that I could return to clinical work, several periods of leave during COVID-19 illness in myself and my family, and the excruciating delays as a consequence of various restrictions in transporting samples and entering laboratories related to COVID-19, the field of diagnostic virology has changed. Enormous research investment and outcomes have been achieved for SARS-CoV-2 in the space of a very short time, and much non-SARS-CoV-2 research globally have fallen by the wayside or been grossly overshadowed. Directly related to my DPhil, the pandemic has led to many more publications on virus neutralisation and mass spectrometry-based proteomics methods. I managed to maintain my originally planned research over this period, albeit at a slower pace and with revisions in some areas. Specifically, after several months of optimising a targeted assay (parallel reaction monitoring, PRM), the work was halted and the made-to-order heavy peptides degraded during storage beyond their expiry date. Additionally, a project that received competitive grant funding faced extraordinary difficulties, elaborated in Chapter 6. However, on a more positive note, my attention and time was redirected towards an entirely new project related to COVID-19 that now constitutes the penultimate chapter (Chapter 8) of this thesis.

Chapter 1

Introduction

Aims of the thesis

My primary research question is whether there is a diagnostic protein signature of Japanese encephalitis (JE) in human cerebrospinal fluid (CSF) that could be harnessed for the development of a rapid diagnostic test (RDT). Secondary aims include strengthening understanding of the host response to Japanese encephalitis virus (JEV) infection *in vivo* and identifying potential novel targets for therapeutics. Lastly, I aim to better understand how proteomics and mass spectrometry (MS) methods could be applied to the diagnosis of other neurological infections and the identification of substandard and falsified (SF) JEV vaccines.

A global issue of undiagnosed neurological infections

A myriad of pathogens infect the nervous system and lead to a diverse range of clinical syndromes (1). In this thesis I will use the term neurological infection interchangeably with central nervous system (CNS) infection, although the former also refers to infections that affect the peripheral nervous system such as leprosy. Neurological infection syndromes may be categorised into acute, subacute and chronic meningitis and encephalitis, space occupying lesions, encephalopathy with systemic infection, toxin-mediated syndromes and post-infectious syndromes (1). Reliable estimates of their epidemiology are patchy; the global burden of disease study draws together information on meningitis and encephalitis (2). These data suggest that the global incidences of meningitis and encephalitis in 2019 were 3.05 (95% confidence interval (CI) 2.64-3.59) and 1.16 (95% CI 0.99-1.64) new cases per 100,000 people respectively, and the rate of disability-adjusted life years (DALYS) was 211.09 (95% CI 178.03-253.44) and 62.00 (95% CI 52.47-82.95) DALYs per 100,000 people (3). There has been a decline in recent years, however these largely preventable conditions continue to account

for a disproportionate amount of death and disability (4). Furthermore, they commonly affect children; in 2019 meningitis was the sixth highest contributor to the burden of disease in children (4).

Identification of an infectious agent is pivotal in tackling the disease, at both patient and population level. However, almost half of the global population has little to no access to diagnostics (5). This is compounded by the fact that obtaining CSF is an invasive procedure that relies on technical expertise and appropriate sterile consumables for the performance of a lumbar puncture (LP). Even in the best-resourced settings, the proportion of patients with suspected neurological infections but without an aetiological diagnosis is reported as one to two thirds (6-10). I have seen this first hand; as a junior doctor I performed audits in three tertiary care hospitals in London (unpublished data and (11)). It has perturbed me that there remains a gap in suspected CNS infection and case ascertainment. This may be associated with worse patient outcomes, as well as considerable uncertainty for the clinical team, patient and relatives. Moreover, these patients are frequently very unwell, requiring specialist neurological critical care input (12). It is for these reasons that we need to keep 'pushing the envelope' as there are no magic bullets (13). A multifactorial approach is needed with improved clinical sampling, including the timing, volume and types of samples (CSF, blood, throat swabs, stool, brain biopsy), multidisciplinary discussion of patients and access to a wider range of diagnostic tests including untargeted approaches such as next-generation sequencing (14, 15). Equally important, the availability, cost and turnaround time of these tests should allow for the results to inform patient management. This is what I and others envisaged when we started a regular encephalitis multidisciplinary meeting at the National Hospital for Neurology and Neurosurgery in February 2018 (16, 17), to provide a platform for the discussion of complex cases. The meeting highlighted the increasing diagnosis of post-infectious and autoimmune disease. Nonetheless, the diagnostic gap remains, even with improved pathogen identification and recognition of non-infectious diagnoses. I propose in Chapter 2 of this thesis that host

biomarkers will be crucial in filling this gap, in patient samples for which the pathogen may have fallen below the limit of detection of available tests or have vanished (18).

In Low- and Middle-Income Countries (LMICs), access to diagnostic tests is even more restricted, and issues with the quality of available diagnostics exacerbates the problem (5). I worked in hospitals in India, Malawi and Laos, and have been struck by the variability in tests available for diagnosing neurological infections. Laos is a unique case; research into neurological infections has been a central theme of the Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (LOMWRU) since its inception in 1999. The Laos CNS infection study has recruited 3,381 inpatients with suspected CNS infections. It represented the first study of the epidemiology of CNS infections in Laos, and one of the largest and most comprehensive studies of the aetiology of CNS infections worldwide (19). In the framework of the Laos CNS infection study, a routine diagnostic service has been provided for Mahosot Hospital and other hospitals which request diagnostic assistance, and LOMWRU and Mahosot Hospital unit have also participated in the South-East-Asia encephalitis (SEAe) study from 2014-2017 (20). This work demonstrated that JEV infection represented the chief identified aetiology, followed by *Cryptococcus* species (spp.), *Orientia tsutsugamushi* (Ot), dengue virus (DENV), *Leptospira* spp., *Rickettsia* spp., *Streptococcus pneumoniae*, *Mycobacterium tuberculosis* (TB), Herpes simplex virus (HSV) 1/2, Human cytomegalovirus (HCMV), enterovirus spp. (EV), varicella zoster virus (VZV), mumps virus and *Plasmodium falciparum* (malaria) (21-32), see Figure 1.1. This is consistent with other studies in similar settings in Asia (33). However, there are large areas of Laos that have no access to any diagnostic tests at all, as is the case in many LMICs (5). It is for this reason that RDTs could be instrumental, as they have been for malaria, DENV and COVID-19, in enhancing diagnosis away from tertiary health facilities where most patients present. I propose in this thesis (Chapters 2-9) that detection of host protein biomarkers in CSF and serum by RDTs will contribute to a paradigm shift in improving patient outcomes in CNS infections.

Figure 1.1 Distribution of clinical presentations in patients with suspected CNS infection, by confirmed aetiology, Laos, January 2003–August 2011, Dubot- Pérès *et al.*, 2019 (19).

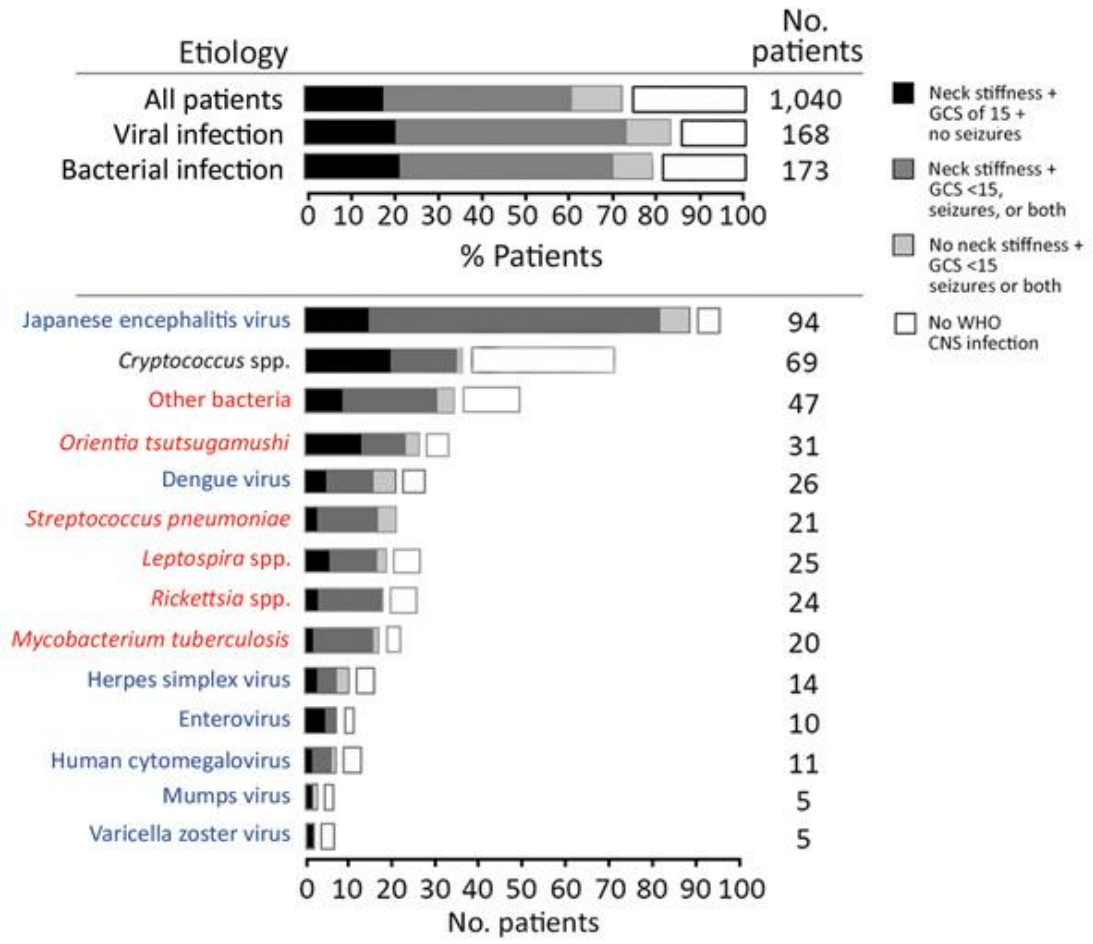


Illustration of key findings from the Laos CNS infection study investigation the aetiology of patients with suspected CNS infection admitted to Mahosot Hospital (Vientiane, Laos) from 2003-2011. 1,065 patients of all ages were recruited, and patient samples were investigated with an extensive panel of laboratory tests including molecular and serological assays. Aetiologies were confirmed in 42.3% of patients, who mostly had infections with emerging pathogens: viruses in 16.2% (mainly Japanese encephalitis virus [8.8%]); bacteria in 16.4% (including Orientia tsutsugamushi [2.9%], Leptospira spp. [2.3%], and Rickettsia spp. [2.3%]); and Cryptococcus spp. fungi in 6.6%, see bottom panel. There was no significant differences in distribution of clinical encephalitis and meningitis by bacterial or viral aetiology, see top panel. Analysis per pathogen includes only patients with single infections. Other bacteria include 7 *Escherichia coli*, 4 *Streptococcus agalactiae*, 4 *Neisseria meningitidis*, 1 *Salmonella enterica* group D, 1 *S. enterica* group B or C, 5 *S. enterica* serovar typhi, 4 *Streptococcus suis*, 3 *Klebsiella pneumoniae*, 7 *Haemophilus influenzae* type b, 5 *Burkholderia pseudomallei*, 6 *Staphylococcus aureus*, and 1 *Morganella morganii*. Blue font indicates viruses, red font indicates bacteria, and black font indicates fungi. CNS, central nervous system; GCS, Glasgow Coma Scale; WHO, World Health Organization.

Japanese encephalitis as a model for host protein biomarker discovery

JEV is a mosquito-borne flavivirus, and a leading cause of neurological infection in Asia. It is of considerable public health importance, with recent estimates based on sparse data suggesting 1.5 billion people are at risk, and 42,000 cases per year (34, 35). It is an emerging disease, with recent evidence of the introduction of JEV in multiple states in Australia leading to serious consequences (36). Patients frequently suffer devastating socioeconomic consequences; JE predominantly affects children in poor rural areas, has a 20-30% case fatality rate, and 30-50% of survivors suffer long-term disability (37). No specific treatment is available; however, several vaccines are available and recommended by the World Health Organization (WHO) (38, 39). Although recent efforts have strengthened JEV vaccination programs, still only 15 of 24 endemic countries include a JEV vaccine in routine immunisation policies, and even then, it is not uniformly available nationwide, with vaccine coverage in targeted areas reported to be as low as 39% (40). Furthermore, the occurrence of SF JEV vaccines represents a risk to vaccination programmes (41, 42), discussed in further detail in Chapter 8. JEV is a zoonosis, there will always be animal reservoirs of infection, and sustained vaccine coverage is essential to control disease.

A fundamental limitation in the control of JEV is the poor accuracy of existing diagnostic tests, requirement for LP and paucity of laboratory capacity for diagnosis (43). This is elaborated in Chapter 2 of this thesis. Surveillance data suggest that only 11 of 24 countries meet the minimum surveillance standards, equivalent to diagnostic testing in a sentinel site (40). This is a threat to vaccine implementation, as accessible and accurate diagnostics are essential to understand epidemiology, effectiveness of vaccination, identify associated research knowledge gaps and facilitate public engagement. This also has implications for appropriate risk-assessment for travellers and whether vaccination is recommended. Aside from JEV control, a diagnosis is crucial for patients, families and health-workers, to be able to institute appropriate supportive care, stop unnecessary antibiotics, or if the test is negative then to prompt further investigation.

The gold-standard JE test is a virus neutralisation test (VNT); however this requires paired acute and convalescent serum, is laborious, time-consuming, requires specialist skills, high-level isolation facilities for viral cell culture and may not define the infecting virus in secondary flavivirus infections (43). This is discussed in Chapter 3, in which I report the VNT experiments that I performed to confirm JE in patient samples, including the development of a novel method of immunoglobulin G (IgG) depletion prior to VNT to improve the interpretability of the test, effectively IgM VNT.

The WHO recommended diagnostic test is the anti-JEV IgM antibody-capture enzyme-linked immunosorbent assay (JE MAC-ELISA) in CSF. There are limited data from field studies comparing CSF JE MAC-ELISA with VNT assays. The manufacturer of the only available commercial kit for diagnosis of JE (InBios) quotes a sensitivity of >90% for well-characterised CSF samples, but sensitivity in the field is as low as 53% (44). There are also increasingly recognised problems with specificity related to prior vaccination and cross-reactivity with other flaviviruses (32, 45). Reported specificity is >90%, however a study by our group demonstrated that 13% of patients with anti-JEV IgM detected in CSF by JE MAC-ELISA had another pathogen detected that may have explained the presentation (32).

Detection of JEV ribonucleic acid (RNA) in CSF and serum would be highly specific, but the period of viraemia is brief and hard to capture clinically, often occurring before the onset of neurological symptoms and signs. Quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR) remains insensitive irrespective of the analytical sensitivity or gene targets (26). For this reason, the application of metagenomics is not likely to significantly improve JEV RNA detection (46, 47). JE provides an ideal model to test the hypothesis that there is a host protein signature in response to a specific infection, and this could be harnessed in an antibody-based point-of-care test. Further, JEV has a predilection for the thalamus and basal ganglia, and the CSF proteome could reflect these lesions (48).

The fundamental role of mass spectrometry in untargeted proteomics

The U.S. Food and Drug Administration (FDA)- National Institutes of Health (NIH) Biomarker Working Group refer to a biomarker as "*a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers. A biomarker is not an assessment of how an individual feels, functions, or survives.*" (49). Molecular biomarkers, in particular, have transformed clinical medicine (50). For instance, the level of lactate detected in a hospital patient's serum is used as a biomarker of severity of illness and informs admission to intensive care worldwide (51). In the widest sense, a biomarker may be a qualitative (present/absent) or quantitative indicator (increased/decreased), it may be derived the host or elsewhere (e.g. a pathogen) and it may be used in determining the diagnosis, prognosis or therapeutic response.

In the last two decades, there has been an exponential increase in publications on biomarker development, associated with the omics revolution in biomedical research (52). However there has also been a great deal of frustration in the low yield of translation of biomarker research and slow pace of implementation into clinical practice. This has been accompanied by a better understanding of the process of biomarker development; the appreciation that biomarker development is a lengthy process that requires sufficient infrastructure and investment, and attention to experimental design such as adequate sample size (53, 54), elaborated in Chapter 4. Specifically for the management of neurological disease, the analysis of CSF has been considered the richest source of potential biomarkers as it is the proximal body fluid to the brain and exists in direct contact with the brain parenchyma. For the purpose of this thesis, with the aim of identifying a host signature for diagnosis of JE in a resource-limited setting, protein biomarker/s would be most easily amenable to translation as a point-of-care test (55). I have focussed on CSF proteomics. A range of proteomics methods exist to detect host markers; however MS is the only method that enables untargeted discovery in clinical samples. Furthermore, as MS detects peptides, it may detect the presence of non-conformational or

degraded proteins, as well as different protein isoforms. I expand on the field of CSF biomarkers in Chapter 4 and Chapter 5, reviewing and critiquing existing literature, to explain the rationale for my experimental methods set out in Chapter 6 and Chapter 7.

Overview of thesis chapters

Chapter 1: In this chapter, I provide an overview of the subject of this thesis, research aims and structure.

Chapter 2: I present the methodology and results of a systematic review that I conducted of all human cases of JE diagnosed by laboratory methods to date, discuss existing diagnostic tests, propose a new criteria for confirmation of JE and highlight the gap that remains in JEV diagnostics.

Chapter 3: Here, I present the methodology and results of IgG ELISA and VNT assays for JEV, DENV, Zika virus (ZIKV) and West Nile virus (WNV) to confirm the Laos patient samples, including a novel method of IgG depletion to effectively perform IgM VNT and improve the interpretability of the diagnostic assay in reference centres. I perform a proof-of-principle study of IgM VNT in samples spotted on filter paper.

Chapter 4: I discuss existing literature on proteomics methods for analysing CSF and present the methodology and results of a systematic review of MS-based proteomics techniques to identify CSF biomarkers for diagnosing suspected CNS infections.

Chapter 5: I discuss existing literature on putative biomarkers of JE, elaborate on the hypothesis that there could be a protein signature of JE in CSF, including the development of an ELISA to detect non-structural protein (NS)-1' antibodies and an integrated approach to support the primary hypothesis.

Chapter 6: This chapter encases the core experimental work performed during the thesis. I present the methodology and results of liquid chromatography (LC)-MS experiments to identify protein biomarkers of JE in CSF. This includes preliminary unfractionated label-free experiments, pilot data dependent acquisition (DDA) experiments using tandem mass tag

(TMT) -11plex labelling and larger DDA experiments using multiplex TMT-16plex labelling. Data analysis is performed using conventional methods, such as differential expression of proteins as well as network analysis and machine learning. I subsequently verified the findings using data independent acquisition (DIA).

Chapter 7: I present the background and aims of work that could not be completed due to COVID-19 induced delays. This includes LC-MS studies to identify protein biomarkers of JE and Ot infection by deep probing in other body fluids - throat, serum and urine samples.

Chapter 8: I introduce the Vaccine Identity and Evaluation (VIE) transdisciplinary research collaboration that I have participated in, and present the methodology and results of experimental work developing methods for the identification of SF JEV vaccines.

Chapter 9: I report my conclusions, public health implications and suggestions for further work.

Concluding remarks

This thesis argues that existing methods of diagnostic techniques for neurological infections used in clinical practice are insufficient, and that innovation is feasible to improve this situation, improving clinical care and reducing the disease burden. I set out the role of diagnostics that incorporate the quantification of combinations of host proteins in parallel, both for RDTs in under-resourced settings and in higher-resourced settings. I test my hypothesis using a wealth of precious CSF samples from Laos, focussing on a leading neurological infection worldwide, JE, and applying cutting-edge LC-MS proteomics laboratory and data analysis methods that constitute the substance of my thesis research. In the penultimate chapter, Chapter 8, I describe research initiated during the COVID-19 pandemic; utilising skills that I learnt during my DPhil, to answer the related question of where there are biomarkers that could be utilised to identify SF JEV vaccines.

Chapter 2

Existing JEV diagnostics

In the introduction of this chapter I provide a general overview of JEV. However, the focus is on a systematic review that I performed at the start of my DPhil, to provide an overview of existing JE diagnostics and foundation for the thesis aims. For this, I extracted data on all laboratory-confirmed human cases of JE with available temporal and geographical details published to date. I explain the rationale for improved diagnostic tests being a crucial part of the public health response and propose a new criterion for confirmation of JE.

Introduction

A historical perspective

Phylogeographical analysis suggests that JEV originated from an ancestral virus in the Indonesia/Malaysia region as recently as two centuries ago (56). The first reports of minor epidemics of encephalitis were seen in the summer in Japan since the 1870s. However, increased attention was drawn to the problem in 1924 when over 6,000 cases were described (57). In lieu of the recent pandemic of encephalitis lethargica (58), the encephalitic illness occurring in Japan was referred to as Japanese 'B' encephalitis (59). The first isolation of JEV was in 1934, when Hayashi demonstrated that a filterable agent inoculated into monkeys produced encephalitis (60). Subsequently, the disease appeared in epidemics every 2-3 years in Japan, China and Korea, and was then described across Asia and the Pacific (56). The US military made a significant contribution to our current knowledge of JEV following their recognition of the virus as a potential threat to servicemen during the Second World War (61). JEV is now endemic in 24 countries in Asia-Pacific region, with over 1.5 billion people living in areas suitable for local transmission (34, 62). The introduction of widespread immunisation

strategies and, to a lesser extent, insecticides in the 1960s have had a considerable impact on control of the disease. However JEV remains a leading cause of neurological infection across Asia (63, 64), and an emerging disease globally (36, 65), with ongoing devastating socioeconomic consequences.

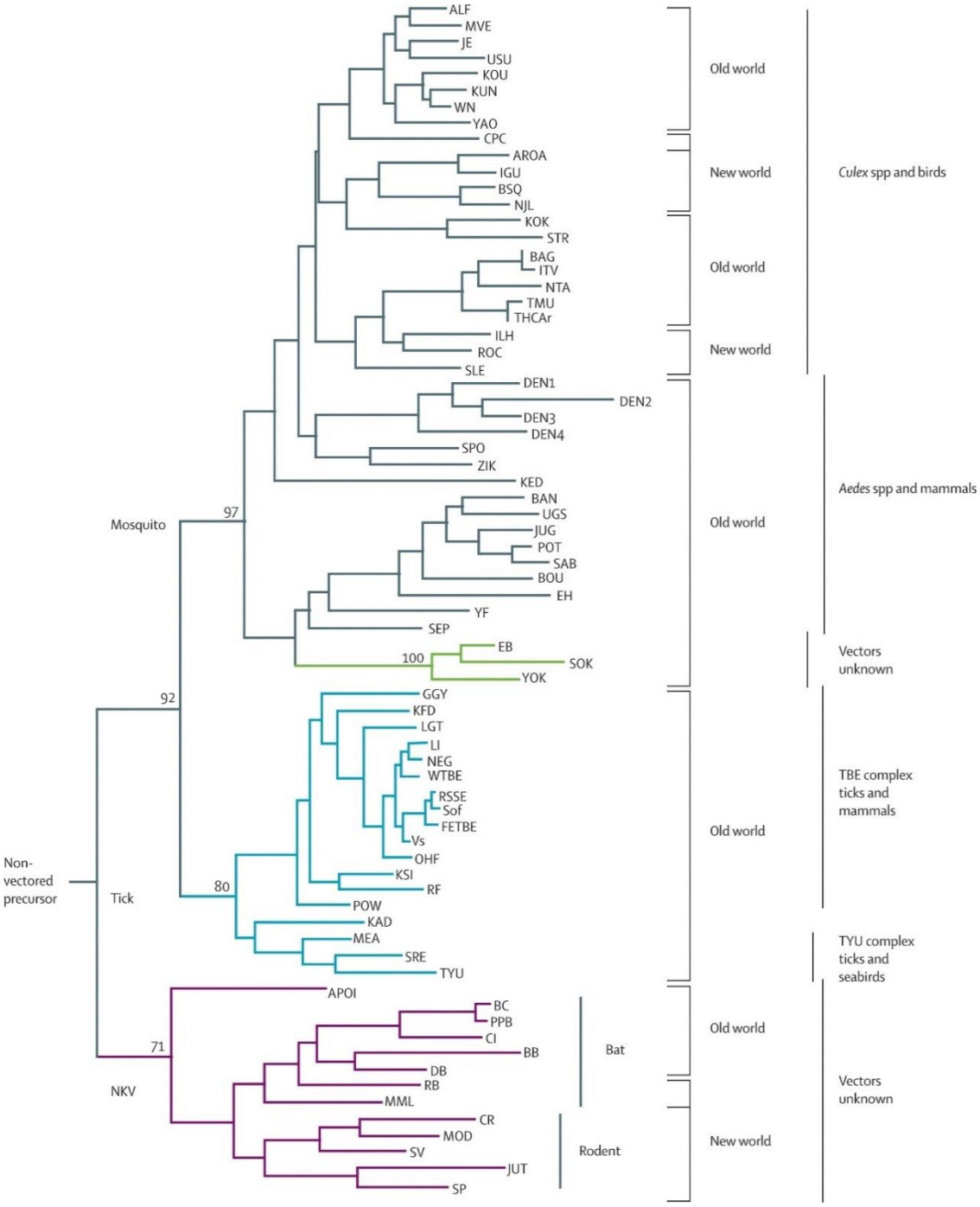
Figure 2.1 Geographic distribution of Japanese encephalitis virus (CDC)

Virology and life cycle

JEV is in the JEV serocomplex, a group of *Culex* (Cx.) spp. mosquito transmitted viruses within the *Flavivirus* genus and the large *Flaviviridae* family, see Figure 2.1 (66). The JEV serocomplex includes several neurotropic and geographically disparate viruses affecting humans. Notably JEV has close antigenic similarities with WNV. JEV is a single-stranded, positive-sense RNA virus, with five genotypes and one serotype identified to date (67). The virus particles are small (~50 nm diameter) and spherical, incorporating a lipid envelope and a single genomic RNA encoding three structural (membrane = M, capsid = C and the envelope = E) and seven non-structural proteins (non-structural = NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), see Figure 2.2 (67). The presence of a ribosomal frameshift in translation also leads to the intriguing presence of a 52 amino acid (aa) C-terminal extension of NS1, termed NS1'. The potential use of NS1 and NS1' as diagnostic biomarkers is discussed in Chapter 5.

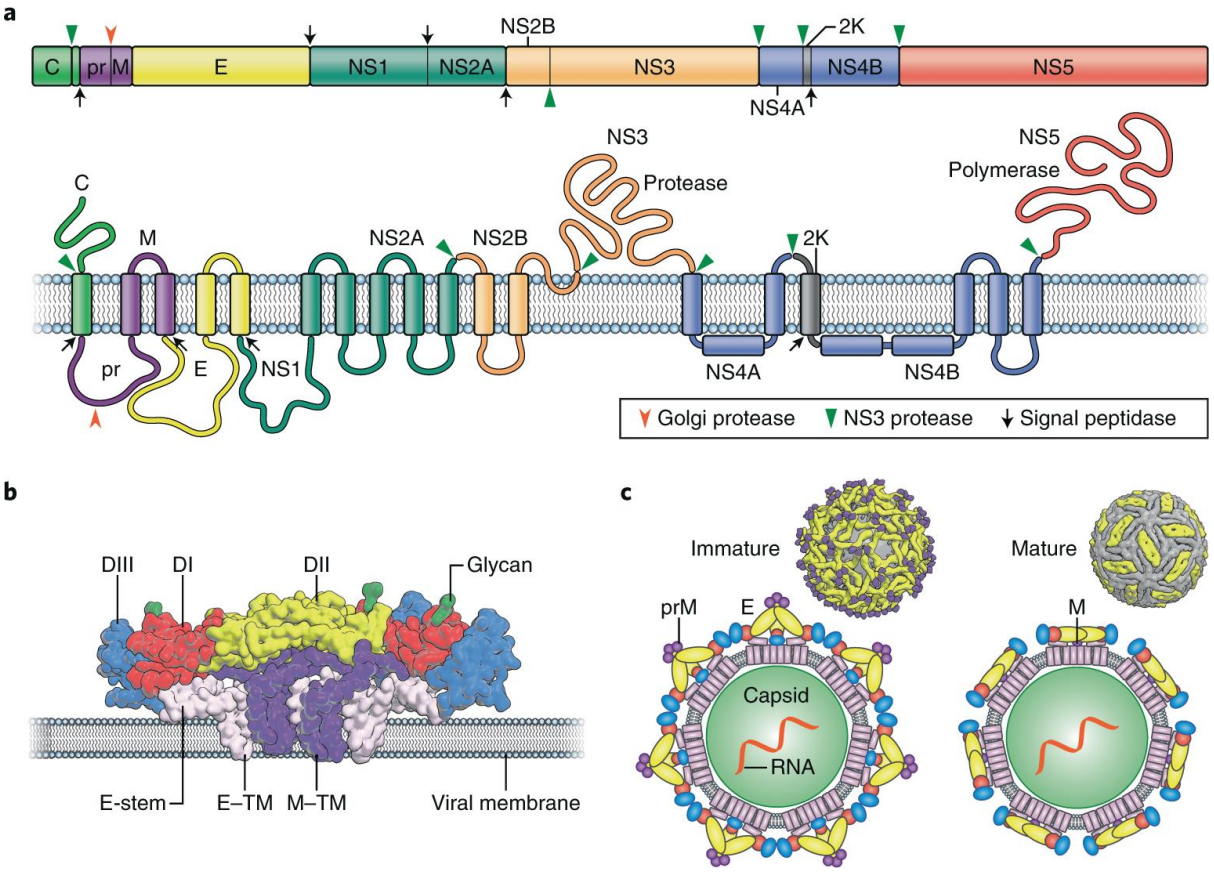
Rural areas with rice and pig farming are the main settings for transmission of JEV (66). Enzootic cycles occur between large waterbirds such as herons and egrets and with pigs. Water-logged fields are breeding grounds for the *Cx.* spp. mosquito, and pigs serve as the primary amplifying host (68). Humans are considered dead-end or accidental hosts and develop only low and brief viraemias. There has been an expansion of JEV into peri-urban and urban settings in recent years, associated with climate change, increasing intensive peri-urban livestock, international trade and travel (66).

Figure 2.2 Phylogenetic tree of the *Flaviviridae* family showing the association of the groups of related viruses with their invertebrate vectors, vertebrate hosts, and geographic distribution., Gould *et al.*, 2008 (69).



ALF=Alfuy. MVE=Murray Valley encephalitis. JE=Japanese encephalitis. USU=Usutu. KOU=Koutango. KUN=Kunjin. WN=West Nile. YAO=Yaounde. CPC=Cacipacore. ARO=Aroa. IGU=Iguape. NJL=Naranjal. KOK=Kokobera. STR=Stratford. BAG=Bagaza. IT=Israel Turkey meningoencephalomyelitis virus. TMU=Tembusu. THCAr=strain of Tembusu. ILH=Ilheus. ROC=Rocio. SLE=St Louis encephalitis. DEN=dengue. SPO=Spondweni. ZIK=Zika forest. KED=Kedougou. UGS=Uganda S. JUG=Jugra. POT=Potiskum. SAB=Saboya. BOU=Bouboui. EH=Edge Hill. YF=yellow fever. SEP=Sepik. EB=Entebbe bat. SOK=Sokoluk. YOK=Yokose. GGY=Gadgets Gully. KFD=Kyasanur Forest disease. LGT=Langat. LI=Louping ill. NEG=Negishi. Sof=Sofjin. FETBE=far eastern TBE. Vs=Vasilchenko. OHF=Omsk haemorrhage fever. KSI=Karshi. RF=Royal Farm. POW=Powassan. KAD=Kadam. MEA=Meaban. SRE=Saumarez Reef. TYU=Tyuleniy. APOI=Apoi. BC=Batu Cave. PPB=Phnom Penh bat. CI=Carey Island. BB=Bukalasa bat. DB=Dakar bat. RB=Rio Bravo. MML=Montana myotis leucoencephalitis. CR=Cowbone Ridge. MOD=Modoc. SV=Sal Vieja. JUT=Jutiapa. SP=San Perlita. TBE=tick-borne encephalitis. WTBE=Western European TBE. RSSE=Russian spring and summer encephalitis. NKV refers to viruses with no known vector.

Figure 2.3 Organisation and structure of the flaviviruses, Pierson *et al.*, 2020 (67).



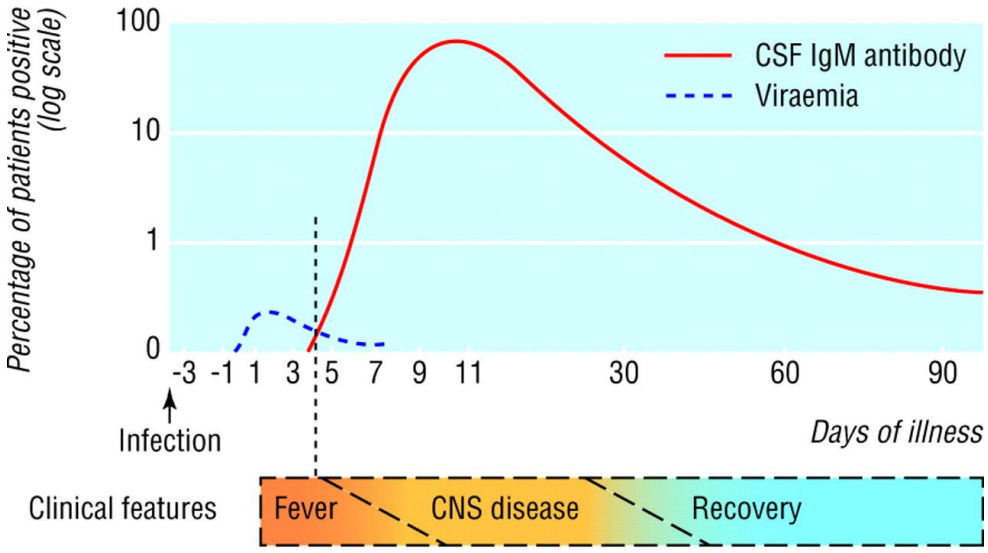
[a] Similar to other flaviviruses, JEV encodes a single open reading frame that is translated in the ER into a polyprotein. This is subsequently cleaved by viral and host cell proteases, resulting in ten functional proteins including the three structural proteins, C, prM and E, and seven non-structural proteins. [b] The E protein is a three-domain structure tethered to the viral membrane by a stem and two transmembrane domains, indicated in red, yellow and blue (DI–III, respectively). The M protein, also attached to the viral membrane by two transmembrane domains, is shown in purple. [c] There is a distinct arrangement of E proteins on immature (left) and mature (right) forms of the virion.

Pathophysiology, clinical presentation and management

Transplacental (70) and transfusion-related (71) JEV transmission have been described in case reports, however the primary route of infection involves the introduction of the virus into the skin following a mosquito bite. The incubation period is typically 4-14 days, see Figure 2.3. It is suggested that the virus replicates in the skin and spreads to local lymph nodes, after which the patient develops a viraemia and the virus may subsequently enter the CNS. The method of entry

to the CNS remains unconfirmed and may involve multiple potential routes (72). In humans, >99% of JEV infections are asymptomatic, however it may also produce a range of syndromes including a mild nonspecific febrile illness, aseptic meningitis, seizures, encephalitis, and poliomyelitis-like flaccid paralysis (73-75). Patients may present with a range of movement disorders, including Parkinsonism, owing to the predilection for the thalamus and basal ganglia (76), discussed further in Chapter 5. Histology demonstrates extensive neuronal degeneration, necrosis, microglial nodule formation, and perivascular and parenchymal leukocyte infiltrates as well as focal haemorrhage (74). Over half of patients die or experience long-term disability. Several randomised controlled trials have been conducted, however no treatment has been identified, and the mainstay of management is supportive care (73).

Figure 2.4 Natural course of JEV infection in humans, Solomon *et al.*, 2003 (77).



Schematic diagram to illustrate the clinical course of Japanese encephalitis: viraemia (blue dashed line), development of IgM (red line) and implications for diagnosis. The first day of fever is taken as first day of illness; most patients are not admitted to hospital until day 3-5 of illness.

Public health perspective and strategies for control

An integrated control programme is proposed by WHO (78, 79), with potential strategies including vector control, see Table 2.1. However, there is little evidence to demonstrate the effectiveness of any control measure other than vaccination. Sustained efforts from international agencies have supported the introduction of immunisation programmes into routine health control schedules in countries with endemic JEV transmission, Table 2.2 (40). Available vaccines and issues of SF vaccines are discussed further in Chapter 8. The evidence suggests that vaccination has had an impact on JE incidence (40, 80-85). However, JEV remains a leading cause of neurological infection in endemic countries, and the Joint WHO/United Nations Children's Fund (UNICEF) surveillance data do not substantiate the improvements cited in the past ten years, with sustained numbers of reported patients over this period, see Figure 2.4.

Table 2.1 Current and potential strategies for controlling Japanese encephalitis and the problems associated with each control method, adapted from Jeffries *et al.*, 2015 (86).

Control Strategy	**Difficulties for Implementation**
Human vaccination	Expensive, multiple doses required, low rates in rural areas
Pig vaccination	Expensive, maternal antibodies reduce efficacy
Insecticide space spraying	Resistance from exposure to pesticides in rice fields, logistically difficult on a large scale
Indoor residual insecticide spraying	*Cx. tritaeniorhynchus* is exophilic
Intermittent irrigation	Logistically difficult in large areas with inadequate infrastructure
Genetically modified mosquitoes	Genetic modification of *Cx. tritaeniorhynchus* required, cost-effectiveness and implementation over large areas
Wolbachia-infected mosquitoes	*Wolbachia* transinfection of *Cx. tritaeniorhynchus* required: long-term effectiveness unknown

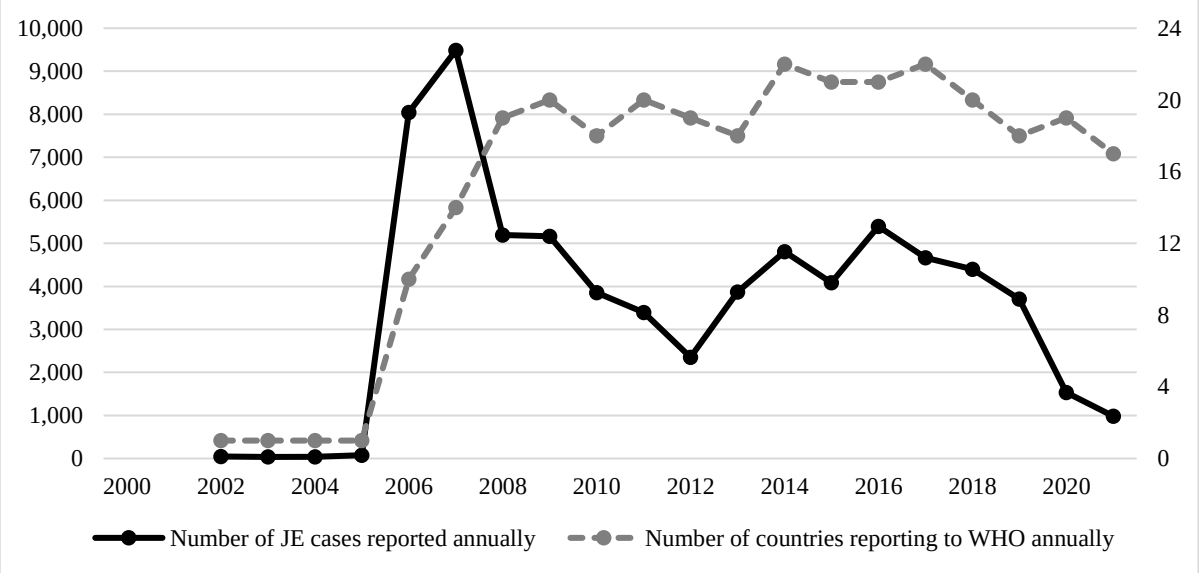
Table 2.2 Country-specific data on the introduction of Japanese encephalitis virus vaccine in JE endemic countries, adapted from the CDC report by Heffelfinger *et al.*, 2017 and updated with WHO surveillance data *(40, 87, 88)*.

Country	WHO region	Vaccine in schedule (2019)	JE immunization program	Year introduced subnationally	Year introduced nationally	Scheduled age (months) for vaccine	Vaccine used in immunisation program
Australia	WPRO	Yes	Risk areas: outer islands of Torres Straits. In addition, in 2022, this has been expanded to risk work including work in piggeries, mosquito surveillance and laboratories (36, 65).	n/a	n/a	12	Live-recombinant and inactivated Vero cell-derived
Bangladesh	SEARO	No	None (89)	n/a	n/a	—	—
Bhutan	SEARO	No	None (90)	n/a	n/a	—	—
Brunei Darussalam	WPRO	No	None (91)	n/a	n/a	—	—
Cambodia	WPRO	Yes	National (92-94)	2009	2015	9	Live-attenuated
People's Republic of China	WPRO	Yes	National; excluding Qinghai, Tibet, Xinjiang, and Hong Kong which do not have endemic transmission (95).	2003*	2008	8	Live-attenuated
DPR of Korea	SEARO	No	None; JEV vaccination campaign in 2016 (96)	n/a	n/a	—	—
India	SEARO	Yes	Subnational (97)	2007	n/a	9–11	Live-attenuated
Indonesia	SEARO	Yes	Subnational: Bali (98)	2018	n/a	—	—
Japan	WPRO	Yes	National (99)	<2002	<2007	6	Inactivated Vero cell-derived
Lao PDR	WPRO	Yes	National (100)	2013	2015	9–11	Live-attenuated
Malaysia	WPRO	Yes	Subnational: Sarawak and Sabah (101)	2002	n/a	9	Live-recombinant
Myanmar	SEARO	Yes	National (102)	n/a	2018	—	—
Nepal	SEARO	Yes	National (103)	2007	2017	12	Live-attenuated
Pakistan	EMRO	No	None (104)	n/a	n/a	—	—
Papua New Guinea	WPRO	No	None (105)	n/a	n/a	—	—

Philippines	WPRO	Yes	Subnational: Regions I-III, and the Cordillera Administrative Region (106)	2018	n/a	—	—
Republic of Korea	WPRO	Yes	National (63, 107)	n/a	<2002	12	Live-attenuated, Live-recombinant, Inactivated Vero cell- and mouse brain-derived
Russian Federation	EURO	No	None (108)	n/a	n/a	—	—
Singapore	WPRO	No	None (109)	n/a	n/a	—	—
Sri Lanka	SEARO	Yes	National (110)	2001	2011	12	Live-attenuated
Republic of China	WPRO	Yes	National (111)	1963	1968	15	Inactivated mouse brain-derived
Thailand	SEARO	Yes	National (112, 113)	n/a	<2002	12	Live-attenuated and Live-recombinant
Timor-Leste	SEARO	No	None (34)	n/a	n/a	—	—
Vietnam	WPRO	Yes	National (114)	<2002	2015	12	Inactivated mouse brain-derived

*According to official WHO data, although it is acknowledged that the People's Republic of China has performed widespread vaccination since 1971(95) . WPRO = Western Pacific Regional Office; SEARO = South-East Asia Regional Office.

Figure 2.5 Number of JE cases reported annually over the last two decades based on WHO/UNICEF surveillance (115). Data include probable* and laboratory confirmed cases reported by JE endemic countries.



The number of JE cases and the number of countries reporting cases annually from the start of reporting in 2002 to 2021 were downloaded from the WHO website https://immunizationdata.who.int/pages/incidence/JAPENC.html?CODE=Global&YEAR= These data represent only reported cases and are not considered to be a true representation of global JE incidence. Weaknesses of these data are discussed in the main text. Prior to 2006, cases were only reported by Thailand. *WHO definition of a probable case (116) = A case that meets the clinical case definition for acute encephalitis syndrome (AES) that occurs in close geographical and temporal relationship to a laboratory-confirmed case of JE, in the context of an outbreak.

JE cases reported to WHO/UNICEF have important limitations. For example, increased awareness of the disease and access to laboratory capacity may contribute to increased case reporting. Conversely, surveillance data are likely to represent only a small proportion of patients (117). This is particularly relevant for JE, occurring predominantly in rural areas lacking diagnostic capacity (118). There are no rapid or point-of-care tests for JE in clinical use (27), and the WHO recommended standard diagnostic assay is an ELISA test that requires trained professionals, appropriate resources and several hours for the results of the tests to be obtained (119). In a survey performed by WHO/UNICEF in 2017, 21 countries responded, of which 11 met the minimum surveillance standards (120, 121). Similarly, there are problems of specificity of the most widely used diagnostic test, JE MAC-ELISA (32, 45, 104, 122). This is a growing issue, with increasing endemicity of other flaviviruses and vaccination coverage.

The reasons for persistence of JE as a public health problem are complex and multifactorial. A key principle that must be kept in mind is that JEV infection is a zoonotic infection, human immunisation will never eradicate it in the natural environment, and therefore sustained vaccination coverage is necessary to protect public health. However, in countries that do have vaccination programmes, they are not necessarily uniformly implemented nationwide, and, in some areas, vaccine coverage is sub-optimal. Whilst there are many reasons for inadequate coverage, this remains a neglected aspect of JE vaccination programmes (123). Furthermore, immunisation strategies are constrained by the absence of adequate diagnostic capacity to investigate the burden of disease, the impact of vaccination (124) and the dynamic epidemiology of JEV infection. For example, in common with other emerging arboviruses (125) JEV has the propensity to emerge and become established in new geographical regions (126). In recent years there have been increasingly frequent reports of cases in peri-urban and urban areas (125, 127), as well as new regions such as Rajasthan in India and more recently (2022) in multiple territories in Australia (65, 128). There have been suggestions of the potential introduction of JEV in Africa (129), with a case report of autochthonous transmission in Angola (130) and seropositivity in Nigeria (131), however neither of these have been substantiated (132). JEV RNA has also recently been detected in birds in Italy (133). This most likely reflects the increased movement globally, via transportation, of animals and goods as well as climate change (134). Increased pig farming in urban areas of Asian countries also impacts on virus amplification. Finally, the live attenuated vaccine in widespread use is based on JEV genotype 3, despite the fact that in recent decades there has been widespread genotype displacement to genotype 1 (135), and evidence of detection of genotype 5 (136).

Accordingly, I aimed to perform a comprehensive review of the evolution of current diagnostic tests for JE. I tackled this by performing a systematic review of published laboratory-confirmed symptomatic cases of JE in humans and extracted data on the laboratory procedures employed. I also appraised novel tests either under development or conceptually applicable for future diagnostic purposes. Data analysis informed discussion on future perspectives for research.

Methods

Searches were performed in the Web of Science and PubMed using the text word term 'Japanese encephalitis' up to 10th May 2022. The abstracts were reviewed and full text was obtained for those potentially containing information on human cases of JE in the English language. The full text articles were then reviewed for those reporting symptomatic human cases of laboratory-confirmed JE. The search was limited to JE cases confirmed during the acute illness or hospitalisation rather than seroprevalence, with geographic information at least to country of onset of illness, and temporal information at least to year of diagnosis. Data were extracted on details of the diagnostic confirmation of the JE cases, with all the data available as Additional material 2.1. A JE case was classified according to the confirmatory level (1-4) developed from existing WHO and Centers for Disease Control and Prevention (CDC) criteria, where 1 provides the highest level of confidence based on the diagnostic test used, as illustrated in Table 2.3.

Table 2.3 Diagnostic criteria used to assess JE laboratory confirmed patients*

Level 1	JEV RNA detected in any specimen by RT-PCR.
	Virus isolation by inoculation of any specimen in cell culture or animal with characteristic CPE and confirmation by detection of JEV RNA or virus antigen.
	JEV virus antigen detected from brain tissue or CSF by immunofluorescence or immunohistochemistry
Level 2	Seroconversion or ≥ X4 rise in anti-JEV Ab by VNT or detection of NAb in CSF; Samples should be tested alongside other endemic flaviviruses (e.g. DENV)
Level 3	Anti-JEV IgM detected in CSF; Samples should be tested alongside other endemic flaviviruses (e.g. DENV)
	Seroconversion or X4 rise in anti-JEV Ab HI, CF, IFA; or seroconversion by ELISA; Samples should be tested alongside other endemic flaviviruses (e.g. DENV).
Level 4	Anti-JEV IgM detected in serum in one sample (acute/convalescent) or VNT tested in serum in one sample or single high titre HI/CF/IFA; Samples must be tested alongside other endemic flaviviruses (e.g. DENV)

RT-PCR = reverse transcription-polymerase chain reaction; RNA = ribonucleic acid; CPE = cytopathic effect; CSF = cerebrospinal fluid; Ab = antibody; DENV= dengue virus; ELISA = enzyme-linked immunosorbent assay; HI = haemagglutination inhibition; CF= complement fixation test; IFA = indirect immunofluorescence assay. *Confirmation of JE is categorised into levels 1-4 based on existing WHO and CDC criteria, such that level 1 provides the highest level of confidence.

Results

The review identified 232 studies in 23 countries in which a total of 24,425 JE patients were confirmed by laboratory tests, see Figure 2.2 for the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement flow diagram. Patients were predominantly diagnosed in Asia, with a suggested case of autochthonous transmission diagnosed in Angola. The studies incorporated a variety of methods for the diagnostic tests, including conventional and novel methods, as summarised in Table 2.4. The data do not provide evidence of change in the certainty of diagnosis through time, see Figure 2.7.

Figure 2.6 PRISMA flow diagram illustrating the systematic review process

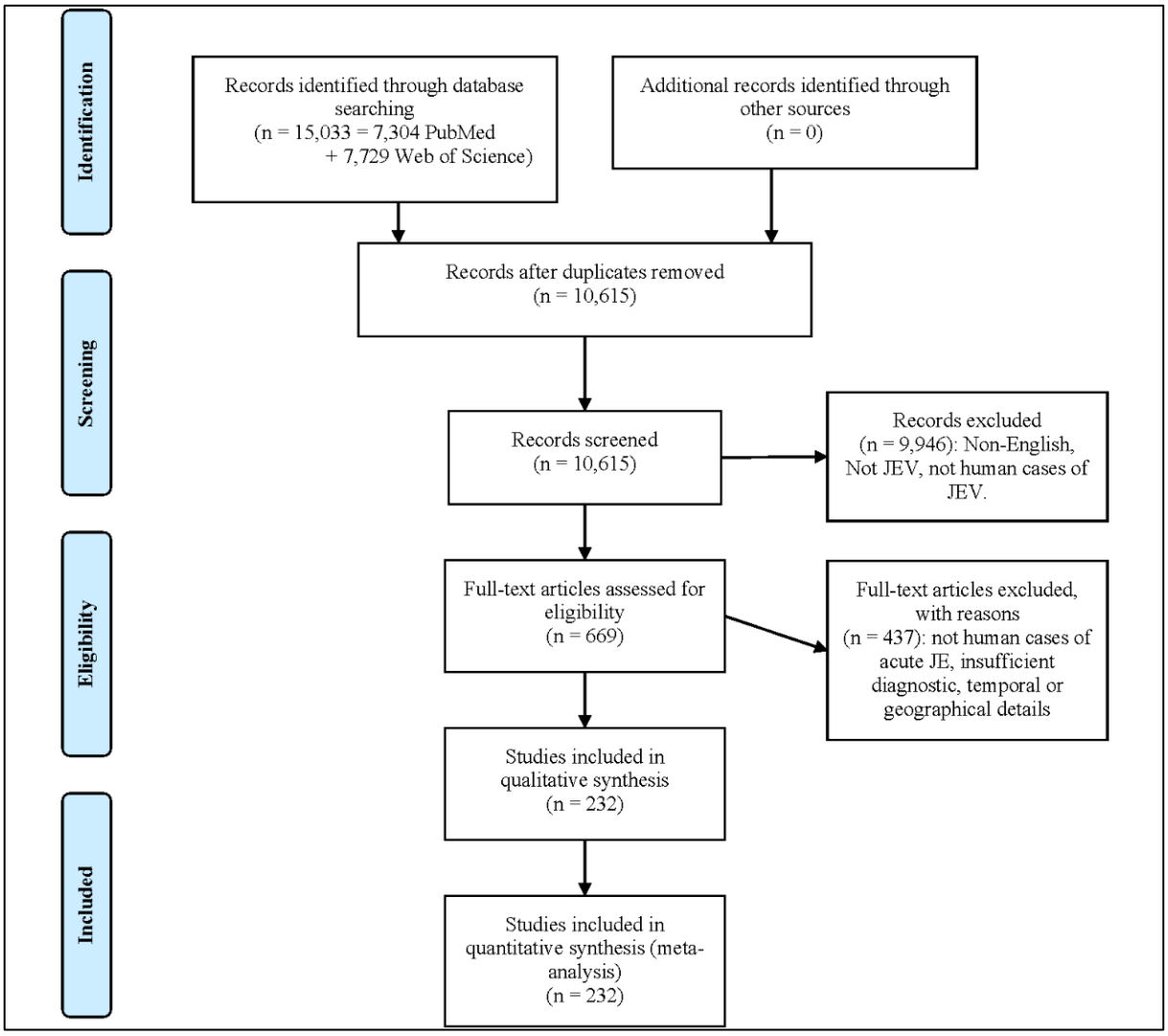
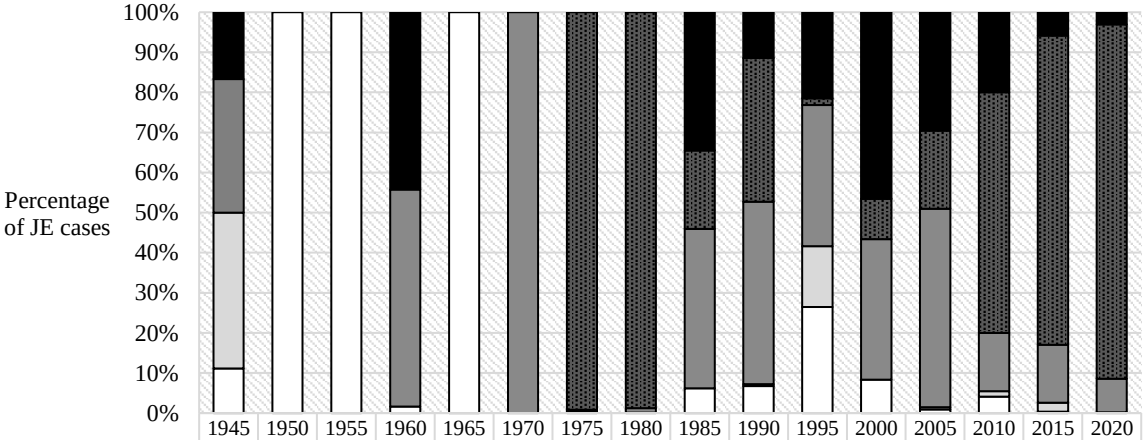


Table 2.4 Diagnostic methods used for evidence of JE

Diagnostic method	Confirmatory level	Advantage	Disadvantage
<u>Virus detection:</u> Virus Isolation: Inoculation of patient sample into animals Inoculation of patient samples onto primary chick or duck embryo cells, and cell lines including Vero, LLCMK, C6/36, MRC and AP61 Inoculation of patient samples into mosquitoes Virus antigen detection: Reverse passive haemagglutination Immunofluorescence microscopy Staphylococcal coagglutination tests using polyclonal or monoclonal antibodies Monoclonal antibody/immunogold/silver-staining (M-IGSS) ELISA to detect viral protein (NS1)	Level 1	Direct detection of the virus or viral protein and high specificity Viral isolation provides molecular epidemiological data	Low sensitivity, laborious and viral isolation requires biosafety 3 laboratory capacity
<u>Molecular detection:</u> Conventional RT-PCR Real-time RT-PCR Nested PCR Specific vs pan-flavivirus Multiplex PCR Next-generation sequencing	Level 1	Direct detection of viral genome, provides high specificity and additional molecular epidemiological data	Low sensitivity
<u>Antibody detection:</u> VNT IgM antibody capture ELISA Avidin biotin system Biotin labelled immunosorbent assay Nitrocellulose membrane-based immunoglobulin M capture dot enzyme immunoassay Haemagglutination inhibition +/- sucrose gradient density centrifugation and 2-mercaptoethanol treatment (2-ME) to detect IgM Complement fixation test Single radial haemolysis	Level 2 for seroconversion demonstrated by VNT Level 3 for detection of IgM in CSF and for seroconversion or ≥4x rise in Ab titre; Level 4 for detection of Ab detection in single sample	Good sensitivity Good specificity for primary infection Commercial kit available Good sensitivity	Cross-reaction with other flaviviruses Requires paired samples Laborious Difficult to interpret in secondary infection Limited specificity Cross reaction with other flaviviruses Requires paired samples Difficult to interpret in secondary infection

Figure 2.7 Temporal changes in JE diagnostic confirmatory level. Percentage of symptomatic human JE cases reported in the literature in English language, in blocks of five years, that were confirmed by laboratory testing Level 1-4*.



	1945-1949	1950-1954	1955-1959	1960-1964	1965-1969	1970-1974	1975-1979	1980-1984	1985-1989	1990-1994	1995-1999	2000-2004	2005-2009	2010-2014	2015-2019	2020-2022
Level 4	3	0	0	54	0	0	0	0	139	118	91	562	365	1772	301	142
Level 3 or 4	0	0	0	0	0	0	1047	824	79	372	7	122	240	5334	3986	4123
Level 3	6	0	0	66	0	70	5	9	160	471	149	423	611	1297	746	391
Level 2	7	0	0	0	0	0	4	0	0	5	64	0	7	120	114	4
Level 1	2	3	1	2	1	0	0	1	25	70	112	101	11	362	20	4

* A total of 24,425 laboratory-confirmed JE cases were identified. Data are reported according to year of publication. Inclusion criteria also required geographical (country) and temporal (year) data. Confirmatory levels of JE diagnosis detailed in Table 2.3; level 1 provides the highest level of confidence. Level 3 or 4 refers to cases that were reported as IgM detected in CSF and/or serum.

Overview of diagnostic testing

Early studies relied on clinicopathological correlates in infected humans, when compared with those observed following animal inoculation of post-mortem samples. Subsequently, hamster, porcine, and human cell culture systems were developed which revealed cytopathic effects when inoculated with JEV-infectious specimens (137, 138). As these procedures improved, mosquitoes and mosquito-cell cultures were added to the resources for isolation and identification of JEV (139, 140). CSF and other body fluids were also included for analysis (141, 142). Subsequently, JEV antigen detection procedures including complement fixation (CF), immunofluorescence (IFA) microscopy of cells in CSF, reverse passive haemagglutination and staphylococcal coagglutination (143-146) were added to the list of

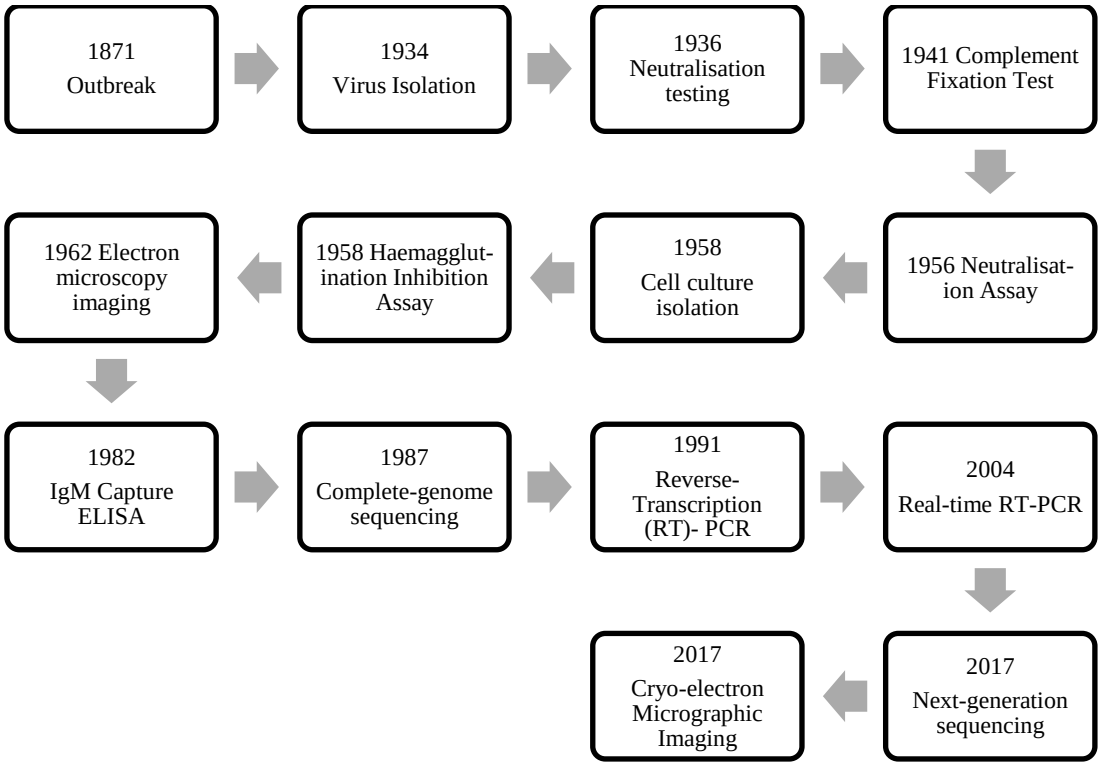
diagnostic tests. Nonetheless, assays involving direct virus detection are minimally useful for the diagnosis of JE as the level of viraemia is usually low and the virus is detectable only briefly early in the infection (147).

In the mid-twentieth century, investigation of the antigenic properties of JEV soon led to the development of serological assays including CF (148), inhibition of haemagglutination (HI) (149), and VNT (150, 151). Early reports of human infection in 1947 used a VNT technique in which a patient sample was mixed with virus and inoculated into mice (150, 152, 153). In 1941, Casals and Palacios published a report on the application of CF (154). The method was used for many years, although it was insensitive, particularly during the acute illness (155). Accordingly, in 1958, Clarke and Casals published a report on the application of the HI (149). The principle exploits the fact that JEV envelope protein agglutinates erythrocytes. Anti-JEV antibodies, developed following infection, bind to JEV protein and thus prevent erythrocyte agglutination, hence the term haemagglutination-inhibition. This remained the method of choice for JE diagnosis, by serological methods, for many years (156-159) and was subsequently adapted as a more convenient microtiter method (139, 151, 160). However, limitations in the sensitivity and specificity of the assay were recognised by Clarke and Casals (149). Moreover, the test relies on the combined paired results obtained from acute and convalescent serum samples, thus taking weeks for confirmation (161). Other serological methods such as single-radial haemolysis (162-164) were also introduced. However, inadequacies were readily acknowledged and it was accepted practice to perform these tests in parallel with others, thus increasing the workload and extending the time for results to be obtained (165, 166).

The plaque-reduction neutralisation test (PRNT) was subsequently developed as the gold-standard for JE diagnosis, using paired acute and convalescent sera and comparison with other endemic flaviviruses, and it remains so today (167). The demonstration of increasing anti-JEV neutralising antibody (NAb) titre in the convalescent serum and the absence or at least fourfold lower titre for NAb against control related flaviviruses, provides a robust diagnosis. However this is laborious, time-consuming and requires high level containment facilities for safe manipulation of infectious JEV in cell culture. Largely for these reasons, JE MAC-ELISA was

developed during the 1980s and has been incorporated as the WHO standard procedure for JE diagnosis (168, 169). Although commercial JE MAC ELISAs are manufactured, they may be hard to access in endemic countries (for example, there is no supplier in Laos), are relatively expensive and require costly ELISA readers and significant technical training. Nevertheless, performance of the kit still requires specialised laboratory equipment and they are by no means point-of-care tests. Moreover, field studies suggest that the sensitivity is 50-70% (44), and concerns have been raised regarding the diagnostic specificity (32). In the last two decades, there has been increased availability of molecular testing, providing crucial data on molecular epidemiology. Nonetheless, the aforementioned low and brief viraemia limits the role of testing for JEV RNA for diagnostic purposes. Similarly, advances in techniques such as near-atomic resolution cryo-electron microscopy contribute to our understanding of detailed viral structure, but not to routine detection of human infection.

Figure 2.8 Chronological representation of discoveries related to the detection of JE ***(137, 144, 148, 151, 153-156)***



Specific findings of the review

Studies reporting on the use of VNT, IgM ELISA and RT-PCR are discussed below, since these assays are, at present, the ones most widely incorporated into clinical diagnostics.

VNT: Thirty-five of the included articles identified evidence of JE using VNT, see Table 2.6, of which 16 clearly performed tests on acute and convalescent sera, indicating seroconversion (166, 170-185). These were largely PRNT (17 studies), and/or a microtitre modification in a 96-well plate (4 studies). Three studies performed focus-reduction seroneutralisation tests (FRNT), a high-throughput modification of the PRNT involving 96-well plates and an immuno-colorimetric assay for end-point determination. Other studies did not describe their methods in detail, or cite references to support them. Twelve reported the JEV strain used (150, 166, 174, 179, 186-193): they were all genotype 3 viruses, except for one that reported the use of genotype 1 and 3 strains to enable VNT-based genotype differentiation (186). Six studies indicated the JEV inoculating dose: the end-point was identified by visual inspection of cytopathic effect (CPE), staining, or IFA. All reports appeared to use two-fold serial dilutions of serum samples, between 1:2 to 1:640 or higher. In terms of quality control, three studies detailed other viruses used, and the use of replicates. Eight studies (150, 166, 174, 188, 191) included the use of other flaviviruses such as DENV, WNV or yellow fever virus (YFV). Studies followed different algorithms for including VNT in patient testing, but it was largely performed to confirm equivocal cases in other serological tests. Acute and/or follow-up serum and/or CSF were tested. For studies that did report individual results, confirmation was rarely achieved as there was either insufficient sera, lack of collection of convalescent sera, failure to detect a four-fold rise of antibody titre in the convalescent serum, or cross-reactivity was detected with related viruses included as controls in the tests.

Table 2.5 Details of virus neutralisation tests.

Reference	Country sampling	Country testing	Technique	JEV Strain	Other viruses tested	Cells	Virus Dose	End-point	Samples tested	Algorithm for VNT
Sabin 1947 (194)	Republic of Korea	Japan	Mice inoculation	G3 (Nakayama), human brain, Tokyo, Japan, 1935 (EF571853)	NR	Intracerebral and intraperitoneal inoculation in mice	NR	NR	Acute and f/up serum, and CSF	All samples
Sabin 1947 (191)	China	Japan	Mice inoculation	G3 (Nakayama), human brain, Tokyo, Japan, 1935 (EF571853)	NR	Intracerebral and intraperitoneal	NR	NR	Acute and f/up serum, and CSF	All samples
Sabin 1947 (150)	Japan	Japan	Mice inoculation	G3 (Nakayama), human brain, Tokyo, Japan, 1935 (EF571853)	NR	Intracerebral and intraperitoneal	NR	NR	Acute and f/up serum, and CSF	All samples
Edelman 1973 (195)	Vietnam	Vietnam	PRNT	NR	DENV	NR	NR	NR	NR	NR
Benenson 1975 (196)	Thailand	Thailand	PRNT	G3 strain (Nakayama), human brain, Tokyo, Japan, 1935 (EF571853)	DENV 4	LLC-MK2 cells	50-100 PFU	NR	Acute and f/up serum	NR
Hoke 1988 (197)	Thailand	Thailand	NR	NR	NR	NR	NR	NR	NR	NR
Cardosa 1991 (166)	Malaysia	Malaysia	PRNT	G3 strain (Nakayama), human brain, Tokyo, Japan, 1935 (EF571853)	DENV 2 (16681 strain)	*Aedes albopictus* C6/36 cells	NR	50%*	Acute and f/up serum	Confirmatory testing after positive MAC-ELISA
Peiris 1992 (170)	Sri Lanka	Sri Lanka	Microtitre VNT	NR	NR	Porcine stable (PS) kidney cells	NR	80%*	Serum (NR if acute and/or f/up)	NR
Wittesjo 1995 (198)	Indonesia	Sweden	PRNT	NR	NR	NR	NR	80%*	Acute and f/up serum	NR
Hennessy 1996 (175)	China	U.S.A.	PRNT	NR	NR	NR	NR	NR	CSF, Acute and f/up serum	All samples
Desai 1997 (189)	India	India	Microtitre VNT	G3 (P20778/P20) human brain, Vellore, India, 1958 (AF080251)	NR	Porcine stable (PS) kidney cells	100 TCID 50	100%*	CSF	All samples
Saito 1999 (182)	Japan	Japan	FRNT	NR	YFV	BHK-21 cells	NR	50%*	CSF, Acute and f/up serum	All samples
Tiroumouro-ugane 2003 (199)	India	India	NR	NR	NR	NR	NR	NR	NR	NR

Cutfield 2005 (200)	China	New Zealand	NR	NR	NR	NR	NR	NR	Acute and f/up serum	NR
Hashisaki 2005 (171)	Thailand	U.S.A.	NR	NR	NR	NR	NR	NR	Acute and f/up serum	NR
Ompusunggu 2008 (181)	Indonesia	Indonesia	PRNT	NR	NR	NR	NR	NR	Serum (NR if acute and/or convalescent)	NR
Lehtinen 2008 (201)	Thailand	Finland	PRNT	NR	DENV 2	NR	NR	NR	Acute and f/up serum	NR
Ravi 2009 (188)	India	India	PRNT	ChimeriVax™-JEV	ChimeriVax™-DENV 2	Vero cells	NR	NR	CSF	Confirmatory testing after positive or equivocal MAC-ELISA
Touch 2009 (185)	Cambodia	Cambodia	PRNT	NR	NR	Vero cells	NR	NR	NR	NR
Anga 2010 (173)	Papua New Guinea	Australia	PRNT	NR	NR	NR	NR	NR	NR	NR
Hossain 2010 (176)	Bangladesh	U.S.A.	PRNT	NR	NR	NR	NR	90%*	NR	NR
CDC 2010 (172)	U.S.A. (Travellers to Philippines and Thailand)	U.S.A.	NR	NR	NR	NR	NR	NR	CSF	NR
Borah 2011 (192)	India	India	Microtitre VNT	G3 strain (P20778/P20) Vellore, India in 1958 human brain (AF080251)	NR	BHK-21 cells	100 TCID50 in 50 µL	50%*	Acute and f/up serum	Patients with paired serum available after MAC-ELISA tested
Lee 2012	Republic of Korea	Republic of Korea	NR	Not reported	NR	NR	NR	NR	Acute and convalescent serum	Confirmatory testing after positive MAC-ELISA/HI/IIF.
Langevin 2012 (177)	Canada (Traveller to Thailand)	Canada	NR	NR	WNV and DENV	NR	NR	NR	CSF, Acute and convalescent serum	All samples
Hills 2014 (202)	China, Taiwan, Republic of Korea	U.S.A.	NR	NR	NR	NR	NR	NR	Acute and f/up serum	NR
Anukumar 2014 (174)	India	India	Microtitre VNT	G3 (P3) human brain, Bankura, India 1973 (AB379813/Z34095)	WNV	Porcine stable (PS) kidney cells	100 TCID50	50%*	Acute serum	All acute serum
Rayamajhi 2015 (203)	Nepal	U.S.	PRNT	NR	DENV, WNV and Powassan viruses.	NR	NR	NR	NR	Confirmatory testing after positive or equivocal MAC-ELISA

Saito 2015 (204)	Laos	Japan	FRNT	Nakayama (Tokyo, Japan, human brain, 1935, G3), Beijing-1 (Beijing, China, human brain, 1949, G3), P19-Br (Chiang Mai, Thailand, human brain, 1982, G1), LaVS56 (Vientiane, Lao PDR, swine sera, 1993, G1), and LaVS145 (Vientiane, Lao PDR, swine sera, 1993, G1)	DENV 1 (Hawaiian), 2 (New Guinea B), 3 (H-87), and 4 (H-241) and WNV	BHK-21 cells	NR	50%*	Acute and f/up serum	All samples
Li 2016 (179)	China	China	PRNT	G3 strain (733913) Beijing, China 1949, human serum (AY243805/AY243844)	NR	BHK-21 cells	100 PFUs	90%*	Acute and f/up serum	All serum
Sunwoo 2016 (184)	Republic of Korea	Republic of Korea	NR	NR	NR	NR	NR	NR	NR	NR
Kyaw 2019 (190)	Myanmar	Myanmar	FRNT and PRNT	G3 strain (JaOrS982) mosquitos, Japan, 1982 (NC_001437)	DENV 1-4	NR	NR	NR	CSF	NR
Fatima 2020 (104)	Pakistan	U.S.A.	PRNT	Not reported	DENV	Vero cells	Not reported	90% PRNT	CSF and acute serum	Confirmatory testing on patients with positive serology by MAC-ELISA
Liu 2020 (205)	China	China	PRNT	G3 strain (P3) isolated from human brain in Bankura, India 1973 (AB379813/Z34095)	WNV	BHK-21 cells	200 PFU/ 100 µL	90% PRNT	Acute and f/up serum	Confirmatory testing on patients with positive serology by MAC-ELISA
Paul 2020 (206)	Bangladesh	U.S.A.	PRNT	Not reported	DENV 1 and 2	Not reported	Not reported	80% PRNT	Serum (Not reported if acute and/or f/up)	Confirmatory testing on patients with positive serology by MAC-ELISA

G1 and 3 = genotype 1 and 3; NR = not reported; CSF = cerebrospinal fluid; PRNT = plaque reduction neutralisation test; DENV = Dengue virus; VNT = viral neutralisation test; FRNT = focus reduction neutralisation test; TCID = median tissue culture infectious dose. *titre required to reduce dengue viral plaques/focus/CPE by 50%, 80% or 90%. MAC-ELISA = IgM antibody capture enzyme-linked immunosorbent assay, HI = haemagglutination assay, IIF = Indirect immunofluorescence assay.

IgM ELISA: One hundred and ninety (82%) studies reported the results of tests using JE MAC-ELISA methods. Notably, 125 of these studies tested both CSF and serum samples, and presented results for the different body fluids separately. One hundred and thirty (68%) reported the method, of which 85 (65%) used in-house methods and 45 (35%) used commercial kits. The main in-house methods involved those described by Burke *et al.* 1985 (168), Innis *et al.* 1989 (207), the National Institute of Virology, Pune (208). Commercial kits were purchased from PanBio (185), Venture Technologies (209), XCyton Diagnostics Ltd. (210) and Shanghai B&C Biological Technology Co. Ltd. (211). There was minimal reporting of quality control measures such as control specimens and repeat testing of positives or reporting of following the manufacturer's instructions. In total, 16,134 JE patients (66%) were diagnosed by JE MAC-ELISA in serum and/or CSF, i.e. results for the different body fluids were not reported separately, with 4,404 (18%) positive in CSF alone. Ninety-nine (52%) studies testing JE MAC-ELISA also reported testing for DENV infection to confirm specificity for JEV, i.e. they were not cross-reactive with DENV.

Molecular tests: Forty-four studies (19%) reported the use of RT-PCR, of which 24 (55%) described the methods used or cited corresponding references. These targeted various regions of the JEV genome, including C, pre-membrane (prM), E, NS2A, NS3, NS5 and the untranslated regions (UTRs). The studies reported the use of conventional RT-PCR either standard, with nested or semi-nested techniques, and RT-qPCR using hydrolysis probes or SYBR green. Two studies reported the use of commercial kits, and 25 reported the use of in-house methods. Most of these studies involved the use of serum and/or CSF, although there were reports of testing other samples such as urine and throat swab samples. In total, 290 (9.8%) patients were positive when tested by RT-PCR.

Other tests: Sixty-three (27%) studies reported isolation of JEV *in vivo* or *in vitro*. Forty-two (18%) performed HI, 14 (6%) performed CF and 7 (3%) performed IFA. Three (1%) studies diagnosed cases by next-generation sequencing.

Discussion

This review reveals that current JE diagnostic techniques are largely confined to those with a low confidence level, i.e. anti-JEV IgM detected in serum samples, or in which reported results do not differentiate between detection of anti-JEV IgM in CSF and serum. There is no doubt that the introduction of IgM ELISA testing in serum samples represents real progress. However, we are now much better informed about the limitations of relying on this method of detection (32, 45, 104, 122). It is also acknowledged as a limitation that the studies included in the review were performed in different settings, with different constraints, financial limitations, and resources available. Nonetheless, the findings highlight the need for both the improvement in accuracy of routine laboratory diagnostics, and also the development of point-of-care tests to confirm cases in JE endemic areas frequently devoid of laboratory capacity. Below, existing assays are discussed in more detail.

VNT: VNT, the focus of Chapter 3, is considered the gold standard for diagnosis of infections due to pathogenic viruses such as JEV, but in the published literature cited herein it was only performed in approximately 15% (35/232) of studies as laboratory confirmation. This is probably due to the fact that performing VNT is technically demanding and requires sufficient volumes of serum/CSF to enable the inclusion of control viruses and duplicates of each titration. Since JEV is a human pathogen with high individual risk, VNT has to be performed in a biosafety level 3 laboratory, placing additional burdens on time, cost and qualified personnel. Another potential complication may arise when sera from patients who have previously been exposed to JEV-related flaviviruses may contain higher titres against the closely related flaviviruses than against JEV ("doctrine of original antigenic sin") (212). This is clearly an issue for DENV, endemic in all JEV endemic areas, but also increasingly for ZIKV (213) and WNV (214-216). For example, the titres of anti-YFV NAb were higher than anti-JEV NAb in JE patients who had previously received the yellow fever vaccine (182). Similarly, in a study testing WNV and JEV, 18 patients' data remained equivocal due to high levels of antigenic

cross-reactivity between these viruses (174). VNT may only be strictly applicable as the gold-standard for vaccine efficacy studies, in which a baseline serum sample is compared with a convalescent sample taken at a fixed interval 1-3 months later. For the purpose of confirming acute JEV, VNT is an imperfect gold standard. Severe constraints on being able to arrange for sample testing by VNT, and the results being interpretable without cross-reactive positivity due to other flaviviruses (which is relatively rare in JEV endemic areas), impede 'neutralisation confirmation'. The NAb titres obtained may be affected by the particular strain of challenge virus utilised (217). A final issue with the VNT is the inability to detect non-neutralising antibodies, thus potentially reducing the analytical sensitivity (218). Therefore, the practicalities of PRNT and diagnostic yield when testing field samples can be low, although the specificity is potentially high.

IgM ELISA: Anti-JEV IgM detection by JE MAC-ELISA is the WHO recommended standard diagnostic test, and 82% (190/232) of studies reported the use of a MAC-ELISA. It is recognised that JEV diagnosis by testing CSF provides considerably stronger confirmation than the use of serum (219). However, obtaining acute and convalescent CSF and serum samples can be difficult, particularly in rural Asia where access is logistically difficult and personnel and appropriate facilities are limited. Only 99 (52%) studies reported contemporaneous testing for anti-DENV-specific antibodies. There are issues in the accurate full reporting of results, both for the breakdown of which patients were diagnosed by testing CSF and/or serum, and contemporaneous testing for DENV-specific antibodies.

RT-PCR for detection of JEV RNA: Diagnosis by the detection of viral genome by deoxyribonucleic acid (DNA) amplification generated by RT-PCR is a valuable addition to diagnostic procedures for RNA viruses. The test has high analytical sensitivity, is very specific, and can provide additional information that can be exploited to understand the molecular epidemiology of the detected virus. Nonetheless, JE cases are rarely confirmed (9.8% in this review but likely overestimated due to reporting bias) using RT-PCR technology, although this will undoubtedly increase in usage as point-of-care and automated methods are developed. Poor

reporting of techniques used in many publications hinders the ability to make comparisons of the efficacy of the different methods (see Bharucha *et al.*, 2018) (26). However, there does appear to be higher analytical sensitivity in studies that used nested and hemi-nested techniques as compared with single RT-PCR, but these techniques are notoriously prone to contamination causing false positive results. It is also recognised that the sensitivity of nucleic acid detection (and protein) detection will continue to increase as technology improves (220, 221). Evidence to suggest that this will be the case arises from the high cycle threshold (Ct) of patients that are confirmed, and the fact that blood donor transmission has been seen in WNV patients who were negative by RT-qPCR tests (222). Recent detection of JEV RNA in throat swabs of JE patients suggests that this non-invasive sample may marginally improve diagnostic yield (223). There have now been three cases of JE, confirmed by RT-PCR, that were first identified by metagenomic next-generation sequencing (mNGS) (224); including in a CSF sample, the first detection of JEV RNA in human urine and JEV detection in serum from an African patient with a co-YFV infection. The latter was not confirmed by an orthogonal method and remains questionable. Nonetheless, unbiased mNGS technology (see below), application and reporting will continue to improve, and could potentially detect JEV in novel locations (14, 46).

Requirements of a new test for the detection of JEV infection: CNS infections are challenging syndromes to diagnose and treat, even in the most highly-resourced centres (225, 226). It is estimated that they may be caused by >100 different pathogens, including novel and emerging pathogens (7). Current approaches to diagnosis in routine clinical practice involves targeted approaches, suggesting that some (potentially treatable) infectious aetiologies are missed (14). Clinical diagnosis is rarely absolute and confirmation requires access to appropriate laboratory facilities and personnel, lacking in many areas worldwide (227). Whilst analysis of brain biopsy material is the gold standard, it is not possible in most cases. Aetiological diagnosis usually involves an invasive LP to obtain CSF, that requires the appropriate clinical skills, infrastructure and patient acceptability. Diagnostic assays are frequently difficult to interpret, may demonstrate poor accuracy, and poor discrimination between previous vaccination or non-

neurological JEV infection (219). Targeted research is needed to raise the bar for both the improvement in laboratory diagnostics as well as development of point-of-care tests (228).

In clinical and epidemiological situations, the detection of JEV RNA can provide an invaluable indication of infection. The sensitivity of this test is to a large extent limited by a combination of the short period of viraemia, the relatively low concentration of virus in CSF and the fragility of RNA (229-238). Introduction of highly sensitive point-of-care tests that may be used to analyse multiple body fluids in parallel would partly resolve these challenges. During recent years, there have been significant developments in highly sensitive molecular point-of-care tests for flaviviruses, such as RT-loop-mediated isothermal amplification (LAMP) (239, 240), RT-recombinase polymerase amplification (RT-RPA) (241), nucleic acid sequence-based amplification (NASBA) (242), transcription-mediated amplification (TMA) , helicase-dependent amplification (HDA), and nicking and extension enzyme amplification reaction (NEAR) (243-252). Microfluidics, chips, paper-based devices and biosensors are also being developed (253-257).

For the time being, we will need to rely on serology for diagnostic confirmation. During the past three years, with the international focus on emerging viruses following the Chikungunya virus, ZIKV and SARS-CoV-2 global epidemics, there have been intensified efforts to reduce cost, increase throughput and improve specificity. These include the analysis of immunoglobulin A (IgA) (258-267) and IgG subclasses (261), antibody avidity (258, 268-271), incorporation of blocking agents, IgG depletion (272) and production of specific monoclonal antibodies for identification of specific viral epitopes (273-277). This recent work highlights the inherent challenges of serological techniques for JE identification. As Lindsey *et al.* describe, antigenic cross-reactivity between related viruses can make it virtually impossible to distinguish the cause of the infection (278). For example, cross-reactive IgM class antibodies may not be stimulated during a related secondary flavivirus infection. On the other hand, IgA may be produced during a secondary flavivirus infection and a laboratory-defined ‘seroconversion’ might be detected following a secondary flavivirus infection by a related flavivirus.

Evidence suggests that the secreted viral JEV non-structural protein 1 (NS1) is present at very low concentrations in serum or CSF, unlike in DENV (279, 280). A novel alternative approach would be to analyse the host response, using transcriptomics or proteomics, the latter being the thrust of this thesis research. However, I proceeded with this work with the acknowledgements of concerns regarding the specificity of relying on the host response, and also how these would be translated into point-of-care tests.

In summary, whilst the diagnosis of JE has been possible for many years, it still requires specialised high containment laboratories and appropriately trained scientists and therefore cannot be reliably carried out in many resource-limited regions where JEV is endemic. A fundamental prerequisite in the public health strategy for the control of JE is lacking, that of a reliable and simple diagnostic procedure that can be adapted for point-of-care tests, and readily available for use throughout JEV endemic regions. Improved diagnostic capabilities (281) will not only benefit individual patients (through accurate diagnosis) but lead to higher quality surveillance data and better understanding of the distribution of JE risk, enabling improved targeting and evaluation of interventions. The lack of diagnostic capabilities for JE is a barrier to understanding the true disease burden and impact of public health strategies.

Publications arising from this chapter

The substance of this chapter has now been published (43). In the process of writing up the chapter, I updated the published review to include an additional 27 articles (10th May 2022). The insights gained from the data discussed in this chapter provided impetus for a published letter (132).

Chapter 3

Improving the JE reference test - JEV IgM-seroneutralisation

In this chapter, I present the methodology and results of anti-JEV IgG ELISA and VNT assays for JEV, DENV (1-4), ZIKV and WNV for the suspected JE patient samples from Laos prior to LC-MS. I describe a novel method of IgG depletion to effectively test for JEV IgM NAb and improve the interpretability of the diagnostic assay in reference centres. I also conducted a proof-of-principle study of VNT performed from samples spotted on filter paper, both dried blood spots (DBS) and dried CSF spots (DCS).

Introduction

Existing assays for diagnosing JE are detailed in Chapter 2. Gold-standard serological confirmation of JEV infection involves assessment of NAb titres using VNT. This is more specific (282, 283) than JE MAC-ELISA, and is recommended to be performed as part of diagnostic algorithms to confirm JE (96, 218). VNT confirmation is increasingly important, as part of implementation of vaccination programmes and with the expected reduction in JE incidence. For my thesis research it was crucial to test all the JE patient samples with the highest confidence; after identifying potential JE patients by JE MAC-ELISA, I confirmed them by VNT, to be able to improve the existing standard JE MAC-ELISA.

Conventional VNT methods involve a PRNT. However, laboratories are increasingly adopting high-throughput 96-well formats with comparable results (43). The high VNT requirements limit implementation: testing involves relatively large (>150 µL) sample volumes, need for paired samples, biosafety 3 category laboratories, reference virus and cell strains, and technical expertise. Indeed, interpreting VNT results are challenging due to cross-reactivity – attributable

to anamnestic responses related to immunological responses against a previously-encountered flavivirus (272). As there is major overlap in distribution of JEV and other flaviviruses, contemporaneous VNT for other endemic flaviviruses is required. In Asia, this involves testing for DENV serotypes 1 to 4, ZIKV, and in some areas WNV (284). All of these viruses can manifest with neurological complications (285).

Multiple methods have been attempted to mitigate cross-reactivity and anamnestic response interference in serological testing for non-JEV flaviviruses. Detailed in Chapter 2, these include analysis of IgA (258-267), IgG subclasses (261), antibody avidity (258, 268-271), incorporation of blocking agents (270, 286) and production of specific monoclonal antibodies for identification of specific viral epitopes (273-277). A modification of VNT, involving prior depletion of IgG, has been successfully performed for ZIKV (272) and DENV infections (287, 288). The underlying principle is that long-lasting IgG responses from vaccination and previous infection are major contributors to non-specific VNT results. IgG removal results in better detection of neutralising IgM antibodies which are markers of acute infection.

In settings with poor access to laboratories, the use of DBS on filter paper is now a well-established diagnostic tool for storing and transporting blood (289). A number of diagnostic assays have been performed downstream of blood eluted from DBS; most widely ELISA techniques, but also molecular assays and other serological tests. There are a few reports of VNT from DBS (290, 291). Dried spots have also been used, to a lesser extent, for analysing other body fluids including CSF (292). In the absence of rapid tests, collection of blood and CSF from suspected JE patients in remote settings could be used to confirm cases by VNT.

I performed a pilot study to evaluate the utility of IgG depletion prior to VNT 'IgM VNT' to detect anti-JEV IgM neutralising antibody for confirming acute JEV infection. I also applied the method on samples spotted onto filter paper and stored at room temperature for 3 months.

Methods

Patient samples

A prospective study of central nervous system (CNS) infection has been conducted at Mahosot Hospital, Vientiane, Laos, since 2003. Methods and results from 2003-2011 have been described (293). Patients from 2014-2017 were part of the Southeast Asia Encephalitis Project (294). The laboratory also receives samples from patients from other hospitals around Vientiane; Friendship, Children's and Setthathirat Hospitals. Written informed consent was obtained from patients or responsible guardians. Anti-JEV and anti-DENV IgM were detected by the Japanese Encephalitis/Dengue IgM combo ELISA (Panbio Inc., Brisbane, Australia, now Alere Inc.) until July 2014, for which result interpretation included a ratio between DENV and JEV results. After August 2014, as per WHO recommendations, the JEV Inbios IgM ELISA (Washington, U.S.A.) was utilised. All samples used were aliquoted and stored at -80˚C. This pilot study involved a convenience sample of consecutive patients with available samples to be tested; hence, a sample size calculation was not performed.

Suspected JE patients had anti-JEV IgM detected by JE MAC-ELISA in CSF or seroconversion between acute and follow-up serum, no other pathogen detected in other body fluid and sufficient volume of acute +/- follow-up serum for VNT. Patients with DENV and JEV RNA or DENV non-structural protein 1 (NS1) in serum or CSF were excluded.

Negative controls included samples from three groups. 1) healthy flavivirus naïve blood donors living in Puy-de-Dôme, in central France, left-over sera (no specific blood sampling) from blood donations. All procedures relating to the conduct, evaluation and documentation of the study were conceived in agreement with the good clinical practices and ethical principles of the Helsinki declaration. Written informed consent was obtained from all subjects included in the study. The protocol is in agreement with the national regulations on personal data (Commission Nationale Informatique et Liberté, France). The collection of biological samples was declared to

the French Ministry of Research. All data and samples were anonymised. 2) ZIKV VNT confirmed sera, collected in Peru in the framework of a seroprevalence study (Cachay *et al.* in prep (295)). All participants signed informed consent for blood sample collection and serological analysis. 3) DENV infection patients from the Laos CNS study (study details reported in the section on suspected JE patients above), confirmed by IgM and/or NS1 ELISA and negative for JE IgM.

Ethical approval

Ethical clearance for the Laos CNS study was granted by the Ethical Review Committee of the Former Faculty of Medical Sciences, National University of Laos (now University of Health Sciences) and the Oxford University Tropical Ethics Research Committee (OXTREC), Oxford, UK. For the SEAe study, ethical approval was granted by OXTREC and the Health National Ethics Committee for Health Research, National Institute of Public, Ministry of Health, Laos. For the blood donor samples, the protocol was presented to an Ethical Committee (Comité de Protection des Personnes Sud Méditerranée I) and because no additional blood sampling was required out of the blood donation, the committee agreed that ethical approval was not required. The protocol is in agreement with the national regulations on personal data (Commission Nationale Informatique et Liberté), the collection of biological samples was declared to the French Ministry of Research and all data and samples were anonymised. For the Zika sera, ethical agreement was granted from Institutional Ethics Committee of the Universidad Peruana Cayetano Heredia (SIDISI 103488).

Anti-JEV IgG ELISA

Anti-JEV IgG was detected using the Euroimmun ELISA kit (Lubeck, Germany) according to manufacturer's instructions. A standard curve using 3 calibration samples provided was used to calculate concentration of antibodies in relative units (RU)/mL for each sample using optical

density results; <16 RU/mL was negative, ≥16 to <22 RU/mL equivocal, and ≥22 RU/mL positive.

IgG depletion

IgG depletion was performed using Protein G HP SpinTrap/ Ab Spin Trap columns (Cytiva 28-4083-47). These contain recombinant Protein G, a protein present in group G *Streptococcus* with high-affinity for IgG. An in-house method developed by the French National Centre for Arboviruses was used, substituting commercial binding buffer by phosphate buffered saline (PBS). Two IgG depletion columns were used for between 100 to 150 µL sample serum. Columns were inverted 3 times and briefly vortexed. Each column was inserted in a 2 mL tube and centrifuged. All centrifugation was performed at 500g for 2 minutes. The subsequent eluate was discarded, 600 µL PBS added to each column and centrifuged again. Columns were transferred to clean 2 mL tubes, 100-150 µL sample added to one column and incubated at room temperature for 4 minutes before centrifugation. The eluate was transferred to the second column, incubated at room temperature for 4 minutes and centrifuged again. The final eluate was stored at -20˚C until VNT.

Virus neutralisation test (VNT)

Two-fold dilutions from 1/20 to 1/2,560 of each serum sample were tested in duplicate by VNT for JEV, DENV1 to 4, ZIKV and WNV. Serum dilutions from 1/10 to 1/1,280 were prepared and mixed 1:1 ratio with 100 median tissue culture infective dose $(TCID)_{50}$ viral suspension (Figure 3.1) using epMotion® 5075 (Eppendorf) in 96-well microplate (Figure 3.1). Negative controls containing minimum essential medium (MEM), with or without serum, were included in each microplate. Plates were incubated at 37°C for 2 hours. A 100 µL suspension of Vero cells (ATCC CCL-81) containing approximately $2x10^5$ cells/mL, was added to each well using the epMotion® 5070 (Eppendorf) and incubated at 37°C in 5% CO_2 incubator. After five to

seven days microplates were read under an inverted microscope. Two investigators read the results for each replicate to identify the end dilution at which there was no cytopathic effect, with a third investigator to resolve disagreement. For duplicates, geometric mean of end dilutions was calculated and reported as NAb titre and ≥40 considered as positive (296, 297). Suspected JE patients were categorised into acute JE positive, 'confirmed' or 'compatible', JE negative and 'unknown', according to criteria in Figure 3.2.

Figure 3.1 Schematic diagram of the plate preparation and steps involved in virus neutralisation test

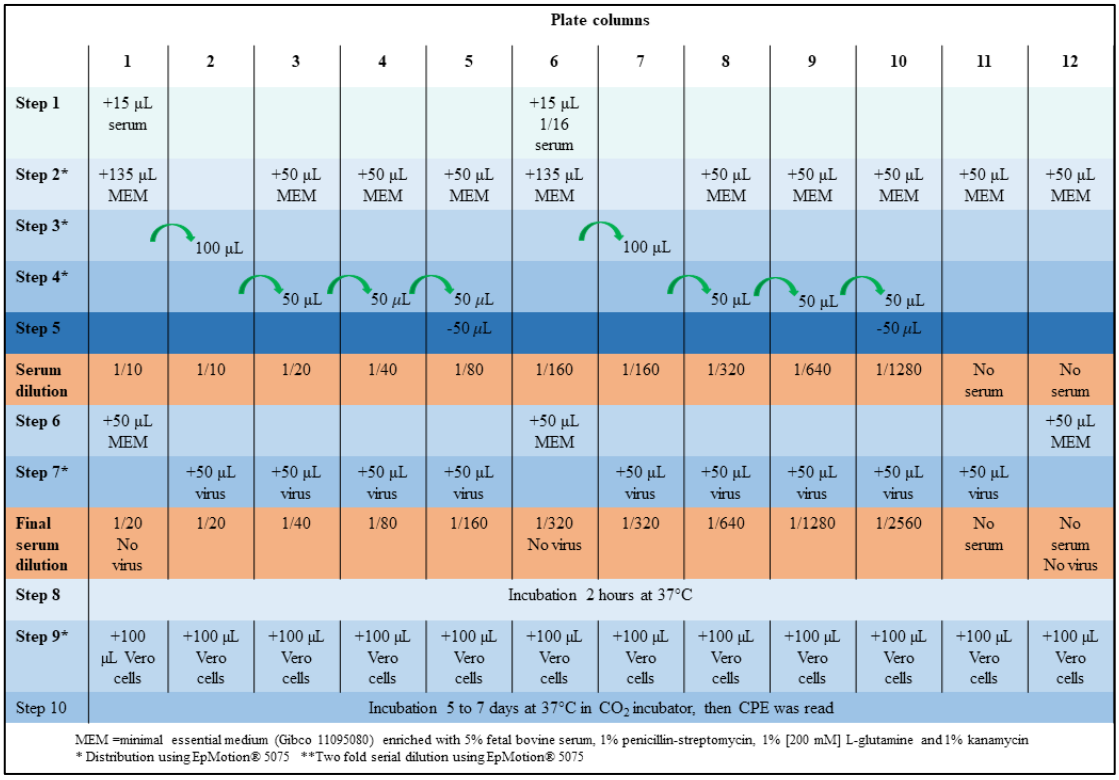


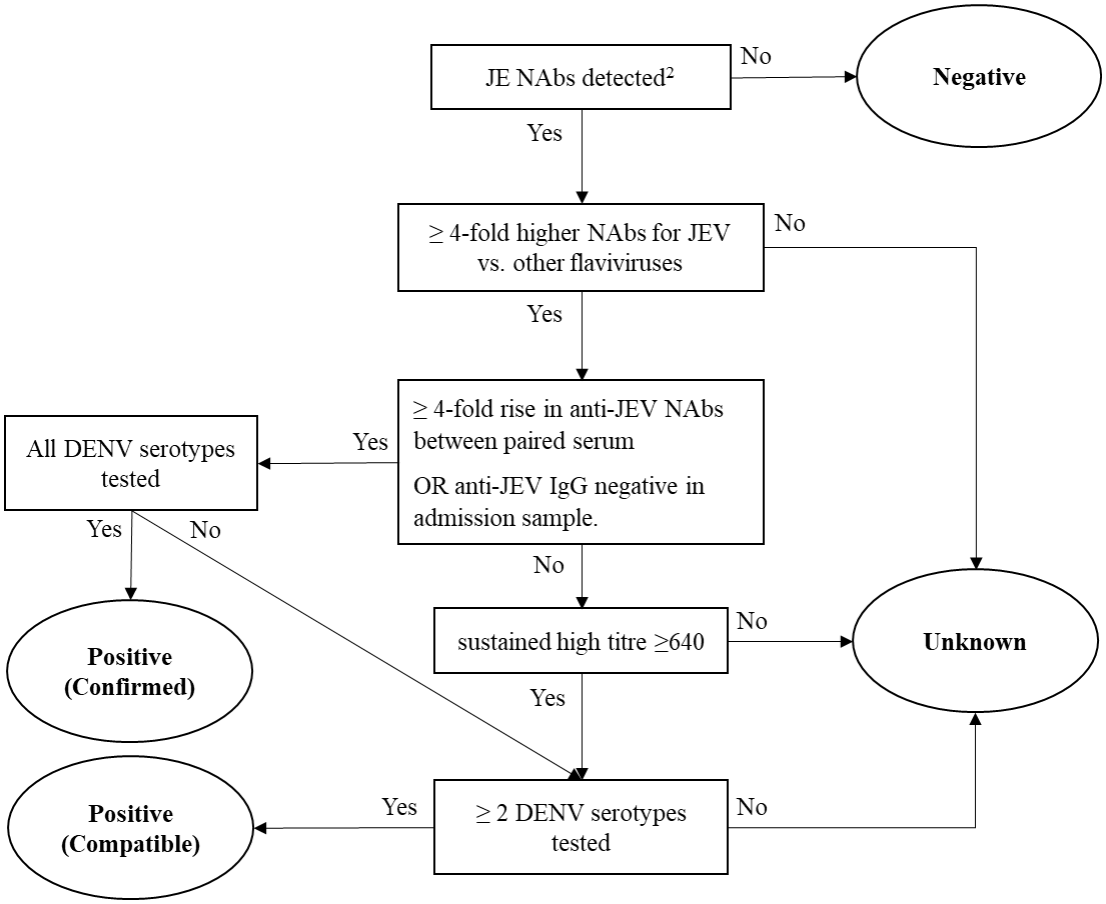
Table 3.1 Virus strain used in virus neutralisation test

Virus	Strain	GenBank Number	EVAg[1] number	Titre $TCID_{50}$/mL	Day Read
JEV	Laos 2009	KC196115	001V-02217	$2x10^9$	5
WNV	UVE/WNV/2008/US/R94224	-	001V-02224	$2.1x10^7$	5
ZIKV	ZIKV strain H/PF/2013 French Polynesia	KJ776791	-	$3.7x10^6$	5
DENV-1	DENV1 2012	VC16692	001V-02335	$3.1x10^7$	7
DENV-2	UVE/DENV-2/1998/MQ/703	AF208496	-	$6.7x10^4$	5
DENV-3	UVE/DENV-3/2001/MQ/2023	AH011666	-	$4.5x10^5$	6
DENV-4	UVE/DENV-4/1998/ID/814	-	-	$3x10^6$	6

[1]EVAg = European Virus Archive – GLOBAL. TCID50 = 50% tissue culture infective dose

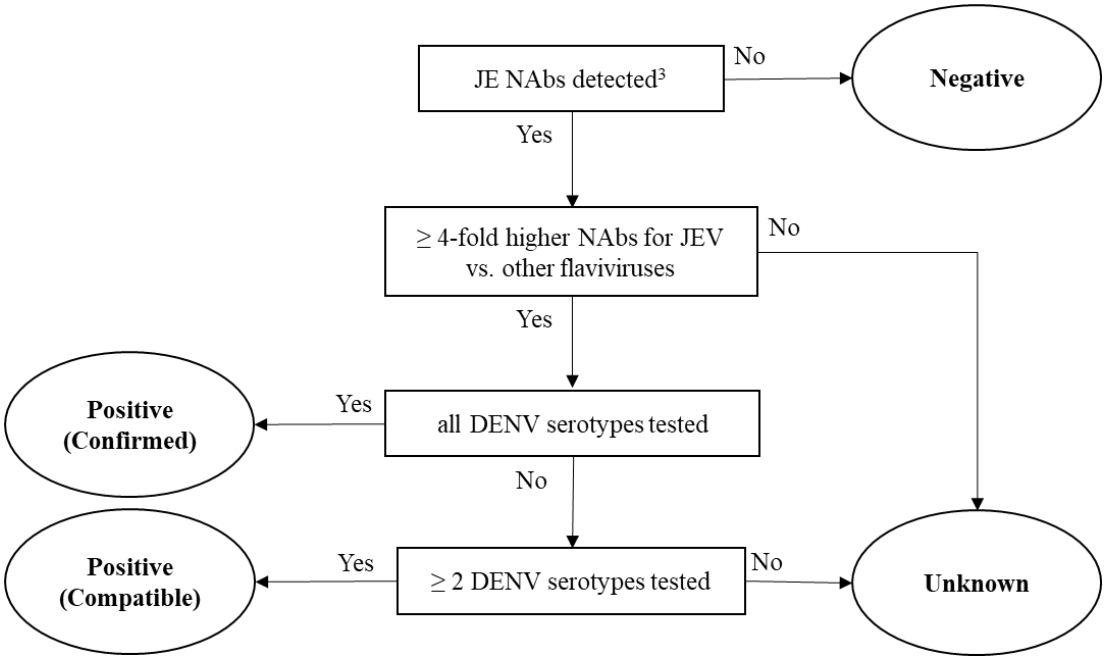
Figure 3.2 Criteria for interpretation of the results and patient categorisation for JE status

A. Standard virus neutralisation test results interpretation for paired sera:



1. All patients included in the study had anti-JEV IgM detected in CSF or IgM seroconversion from acute to follow-up serum samples, JEV RNA not detected, DENV RNA or NS1 not detected, and no other pathogen detected by extensive molecular and serological testing (293).
2. IgM VNT consists in the performance of VNT on serum samples after IgG depletion confirmed by a negative anti-JEV IgG ELISA result.
3. Nab titre ≥40 considered as positive. It would be advised that patients categorised as JE negative by this algorithm should ideally have a serum sample tested at 28 days post symptom onset.

B. Standard virus neutralisation test results interpretation for a single serum:



1. All patients included in the study had anti-JEV IgM detected in CSF or IgM seroconversion from acute to follow-up serum samples, JEV RNA not detected, DENV RNA or NS1 not detected, and no other pathogen detected by extensive molecular and serological testing (293).
2. IgM VNT consists in the performance of VNT on serum samples after IgG depletion confirmed by a negative anti-JEV IgG ELISA result.
3. Nab titre ≥40 considered as positive. It would be advised that patients categorised as JE negative by this algorithm should ideally have a serum sample tested at 28 days post symptom onset.

Filter paper

3MM Chr Blotting paper (Whatman, GE Healthcare Life Sciences, UK) is available as large sheets, is relatively inexpensive and suitable for wide application in resource limited settings. Pre-cut filter paper was prepared and processed as per a previous publication (24). In brief, 250 µL serum or CSF was spotted onto a 1.49 cm diameter pre-cut circle of filter paper and left to dry overnight. The filter paper was put into individual labelled 1.5 ml Eppendorf tube with approximately 10 silica beads as a desiccant.

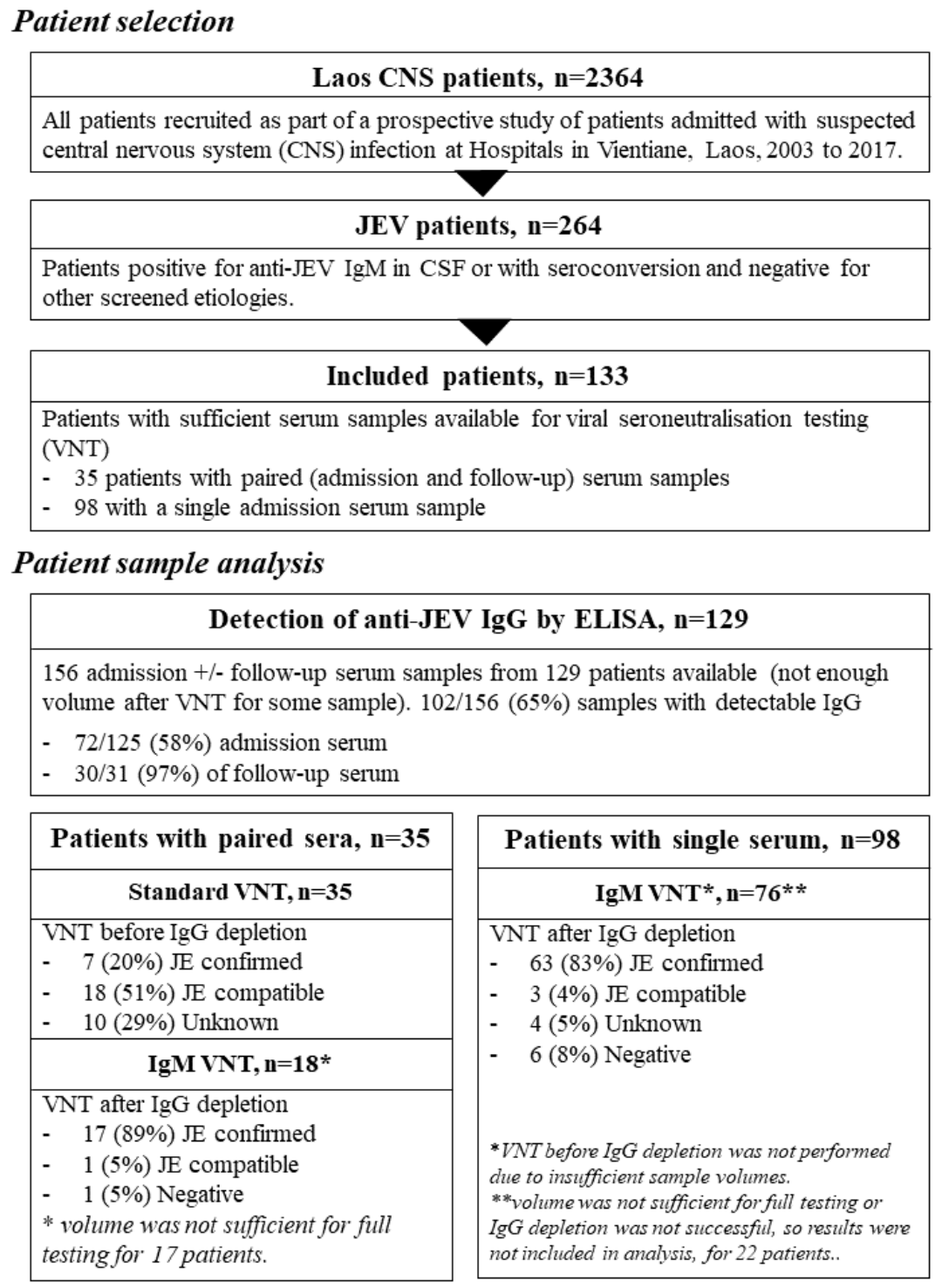
For elution after storage at room temperature for 3 months, the silica was removed, and 200 µL PBS/0.1%Tween-20 added. The tubes were vortexed every 20 mins for 1 hour, incubated at room temperature for 6 hours and centrifuged for 10 mins at 3,000 g. Finally, the liquid was removed and stored in a clean 1.5 µL Eppendorf tube for downstream anti-JEV IgG ELISA, IgG depletion and VNT.

The results of IgG ELISA and VNT were used to categorise the patients as per the criteria reported in 3.2. Agreement between neat sample as compared to filter paper was defined as the patient being categorised in the same way.

Results

From 2003 to March 2021, 264 patients with suspected CNS infection were positive for anti-JEV IgM (in CSF or with seroconversion) and negative for other screened aetiologies (293), see Figure 3.3. Paired serum samples (admission and follow-up) were available for 35 patients, and a single acute sample for 98 patients. Among these 133 included patients, 130 (98%) had anti-JEV IgM detected in CSF, and three (2%) demonstrated IgM seroconversion only (no anti-JEV IgM in CSF) in paired sera. Median age of the patients was 11 (6-20 interquartile range, IQR) years, and 32% (43/133) were female. Median duration of illness on admission was 5 (4-6 IQR) days and median time between admission and follow-up serum collection was 14 (10-25 IQR) days.

Figure 3.3 Summary of the suspected JE patient samples tested



IgG depletion

102/156 (65%) serum samples, including 72/125 (58%) of admission sera and 30/31 (97%) of follow-up sera, were anti-JEV IgG positive by ELISA before IgG depletion. Seventy samples had sufficient volumes to be tested for anti-JEV IgG by ELISA after IgG depletion. Fifty-nine (84%) were negative or equivocal after IgG depletion. Six samples were equivocal before IgG depletion, and all of these were negative after IgG depletion. Samples that remained positive after IgG depletion demonstrated a decline in titre, however the starting anti-JEV IgG result in these cases was high, all above 125 RU/mL (positive=>22 RU).

VNT for the patients with paired serum samples

VNT results prior to IgG depletion enabled classification of 25/35 (71%) as JE positive, 7 (20%) confirmed and 18 (51%) compatible; and 10 (29%) as unknown (Table 3.2 & Appendix 3.1). Eighteen of these patients had sufficient serum available for IgM VNT in at least one sample. The results enabled reclassification through the removal of cross-reactive IgG to other viruses and the specific detection of anti-JEV IgM, such that 17 (94%) were classified as JE positive, 16 (89%) confirmed and 1 (6%) compatible; and 1 (6%) as JE negative. Five patients classified as unknown by VNT did not have sufficient acute and/or follow-up sample to perform IgG depletion and/or anti-JEV IgG ELISA testing.

For the subset of 32 patients classified as JE positive, confirmed or compatible (before or after depletion), the median duration of onset of illness was 5 (4-7 IQR) days, and median duration between paired serum samples was 14 (11-24 IQR) days. 17/24 (71%) of these patients had detectable anti-JEV IgG, before IgG depletion, in the admission serum, and all (23/24, 96%) had detectable anti-JEV IgG in the follow-up sample.

Table 3.2 Virus neutralisation test antibody titre in acute and follow-up serum samples for patients with positive anti-JEV IgM antibody-capture ELISA

Patient number	Sample type	Days of Illness	Before IgG depletion = Standard VNT								After IgG depletion = IgM VNT								
			Class	JEV IgG	NAb titre						Class	JEV IgG	NAb titre						
					JEV	D1	D2	D3	D4	ZIK	WN			JEV	D1	D2	D3	D4	ZIK	WN
1597	Adm	5	Conf	-	1280	neg	neg	neg	neg			Conf	-$	160*	neg*	neg*	neg*	neg*		neg*
	FU	59		+	2560	14	14	neg	20					640*	neg*	neg*	neg*	neg*		
1704	Adm	5	Conf	+	640	20	neg	20	28			Conf	-	640*	neg*	neg*	neg*	neg*	neg*	neg*
	FU	13		+	2560	40	neg	28	80					2560*	20*	neg*	neg*	neg*	neg*	neg*
829	Adm	4	Conf	-	1280	neg	neg	neg	neg	neg	14	Conf	-	640*	neg*	neg*	neg*	neg*	neg*	neg*
	FU	21		+	2560	neg	neg	neg	14	neg*										
908	Adm	4	Conf	+	40	160	160	40	113	neg		Conf								
	FU	14		+	2560	20	neg	neg	14				-	2560	neg*	neg*	neg*	neg*	neg*	
928	Adm	5	Conf	-	1810	20	neg	neg	neg	neg	56	Conf	-	2560*	neg*	neg*	neg*	neg*	neg*	neg*
	FU	44		+	2560	neg	neg	neg	14				-	2560	neg*	neg*	neg*	neg*	neg*	neg*
2078	Adm	7	Conf	eq	160	20	neg	neg	neg			Conf	-							
	FU	17		+	2560	neg	neg	20	20				-	≥2560	neg	neg	neg	neg		
101	Adm	4	Conf	-	2560	neg	neg	neg	neg			Conf	-	453	neg	neg	neg	neg		
	FU	6		-	2560	neg	neg	40	neg				-	≥2560	neg	neg	neg	neg		
1610	Adm	6	Comp	+	2560	14	neg	neg	20			Conf	-	1280*	neg*	neg*	neg*	neg*	neg*	neg*
	FU	40		+	2560	160	20	neg	113					2560	neg	neg	neg	neg	neg	neg
483	Adm	7	Comp	+	2560	neg	neg	neg	neg			Conf	-	2560*	neg*	neg*	neg*	neg*	neg*	neg*
	FU	21		+	2560	20	20	neg	20	neg	neg			2560*	neg*	neg*	neg*	neg*	neg*	neg*
884	Adm	5	Comp	+	2560	40	neg	neg	40	neg	40	Conf	-	1280*	neg*	neg*	neg*	neg*	neg*	neg*

	FU	19		+	2560	neg	neg	neg	20				-	2560*	neg*	neg*	neg*	neg*	neg*	neg*
1074	Adm	6	Comp	eq	452		neg*	neg	neg			Conf								
	FU	84		+	1810	40	40	neg	80				-	1280*	neg*	neg*	neg*	neg*		neg*
1180	Adm	7	Comp	+	1280	452	640	452	160	neg		Conf	-	1280*	neg*	neg*	neg*	neg*		neg*
	FU	13		+	2560	226	226	160	160	20	80									
2053	Adm	5	Comp	+	2560	20	neg	neg	neg			Conf	-	2560	neg	neg	neg	neg		
	FU	18		+	1280	80	neg	40	neg				-							
775	Adm	3	Comp	-	320	neg	neg		neg			Comp	-$	113	neg	neg	neg			
	FU	12		+	640	neg	neg		40					640	neg	neg	neg			
5149	Adm	1	Ukn	+	2560	2560	2560	2560	2560			Conf		1280*	20*	20*	20*	neg*		
	FU	28		+	2560	2560	2560	2560	2560				-	2560*	40*	20*	40*	20*		neg*
1056	Adm	3	Ukn	+	320	1810	320	320	320	28	40	Conf	-	640*	20*	neg*	neg*	neg*		neg*
	FU	28		+	2560															
1917	Adm	7	Ukn	+	2560	2560	2560	neg	453			Conf	-	2560	neg	neg	neg	neg		
	FU	12		+	1280	2560	2560	neg	320				-	1280	neg	neg	neg	neg		
1036	Adm	4	Ukn	+	80	2560	226	320	226	neg	20	Neg	-	neg*	80*	neg*	20*	neg*		neg*
	FU	16		+	640	2560*	1280	2560												

Adm= serum on admission; FU=serum at follow-up; NAb titre=Neutralising antibody titre assessed by virus neutralization test (VNT), geometric mean calculated from duplicate results, **=indeterminate, NAb titre underlined to indicate the maximum dilution tested, neg=no NAb detected in duplicate samples (observation of cytopathic effect) for all serum dilutions tested (lowest one=20); NAb titre ≥40 considered as positive; JEV=Japanese encephalitis virus; D1-4=Dengue virus 1-4; ZIK=Zika virus; WN=West Nile virus; Class=classification for JE status according to criteria in Figure 3.2; Conf=Confirmed; Comp=Compatible; Ukn=Unknown; JEV IgG=anti-JEG IgG detection by ELISA (Euroimmun); +=Positive; eq=Equivocal; -=Negative. *Only one replicate tested or interpretable, the other samples were tested in duplicate. [+] This sample would be classified as confirmed JE based on a single sample, however as the FU sample is negative it is more logically classified as Ukn. -$ JEV IgG negative before depletion

Negative control sera

IgM VNT was performed on three other groups of negative control sera to assess the specificity of the novel method. JEV NAb was not detected by IgM VNT or VNT in the healthy flavivirus naïve blood donors (n=10) or ZIKV infection sera (n=4), see Table 3.3 and Appendix 3.2. In the DENV patient sera, 2/12 (17%) did not have detectable JEV NAb, and for both of these patients IgM VNT was performed and was also negative. In the 10/12 (83%) patients with DENV infection with JEV NAb detected by VNT, 8/10 (80%) did not have detectable JEV NAb after IgG depletion. For the two remaining, one did not have a result for IgM VNT and the other showed negative JEV VNT for admission serum and a low JEV NAb titre of 40 in follow-up serum. There were not sufficient samples volumes available to perform DENV VNT.

Table 3.3 JEV virus neutralisation test antibody titre for negative controls

			Before IgG depletion		After IgG depletion	
Patient number	**Category**	**Sample type**	**JEV IgG**	**JEV NAb titre**	**JEV IgG**	**JEV NAb titre**
Sera_M00212_A2	Healthy flavivirus naïve serum from France	Blood donor	-	neg	-	neg
Sera_M00212_A3	Healthy flavivirus naïve serum from France	Blood donor	-	neg	-	neg
Sera_M00212_A5	Healthy flavivirus naïve serum from France	Blood donor	-	neg	-	neg
Sera_M00212_A9	Healthy flavivirus naïve serum from France	Blood donor	-	neg	-	neg
Sera_M00212_A11	Healthy flavivirus naïve serum from France	Blood donor	-	neg	-	neg
Sera_M00212_B1	Healthy flavivirus naïve serum from France	Blood donor	-	neg	-	neg
Sera_M00212_B2	Healthy flavivirus naïve serum from France	Blood donor	-	neg	-	neg
Sera_M00212_B3	Healthy flavivirus naïve serum from France	Blood donor	eq	neg	-	neg
Sera_M00212_B4	Healthy flavivirus naïve serum from France	Blood donor	-	neg	-	neg
Sera_M00212_B9	Healthy flavivirus naïve serum from France	Blood donor	-	neg	-	neg
ZIKA_2	Zika convalescent serum from South America	FU	+	neg	-	neg
ZIKA_23	Zika convalescent serum from South America	FU	-	neg	-	neg
ZIKA_95	Zika convalescent serum from South America	FU	+	neg	-	neg
ZIKA_149	Zika convalescent serum from South America	FU	+	neg	-	neg
1443	Dengue acute serum from Laos CNS study	Adm FU	- -	20 28	- -	neg neg
869	Dengue acute serum from Laos CNS study	Adm	-	neg	-	neg

		FU	-	neg	-	neg
1103	Dengue acute serum from Laos CNS study	Adm	-	160	-	neg
		FU	+	905	-	neg
949	Dengue acute serum from Laos CNS study	Adm	+	160	-	neg
		FU	+	113	-	neg
951	Dengue acute serum from Laos CNS study	Adm	+	neg	-	neg
		FU	+	453	-	neg
1301	Dengue acute serum from Laos CNS study	Adm	+	57	-	neg
		FU	+	160	-	neg
L170	Dengue acute serum from Laos CNS study	Adm	+	160	-	neg
		FU	+	226	-	neg
953	Dengue acute serum from Laos CNS study	Adm	+	113	-	neg
		FU	+	80	-	neg
L37	Dengue acute serum from Laos CNS study	Adm	+	160	-	neg
		FU	+	320	-	neg
L90	Dengue acute serum from Laos CNS study	Adm	+	453	-	neg
		FU	+	905	-	neg
790	Dengue acute serum from Laos CNS study	Adm	+	453	-	28
		FU	+	1280	-	40
L221	Dengue acute serum from Laos CNS study	Adm	+	neg	-	**
		FU	+	226	-	**

Adm= serum on admission; FU=serum at follow-up; NAb titre=Neutralising antibody titre assessed by virus neutralization test (VNT), geometric mean calculated from duplicate results, NAb titre underlined to indicate the maximum dilution tested, neg=no NAb detected (observation of cytopathic effect) for all serum dilutions tested (lowest one=20), NAb titre ≥40 considered as positive; JEV=Japanese encephalitis virus; JEV IgG=anti-JEG IgG detection by ELISA (Euroimmun); +=Positive; eq=Equivocal; -=Negative; ** Neither replicate tested or interpretable.
Samples with no detection of anti-JEV antibody are highlighted in green, and the ones with anti-JEV antibodies detected (either by ELISA or VNT) are highlighted in blue.

Positive, negative and overall percentage agreement

The new test, IgM VNT, was compared to the reference standard VNT. This was based on results for patients classified as JE positive or negative by standard VNT and with sufficient sera to complete IgM VNT, that is VNT performed after IgG depletion and IgG ELISA to confirm IgG depletion. This included 14 JE positive and 16 JE negative patients. Positive, negative and overall percentage agreements (PPA, NPA and OPA) were all 100%, see Table 3.4.

Table 3.4 2x2 table of the results of IgM virus neutralisation test (VNT) as compared to standard VNT

Number of patient samples		Reference test – standard VNT		
		JEV positive*	JEV negative*	Total
IgM-VNT	JEV positive*	14	0	14
	JEV negative*	0	16	16
	Total	14	16	30

*The classification of patients followed the criteria set out in Figure 3.2.

VNT after IgG depletion for patients with single acute serum

76/98 (78%) patient samples had sufficient volumes for IgG depletion, confirmatory IgG ELISA testing and then VNT, 'IgM VNT'. Results allowed classification for 72/76 (95%) patients; 70 (92%) JE, 63 (83%) confirmed and 3 (4%) compatible, 6 (8%) negative and 4 were (5%) unknown (Table 3.5 and Appendix 3.3).

Table 3.5 Virus neutralisation test antibody titre for patients with only a single acute serum sample

Patient num-ber	Days of Illness		Before IgG depletion	After IgG depletion = IgM VNT						
		Class		JEV IgG	NAb titre						
			JEV IgG		JEV	D1	D2	D3	D4	ZIK	WN
34		Conf	-	-	160	neg	neg	neg	neg	neg	neg
37	3	Conf	-	-	640	neg	neg	neg	neg	neg	neg
38	2	Conf	-	-	57	neg	neg	neg	neg	neg	
40	14	Conf	-	-	80	neg	neg	neg	neg	neg	neg*
44	4	Conf	-	-	57	neg	neg	neg	neg	neg	neg
47	4	Conf	-	-	320	neg	neg	neg	neg	neg	neg
52	4	Conf	-	-	80	neg	neg	neg	neg	neg	neg
53	4	Conf	-	-	57	neg	neg	neg	neg	neg	neg
59	4	Conf	-	-	320	neg	neg	neg	neg	neg	neg
60	1	Conf	-	-	160	neg	neg	neg	neg	neg	neg
64	3	Conf	-	-	80	neg	neg	neg	neg	neg	neg
57	6	Conf	-	-	640	neg	neg	neg	neg	neg	neg
66	8	Conf	-	-	40	neg	neg	neg	neg	neg	neg
73	5	Conf	-	-	1810	neg	neg	neg	neg	neg	neg
76	5	Conf	-	-	320	neg	neg	neg	neg	neg	neg
87	4	Conf	-	-	57	neg	neg	neg	neg	neg	neg
88	6	Conf	-	-	160	neg	neg	neg	neg	neg	neg
92	3	Conf	-	-	905	neg	neg	neg	neg	neg	neg
98		Conf	-	-	320	neg	neg	14	neg	neg	neg
101		Conf	-	-	1280	neg	neg	neg	neg	neg	neg
102		Conf	-	-	640	neg	neg	28	neg	neg	neg
103		Conf	-	-	320	neg	neg	neg	neg	neg	neg
104		Conf	-	-	226	neg	neg	neg	neg	neg	neg
105		Conf	-	-	160	neg	neg	neg	14	neg	neg
111		Conf	-	-	452	neg	neg	neg	neg	neg	neg
112		Conf	-	-	160	neg	neg	neg	neg	neg	neg
127		Conf	-	-	640	neg	neg	neg	neg	neg	14
118		Conf	-	-	640	neg	neg	neg	14	neg	neg
89	3	Conf	-	-$	160	neg	neg	neg	neg	neg	neg
97		Conf	-	-$	640	neg	neg	neg	neg	neg	neg
94	3	Conf	-	-$	640	neg	neg	neg	neg	neg	neg
110		Conf	-	-$	160	neg	neg	neg	neg	neg	neg
121		Conf	-	-$	160	14	neg	neg	neg		

54	3	Conf		-	57	neg	neg	neg	neg	neg	neg
128		Conf	eq	-	640	neg	neg	neg	neg	neg	neg
51	5	Conf	eq	-	905	neg	neg	neg	neg	neg	neg
33		Conf	eq	-	452	neg	neg	neg	neg	neg	neg
58	5	Conf	eq	-	2560	neg	neg	neg	neg	neg	neg
62	6	Conf	eq	-	226	20	neg	neg	neg	neg	neg
35	4	Conf	+	-	160	neg	neg	neg	neg	neg	neg
36		Conf	+	-	40	neg	neg	neg	neg	neg	neg
41	4	Conf	+	-	320	neg	neg	neg	neg	neg	neg
65	13	Conf	+	-	80	14	neg	14	neg	neg	neg
67	4	Conf	+	-	1280	neg	neg	neg	neg	neg	28
68	6	Conf	+	-	2560	neg	neg	neg	neg	neg	neg
69	5	Conf	+	-	452	neg	neg	neg	neg	neg	neg
70	6	Conf	+	-	640	neg	neg	neg	neg	neg	neg
71	8	Conf	+	-	640	neg	neg	neg	neg	neg	14
74	4	Conf	+	-	226	neg	neg	neg	neg	neg	neg
75	14	Conf		-	905	neg	neg	neg	neg	neg	neg
79	5	Conf	+	-	640	neg	neg	neg	neg	neg	neg
80	7	Conf	+	-	226	neg	neg	neg	neg	neg	neg
81	6	Conf	+	-	640	neg	neg	neg	neg	neg	neg
82	6	Conf	+	-	905	neg	neg	neg	neg	neg	neg
86		Conf	+	-	2560	neg	neg	neg	neg	neg	20
91	7	Conf	+	-	1280	neg	neg	neg	neg	neg	neg
95	4	Conf	+	-	320	neg	neg	neg	neg	neg	14
99		Conf	+	-	640	40	neg	neg	20	neg	neg
108		Conf	+	-	320	neg	neg	neg	40	neg	neg
109		Conf	+	-	113	neg	neg	neg	neg	neg	neg
116		Conf	+	-	113	neg	neg	neg	neg	neg	neg
122		Conf	+	-	1280	neg	neg	neg	neg	neg	neg
125		Conf	+	-	160	neg	neg	14	neg	neg	neg
31		Comp	-	-	226		neg	neg	neg*		
32		Comp	-	-	226		neg	neg	neg*		
45	10	Comp	-	-	80		neg	neg	neg		
56	6	Comp	+	-	80	neg	40	20	neg	neg	neg
77	6	Comp	+	-	80	neg	28	neg	neg	neg	neg
85		Comp	+	-	320	98	160	57	20	neg	neg
78	4	Comp	+	-	160	neg	neg	neg	neg	80	neg
39	3	Neg	-	-	neg	neg	neg	neg	neg	neg	neg
83	5	Neg	-	-	neg	neg	neg	neg	neg	neg	neg

48	3	Neg	+	-	neg	20	neg	neg	neg	neg	neg
61	14	Neg	+	-	neg	14	14	neg	14	neg	neg
49	10	Neg	+	-	neg	14	neg	neg	neg		
120		Neg	+	-	20	40	neg	20	14	neg	neg

NAb titre=Neutralising antibody titre assessed by virus neutralization test (VNT), geometric mean calculated from duplicate results, NAb titre underlined to indicate the maximum dilution tested; neg=no NAb detected in duplicate samples (observation of cytopathic effect) for all serum dilutions tested (lowest one=20); NAb titre ≥40 considered as positive; JEV=Japanese encephalitis virus; D1-4=Dengue virus 1-4; ZIK=Zika virus; WN=West Nile virus; Class=classification for JE status according to the criteria set out in Figure 3.2; Conf=Confirmed; Comp=Compatible; Ukn=Unknown; JEV IgG=anti-JEG IgG detection by ELISA (Euroimmun); +=Positive; eq=Equivocal; -=Negative; *Only one replicate tested or interpretable, the other samples were tested in duplicate. Grey shading=test not performed. -$ JEV IgG negative before depletion.

Filter paper

Testing was also performed to evaluate the VNT from the eluate of samples spotted on filter paper. Eight follow-up serum and 8 CSF samples were spotted on pre-cut circles of filter paper, and the eluate was used for anti-JEV IgG ELISA, IgG depletion and VNT starting from 1/10 dilutions, see Table 3.6. There was 14/16 (88%) overall agreement in the results of JE IgG testing filter paper eluate vs neat samples, for follow-up serum or CSF. For the follow-up serum, there was sufficient volume (> 400 μL) to perform IgG immunodepletion and VNT testing of filter paper eluate vs neat serum, and there was 6/7 (86%) overall agreement, although the NAb titres were high and end-point (>1,280) was reached for 6/7 of the samples. For the CSF, there was not sufficient sample volume to perform VNT on neat CSF alongside DCS, however there was 6/8 (75%) overall agreement for DCS compared to results of neat serum VNT. For the three patient samples for which neat and dried spots did not agree, the VNT results were not interpretable.

Table 3.6 Virus neutralisation test antibody titres in neat and filter paper samples

Patient number	Sample type	Days of Illness		Before IgG depletion	After IgG depletion = IgM VNT							Agreement neat and filter paper
			Class	JEV IgG	JEV IgG	NAb titre						
						JEV	D1	D2	D3	D4	ZIK	WN	
1327	Adm-Neat	4	Conf	-	-	57	neg	neg	neg	neg	neg	neg	Yes
	CSF-Neat	4	nd	-	-$	nd	nd	nd	nd	nd	nd	nd	
	CSF-DCS	4	Conf	-	-	20	nd	neg	neg	neg	nd	nd	
1331	Adm-Neat	3	Conf	nd	-	57	neg	neg	neg	neg	neg	neg	No
	CSF-Neat	3	nd	-	-$	nd	nd	nd	nd	nd	nd	nd	
	CSF-DCS	3	Ukn	-	-	**	nd	neg	neg	neg	nd	nd	
1346	Adm-Neat	6	Conf	-	-	640	neg	neg	neg	neg	neg	neg	Yes
	CSF-Neat	6	nd	-	-$	nd	nd	nd	nd	nd	nd	nd	
	CSF-DCS	6	Conf	-	-	160	nd	neg	neg	neg	nd	nd	
1354	Adm-Neat	4	Conf	-	-	320	neg	neg	neg	neg	neg	neg	Yes
	CSF-Neat	4	nd	-	-$	nd	nd	nd	nd	nd	nd	nd	
	CSF-DCS	4	Conf	-	-	320	nd	neg	neg	neg	nd	nd	
1427	Adm-Neat	1	Conf	-	-	160	Neg	neg	neg	neg	neg	neg	Yes
	CSF-Neat	1	nd	-	-$	nd	Nd	nd	nd	nd	nd	nd	
	CSF-DCS	1	Conf	-	-	160	nd	neg	neg	neg	nd	nd	
1467/2	Adm-Neat	6	Conf	eq	-	226	20	neg	neg	neg	neg	neg	Yes
	CSF-Neat	6	nd	-	-$	nd	nd	nd	nd	nd	nd	nd	
	CSF-DCS	6	Conf	-	-	20	nd	10	neg	neg	nd	nd	
1488	Adm-Neat	3	Conf	-	-	80	neg	neg	neg	neg	neg	neg	Yes
	CSF-Neat	3	nd	-	-$	nd	nd	nd	nd	nd	nd	nd	
	CSF-DCS	3	Conf	-	-	320	nd	neg	neg	neg	nd	nd	
1490	Adm-Neat	13	Conf	+	-	80	14	neg	14	neg	neg	neg	No
	CSF-Neat	13	nd	-	-$	nd	nd	nd	nd	nd	nd	nd	
	CSF-DCS	13	Ukn	-	-	**	nd	neg	neg	neg	nd	nd	
1006	FU-Neat	Ukn	Neg	+	-	>1280	nd	neg	neg	neg	nd	nd	No
	FU-DBS		Ukn	+		**	nd	10	neg	neg	nd	nd	
152	FU-Neat	19	Conf	+	-	>1280	nd	nd	nd	nd	nd	nd	Yes

	FU-DBS		Conf	+		>1280	nd	neg	neg	neg	nd	nd	
184	FU-Neat	13	Conf	+	-	>1280	nd	160	neg	nd	nd	nd	Yes
	FU-DBS		Conf	+	-	>1280	nd	**	neg	neg	nd	nd	
297	FU-Neat	32	Conf	eq		>1280	nd	40	neg	neg	nd	nd	Yes
	FU-DBS		Conf	+	-	>1280	nd	10	neg	neg	nd	nd	
503	FU-Neat	110	Conf	+	-	>1280	nd	160	neg	nd	nd	nd	Yes
	FU-DBS		Conf	+		>1280	nd	neg	neg	neg	nd	nd	
509	FU-Neat	14	Conf	+	-	>1280	nd	640	20	40	nd	nd	Yes
	FU-DBS		Conf	+	-	>1280	nd	320	40	40	nd	nd	
651	FU-Neat	41	Conf	-	-$	>1280	nd	neg	neg	neg	nd	nd	Yes
	FU-DBS		Conf	+		>1280	nd	neg	neg	neg	nd	nd	
910	FU-Neat	13	Conf	+		640	nd	neg	neg	neg	nd	nd	No
	FU-DBS		Conf	+	-	>1280	nd	neg	neg	neg	nd	nd	

CSF= cerebrospinal fluid; Adm= serum on admission; FU=serum at follow-up; DCS=Dried CSF spot; DBS=Dried blood/serum spot; NAb titre=Neutralising antibody titre assessed by virus neutralization test (VNT), geometric mean calculated from duplicate results, **=indeterminate, NAb titre underlined to indicate the maximum dilution tested, neg=no NAb detected in duplicate samples (observation of cytopathic effect) for all sample dilutions tested (lowest one for serum=20 and CSF=10); NAb titre ≥40 considered as positive; JEV=Japanese encephalitis virus; D1-4=Dengue virus 1-4; ZIK=Zika virus; WN=West Nile virus; Class=classification for JE status according to criteria in Figure 3.2; Conf=Confirmed; Comp=Compatible; Ukn=Unknown; JEV IgG=anti-JEG IgG detection by ELISA (Euroimmun); +=Positive; eq=Equivocal; -=Negative. *Only one replicate tested or interpretable, the other samples were tested in duplicate. [+] This sample would be classified as confirmed JE based on a single sample, however as the FU sample is negative it is more logically classified as Ukn. -$ JEV IgG negative before depletion. There was a mishap with the Dengue 1 VNT plate, no results were obtained, and there were insufficient sample volumes to repeat this plate.

Discussion

This pilot study included a large set of well-characterised patients recruited prospectively in clinical studies, with extensive VNT for JEV, DENV 1-4, ZIKV and WNV. I show that the implementation of IgG depletion prior to VNT performed on par with standard VNT (100% PPA, NPA and OPA) and resulted in a significantly higher proportion, compared with standard VNT, of patients being classified. Of the patients with paired sera tested to confirm acute JE infection, 74% (26/35) were classified without an IgG depletion step, in contrast to 100% when IgG depletion was included. Furthermore, IgG depletion improved the diagnostic confidence of patients classed as JE positive, from 7/26 (27%) 'confirmed' and 19/26 (73%) 'compatible' with standard VNT to 16/17 (94%) 'confirmed' and 1/17 (6%) 'compatible' with IgM VNT. Depleting IgG also enabled a diagnosis of JE in 95% of patients for whom only a single sample was available, allowing for specific neutralisation of the IgM remaining in the sample.

The high proportion of patients presenting with detectable anti-JEV IgG before depletion and reduction in DENV neutralisation titres after depletion strengthen the underlying premise of this study, that IgG complicates discrimination by VNT, especially in areas with high endemicity of other flaviviruses and increasing utilisation of JEV vaccination.

A limitation is that there were not sufficient sample volumes available to perform standard and IgM VNT in all samples. However, the testing was retrospectively performed on a relatively large number of very precious samples. It would be realistic in clinical practice to secure the serum volume (400 µL) needed for prospective IgM VNT testing. This is one of the advantages of the new technique, that it relies on a single serum sample rather than paired sera or CSF. The efficiency of the IgG depletion was evaluated using anti-JEV IgG ELISA. I found that 84% of the anti-JEV IgG ELISA positive sera became negative after IgG depletion. All samples with an anti-JEV IgG ELISA result <125 RU/mL were negative after IgG depletion, suggesting IgG depletion was probably incomplete in samples with high titres. Further optimisation is required

to ensure that depletion is fully effective, perhaps with alternative methods depending on initial anti-JEV IgG result such as the use of three rather than two IgG depletion columns.

The principle of removing IgG, and the use of IgM as a biomarker for confirming acute infection is by no means novel. In 1973, Edelman and Pariyanonda reported a modified haemagglutination inhibition involving depletion of IgG, by sucrose density gradient centrifugation of whole serum and 2-mercaptoethanol treatment (195). The improved discrimination of evidence for acute JE in patient samples gave rise to further work developing the widely used JE MAC-ELISA (168, 298). However, with evidence suggesting sub-optimal performance of JE MAC-ELISA (32), the increasing use of the JEV vaccine, as well as hyperendemicity of DENV serotypes, the requirement for accurate diagnostic confirmation becomes even more pertinent. Although the performance of contemporaneous anti-DENV IgM ELISA and calculation of a JEV:DENV IgM ratio has improved specificity (299), the combination of VNT and IgG depletion (IgM VNT) permits IgM detection with higher specificity than by using JE MAC-ELISA alone.

Calvert *et al.* (272) showed that IgG depletion prior to neutralisation testing considerably improved the differentiation of acute ZIKV from DENV infections, 15% before to 77% after IgG depletion. This has also been demonstrated for differentiating DENV serotype infections (287, 288). It is notable that patients with JE typically present after the virus has entered the CNS, by the time of clinical presentation anti-JEV IgM and IgG is detectable in a larger proportion of patients than those with non-neurological flavivirus infection. Therefore, the use of the IgM VNT method for JE confirmation is a logical approach.

The humoral responses to JEV infection are directed mainly against antigenic epitopes on the viral envelope protein. There is major cross-reactivity with other endemic circulating flaviviruses and therefore it was crucial to test for all DENV serotypes (300, 301), ZIKV (302) and WNV (293) where they are sympatric. Likewise, IgG depletion and seroneutralisation might play a role in the diagnosis of DENV neurological infections for which there is considerable diagnostic uncertainty (76).

A diagnostic accuracy study should ideally have been performed with an *a priori* sample size calculation, prospectively testing consecutive patients with suspected neurological infection by the reference standard VNT to ascertain JE positive and negative patient samples. However, I was not able to conduct this in this pilot study, and flavivirus naïve patients from France were included as an additional category of negative controls. The fact that the suspected JE patients already had anti-JEV IgM detected in CSF or experienced JEV seroconversion reflects the role of VNT within reference centres. Further limitations include missing data due to limited sample volumes and that the dilutions were 1/20 to 1/2560 for the sera. Ideally, serum should be tested to the end-point of dilution, that is, diluting a positive sample until the first dilution that is negative. IgM VNT is a diagnostic test suited for reference centres and optimisation will be required to adapt the technique to be high-throughout, using protein G slurry and an automatised format for VNT testing 1/20 to 1/5120. Additionally, not all the virus strains used were sourced from the countries that the samples were derived from; the DENV strains isolated from Laos did not provide sufficient CPE for the assay, and neither ZIKV nor WNV have been isolated from patients in Laos.

The filter paper work provided a proof-of-principle that filter paper could be used for storing and transporting serum and CSF in remote areas for testing for acute JEV infection. Unfortunately, sample volumes available limited the extent of this work. There were three filter paper samples for which VNT results were uninterpretable, potentially due to antibody clumping or inactivation after three months storage at room temperature or due to the Tween-20 used as part of the elution process. It would be worthwhile testing storage on filter paper at shorter intervals, e.g. 1 week, 2 weeks and 1 month, and also incorporating alternative elution methods such as substituting PBS/Tween-20 with tissue culture media.

In conclusion, measurement of anti-JEV IgG and the performance of IgM VNT improved performance and allowed the use of single serum sample instead of paired sera for JE confirmation. This innovation holds promise for wider incorporation into testing algorithms in the reference confirmation of JE and DENV neurological infections. While filter paper spotting

and elution, and IgG depletion may be considered laborious, it could be implemented as part of an algorithm, discussed further in the final chapter of this thesis, Chapter 9.

Publications arising from this chapter

This substance of this chapter has now been published (303).

Chapter 4

Mass-spectrometry methods for identification of protein biomarkers in cerebrospinal fluid

In this chapter, I discuss existing literature on proteomics methods for analysing CSF and present the methodology and results of a systematic review of MS-based proteomic techniques to identify CSF biomarkers for diagnosing suspected CNS infections. This provides a background to the LC-MS methods applied in Chapter 6.

Introduction

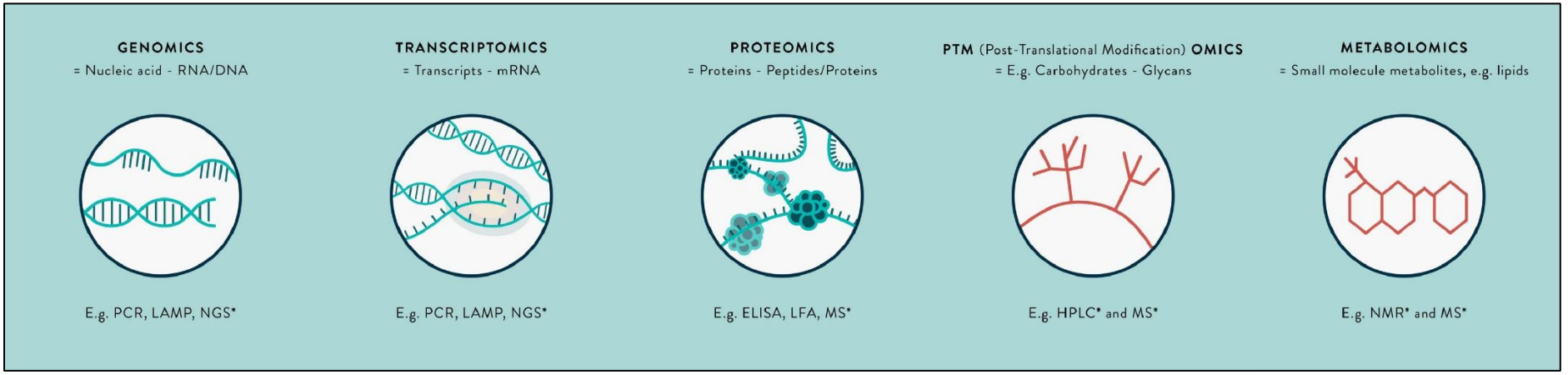
Introduced in Chapter 1 of this thesis, CNS infections account for a large number of DALYs worldwide every year (304). This group of diseases also unduly affects the poorest, marginalised communities, with limited access to healthcare (228). An urgent research priority is scaling up diagnostic capacity, particularly in resource-constrained settings, and introduction of point-of-care tests (227, 228, 305, 306). Diagnostics are fundamental for individual patient management decisions, estimations of disease burden, vaccine effectiveness and treatment trials.

Development and implementation of diagnostics for CNS infections is fraught with difficulties irrespective of the resources available (307, 308). Although brain biopsy is the gold standard for diagnosis, this is rarely performed (219). Clinicians rely on LP to obtain CSF to be tested as a surrogate specimen to test for markers of CNS infection. Furthermore, although currently available methods involve a targeted approach, 'you need to know what you are looking for', and the list of potential pathogens implicated in CNS infections is extensive, geographically

diverse, and dynamic (14). The targeted methods largely rely on molecular assays (mainly PCR) or serology (mainly ELISA). There are limitations in the accuracy of these tests; broadly, PCR has variable sensitivity in CSF due to low pathogen concentrations, whilst serology may have poor specificity. Even in the best-resourced centres worldwide, suspected cases of CNS infections remain undiagnosed in one to two-thirds of patients (7, 308).

Metagenomics techniques (Figure 4.1), such as mNGS, are emerging as a novel useful untargeted, 'you don't need to know what you are looking for' approach to diagnosing CNS infections (6, 14, 309). In particular, they have a role in identifying pathogens for which there are mutations in the PCR primer or probe binding sites on the genome (310), or those that are rare or novel causes and not routinely tested (311-315). Currently, evidence from metagenomics is limited to case reports or case series, and testing requires considerable resources and expertise. Furthermore, there are infections, for example JE, in which the window for pathogen detection is narrow, and a diagnostic test based on identifying genetic material has limited use. Advances in molecular technology are likely to reduce the proportion of undiagnosed CNS infections, however it is likely that at least 10-20% will remain undiagnosed by these methods (14).

Figure 4.1 The omics revolution in biomarker discovery – a summary of key terms



*Untargeted -omics techniques. Abbreviations: RNA=Ribonucleic acid; DNA=Deoxyribonucleic acid; mRNA=Messenger RNA; PCR=Polymerase chain reaction; LAMP=Loop-mediated isothermal amplification; NGS=Next-generation sequencing; ELISA=Enzyme-linked immunosorbent assay; LFA=Immunochromatographic lateral flow assay; MS=Mass spectrometry; NMR=Nuclear magnetic resonance.

Alternative methodological approaches involve transcriptomics, proteomics and metabolomic techniques (see Figure 4.1) (309, 316, 317). These may identify pathogen-derived or host markers of infection as messenger RNA (mRNA) transcripts, proteins or metabolites, respectively, that are altered in expression in certain CNS infections. These biomarkers represent fundamental processes occurring in cells, that is, the functional elements of the genome. The transcriptome is highly dynamic, affected by external perturbations, and mRNA as a biomarker is relatively easily degraded in the extracellular environment (318, 319). There is minimal literature on the CSF transcriptome. However, studies in whole blood show discrepancies between findings in different studies. Importantly, mRNA detection is not currently as amenable for translation to the bedside as that for a protein biomarker, for which immunochromatographic antibody-based assays 'pregnancy test-style dipsticks' may be produced. There are similar issues with metabolomics, the study of substrates and products of metabolism, and sample preparation is more complex (316, 320). For these reasons, this review is focussed on proteomics, although the potential of transcriptomics and metabolomics in this field remains to be explored. An illustration of the potential impact of a protein biomarker is the detection of NS1 in suspected dengue virus infection. The window for detection of NS1 protein is longer than that of DENV RNA, and the protein has been harnessed for point-of-care testing (321). NS1 protein ELISA and rapid tests have been introduced worldwide for testing serum and CSF, as recommended by the World Health Organisation (322), with data suggesting a reduction in unnecessary antibiotic prescriptions (323). Similar successes for other pathogens could have significant incremental public health impact.

Analysis of CSF total protein concentration is one of the cornerstones of conventional diagnostic techniques, used to classify CNS infections into bacterial, tuberculous and viral categories (324, 325). However, beyond serological testing, to date there has been limited unbiased profiling of the CSF proteome in infections (326). One of the reasons for the lack of

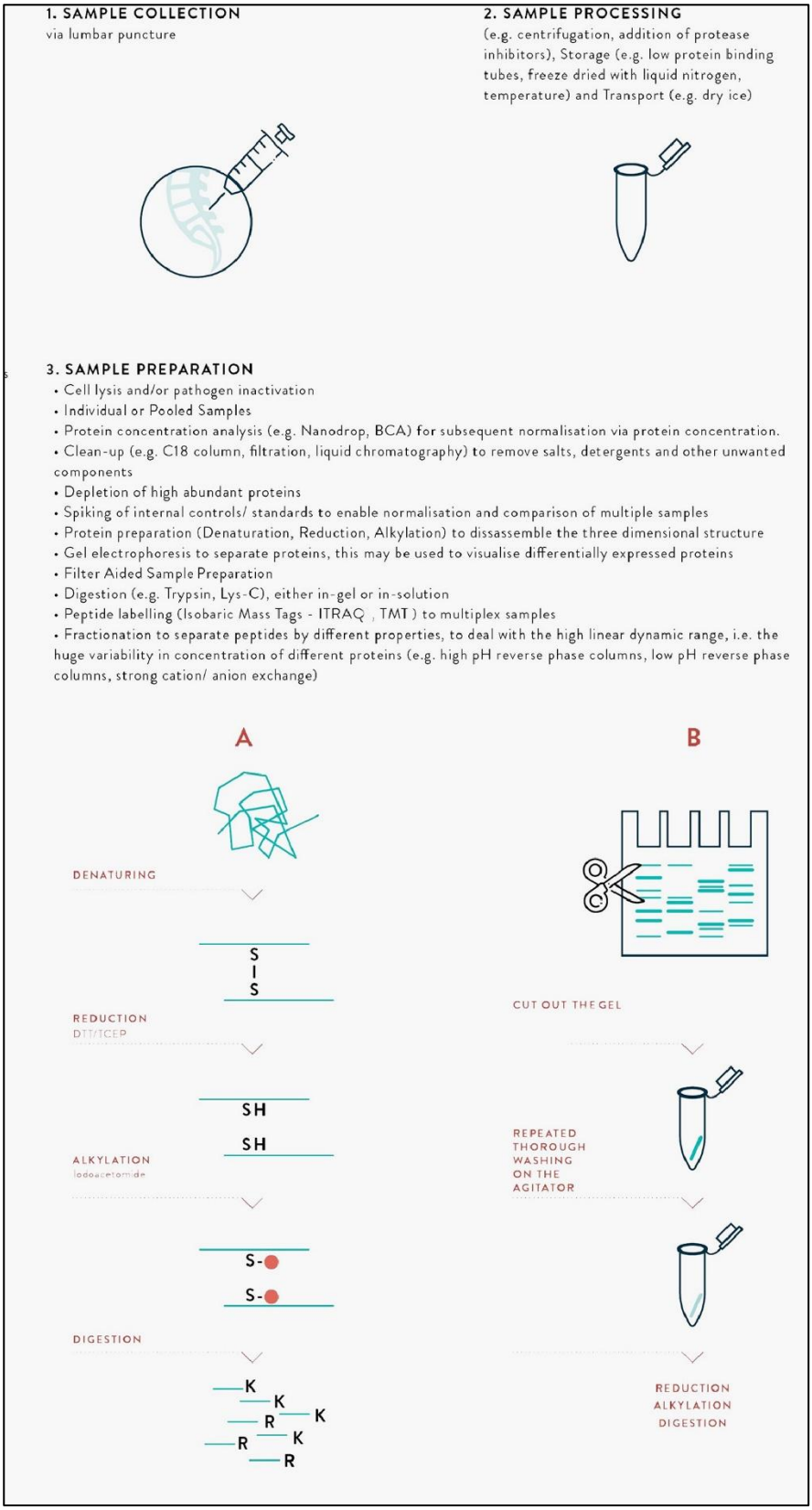
knowledge in the field of protein biomarker development has been the confines of available technology, including challenges of the substantial variability in concentration of different proteins in clinical samples and low concentration of potential biomarkers, i.e. ‘trying to find the needle in a haystack’. Steady maturation of MS instruments and workflows has transformed proteomics applications, which means that we are now in the position to address these issues. MS proteomics techniques facilitate uniquely unbiased, sensitive and quantitative analysis of body fluids and tissues. This is evident from the enormous investment in proteomics biomarker discovery for neurodegenerative diseases and cancer (327).

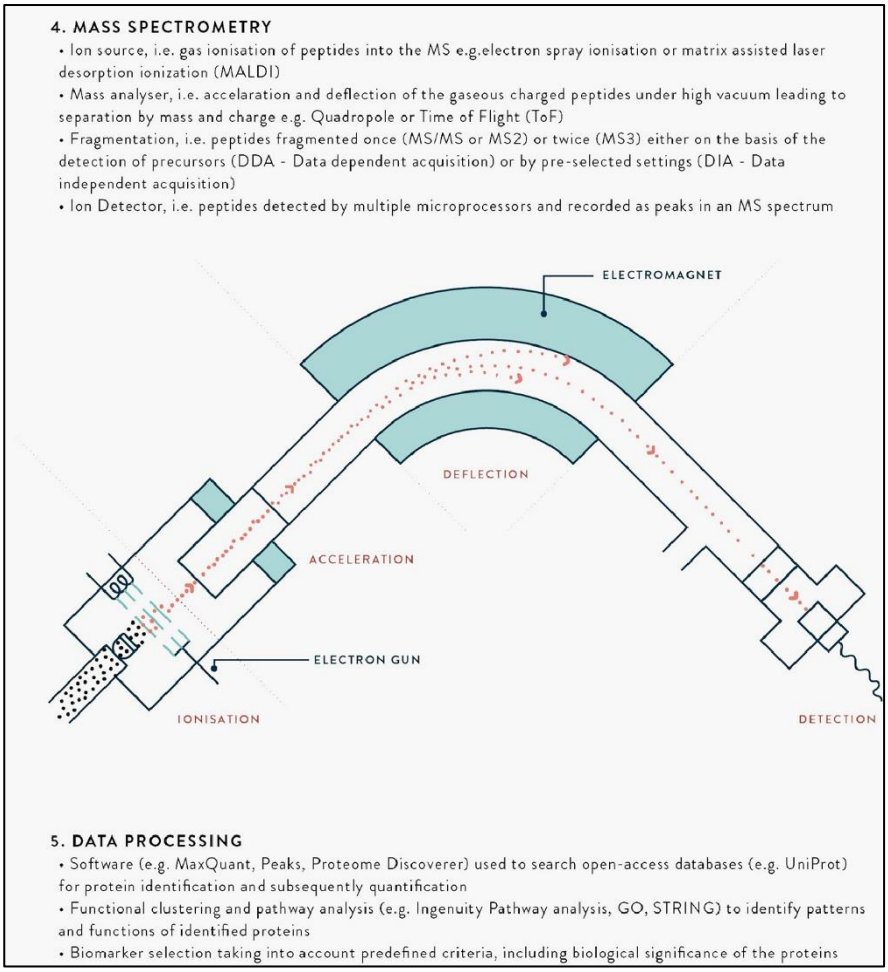
There are a wide range of MS proteomics methods involving intact protein analysis, ‘top-down’, ‘middle-down’ or ‘bottom-up’ (protein digestion and sequencing) approaches. A glossary of MS terminology is presented in Table 4.1, with schematic illustration of the processes in Figure 4.2. The application of matrix assisted desorption ionisation – time of flight (MALDI-TOF) has revolutionised the medical microbiology laboratory due to the speed, accuracy and high-throughput, although a limitation is that it is currently only used in clinical practice for pathogens included in the database (328). For biomarker discovery, the principal approach incorporates bottom-up MS, typically coupled with nanoflow liquid chromatography to separate samples into fractions and address the substantial differences in abundances of different proteins, referred to as the high dynamic range (329). An important technique has been the introduction of peptide labelling, allowing introduction of multiple samples each with different labels during a single MS run, improving comparison of relative protein abundance between samples. There have also been considerable advances in separation methods to enable deeper probing of the proteome (330).

Table 4.1 Glossary of MS terminology

Term	Explanation
Mass spectrometry (MS)	The basic principle underlying MS is the identification of peptides or proteins based on separation by mass and charge. Peptides or proteins are ionised, accelerated and usually deflected by a strong electromagnetic field, such that they reach a detector at different times based on their mass and charge. Detection of a peptide or protein is recorded as a peak and used to determine the peptide sequence and identity. This may also be termed peptide mass fingerprinting, or peptide sequencing.
Types of Ionisation; ESI, MALDI and SELDI.	Introduction into the mass spectrometer of sample containing proteins or peptides of interest requires ionisation. Two key methods include 1. electrospray ionisation 'ESI' and 2. laser desorption ionisation (matrix-associated 'MALDI' and historically surface-enhanced 'SELDI').
Tandem MS; MS2 and MS3	MS may also involve the fragmentation of peptide or protein ions, such that a precursor peak is recorded along with fragment peaks (MS2) or fragments of fragments (MS3). This improves selectivity, identification and detection.
Types of Mass Analysers; Quadrupole, ToF and Orbitrap	Mass analysers separate peptide or protein ions within the MS. Two key methods include 1. quadrupole, four metal rods with alternating electromagnetic fields, and 2. time of flight, based on time alone. 3. Orbitrap, based on ionised ions rotate around a central rod at high voltage and vacuum. MS may involve a single or combination of methods.
Native, Top-down, Middle-down vs Bottom-up protein analysis	Prior to MS, samples may be digested with an enzyme (usually trypsin) into peptide fragments (up to 3 kDa). This is termed bottom-up MS. In a middle-down approach, restricted protein digestion is performed using specific enzymes, resulting in larger peptides (3-10 kDa) as compared to bottom-up proteomics. In contrast, analysis may involve intact protein analysis no digestion, top-down MS. Bottom-up is more common, however there are instances when top-down is required, for example for analysis of proteins in their native structure.
Unbiased vs Targeted (e.g. PRM, SRM, MRM)	MS performed to identify specific pre-selected proteins or peptides is termed targeted, whereas discovery MS performed to identify any/ novel proteins or peptides is termed unbiased or shotgun 'you don't need to know what you are looking for'. Targeted MS is usually more sensitive than unbiased, and also allows high-throughput, e.g. parallel reaction monitoring (PRM), selected reaction monitoring (SRM) and multiple reaction monitoring (MRM).
Dynamic range	The range in concentrations of proteins present in a sample. This may vary by many orders of magnitude, leading to difficulties in identifying low abundant proteins of interest.
Sample preparation	Steps involved in preparing a sample prior to introduction in the MS. This usually involves disassembly of the 3D structure with denaturation (unfolding; e.g. urea), reduction (breaking disulphide bonds in the secondary structure e.g. dithiothreitol) and alkylation (capping free thiol chains to prevent reformation of disulphide bonds). The protein sheets are then digested into smaller (e.g. 9 amino acid chain) peptide fragments.
Depletion	Removal of high abundant proteins (e.g. albumin and IgG), to improve MS identification of low abundant proteins of interest. Typically involves immunodepletion, i.e. antibody methods.
Pooling	Combining several samples in a single analysis.
Labelling	Addition of isobaric mass tags to peptides during sample preparation, e.g. iTRAQ or TMT, to enable the analysis of multiple samples in a single MS run. This allows relative quantitation of peptides or proteins, reduces the time and obviates bias due to inter-run variation.
Separation and Fractionation; Liquid-chromatography (LC)	In order to deal with the high linear dynamic range in clinical samples, proteins are separated by various methods based on different properties of the proteins such as their mass, charge or hydrophobicity. Multidimensional separation refers to multiple methods being performed on the same sample, and orthogonal when the methods are based on different properties. These methods may be prior to digestion, such as by gel-electrophoresis, after which the gel is cut up and prepared to be loaded on the MS. There are various methods coupled with the mass spectrometry machine, e.g. liquid chromatography. e.g. strong cation exchange, high pH or low pH reverse-phase and C18 analytical column.
Functional analysis or Clustering	Identified proteins are investigated using programs that analyse the subcellular location, biochemical pathways and functions. Clustering refers to the mapping of proteins by their reported functions, to examine whether multiple proteins are involved in similar pathways, as this may strengthen evidence for their roles.

Figure 4.2 Schematic diagram of the key steps in mass spectrometry CSF biomarker identification

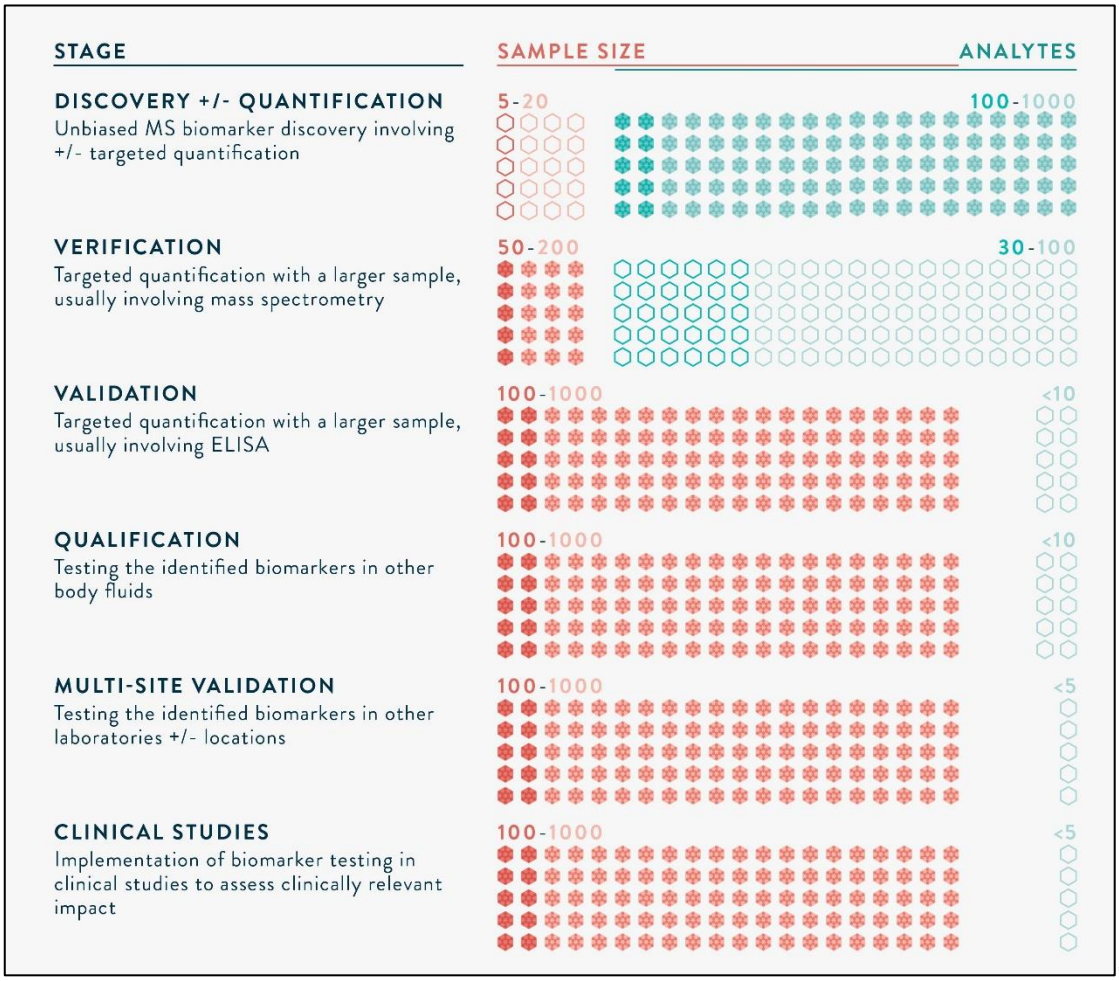




MS-based proteomics biomarker development has aptly been compared to the drug development pipeline. As illustrated in Figure 4.3, a series of well-designed studies are needed, from biomarker discovery through to implementation in clinical practice (331). Appropriate sample size calculations are required to ensure that inferences are valid. A discovery study typically incorporates a small number (e.g. 5-20) of patient samples (carefully selected cases and controls) 'biological replicates' and unbiased, highly sensitive MS workflows. Candidate biomarker(s) identified are then verified in a larger group of patient samples (e.g. 50-200) using high-throughput techniques such as parallel reaction monitoring. Machine learning algorithms, such as principal component analysis, are frequently applied to identify biomarker(s) that best differentiate between cases and controls (332). Based on calculations of receiver operating characteristics (ROC) curves, with targets for sensitivity and specificity, a biomarker or a

combination of biomarkers are selected. The proteins are usually utilised to develop an antibody-based test, such as an ELISA or immunochromatographic assay. Validation involves testing in an even larger group (e.g. 100-1000), in multiple sites, and may also be qualified in other body fluids. Prior to implementation in clinical practice, well-designed studies are also needed to demonstrate clinical impact and/or cost-effectiveness. As with drug-development, many candidate biomarkers do not reach clinical practice, for a variety of reasons such as lack of sensitivity, specificity or reproducibility, however this is an inevitable part of the process.

Figure 4.3 The long road to clinical application of biomarkers *(331)*



*Sample sizes are informed by appropriate statistical tests, and numbers provided are rough estimations of those required.

I performed a systematic review evaluating the use of MS-based proteomics techniques to identify biomarkers in CSF for the diagnosis of suspected CNS infections. The review covered all patient ages, and included clinical studies from biomarker discovery through to validation. The aim was to summarise the state of this promising technology, to identify problems and focus on research needed to improve its implementation into clinical practice.

Methods

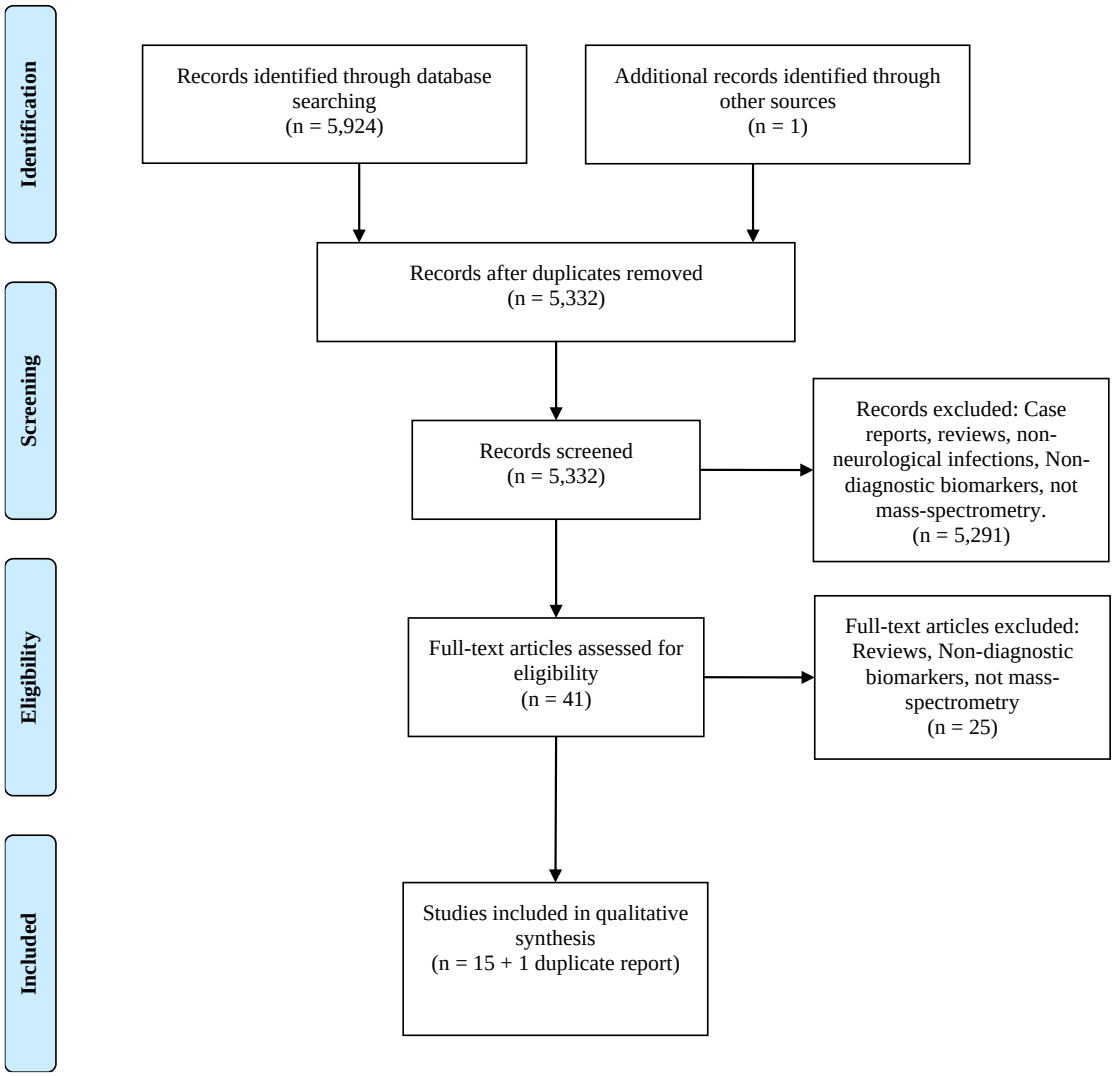
The study protocol was registered with PROSPERO, study ID CRD42018104257. References for this review were identified through searches of PubMed, Embase, Web of Science, and Cochrane for articles published from 1st January 2000 to 17th May 2022, by use of the Medical Subject Headings (MeSH) and text word terms ("cerebrospinal fluid" or "csf"), ("mass-spectrometry" or "proteom*" or "biomarker*") and "infectio*". Criteria for study inclusion involved all primary research except case reports, on the diagnosis of infectious diseases except human immunodeficiency virus (HIV) or nosocomial infections, applying MS to human CSF samples and reporting in English language. Articles were imported into EndNote, and then de-duplicated. Abstracts were screened for the study criteria. Two authors (myself and Bevin Gangadharan) independently performed a review of full articles to identify eligible articles using the study criteria, and then used standardised proformas to perform quality assessment and data extraction. A third author (Abhinav Kumar) resolved any disagreement. Additional articles were identified from hand-searching references in relevant articles, searching Web of Science for publications citing the articles, and by emailing experts in the field. Quality assessment was performed in accordance with the guidelines, 'Recommendations for Biomarker Identification and Qualification in Clinical Proteomics' (333) using the standardised eight-point checklist. Another reporting guideline has also been utilised in the discussion, the 'Guidelines for uniform reporting of body fluid biomarker studies in neurologic disorders' (334), and relates to mature biomarker candidates (335). Data were extracted using criteria developed by the authors in reference to available guidelines (331, 333, 334, 336-338). During the data extraction, authors of relevant articles were contacted for further details, if required.

Results

Study selection

A PRISMA Flow diagram is presented in Figure 4.4, and summarises the results at each stage in the process of study selection. Database searches identified 5,924 papers (PubMed=1,769, Embase=3,071, Web of Science=1,084, Cochrane=0). De-duplicating and initial screening of abstracts as per the inclusion criteria identified 41 papers. The majority of the identified papers were excluded during screening as they did not involve primary research, neurological infections or MS. For instance, many papers focused on cell culture proteomics or multiplex ELISA techniques. Full-text screening was performed using the same inclusion criteria, and led to the inclusion of 16 papers for quality assessment and data extraction (339-350). Articles excluded during the full-text analysis were reviews, involved ELISA or protein microarray techniques, or were not confined to diagnostic biomarkers. Two studies (344, 350) reported the same patients and MS experiment, with focus on different findings obtained and different confirmatory methods. The two articles are counted as one study for the purposes of calculated percentages below.

Figure 4.4 PRISMA flow diagram of the number of records identified, included and excluded, and the reasons for exclusions.

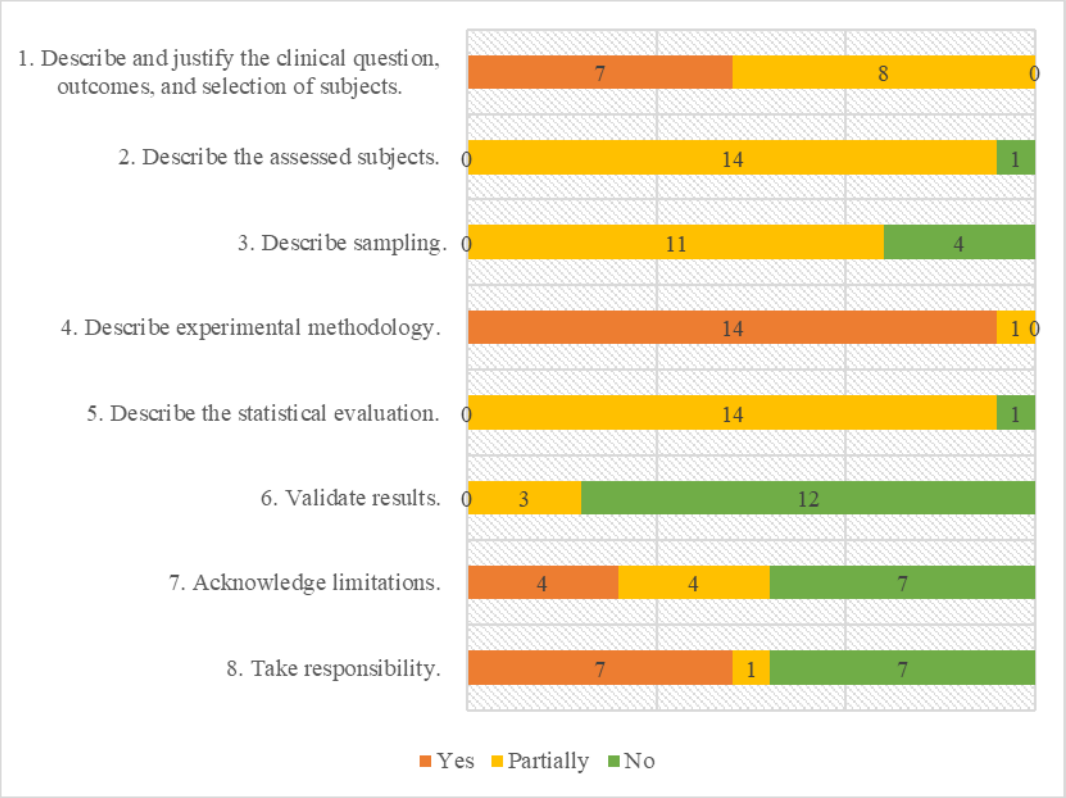


Quality Assessment

The quality of the studies was assessed using the eight criteria set out by the guidelines of Mischak *et al,* 2010 (333) (Figure 4.5, Additional material 4.1). None of the studies fulfilled all the criteria. Eight studies (53%) did not report clear questions, outcomes, or selection of subjects. One study did not report the patient age, one study reported the age range, and four studies reported mean ages while it is more appropriate to report median ages given the small sample size. Sex was reported, and this were broadly matched, however there was no discussion

on this potential effect. Ethnicity was not reported in three quarters of articles. None reported comorbidities, pregnancy or routine medications.

Figure 4.5 Quality assessment of included studies



There was minimal data on the methods of sample collection or duration of sample storage. However, the studies provided data on sample storage, preparation, and the technical aspects of MS. None of the studies reported sample size calculations, and there was inconsistent reporting of statistical methods used. Although the studies uniformly reported the potential role of identified proteins/ peptides, the limitations of the study were only reported in one third of studies.

A fixed quality cut-off for including studies was not set, and all the studies were included in the data extraction. Nonetheless, it is notable that nine (60%) studies failed 6 or more of the 8-point

quality assessment measure. This may partly be due to insufficient awareness or adherence to reporting guidelines. However, it also suggests a high-risk of bias across the studies.

Data Extraction

The data extracted from the studies are summarised in Table 4.2, with further detail in Additional material 4.2. All the identified studies involved biomarker discovery work, and five studies also performed further work termed verification or validation. The studies were published between 2011 and 2022 and seven (47%) were from the last five years. There was a wide geographical spread of locations of MS experiments; six performed in Asia, six performed in Europe, one in North America, one in South America and one in Africa. Most of the samples were collected in the same country in which MS experiments were performed, except for four in which samples were transported from Angola, Greece, and the Netherlands to France (342), from Malawi to the UK (343), from Angola, Chad, and the Democratic Republic of the Congo to Switzerland (351), and from Vietnam to the UK (352). It was not possible to perform a meta-analysis of included studies due to incomplete reporting, and low responses (2, 18%) to email requests sent to corresponding authors.

Table 4.2 Summary of included studies

Study	Site[a]	Study Groups	Sample Size[b]	Methods Summary	Body Fluid	MS	Data Processing		Biomarkers	
							Database	Software	Criteria	Results
Angel 2012	USA	Early-disseminated Lyme disease vs CSF inflammation	26 vs 19	Biomarker Discovery: 1) CSF from 9 pooled cases vs 12 pooled controls, fractionated using offline strong cation exchange (SCX) into 35 vs 25 fractions and analysed label-free by reverse-phase capillary LC-MS; 2) To enable analysis of a larger group including samples with insufficient volume for fractionation, CSF from 26 cases (including the same as the first group) and 7 controls were analysed individually by unfractionated label-free LC-MS.	CSF	LTQ	Human and Pathogen	Identification with SEQUEST, quantification & statistical analysis with in-house DAnTE.	ANOVA (P value <0.05); AUROC >=0.8.	13 host proteins differentially expressed between cases vs controls: Secreted phosphoprotein 1, EGF-containing fibulin-like extracellular matrix protein 1, Myoglobin, POTE ankyrin domain family member I, Actin, cytoplasmic 2, Lysozyme, Complement C1q C & B, Ig κ variable 3D-15 & 3-20, IgG Fc binding protein, Vitronectin, α-2- macroglobulin.
Asano 2011	Japan	Paediatric acute encephalopathy vs febrile seizures	13 vs 42	Biomarker Discovery: 1) CSF from 8 cases vs 28 controls was applied to a 96-well ProteinChip and analysed by SELDI-ToF MS to identify differences in peaks; 2) This was repeated in a different group of samples (referred to as validation) with 5 cases vs 14 controls; 3) The peak with the best separation between the groups was analysed by tandem MS (MALDI-QSTAR) to enable protein identification.	CSF	SELDI-TOF and MALDI-QSTAR	Human	Identification with Mascot, quantification and statistical analysis with ProteinChip Data Manager software	Kruskall-Wallis H test, Mann-Whitney test with Bonferroni-Dunn correction (P value <0.05).	1 host protein differentially expressed in between cases vs controls: Peptide fragment from the neurosecretory protein VGF precursor. Note – 15 peaks were different in the first experiment, and of these 4 were confirmed as being different in the second, but only one of these was subject to tandem MS to enable protein identification.
Bakochi 2021^	Sweden	Acute bacterial vs viral meningitis vs neuroborreliosis vs non-infectious	35 vs 21 vs 7 vs 49	Biomarker Discovery: 1) A merged assay library was constructed from DDA LC-MS runs of each sample, bacterial isolates combined with previously established data from healthy human organs and primary cell lines. SWATH-MS (DIA) was performed, with protein identification based on the assay library.	CSF	Q Exactive Plus Orbitrap	Human and Pathogens	Identification with DIA using Diana	LASSO regression and cross-validation, AUROC >0.8.	18 host proteins identified with good discriminatory performance, AUROC >0.9. A1AT, A2GL, ANT3, APOE, CATD, CD14, CFAH, CLUS, DKK3, ENPP2, FCGBP, GELS, HEP2, HPT, ITIH2, PROS, SCG1, TTHY.
Bonnet 2018	France	*T.b.gambiense* early stage vs late stage vs controls (CSF <5 WCCs and no trypanosomes)	3 vs 4 vs 3	Biomarker Discovery: 1) CSF from 2 case groups vs controls (3, 4, 4) compared by LC-MS, involving abundant protein depletion and concentration by filtration, and then analysed by label-free LC-MS. Biomarker Verification: A larger group (23, 43 vs 14) used to verify potential biomarkers using label-free LC-MS and then ELISA to detect neuroserpin, neogenin and secretogranin 2.	CSF, urine and saliva.	Q Exactive Orbitrap	Humans and Pathogen	Identification with Proteome Discoverer, Mascot, quantification and statistical analysis with Progenesis QI.	ANOVA (P value <0.05)	37 host proteins differentially expressed between groups and with a biological role[e]. 3 host proteins were selected for verification: Neuroserpin, neogenin and secretogranin 2; only 1 host protein, neuroserpin, was detected by ELISA, and this differentiated between early and late stage disease.
Cordeiro 2015	Brazil	Pneumococcal vs Meningococcal vs Enterovirus meningitis	3 vs 3 vs 3	Biomarker Discovery: 1) CSF from 6 of each of the 3 types of meningitis and 6 controls (patients without infection, neurodegenerative or psychiatric disease) tested involving abundant protein depletion and then separation with 2D gels; 2) 3 patients from each type of meningitis selected for running as pooled samples on gel, gel-extraction and MS.	CSF	MALDI-ToF/ToF	Human	Identification with Mascot	Qualitative analysis – proteins present in 11/12 gels per group, and not present in another group	4 host proteins differentiated between groups and were used to develop a predictive model of meningitis: Apolipoprotein A-I (present in all causes of meningitis and not controls), C-reactive protein & Complement C3 (present in bacterial meningitis and not viral), Kininogen-1 (present in Meningococcal meningitis).

Fraisier 2014	France	West Nile virus vs Non-WNV infection, headache, idiopathic intracranial hypertension and healthy controls.	8 vs 11	Biomarker Discovery: 1) CSF from 8 pooled cases vs 11 pooled controls (6 with acute headache and 5 with idiopathic intracranial hypertension) analysed using iTRAQ labelling, isoelectric point based separation into 12 fractions and then LC-MS analysis. Biomarker Verification: Secondly, CSF and/or serum was tested for the identified protein Defensin Alpha-1 by an ELISA in 16 cases vs 13 controls.	CSF and Serum	LTQ	Human	Identification, quantification and statistical analysis with Mascot and SEQUEST through Proteome Discoverer.	Kruskal-Wallis and Mann-Whitney U tests, P value <0.05	47 host proteins differentially expressed in cases vs controls during discovery. 1 host protein, Defensin α-1, was selected based on its high fold-change, possible functional association with WNV pathobiology, potential use as severe infection biomarker, and availability of commercial ELISA tests.
Gomez-Baena 2017	UK	Pneumococcal meningitis vs controls (normal CSF)	16 vs 12	Biomarker Discovery: 1) CSF from 16 cases vs 12 controls analysed label-free using LC-MS; 2) CSF from 20 cases and 15 controls used to confirm a subset of proteins by western blotting.	CSF	LTQ-Orbitrap Velos	Human and Pathogen	Identification with Proteome Discoverer and Mascot, quantification and statistical analysis with Progenesis QI.	ANOVA, P value <0.05	134 host proteins and 6 Streptococcus proteins differentially expressed in cases vs controls during discovery. 5 host proteins were selected and confirmed by western blotting based on confidence of protein identification, magnitude of change, extent of protein coverage and putative role in the pathology: Myeloperoxidase, S100 calcium binding protein A9, Cathelicidin antimicrobial peptide, Ceruloplasmin and Cystatin C.
Luo 2022^	China	Paediatric acute bacterial meningitis vs viral meningitis vs non-infectious	8 vs 9 vs 6	Biomarker Discovery: 1) 1) CSF from 8 cases vs 15 (9, 6) controls analysed label-free using LC-MS. Biomarker Verification: Secondly, CSF was tested for the identified proteins CD163, alpha 2-macroglobulin (A2M) and sAPPα by an ELISA in 20 cases vs 42 (22, 20) controls.	CSF	Q Exactive Plus Orbitrap	Human	Identification, quantification and statistical analysis with MaxQuant.	T test, P value <0.05.	171 host proteins differentially expressed in case vs controls. 3 proteins with high discriminatory performance: A2M, APP and CD163.
Mu 2015[c]	China	Tuberculous meningitis vs healthy controls	12 vs 12	Biomarker Discovery: 1) CSF from 12 cases vs 12 healthy controls analysed, including high abundant protein depletion, protein digestion, iTRAQ labelling, SCX fractionation, and LC-MS. Biomarker Verification[d]: CSF from 25 cases and 28 controls tested for Apolipoprotein B (ApoB) and Apolipoprotein E using ELISA.	CSF	Triple-ToF	Human	Identification, quantification and statistical analysis with ProteinPilot.	Not reported, P value <0.05.	81 host proteins differentially expressed in cases vs controls. 2 host proteins were selected for their role in lipid metabolism and confirmed by western blotting and ELISA: Apolipoprotein B & E. Apo B was confirmed, with AUROC 0.91, sensitivity 89.3% and specificity 92%.
Njunge 2017	Kenya	Acute Bacterial Meningitis vs Cerebral Malaria	37 vs 22	Biomarker Discovery: CSF from 37 ABM cases vs 22 CM cases was analysed label-free using LC-MS.	CSF	Q Exactive Orbitrap	Human	Identification, quantification and statistical analysis with MaxQuant and R.	Mann Whitney test, P value <0.05, AUROC >0.9	52 host proteins differentially expressed in the two groups. 2 host proteins were identified with sensitivity >98% and specificity = 1: Myeloperoxidase and Lactotransferrin
Ou 2013	China	Tuberculous meningitis, Cryptococcal meningitis vs Healthy controls	20 vs 20 vs 20	Biomarker Discovery: CSF from 20 pooled TBM vs 20 pooled Crypto cases vs 20 pooled controls, fractionated using SCX chromatography and analysed by LC-MS. Biomarker Verification[d]: CSF from 25 cases vs TBM, 25 Crypto vs 25 controls tested for S100A8 and ApoB using ELISA.	CSF	QSTAR	Human	Identification, quantification and statistical analysis with ProteinPilot.	Not reported, P value <0.05.	9 host proteins differentially expressed in cases vs controls. 2 host proteins were selected for and confirmed by western blotting and ELISA: Apolopoprotein B and S100 calcium binding protein A8

Sengupta 2015	India	Japanese encephalitis virus vs non-JEV Acute Encephalitis Syndrome	10 vs 10	Biomarker Discovery: CSF from 10 pooled cases and 10 pooled controls separated by SDS-PAGE, JE-specific visualised spots excised and analysed by MS.	CSF	MALDI-ToF	Human	Identification, quantification and statistical analysis with ProteinPilot using MASCOT.	Qualitative analysis of proteins visualised only in JEV cases	7 host proteins identified only in cases: Serum albumin, Vitamin D-binding protein, Fibrinogen gamma chain, Fibrinogen beta chain, Complement C3 & C4b, Actin cytoplasmic-1.
Sun 2020^	China	Enterovirus meningitis vs controls of febrile seizures, epilepsy, non-CNS infections	52 vs 53	Biomarker Discovery: 1) A assay library was constructed from DDA LC-MS runs of a pooled CSF sample of all 52 cases and 53 controls with prior offline fractionation into 60 fractions concatenated into 15. Boxcar (DIA) was subsequently performed with protein identification based on the assay library.	CSF	Q Exactive HF Orbitrap	Human and pathogen	Identification, quantification and statistical analysis with MaxQuant.	AUROC >0.9	381 host proteins differentially expressed. 6 proteins with high discriminatory performance: LCP1, ELANE, LCN2, SECTM1, ARHGDIB and CXCL10
Thanh 2020^	U.K.	Tuberculous, acute bacterial, encephalitis (cryptococcal, eosinophilic and anti-NMDA R) and non-CNS infections	20 vs 10 vs 10 vs 5	Biomarker Discovery: 1) CSF from the different groups was analysed by label-free LC-MS. Biomarker Validation: CSF of 364 patients was tested for NGAL (LCN2) by ELISA.	CSF and Plasma	Q Exactive HF Orbitrap	Human	Identification, quantification and statistical analysis with MaxQuant and Perseus.	AUROC, criteria not reported	60 and 19 host proteins were differentially expressed in the CSF in BM and TBM, respectively. 1 protein had the best discriminatory performance Lipocalin 2 (LCN2/NGAL).
Tiberti 2015	Switz-erland	T. bruceii gambiense, T. rhodesiense vs controls (CSF WCC <5 and no Trypanosomes)	3 vs 3	Biomarker Discovery: 1) CSF from 3 cases of T. rhodesiense vs 3 cases of *T* gambiense labelled with TMT, isoelectric point-based separation into 12 fractions and analysed by MS. Biomarker Verification: CSF of 39 and 126 cases, and from 20 non-infected control subjects. In addition to CSF, n = 29 plasma samples obtained from 15 and 14 cases.	CSF and Plasma	LTQ Orbitrap Velos	Human and Pathogen	Identification, quantification and statistical analysis with EasyProt.	Kruskall-Wallis H test, Mann-Whitney test with Bonferroni-Dunn correction (P value <0.05).	11 host proteins differentially expressed between groups. 3 proteins were selected for verification, based on their TMT ratios as well as on their appearance in the pathways of interest: C-reactive protein, Orosomucoid-1 and Complement component 9.

[a]Site refers to the location of the mass spectrometry work. [b]Sample sizes presented include the number of patient CSF samples tested by mass-spectrometry methods, cases vs controls. [c]Yang *et al* 2015 is not presented as it duplicates the work reported by Mu *et al* 2015. [d]Reported as validation, however this is verification. [e]Only CSF biomarkers are reported. ^Study identified following the published review.

There was a range of infections studied, including bacterial, viral and parasitic infections (Table 4.2). Additional body fluid samples corresponding to CSF were analysed in three studies, two articles also described analysis of serum and another two included plasma. Importantly, one study analysed additional non-invasive samples, saliva and urine (353). Non-invasive samples for biomarker detection are likely to be of great importance for improving diagnostic capacity in clinical practice.

The method of collecting CSF was not described in any article. Eleven (73%) articles reported the method of storage, of which four (27%) involved centrifugation, and two (13%) involved snap freezing with liquid nitrogen. Three (20%) studies used immunodepletion to remove highly abundant proteins such as albumin and transferrin. Eleven (73%) studies fractionated the samples, using various combinations of offline and mass spectrometer coupled systems. One (7%) involved gel-extraction methods, while the others were gel-free. Four (27%) used labelling, using isobaric tags for relative and absolute quantitation (iTRAQ) or tandem mass tags (TMT). One study reported the use of an internal control, bovine beta-lactoglobulin (349). All studies involved bottom-up MS approaches. Three (20%) studies used time-of-flight mass-spectrometers, and the other twelve (80%) used orbitrap mass-spectrometers. Extracted data was searched using human proteome databases for all, and six (40%) also searched using pathogen databases.

At the discovery stage, a median (range) of 13 (1-140) potential biomarkers were identified per study. A sub-group of eight studies performed further evaluation, using either targeted MS or antibody-based confirmation, confirming findings in a median (range) of 2 (1-5) proteins. Four studies referred to an additional study group as verification, although two of these studies interchangeably used the term validation for the same analysis (342, 353). The aim of the verification stage is to confirm numerous potential biomarker(s), and reduce the numbers down to a single marker or combination (<10) markers that may be feasible to subsequently test in an antibody-based platform. The sample size for verification is generally 50-200, and the method of detection is usually targeted MS. One study incorporating verification used targeted MS,

while the other three studies reporting verification used ELISA assays to confirm 1-3 biomarkers. Similarly, four studies referred to validation involving ELISA assays to confirm biomarkers in samples of 25-364 cases. There was no sample size calculation reported. Twelve (80%) studies reported investigation of pathway analysis, functional clustering and/or subcellular localisation, with programs such as the Gene Ontology (GO) tool (354), and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (355). Seven (47%) uploaded the data to an open-access database, such as the Proteomics Identification Database (PRIDE) (356). No follow-up publications were found for any of the included studies, or from personal correspondence with authors, such that it appears that all the biomarkers identified await further evaluation, and implementation as diagnostic tests.

It is noteworthy that only one (7%) study investigating pathogen-derived biomarkers identified any (343), suggesting low sensitivity of these techniques for pathogen-derived proteins (339). The biomarkers identified included plasma proteins associated with damage to the blood-brain barrier, immune activation, inflammation, and proteins from brain tissue associated with parenchymal damage (summarised in Table 4.2). Notably, two studies on *Mycobacterium tuberculosis* infection identified apolipoprotein B, and two studies involving *Streptococcus pneumoniae* infection identified myeloperoxidase. However there was also considerable differences between biomarkers identified by different studies (341, 343, 344, 346). The two studies investigating *Trypanosoma spp.* identified different biomarkers, C-reactive protein and ORM1, and neuroserpin (349, 353).

Discussion

This is the first systematic review of the application of proteomics-based MS that could be found for identification of clinical biomarkers for diagnosing CNS infections. Results demonstrate an emerging field, currently largely limited to pre-clinical discovery studies. This is not surprising considering that the human proteomics project was set up over a decade after the human genomics project, and proteomics investigation has lagged behind that of genomics (357).

Fifteen studies were identified that met the eligibility criteria, included in the final quality assessment and data extraction, of which seven (47%) were published within the last five years. Eleven pathogens were studied, and a median (range) of 13 (1-140) potential protein biomarkers identified per study. There was some consistency in biomarkers identified in different studies of the same pathogen. However, none of the biomarkers have been further investigated in subsequent publications or commercialised. It is unclear if this work is currently ongoing, not planned due to a lack of infrastructure and investment to carry the biomarkers through to validation, or it has happened but has not been published due to poor performance in further testing.

Successful application of MS depends on appropriate samples and well-designed experiments (358). For this, interdisciplinary collaboration is key, and the process is a significant undertaking. It is not sufficient for samples to be deposited with a MS laboratory, awaiting output of candidate biomarkers. To fully harness the technology, there need to be clear research questions and criteria for a potential biomarker. In line with this, and providing source for improvement in future studies, quality assessment revealed sources of bias in study design, such as a lack of clear case definitions for the target diseases and controls, explicit sampling methodology, sample size and statistical methods. In particular, there were inconsistencies in matching between cases and controls (359). It is possible that this may partially be explained by standards of reporting, as even though further details were requested, there was a low response

rate (2/11, 18%) to these requests. However, it is also clear that the experiments were frequently conceived retrospectively, using samples remaining from various other studies. The findings also emphasise the challenges of conducting these studies, due to limitations in obtaining sufficient CSF samples, or reaching a consensus on case definitions. To this end, three of the studies reported validation however my impression is that this is not validation due to the small sample size and lack of a sample size calculation. None of the studies took further steps to investigate impact in clinically relevant groups, such as consecutive patients with suspected CNS infections.

While all studies reported that ethical approval was obtained, it is important to recognise the subtle differences in permission that should be sought for a study on biomarker discovery. For example, an unbiased biomarker discovery study may identify a disease in the participant that is unrelated to the project aim. Another ethical issue is the use of CSF from healthy people, as CSF must be obtained by a significantly invasive procedure that does not have zero risk. Three studies involved obtaining CSF samples from healthy participants with informed consent. This would perhaps not be deemed ethical in all countries. It could also be argued that healthy CSF is not necessary, as an appropriate control group would be other CNS infections (333). Additionally, commercial samples marketed as healthy CSF need to be utilised cautiously as clinical characterisation and diagnostic testing may not be comprehensive, or fully disclosed. Sample preparation and MS analysis are dependent on the volumes of CSF and equipment available. The search for biomarkers has been equated to the search for a needle in a haystack (360). The optimal strategy for probing CSF proteins remains elusive due to the high variability in methods used, i.e. there are few studies that systematically investigate the effect of changing one factor in the long series of experiments required to perform LC-MS protein analysis (361). It is clear that a small number of proteins such as albumin and transferrin are highly abundant, and these contribute to the large range of proteins in CSF. Nonetheless, strategies of immunodepletion require large volumes of CSF, and may also remove proteins of interest bound to abundant ones. To this end, only three (20%) studies performed highly abundant

protein depletion. It is unclear if in-gel digestion is superior to in-solution digestion. In-gel digestion would lose smaller <10 kilodalton (kDa) proteins, however there are few relevant proteins of this size, and the impact is estimated to be minimal. Similarly, although filtration devices used to concentrate CSF have been implemented, these may lead to protein losses, and not only proteins below the molecular weight cut-off of the device. The standard enzyme used for digestion is trypsin, however neuropeptides do not terminate in predictable amino-acids, and have more post-translational modifications such as glycosylation and phosphorylation (362). For this reason, the use of Lys-C has been used in addition to trypsin to improve the consistency of protein digestion after lysine residues (363).

In the review, four (27%) studies used isobaric mass-tagging to allow simultaneous analysis of more than one sample in the same MS experiment. This is beneficial as it reduces bias due to the inherent variability between runs, improves relative quantification between samples, speed and equipment costs. Nonetheless, the larger number of steps required during sample preparation may lead to protein losses, and tagging methods are more expensive. Label-free quantification is steadily improving, and was utilised in all the studies published in the last three years after publication of my review. The strategy for separation is very important, and a wide variety of methods are used, both off-line and online (coupled to the MS machine). It is clear that multidimensional orthogonal strategies are necessary, i.e. sequential methods of separation involving different mechanisms such as separation by mass and charge. For instance, a useful strategy has been strong cation exchange coupled with online reverse-phase LC-MS. Different strategies, such as different series of sample preparation methods, and also different protein identification software may reveal different biomarkers (361). For this reason, if there is sufficient CSF it may be useful to try different methods.

Notably, while all studies searched human proteome databases, only four studies also searched pathogen-derived databases. It is possible that researchers were convinced that pathogen searches would be futile. However, for the purposes of diagnostics, pathogen-derived

biomarkers are likely to be more specific than host biomarkers, and it would seem sensible to attempt to look for them during the analysis.

Limitations of this review include the restriction to MS proteomics analysis, English language, non-HIV infection and human participants. HIV infection provides an additional level of complexity due to the high incidence of opportunistic infections in this disease. Studies of animal models may also provide informative data. Publications involving MS that did not involve unbiased discovery work, 'peptide mass fingerprinting' or 'peptide sequencing' were not included. For example MALDI-TOF without protein sequencing was not included. Additionally, the review did not extend to transcriptomics, post-translational-omics or metabolomics research (364).

The discussion has focussed on pre-clinical biomarker discovery, as this has been the focus of studies to date, but clinical validation is an essential part of the process. Immunoassays are more sensitive and easier to use, but have their own problems related to non-specific reactivity (365, 366). A validation study needs a sample size calculation, requiring approximately 100-1,000 patient samples, tested in hospitals, and requires comparison with the gold-standard diagnostic test. The results would need to be presented as sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), with ROC curve analysis and area under the ROC (AUROC) values. These studies are a significant undertaking, and need considerable investment. Until these aspects are acknowledged, and studies designed accordingly, it is unlikely that biomarker discovery will be successfully translated to the bedside.

Lastly, the systematic application of MS methods to identify protein biomarkers for specific infections, or groups of infections, will improve diagnostic yield in the challenging group of CNS infections that remain undiagnosed. Furthermore, strengthening knowledge of these proteins will also inform understanding of pathophysiology of infection, and identify targets for developing therapeutics.

Publications arising from this chapter

This substance of this chapter has now been published (367). In the process of writing up the chapter, I updated the published review to include an additional 4 articles (17th May 2022).

Chapter 5

Putative protein biomarkers of JE

In this chapter, I develop the underlying hypothesis that forms the basis of the thesis, that there could be a protein signature of JEV in CSF and this could be utilised to aid rapid and accessible diagnosis of JE. I discuss different strands of this hypothesis and review their respective evidence base. I report experimental work performed to test one of the strands, distinct from the LC-MS work discussed in the following chapters, Chapter 6 and Chapter 7, aiming to establish an ELISA assay to detect anti-JEV NS1' antibodies in human serum and CSF. Finally, I provide support for integrating the different strands of the hypothesis together using data science.

Introduction

In a healthy person, CSF is a clear colourless ultrafiltrate of plasma that is contained within the ventricles and subarachnoid space of the brain and spinal cord (368). CSF provides protection, nourishment, homeostasis and waste removal for the CNS. In view of its close proximity to the brain, it has traditionally been sampled in the work-up of patients with suspected neurological disease in order to provide insights into the underlying pathophysiology and aetiological diagnosis of their condition (369). CSF sampling involves an invasive procedure, an LP, that is not without risks, but far less than those associated with a brain biopsy. In the field of biomarker research, CSF is considered a rich source of disease biomarkers, and the logical target of early discovery work that is the focus of this thesis (361, 370). However, it is acknowledged that non-invasive fluids are more straightforward for implementation for patient diagnostics, and these are the subject of Chapter 7. In view of the downstream application of my

experimental work, to identify 2-3 proteins for an RDT, CSF proteomics methods have been applied in this thesis, rather than transcriptomics or metabolomics analyses.

Dr Walter Essex-Wynter initiated the performance of lumbar puncture and also of analysing total protein in CSF at University College London Hospital (371). He published results of measuring albumin, specific gravity, glucose, chloride and pH in 1981. Subsequently, Dr Heinrich Quincke refined the technique using a needle (372).

While these practices are now routine, the analysis of CSF proteins is still in a nascent phase (373, 374). The biggest strides have been made in the field of dementia research, and a handful of CSF proteins are now used for diagnosis; 14-3-3 proteins for Creutzfeldt–Jakob disease (CJD), amyloid-β 1–42/1–40 ratio (Aβ42/40), Microtubule associated protein tau (tau/MAPT) and hyperphosphorylated- (p) tau for Alzheimer's disease (AD) (375). In contrast, the study of protein biomarkers of CNS infections has largely been restricted to detection of specific anti-pathogen antibodies (369). The fifteen studies identified in the systematic literature review reported in Chapter 4 on the application of mass-spectrometry for the identification of diagnostic protein CSF biomarkers demonstrate that this is changing. However, CSF protein biomarker research is fraught with difficulties, for example pre-analytical variables, sufficient number of samples, expensive and relatively low throughput research methods, as well as the reliance on sampling patients with rare CNS infections using lumbar punctures makes this even more difficult (334, 374).

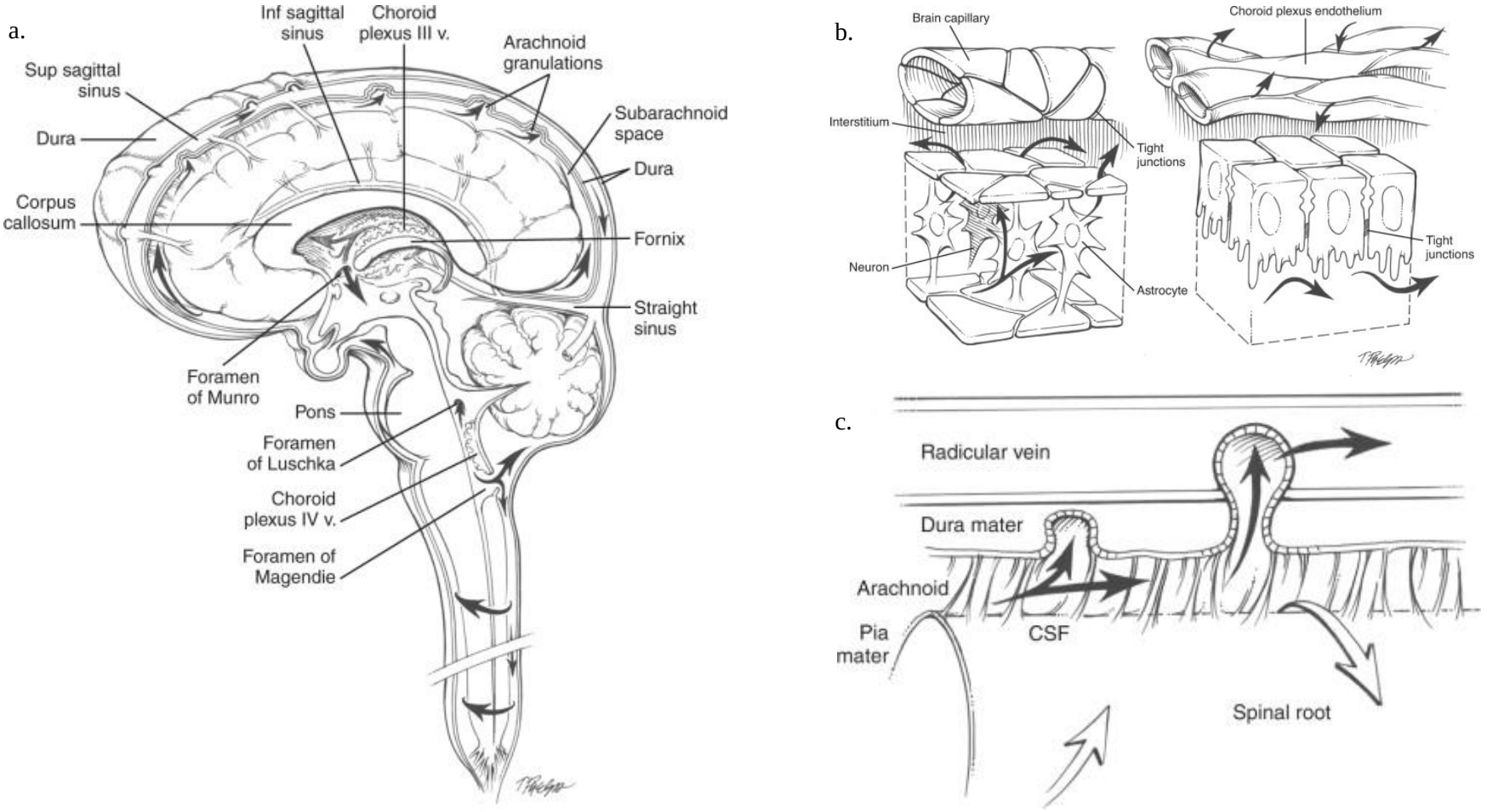
Anatomy and physiology of healthy CSF

The circulation of CSF through the brain and spinal cord is illustrated in Figure 5.1a. CSF is mainly produced by specialised ependymal cells of the choroid plexus in the ventricles of the brain by filtration and active transport, and to a lesser extent exuding from perivascular space of the brain parenchyma, meninges and dorsal root ganglia (376, 377). It flows through the ventricles, and into the subarachnoid space at the Foramen of Luschka and Magendie (378).

Following circulation over the brain and spinal cord, CSF is reabsorbed in the arachnoid villi and granulations, Figure 5.1c (378).

The blood-brain-barrier (BBB) and blood-CSF-barrier (BCB), both illustrated in Figure 5.1b, have been the subject of considerable interest from the early 19th century when Paul Ehrlich demonstrated that intravenously injected dyes did not stain the brain (379). These two barriers are commonly referred to together as the BBB, as will be the case in this thesis. These barriers ensure the complex functions of maintaining homeostasis and facilitating the entry of nutrients into the brain while protecting it from harmful substances including pathogens. CNS disease, particularly CNS infections, are usually associated with disruption of the BBB, discussed further below (380). Notably, as emphasised in E. Thompson's research, the BBB is not a single barrier (or dual barrier incorporating the BCB), there are a number of discrete links that lead to complex dynamics that are played out in the CSF proteome (381).

Figure 5.1 Anatomy of CSF circulation, Hartman *et al.*, 2009 (378)



[a] Route of flow of CSF through the brain from the choroid plexus in the ventricles through to reabsorption in the arachnoid granulations. [b] Location of tight junctions that form the blood-brain-barrier. [c] CSF drainage at the spinal nerve roots.

The total volume of CSF at any one time is approximately 125-150 mL, and this is recycled approximately four times per day or every 6 hours, such that 500-600 mL is produced per day, or 0.3 mL/min (377). This is clearly much more static than the main blood stream; with the total circulatory blood volume of approximately 5 L being replaced once a day. This is an advantage for biomarker discovery, as CSF is less susceptible to systemic clearing than other body fluids (382). Normal CSF is sterile, with <5 /mm^3 white blood cells (WBCs), no red blood cells and 0.50–0.80 g/L glucose (approximately 2/3^{rd} that of blood) (377). In comparison to blood, it has higher sodium, chloride and magnesium and lower potassium and calcium concentrations. The CSF total protein content is 0.5% that of blood, such that the normal range for an adult is 0.15-0.60 g/L (376). It has been demonstrated that there is considerable variability with age, such that it has been suggested that a rule of thumb is that CSF protein is approximately the same as the age of the person in mg/dL. Data from over 6,000 children suggest that in the first 6 months of life the CSF protein is highly variable (0.47 g/L, IQR 0.26-0.65), the upper limits in different age groups are for 6 months-2 years 0.25 g/L, 2-6 years 0.25 g/L, 6-12 years 0.28 g/L, and 12-18 years 0.34 g/L (383). This is suggested to be related to reducing CSF flow rate with increasing age. Furthermore, there are diurnal and rostrocaudal regional differences in total CSF protein.

CSF proteins

The makeup of CSF protein is not well established. Studies of the CSF proteome in healthy people have been performed, however the challenges of CSF sampling restrict the same exponential increase that has been seen for studies of the blood (blood/serum/plasma) proteome, see Figure 5.2 and Table 5.1. It is clear that, like blood, there is a high dynamic range, such that 20-30 proteins constitute 80-90% of the total protein (384). These are considered ‘highly abundant proteins’, mainly blood proteins such as Albumin and IgG, however the rank order of major proteins in CSF is not exactly the same as blood, see Table 5.2. The difference is due to

the selectivity for lower molecular weight proteins entering into CSF (first demonstrated by Klaus Felgenhauer, Figure 5.3 [a] and redrawn by Edward Thompson [b]) and presence of locally released brain proteins (385). It is estimated that 80% of CSF proteins originate in blood, and 20% are brain-derived (386). The latter largely reflect secreted proteins and the extracellular domain of membrane proteins, but may also include proteins released following cell damage or death (387). Extensive analysis of brain tissue, largely based on RNA sequencing (RNA-seq) experiments, suggests that 16,507 of all human protein coding genes (n=20,090) are detected in the human brain (388). Of these, the data suggests that 2,709 genes are classified as brain elevated (normalised transcript expression values in the brain is at least four times the mean of other regions) and 204 genes were only detected in the brain, see Figure 5.4. The rank order of proteins in CSF produced by Stoop *et al.* by LC-MS is presented in Appendix 5.1 (389). To date, the deepest analysis of the CSF proteome to my knowledge is that performed by Macron *et al.* which identified 3,379 proteins (361). Notwithstanding, the complex interplay of serum and brain proteins that constitute the CSF proteome has enormous potential for dysregulation in CNS infections.

Figure 5.2 PubMed records for studies of the CSF and blood proteome over time

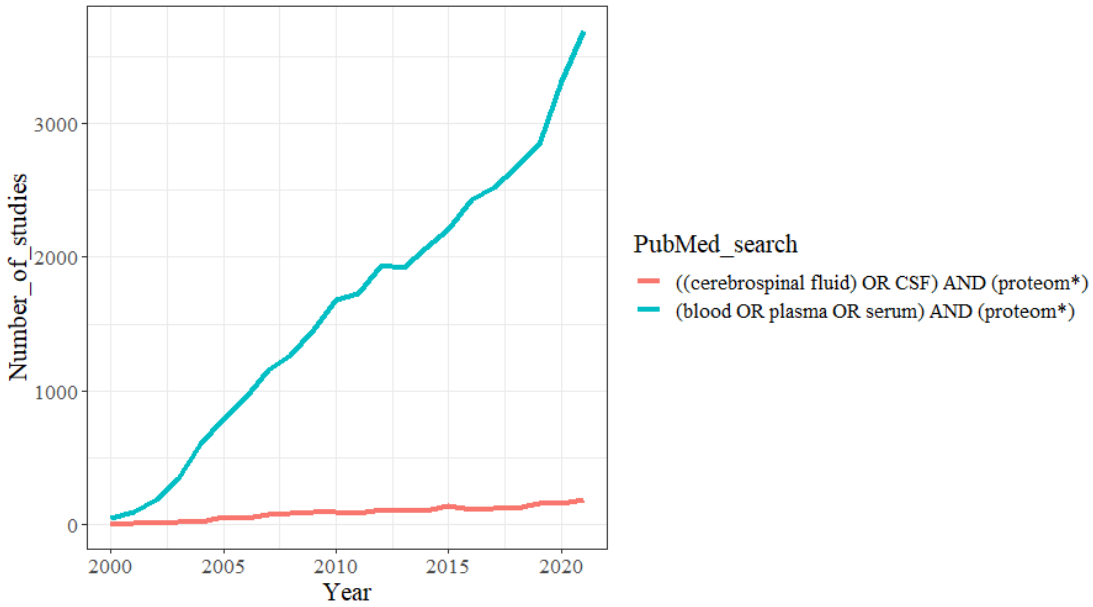


Table 5.1 Proteomic studies of CSF collected from healthy people since 2000, adapted from Macron *et al.*, 2018 (361)

Author and reference	Year of publication	Sample preparation method	LC-MS or MS method	Number of proteins identified
Sickmann *et al.* (390)	2000	2D-gel electrophoresis	MALDI-TOF	21
Yuan *et al.* (391)	2002	Protein precipitation or bio spin column and 2D-gel electrophoresis	MALDI-TOF	22
Sickmann *et al.* (392)	2002	2D-gel electrophoresis	MALDI-TOF	70
Finehout *et al.* (393)	2004	2D-gel electrophoresis	MALDI-TOF	82
Maccarrone *et al.* (394)	2004	Immunodepletion and 2D-gel electrophoresis	2D-LC ion trap (IT)[1]	347
Xu *et al.* (395)	2006	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and SCX-RP–LC	2D-LC IT[1]	466
			2D-LC LTQ-Fourier transform ion cyclotron resonance (FT-ICR)	608
Pan *et al.* (396)	2006	Isolation of N-linked deglycosylated peptides/extraction of glycoproteins	2D-LC IT[1]	216
Zougman *et al.* (397)	2008	SDS-PAGE	1D-LC LTQ-Orbitrap/LTQ-FT-ICR	798
Mouton-Barbosa *et al.* (398)	2010	Peptide ligand library and SDS-PAGE	1D-LC LTQ-Orbitrap	1,212
Schutzer *et al.* (399)	2010	Immunodepletion and SCX-RP-HPLC	1D-LC LTQ-Orbitrap	2,630
Guldbrandsen *et al.* (400)	2014	SDS-PAGE and mixed mode reversed phase-anion exchange for mapping the global CSF proteome, and hydrazide-based glycopeptide capture for mapping glycopeptides	1D-LC LTQ-Orbitrap[2]	3,081
Zhang *et al.* (401)	2015	Immunodepletion and high-pH RP–LC	1D-LC Q-TOF[3]	3,256
Begcevic *et al.* (402)	2016	SCX-LC	1D-LC-Q-Orbitrap[4]	2,615
Macron *et al.* (361)	2018	Immunodepletion, isoelectric focussing fractionation	1D-LC-Orbitrap FL[5]	3,379
Macron *et al.* (403)	2020	Immunodepletion, isoelectric focussing fractionation	1D-LC-timsTOF	3,174

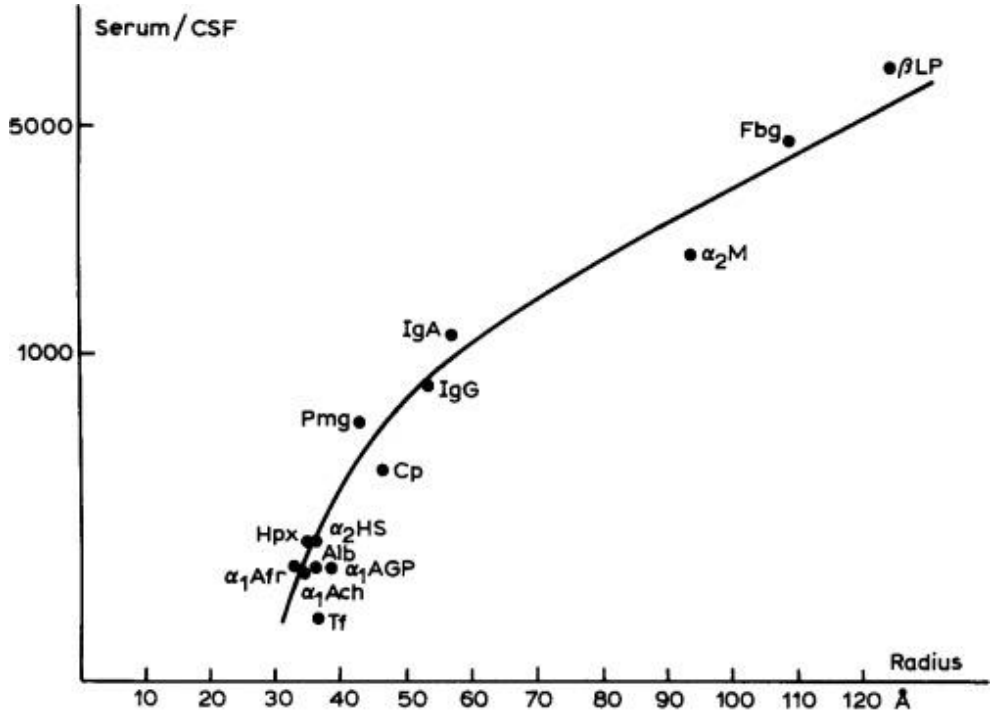
1. LCQ DECA XP-PLUS, 2. LTQ-Orbitrap Velos Pro, 3. Triple TOF 5600, 4. Q Exactive Plus Orbitrap, 5. Orbitrap Fusion Lumos.

Table 5.2 Suggested rank, in order of descending abundance, of the major proteins in CSF vs serum, adapted from Thompson, 2005 (384)

Rank	**CSF**	**Serum**
1	Albumin	Albumin
2	Prostaglandin-H2 D-isomerase	IgG
3	IgG	Apolipoprotein B
4	Transthyretin	Apolipoprotein A
5	Serotransferrin	Fibrinogen
6	Alpha-1-antitrypsin	Serotransferrin
7	Apolipoprotein E	Alpha-1-antitrypsin
8	Cystatin-C	Alpha-2-macroglobulin

Figure 5.3 Molecular selectivity of the blood-brain-barriers.

a. Felgenhauer's original diagram of serum:CSF ratios for 14 highly abundant proteins plotted against molecular size (385)



βLP=Beta lipoprotein, Fbg=Fibrinogen, α_2M=Alpha-2-macroglobulin, IgA=Immunoglobulin A, IgG=Immunoglobulin G, Pmg=Plasminogen, Cp=Caeruloplasmin, α_2HS=Alpha-2 Hermann Schltz, Hpx=Haemopexin, Alb=Albumin, α_1AGP= Alpha-1-acid glycoprotein/ Orosomucoid (ORM), α_1Atr=Alpha-1-antitrypsin, α_1Ach=Alpha-1-antrichymotrypsin, Tf=Tranferrin.

b. Thompson's revised diagram of percentage transfer for the same 14 proteins plotted against molecular size (384).

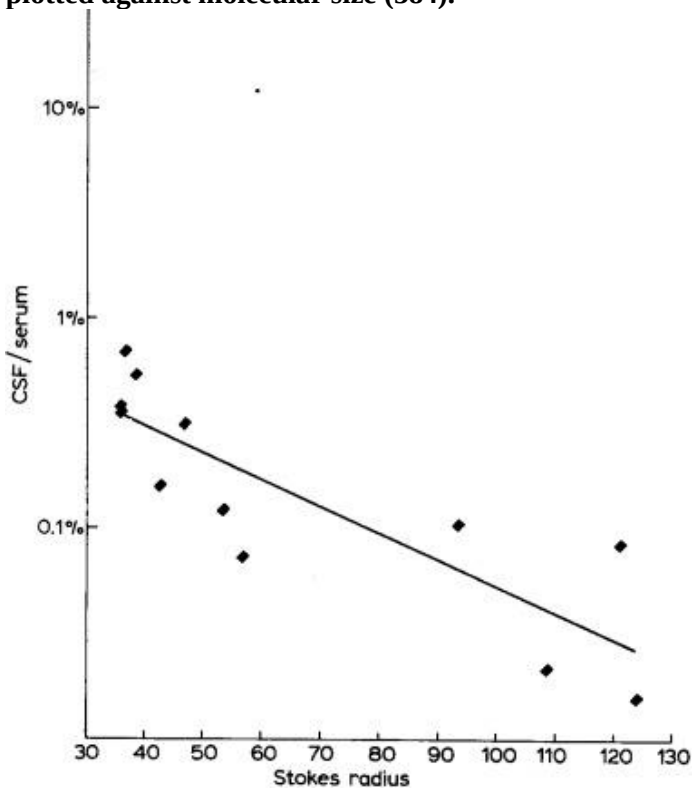
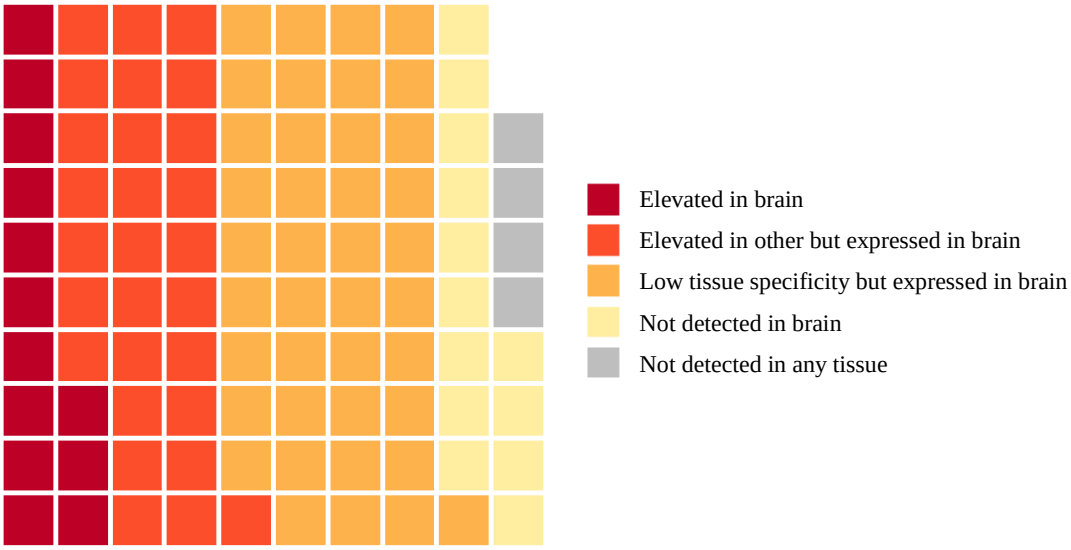


Figure 5.4 Data on brain protein expression from the Human Protein Atlas (388).



The CSF proteome in disease and how this relates to JE

Refining the objective

The ultimate goal for this thesis is to identify 2-3 proteins that could be used in a diagnostic test for JE. However, it is acknowledged that it may not be possible to identify proteins that are pathognomonic of JE. Instead, an RDT could include detection of anti-JEV IgM (this does not exist at the time of writing) together with the detection above a threshold of two proteins with high specificity for JE. The current challenge of JE diagnostics has been elaborated in Chapter 2 and Chapter 3. The possible differential diagnoses (i.e. in geographical areas where JEV is endemic) are wide ranging, and a lengthy but not exhaustive list is presented in Table 5.3. It is not currently possible to make a diagnosis based on clinical presentation, and we rely on laboratory investigations. Important differential diagnoses for JE include DENV and Ot infection (19, 404), which are discussed in more detail below in relation to differentiation from JE.

Table 5.3 Important differential diagnoses for JE (369, 405, 406)

Infections	Bacteria	*Streptococcus pneumoniae*[1]
		Neisseria meningitidis[1]
		Haemophilus influenzae[1]
		Listeria monocytogenes[3]
		Streptococcus agalactiae[3]
		Escherichia coli
		Streptococcus suis[2]
		Mycobacterium tuberculosis[1]
		Brucella spp.[2]
		Klebsiella pneumoniae[3]
		Morganella morganii[3]
		Staphylococcus aureus[3]
		Streptococcus pyrogenes[3]
		Bartonella henselae (cat scratch disease)
		Chlamydophila spp.
		Treponema pallidum
		Borrelia spp.[2]
		Coxiella burnetti
		Erlichia spp.[2]
		Orientia tsutsugamushi[2]
		Rickettsia typhi[2]
		Leptospira spp.[2]
		Salmonella spp.
		Burkholderia pseudomallei (Melioidosis)[2]
		Mycoplasma pneumoniae[3]
	Viruses	Herpes simplex virus 1 and 2
		Varicella zoster virus
		Enterovirus spp.
		SARS-CoV-2
		Human immunodeficiency virus
		Human herpes virus 6[3]
		Human herpes virus 7[3]
		Adenovirus[3]
		Rotavirus[1,3]
		Norovirus
		LCMV
		Cytomegalovirus
		Mumps virus[1]
		Measles[1]

		Influenza virus Respiratory syncytial virus JC virus Dengue virus[1,2] Zika virus[2] Rabies lyssavirus[1,2]
	Fungi	*Cryptococcus neoformans*
	Parasites	*Naegleria fowleri*[2] *Acanthamoeba* spp.[2] *Angiostrongylus cantonensis*[2] *Toxoplasmosis gondii* *Neurocysticercosis* (pig tapeworm larva)[2] *Trypanosoma brucei* (African sleeping sickness)[2] *Plasmodium falciparum* (cerebral malaria)[1,2] *Gnathostoma spinigerum*[2]
Non-infectious conditions	Autoimmune encephalitis Inflammatory disease Cerebrovascular disease Neurodegenerative Malignancy Psychiatric Toxic/Metabolic Epilepsy	

1. Vaccine preventable, 2. Specific geographical distribution, 3. Specific risk groups

Dengue virus (DENV)

DENV is spread by the *Aedes* spp. mosquitos in contrast to *Cx.* spp. mosquitos that transmit JEV (407). Nonetheless, it is hyperendemic in the same geographical areas as JEV. Although it is not a classic neurotropic arbovirus, the very high incidence of DENV of an estimated 100 million per year symptomatic infections means that even 1% associated neurological presentations represents a significant burden of CNS infections (408). Our understanding of the incidence and pathophysiology of DENV neuroinvasion and infection, in contrast to DENV systemic infection with neurological presentations, is not clear (409). Current WHO recommendations advise contemporaneous testing for DENV alongside any case of JE,

including DENV PCR (if available), NS1 and IgM RDT and/or ELISA. While the detection of DENV RNA and NS1 are highly specific, particularly if detected in CSF, they are not frequently detected, and diagnosis relies on anti-DENV IgM that has the same issues as that of anti-JEV IgM. Interpretation relies on imperfect algorithms to support the diagnosis of JE vs DENV (299), for example, when calculating a ratio of the result of JEV/DENV IgM ELISA, a ratio over 1 indicates JE and under 1 DENV.

Analysis of the sequence of DENV isolates from CSF in patients with neurological presentations might provide insight into whether the virus acquires mutations that improve its ability to cause neurological infection. However, in spite of an estimated 1 million patients with DENV neurological presentations, in 2019 there were only two patients with DENV isolated from CSF reported in published literature (an update at the time of writing in May 2022 shows that subsequently three more have been published) (410). Even for these two patients, the red blood cell (RBC) count was not reported and it is possible that the detected virus could have been related to blood contamination associated with a traumatic LP (411). From the Laos CNS infection study, there was a single patient with DENV RNA detected in CSF, however the number of RNA copies was low and sequencing failed.

In the absence of virological characterisation, a systematic review of brain imaging findings in JE vs DENV has recently been published. Neurological infections diagnosed to be in the higher level of categorisation (level 1/2) were more likely to show brain imaging findings similar to JE, such as thalamic lesions. These data suggests that the diagnosis of DENV neurological infection remains inadequate, and that DENV neurological presentations represent a heterogenous group.

These data hold relevance for comparing the CSF proteome in JE vs DENV; it is possible that the rare cases of true DENV neurological infection may have similar CSF findings to JE, however for many patients with DENV infection and neurological manifestations, in whom the underlying pathology may be a systemic insult, cerebrovascular insult or encephalopathy, the

CSF proteome may be sufficiently different to allow identification of diagnostic biomarkers. In particular, it is accepted that DENV is associated with plasma leakage, and one might expect to see more evidence of plasma leakage also into the CSF proteome.

Orientia tsutsugamushi (Ot)

Ot is an obligate intracellular bacterium transmitted via the bite of *Leptotrombidium* mites (chigger) and has an overlapping geographical distribution with JEV, remaining endemic to a region spanning most of Asia to Northern Australia, termed the tsutsugamushi triangle (412). Ot, also termed scrub typhus, is an emerging disease with increasing recognition and diagnosis (413). It must be noted that in contrast to DENV and JE, Ot is potentially treatable with the low cost and widely available antibiotic doxycycline (414).

In the human host, Ot targets endothelial cells and phagocytes for replication leading to pathology in highly vascularised organs such as the brain (415). The word typhus is derived from typhos meaning stupor, and neurological presentations are seen in an estimated 20-50% of patients (416). Diagnosis of Ot also relies on serological tests, IFA and more recently ELISA, that has similar issues as those discussed above for JE and DENV (417-425). There are patchy data on brain imaging in Ot; case reports have described white matter lesions and microhaemorrhages, supporting the underlying pathophysiology of a vasculitic process (426). Mouse models of Ot infection corroborate this picture, with upregulation of markers of endothelial activation (L-selectin, Intercellular adhesion molecule 1, Platelet endothelial cell adhesion molecule, and integrin up-regulation) (415).

Dittrich *et al.* performed an analysis of the BBB function in patients from the Laos CNS infection study, including ELISA detection of tau/MAPT, GFAP, S100b and NSE (427). The study categorised patients into bacterial meningitis, tuberculosis meningitis, JE, Ot and other rickettsial infections. Interestingly, the patients with JE had higher levels of tau/MAPT detected in CSF, suggesting that there are differences in JE vs Ot. Unfortunately, there were no patients

with non-JEV viral infections (e.g. DENV) included in the study. Another study in patients with fever showed that markers of coagulation predict the diagnosis of Ot in serum, however this would need to be investigated in CNS infection patients (428). These data hold promise for more extensive profiling of CSF proteins, as is reported in the next chapter, Chapter 6.

Potential diagnostic protein biomarkers of JE

Broadly, the CSF proteome during a CNS infection could contain pathogen and/or differentially regulated human/host proteins. A pathogen protein would be highly specific, but the low concentrations in CSF mean that these proteins are likely to fall below the limit of detection of any assay. With regards to host proteins, in view that 80% of the CSF proteome represents transfer from the serum, the CSF proteome partially reflects the serum proteome. In an infection, we would expect upregulation of acute phase proteins such as C-reactive protein (CRP), proteins related to liver or renal derangement, activation of the clotting cascade and the immune response. It might seem foolish and convoluted to measure serum proteins such as CRP or procalcitonin in precious CSF samples. However, as described above, the selective entry of low molecular weight proteins, and the complex dynamics of serum proteins entering CSF compared to each other as well as brain proteins may prove to be useful diagnostic biomarkers (429).

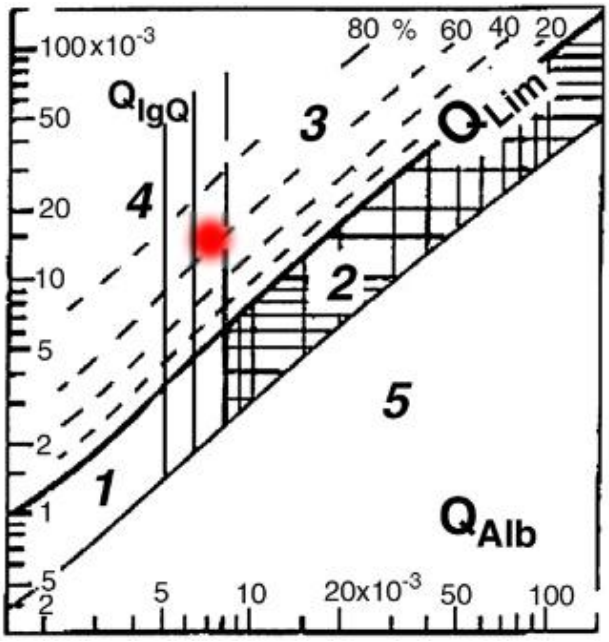
Routine laboratory analysis of CSF relies on the quantitation of total protein in CSF. In viral infections such as JE, the total protein is frequently raised (369). While the total protein in itself does not serve as an accurate biomarker, it suggests that there are alterations in the CSF proteome. Primarily, the increase in total protein is a reflection of BBB damage (380). This BBB damage is also accompanied by an alteration in the selectivity of the BBB, such that the relative amounts of proteins change dependent on other factors such as the protein's size, charge and glycosylation (384).

The ideas raised in the last two paragraphs highlights the important concept of analysing the relative amount of proteins in CSF and serum, and in comparison to each other (382). A number of different terms have been used to investigate this, with suggested terminology defined in Table 5.4, however it must be noted that this is not used consistently in the literature. Hansotto Reiber was one of the first scientists to investigate this, primarily looking at immunoglobulins. He studied 4,300 patient paired CSF and serum samples and proposed the interpretation of whether there is intrathecal immunoglobulin synthesis using a Reibergram, plotting the immunoglobulin quotient against the albumin quotient, see Figure 5.5 reproduced from a publication illustrating the application of a Reibergram for investigating a case of suspected suspected subacute sclerosing panencephalitis (430, 431). Reiber proposed that CSF proteins cannot be studied without looking at their corresponding serum levels. Thompson proposed the term percentage transfer to describe the ratio of the concentration of a protein in CSF to that of the same protein in serum and reported as a percentage (384). The term 'percentage transfer' may be somewhat misleading, as it implies that the amount refers to the proportion of the protein that has been transferred from serum to CSF and this is not the intention. Nonetheless, the utility of the value is clear, particularly as Thompson shows that proteins with 0.4-1% percentage transfer are unlikely to be produced by local brain synthesis. A logical approach that is used today, but not sufficiently widely, is the relative immunoglobulin index. For example, the quotient of a specific pathogen IgG is compared to the quotient of total IgG or better still the quotient of a specific pathogen IgG that is in high abundance but not considered to be secreted intrathecally, e.g. measles IgG in a patient who has been immunised. The subsequent paragraphs describe potential JE diagnostic CSF protein biomarkers in more depth.

Table 5.4 Terms used to describe quantities of CSF proteins, adapted from Thompson 2005 (384).

Ratio	The ratio of the concentration of a protein to the concentration of another protein
Quotient	The ratio of the concentration of a protein in CSF to the concentration of the same protein in serum
Percentage transfer	The percentage ratio of the concentration of a protein in CSF to the concentration of the same protein in serum
Index	The ratio of the quotient of a protein to the quotient of another protein, typically albumin
Relative specific antibody	The ratio of the quotient of a specific immunoglobulin to the quotient of total IgG
Selectivity	The slope of the line derived from plotting proteins against their molecular sizes

Figure 5.5 Reibergram, Winchester and Brown, 2011 (431).



As described in Winchester and Brown, 2011: "A serum/CSF quotient diagram for IgG with hyperbolic curves. The upper hyperbolic curve (thick line) of the reference range represent the discriminant lines between brain-derived and blood derived immunoglobulin fractions in CSF, called Q_{Lim}. The dotted lines above Q_{Lim} represent intrathecal fractions (IF) as percent of total CSF concentration as IgG_{IF}. The limit of the reference range for Q_{Alb} between normal and increased CSF protein concentration (blood-CSF barrier dysfunction) is indicated by the age-dependent vertical lines: these are present at $Q_{Alb}=8x10^{-3}$ (up to 15 years), $Q_{Alb}=6.5x10^{-3}$ (up to 40 years) and $Q_{Alb}=8x10^{-3}$ (up to 60 years). The diagram depicts five ranges: 1. normal; 2. pure blood-CSF barrier dysfunction (i.e. reduced CSF turnover); 3. intrathecal Ig synthesis with a reduced CSF turnover; and 4. Intrathecal Ig synthesis without change in CSF turnover. Values below the lower hyperbolic line, in range 5, suggest a methodological fault. Plotting the data from the patient shows that there is no evidence of increased CSF protein concentration (the Q_{Alb} limit for a 42-year-old woman is $6.8x10^{-3}$). However the Q_{IgG} is greater than Q_{Lim} indicating intrathecal IgG synthesis."

JEV proteins

There are clear advantages in the specificity of a viral protein as a diagnostic biomarker of infection, as compared to a host protein. E and NS1 proteins are potential targets, as these are the proteins entering the extracellular milieu, and are recognised as the principle antigenic proteins with detectable antibodies being anti-E or anti-NS1 (432). E is a 53-54 kDa outer structural glycoprotein that functions in multiple steps of the JEV life cycle, including assembly, budding, attachment and membrane fusion with the target cell (433). NS1 is a multifunctional, 46–55 kDa protein, with molecular weight depending on the extent of glycosylation (434). NS1 exists as a monomer, a dimer (membrane-bound protein, mNS1) and a hexamer (secreted protein, sNS1). Intracellularly, NS1 is involved in viral replication and assembly, whereas the secreted as well as the membrane-bound NS1 play a role in immune evasion and disease pathogenesis (435).

To date, studies performing gel electrophoresis and/or western blotting of CSF samples have not identified JEV proteins (436, 437). Secreted NS1 would be the most likely pathogen protein to reach sufficient levels to represent a diagnostic biomarker of JEV. Furthermore, bioinformatic analysis suggests that NS1 has less cross-reactivity with other flaviviruses than E (432). The detection of dengue NS1 is an established diagnostic marker of DENV infection, as it is specific and sensitive (438). This has not been demonstrated for JEV, with two studies testing JEV NS1 in human samples, of which testing CSF in humans showed a sensitivity of 10.5% and 52%, respectively, in CSF in clinical samples, with the latter study only testing JEV RNA positive samples (279, 280, 439). The low sensitivity could be due to the early elimination of JEV and viral proteins. It is well-established that JEV RNA is rarely detected by the time patients come into hospital (26), and the level of NS1 in patients in whom it was detected was very low (279). However, there may be other important factors. NS1 is a 352 aa long protein that occurs in two forms - a membrane-associated dimer (~49 kDa per monomer), found on the endoplasmic reticulum (ER) surface and the plasma membrane, and a secreted hexamer (52 to 55 kDa per monomer) (20). The masses of the two NS1 forms are different due to differential

glycosylation, and the secreted form has two N-glycosylation sites (in comparison to other viruses in the JEV serocomplex that have a third site). The NS1 was produced using the Nakayama JaOArS982 strains (genotype 3), whereas it is known that genotype 1 is more widespread. These may lead to alternative glycoprotein isoforms, and alternative antibody binding due to sequence mismatch or different protein folding. Equally, there may be post-translational modifications that may result in alternative glycosylation, particularly as the NS1 was produced in Drosophila S2 and C6/36 cells. Lastly, it is known that there is a ribosomal frameshift during the translation of NS1 leading to the production of NS1', suggested to occur in 30-50% of translation events, and caused by a slippery heptanucleotide sequence or 3' pseudoknot structure (440). This leads to the addition of 52 aa after the frameshift (441). This is important, as NS1' is associated with neurovirulence in JEV, and an NS1' is also produced by other viruses in the JEV serocomplex notably WNV (442). NS1' is 55 kDa, as compared to 43 kDa for NS1 (441). NS1' sequence analysis suggests that it may also have additional N- and O-glycan sites that could mask antigenic epitopes and lead to different conformation (443).

Table 5.5 A critical review of studies that have developed ELISA assays for JEV NS1

Kumar *et al*, 2011 (280)	• Development of NS1 capture ELISA • Extracted and amplified the full-length NS1 genomic region from JaOArS982 (genotype 3) in mosquito (C6/36) cell culture. • Cloned and expressed the purified PCR product into His tag pQE-30UA expression vector. Western blot – 44kDa • Injected intravenously into 8-week-old rabbit to produce hyperimmune serum. • Injected intraperitoneally into 4 weeks old BALB/c mice. The mouse with the highest titre of antibodies was chosen to harvest splenocytes. Separated splenocytes were fused with SP2/0 myeloma cells. High-affinity MAB (G7) was selected on the basis of strong positive reactions with recombinant JEV NS1 proteins by ELISA. This was an IgA antibody. • Goat anti-mouse IgG HRP conjugate, then TMB with ELISA plate reader to detect OD. • LOD 1ng/ml • Evaluation in clinical samples: • 120 serum and 80 CSF samples; 101 samples positive for JEV RNA by RT-qPCR • The antigen was detected in 47% of sera and 52% of CSF samples testing positive for JEV RNA by RT-qPCR; 3 samples were false negatives and there were no false positives. For one patient, NS1 could be detected for longer than JEV RNA (up to Day 9 post onset of symptoms). In addition, recombinant NS1 sandwich ELISA did not yield positive results for infected culture fluid (ICF) of the closely related flavivirus and alphavirus members; DENV, WNV, YFV, SLE, Chikungunya, and Ross River viruses. • Note, the reference standard was detection of JEV RNA, but this is rarely detected in clinical practice.
Li *et al*, 2012 (279)	• Development of NS1 capture ELISA • Extracted and amplified full-length NS1, N- and C-terminus regions from Nakayama strain (genotype 3) in Vero cell culture.

	• PCR products inserted into pMT/BiP/V5-His A vector and co-transfected into Drosophila S2 cells. • S/C infection into 1-month-old BALB/c female mice. Splenocytes harvested and fused to SP2/0 myeloma cells. MAbs purified. • NS1 protein migration showed three peaks, one >670 kDa that corresponded to aggregates or a multimer, one between 670 and 158 kDa that corresponded to the hexameric form of NS1, and one between 158 and 47 kDa that corresponded to the dimeric form. • Two MAbs from a panel of 18 were selected because they showed the highest affinity for JEV NS1 hexamer - 3E10 and 8F1 • MAb mapping was performed with NS11–352, and N-terminal NS11–143 and C-terminal NS1244–352 fragments. MAb 3E10 recognized a conformational epitope and that MAb 8F1 reacted with a linear epitope located in the C-terminal fragment of the protein. • MAb 8F1 recognized NS1 in cells infected with JEV, WNV, MVEV, YFV and DEN-2V but not with tick-borne encephalitis virus, whereas 3E10 showed a positive fluorescence only with cells infected with JEV • An NS1 antigen-capture assay with MAb 8F1 as capture antibody and biotin-labelled 3E10 MAb as revealing antibody. • LOD 0.2 ng/ml • Evaluation in clinical samples • JEV NS1 antigen was detected in 10 of 42 IgM-positive serum samples (23.8%) and in four out of 38 IgM-positive CSF samples (10.5%). All were negative for DENV NS1.
Potential for further work	Target JEV genotype 1 Synthesise NS1 in human cells Utilise the secreted hexameric NS1 Utilise a more sensitive assay format such as a Luminex assay(444)

In summary, evidence to date suggests that JEV proteins are not suitable diagnostic biomarkers of JE, mainly due to the low concentrations in human infection. Detection of viral proteins is not the same as designing PCR primers and probes for nucleic acid amplification of a target sequence. For proteins, there are putative genotype variants, secondary structures, post-translational modifications, peptide fragments rather than the entire protein. Nonetheless, in my LC-MS work, I ensured that JEV pathogen sequences were included in the search database, to attempt to detect viral proteins.

BBB dysfunction

The tightly regulated BBB breaks down during systemic inflammation and infection (380, 445). The underlying pathophysiology involves a variety of molecular mechanisms, some of which are pathogen specific. This is accompanied by changes in the CSF proteome (446). There is an increase in the entry of serum proteins, although there are still differentiated changes related to the protein's molecular size and charge. A recent study used an albumin quotient of >9 as a definition of BBB dysfunction/impairment (BBBI), and applied lasso regression modelling to

identify a diagnostic protein signature in CSF of BBBI including Alpha-1-B glycoprotein (A1BG), Afamin (AFAM), Carboxypeptidase B2 (CBPB2), Dermcidin **(**DCD), Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (ENPP2), Alpha-2-HS-glycoprotein (FETUA), Helicase with zinc finger domain 2 (HELZ), Insulin-like growth factor-binding protein 7 (IBP7), Peroxiredoxin-2 (PRDX2), Serum amyloid-p component (SAMP), Transthyretin (TTHY) and Vitamin D-binding protein (VTDB) (446).

For JEV, it is not entirely clear whether BBBI occurs before or after neuroinvasion, but BBBI has been suggested to be associated with upregulation of proinflammatory cytokines, nitric oxide and matrix metalloproteinases (447-449). It has also been recently demonstrated that flavivirus NS1 induces endothelial hyperpermeability in a tissue-specific manner, such that JEV and WNV NS1 induce hyperpermeability in brain endothelial cells (435). While these data support the hypothesis that BBBI could be a factor in alteration of the CSF proteome in JE, this may not represent specific biomarkers for disease. To this end, a study measuring the albumin quotient (referred to by the authors as the albumin index) in JE confirms BBBI, but this did not enable sufficient differentiation in comparison to other important categories of neurological infections (427).

Brain damage

It would be expected that the CSF proteome in JE would also reflect cellular and molecular markers of brain damage (450-455). The brain consists of an approximately equal number of neurones and glia, with glia including astrocytes, microglia, ependymal cells and oligodendrocytes. CSF protein biomarkers of each of these cells have previously been described, see Table 5.6. More specifically, the proteins may provide insight into the underlying pathophysiology - for instance, biomarkers of neurofilaments, intermediate filaments or microfilaments, or biomarkers of synaptic damage or protein aggregation (456-458). Brain enriched proteins may be more informative. Additionally, there is breakdown of the

extracellular matrix. In JEV, there is predilection for neurones rather than glia. This is clear from virus infection in cell culture, in which CPE is seen very quickly in neuronal cell lines. My hypothesis would be that this is reflected in the CSF proteome, with a higher concentration of neuronal markers compared to other CNS infections, and a higher ratio of neuronal markers as compared to glia.

Table 5.6 Cellular and molecular protein biomarkers of brain damage

Cell type	Examples of CSF protein biomarkers
Neurones	Microtubule associated protein tau (tau) Neurone-specific enolase (Gamma-enolase) Neurofilament light
Glia	
- Astrocytes	Glial fibrillary acidic protein
- Microglia	Triggering receptor expressed on myeloid cells 2
- Oligodendrocytes	Myelin basic protein
Endothelial cells	Intercellular adhesion molecule 1 Vascular adhesion molecule 1

The CSF proteome might also reflect the predilection of a pathogen or disease for specific brain regions (459, 460). In JE, there is predilection for the thalamus and basal ganglia, although these are not pathognomonic of the disease (76). I hypothesise that the CSF proteome could reflect pathology in specific brain regions.

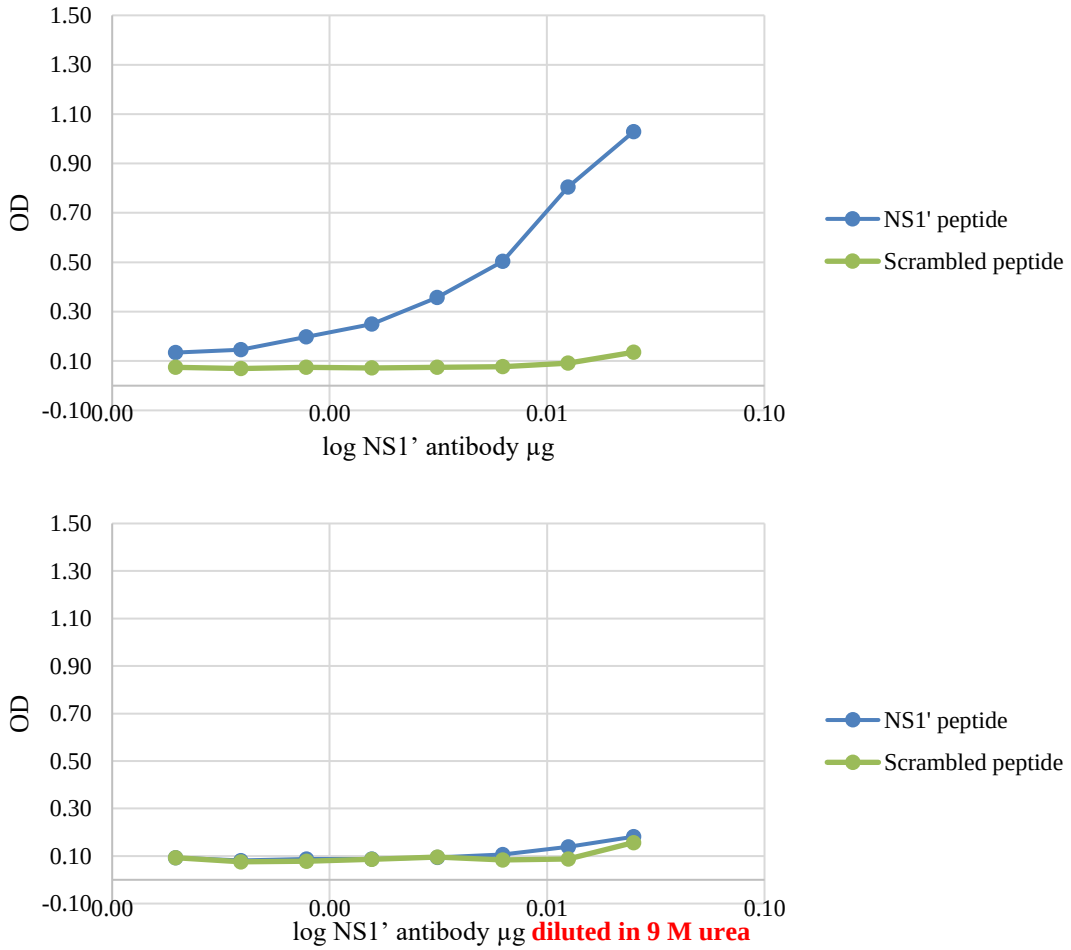
Host response

A plethora of proteins related to the host response would be expected to demonstrate differential expression in patients with JE vs healthy controls. In the course of JE, there is activation of the innate immune response, associated with upregulation of interferon stimulating genes (ISGs), secretion of cytokines and chemokines, infiltration of immune cells and secretion of antibodies (461). There have been a handful of transcriptomics and proteomics studies in cell culture (462-473), mice (471, 474-476) and humans (477) that support this. Functional analysis suggests enrichment in proteins associated with a variety of pathways including apoptosis, heat shock response and ER stress (478, 479).

In current clinical practice, anti-JEV IgM in CSF by ELISA has been the mainstay of diagnosis. These assays frequently detect anti-JEV E antibodies as these are produced in the highest concentration. NS1 has been considered to have less cross-reactivity with other flaviviruses, and also more specificity as anti-NS1 would not be expected to be produced in patients that have received certain types of vaccines (i.e. vaccines other than the live-attenuated vaccine). As discussed above, NS1' is only produced in neurotropic flaviviruses (i.e. not dengue) and so detecting specific antibodies against the 52 aa region only present in NS1' could be a highly specific method of detection.

I developed an ELISA to detect anti-JEV NS1' antibodies, that was specific for NS1' as compared to NS1. Details are shown in Appendix 5.2. The ELISA format involved coating a hydrophilic ELISA plate with a linear sequence of 52 aa from the C terminal end of JEV NS1' Laos 2009 strain genotype 1, i.e. the additional sequence that is not present in NS1. The plate was then blocked and the sample added, followed by anti-IgM/ anti-IgG antibody. The ELISA worked for the positive control, shown in Figure 5.6a, however it did not work for the CSF samples diluted in urea. An additional experiment showed that the addition of 9 M urea to the positive control led to a negative result with the ELISA demonstrating that detection is conformation dependent. As all my CSF samples were diluted in urea this could be the reason for the negative result. I subsequently tested a single serum sample from a patient with JE (kindly provided by Lance Turtle), but this was negative. Due to COVID-19, I was not able to pursue this project, however further work could involve expressing NS1', to perform structural studies to understand its role, to investigate its post-translational modifications (glycan site analysis suggests that there are glycosylation sites on the 52 aa C terminal end of NS1' that could shield it from antibodies and this could be the reason why no antibodies are detected against this stretch of aa), and also to develop an alternative ELISA format in a double-antigen binding assay (DABA) ELISA.

Figure 5.6 Development of an anti-JEV NS1' antibody ELISA using a positive control of an anti-JEV NS1' mouse monoclonal IgG antibody.



A note on contamination

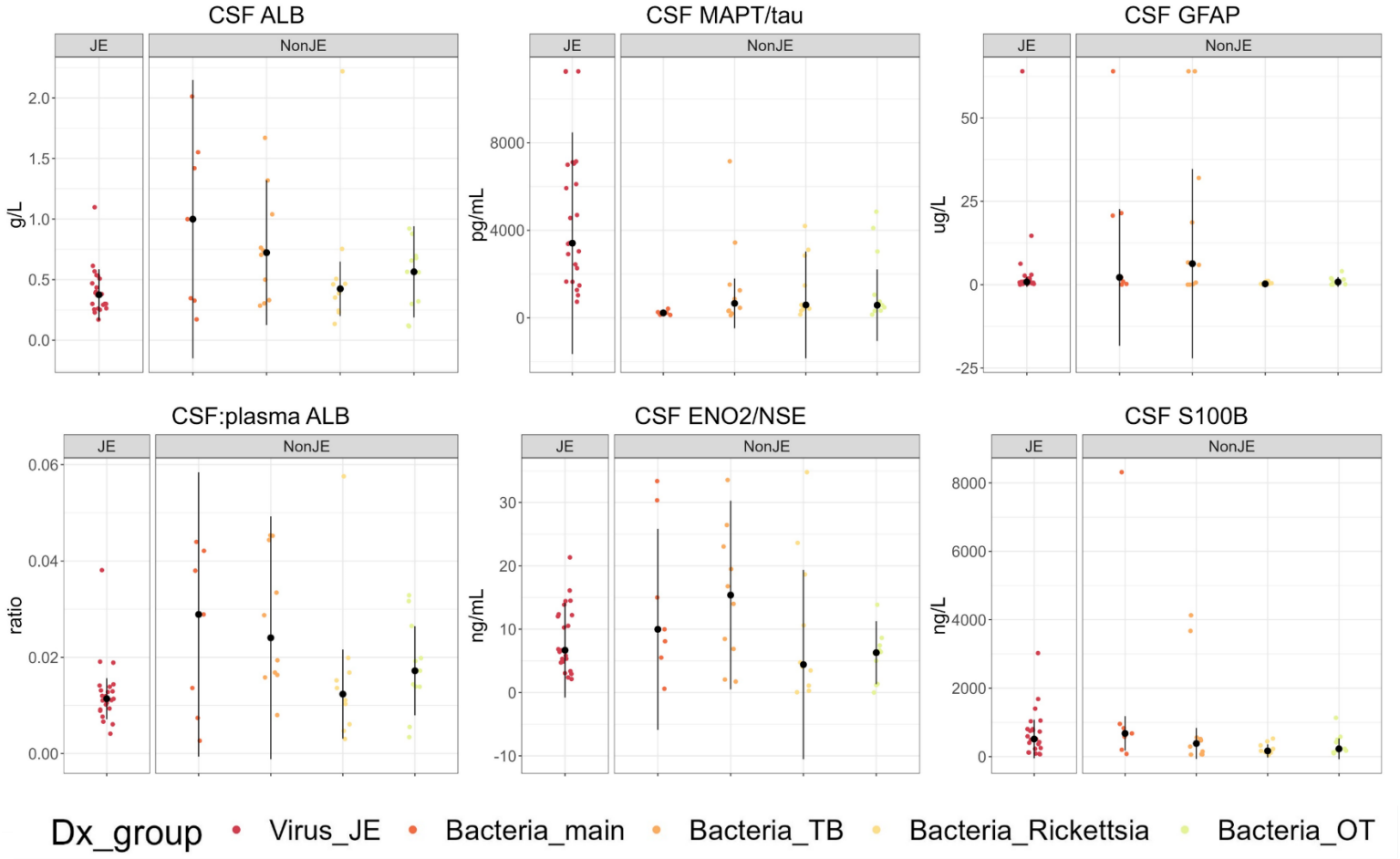
Contamination of CSF by blood during an LP procedure is a relatively common issue. It is suggested that the total cell count is interpreted by considering the LP CSF blood contamination (reduction of 1 leukocyte/µL per 1000 erythrocytes/µL in the CSF). It has also been suggested that the first 2 mL of CSF is not used for CSF proteomics (374), but this is not always easy to follow in practice. Even blood contamination below the limit of detection of RBCs can bias results and needs to be taken into account, including in the interpretation of Reibergrams. This has been studied, and various proteins biomarkers of blood contamination have been identified.

A targeted LC-MS (parallel reaction monitoring, PRM) assay has been developed to detect blood contamination, and urine dipsticks have also been used for the same purpose (480). Additionally, MS experiments are very prone to skin contamination (e.g. keratin), and an exclusion list of potential skin contaminants is frequently applied (481).

An integrative approach to identifying a JE protein signature using data science

I finish this chapter by utilising data to illustrate the summary above, that is, the potential utility of identifying a group of CSF proteins that might together represent a specific diagnostic test for JE. I was kindly provided with the raw data for the study by Dittrich *et al*. published in 2016 (29). In the study, 66 patient samples from the Laos CNS infection cohort were tested for serum and CSF albumin, gamma-enolase/neuron-specific enolase (ENO2/NSE), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B) and tau/MAPT by ELISA, see Figure 5.7, of which 15 patient samples are included in the LC-MS/MS analysis presented in Chapter 6. To this end, these protein biomarkers provide a measure of BBB impairment (albumin index), neuronal damage (tau/MAPT and NSE) and glial damage (GFAP and S100b). I included these data in addition to the patient's age, from the patients with a single confirmed infection and no missing data (n=56), in a random forest model in R, detailed in Appendix 5.3, with results suggesting a 96.7% (95% CI 88.5-99.6) specificity for diagnosing JE. These data provide support to the different strands of the hypothesis, and how an integrated approach provides high specificity.

Figure 5.7 Re-analysis of previously published CSF protein data from the Laos CNS infection study (29)



Albumin index (CSF:plasma albumin) as a measure of blood–brain barrier (BBB) function, the correlation between AI and CSF biomarkers and levels of biomarkers of neurological injury, comparing different types of CNS infection (n=66).

Conclusions

In summary, the CSF proteome during infections, including that in JE, is not well-characterised.

1. Pathogen proteins may be present, most likely secreted proteins or extracellular domains of membrane proteins, however by the time a patient presents to hospital and an LP is performed the concentration is frequently too low to be detected by any method.
2. 80% of the CSF proteome is derived from ultrafiltration of serum, with the caveat that there is some selectivity in the ultrafiltrate for lower molecular weight and potentially acidic and glycosylated proteins. To this end, in an infectious disease, the CSF proteome partially reflects that of serum; there is an upregulation of the acute phase response for example CRP, immune cells and immunoglobulins. An additional complexity is that the BBB may be impaired to different extents in diseases, and the selectivity may also be impaired, such that there may be an increase in all serum proteins, and potentially also in higher molecular weight proteins that would otherwise not have been present.
3. Brain proteins represent a relatively small proportion of the CSF proteome but are important for the prospects of biomarker discovery, to provide insight into the true brain functioning, as a "window into the brain". RNA-seq analysis of brain tissue suggests that 16,507 of all human protein coding genes (n=20,090) are expressed by the brain. However the high dynamic range (difference in concentration between the high abundant proteins such as albumin and IgG and that of most brain proteins) and low abundance of these poses a challenge for research. Brain proteins reflect the various sources; brain, meninges and dorsal root ganglion, and reflect activity of different cells of the brain, predominantly (%) neurones and also (%) glia. The CSF proteome in CNS infections may reflect the host response to infection, cellular death and dysfunction, and potentially also regional brain damage.
4. The complex interplay between these different aspects, and how this manifests *in vivo* in JE, as compared to other CNS infections remains to be determined. Studies of brain imaging, or proteomics studies *in vitro* and in animal models of JE have been performed and provide

preliminary insights. However, much remains to be performed for study in human patients, as an essential piece to the puzzle, and is the subject of Chapter 6.

Publications arising from this chapter

The hypothesis regarding detectable CSF proteins related to brain regions affected in different diseases gave rise to the study question for an MSc student, Thomas Pichl's, LSHTM MSc dissertation project that I supervised and co-authored (76).

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Chapter 6

LC-MS/MS studies to identify protein biomarkers of JE in cerebrospinal fluid

In Chapter 5, I detailed the basis for my hypothesis that there is a detectable diagnostic protein signature of JE in patient CSF samples. This chapter encompasses a rigorous experimental analysis of this hypothesis. The method involved tandem or MS2 or MS/MS, that is, there is fragmentation of peptides inside the mass spectrometer. For this reason, I use the more specific abbreviation LC-MS/MS in this chapter and Chapter 7. I describe the methodology and results of LC-MS/MS experiments to identify protein biomarkers of JE in CSF. This includes preliminary unfractionated label-free experiments, pilot DDA experiments using TMT-11plex labelling, larger DDA experiments using multiplex TMT-16plex labelling and DIA verification. DIA analysis was label free and not subject to the same batch effects seen with TMT-DDA data. Data analysis is performed using conventional methods such as differential expression of proteins as well as network analysis and machine learning.

Introduction

In Chapter 4 and Chapter 5, I introduce proteomics methods that have been applied to the study of CSF. LC-MS methods are unrivalled in the capacity to perform de novo peptide sequencing and untargeted analysis. A review of previous studies on LC-MS methods for analysing CSF samples presented in Chapter 4 demonstrated the diversity of methods that are used (395, 482-513). Frustratingly, it is not possible to compare the processing pipelines as they each involve multiple steps: sample collection, processing, offline separation, online separation and mass spectrometry. However, it is evident that there are issues with existing LC-MS methods for application in clinical proteomics analysis. Notably, the cost, sensitivity, dynamic range,

reproducibility and throughput are major limitations and experimental approaches typically involve trade-offs between each other. For instance, increasing sensitivity enables deeper probing of the proteome, with associated increasing detection of the interesting low abundant brain-derived proteins. Higher sensitivity might be obtained by large sample volumes, immunoaffinity depletion of the most highly abundant proteins, extensive multiple dimensional fractionation (e.g. performing successive LC separation using orthogonal methods) and long online LC gradients coupled to the most sensitive mass-spectrometers (e.g. the Orbitrap Eclipse Tribrid). However, a single sample separated into 100 fractions and analysed with four-hour gradients would take seven days to run and cost approximately £50,000 in the university's proteomics facility. Furthermore, in view of the diversity of the host response, analysis of the host proteome requires a relatively large sample size (482).

My colleagues had already performed a pilot study of 6 JE patient CSF samples and 4 healthy CSF samples as controls, and identified approximately 500 proteins using label-free LC-MS/MS methods, summarised in Appendix 6.1 (unpublished data Gangadharan *et al.* 2017). I systematically interrogated the different possibilities for each step in the experimental process to improve the sensitivity while considering the cost and throughput implications and performed preliminary work summarised in Table 2.1 in the results and Appendix 6.2-6.4. The samples that I used were already denatured in 9 M urea, to inactivate any pathogens and render the sample acellular, and could not be processed by immunodepletion or immune-enrichment. Although, on balance, there is evidence that immunodepletion leads to the loss of low abundant brain-derived proteins and additional sample processing steps may affect downstream quantitation (483, 484). Whilst I had a relatively large volume of CSF per patient sample (50 µL), the total protein content that could be used depended on the protein concentration of the samples. Multiple offline (prior to LC-MS/MS) separation methods have been described, including both gel-based and in-solution methods. Gel-based methods are more traditional and have been used frequently (485-491), however they are laborious, less reproducible, eliminate proteins of molecular weight <10 kDa and >120 kDa, may lead to the loss of proteins that could

be valuable biomarkers; there is also the suggestion that they lead to fewer protein identifications than in-solution methods (492). I did perform preliminary work investigating protein concentration methods using molecular weight cut-off (MWCO) filters and gel based methods prior to label-free LC-MS that are in keeping with previous literature, presented in Appendix 6.3. I chose to focus on in-solution methods, although there are important roles for gels, for instance in studying different protein isoforms or post-translational modifications. The choice of offline in-solution separation was more difficult, for instance whether to perform a single method (one-dimensional, 1D) or multiple successive orthogonal methods (two-dimensional, 2D or three-dimensional, 3D), that is, one that was based on separating on the basis of molecular weight, the other by isoelectric point or hydrophobicity. My analysis of the literature suggested that there was no clear evidence of the advantage of one over another (395, 482, 486, 493-509), and after a trial of separation with HILIC detailed in Appendix 6.2, I decided to perform a method that is relatively established, high pH reverse phase (RP) fractionation (502, 505, 510-513). Online low pH RP fractionation is widely used, and I optimised a 2-hour gradient; although considering equilibration and washes the total run time was 171 minutes. I was fortunate to have access to a Q Exactive benchtop hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific) "QE classic", the basic orbitrap instrument, with good sensitivity and throughput, capacity to analyse TMT labelled samples, but admittedly not with the highest performance. It was not fast enough for reliable DIA analysis that could improve coverage, the proportion of the proteome that is detected (514). For these reasons, it was clear that I needed to perform TMT labelling, as the LC-MS/MS throughput would have severely limited the number of samples that could be analysed. TMT 11-plex allowing multiplexing of 11 samples was released at the beginning of my DPhil in 2018, and TMT 16-plex (TMTpro) in 2020. However there has been a body of criticism of TMT labelling, including reports that it leads to false positives due to co-fragmenting peptides causing distortion of peptide quantitation termed 'ratio compression', dependence on top n peptide fragmentation and accumulation of technical variance during peptide labelling (515, 516). In

view of these potential issues, as a verification step, I processed 10% of the samples by DIA in collaboration with the Fisher laboratory at the Target Discovery Institute, University of Oxford. Proteomics data analysis is an evolving field and presents additional challenges beyond those discussed above related to the laboratory methods (517-519). Network science and machine learning are two complementary disciplines enabling insights into complex high dimensional data (520, 521). Networks, comprised of nodes and links, are naturally attuned to problems where features have a relational structure (522) and have a track record of success in understanding networks of biological interactions (523) . On the other hand, machine learning can uncover signals in data related to outcome variables and identify predictive markers of disease, a vital exploratory process for constructing diagnostics (524). Used in conjunction, network science and machine learning provide novel characterisation of disease states and can identify robust predictive markers of disease (525).

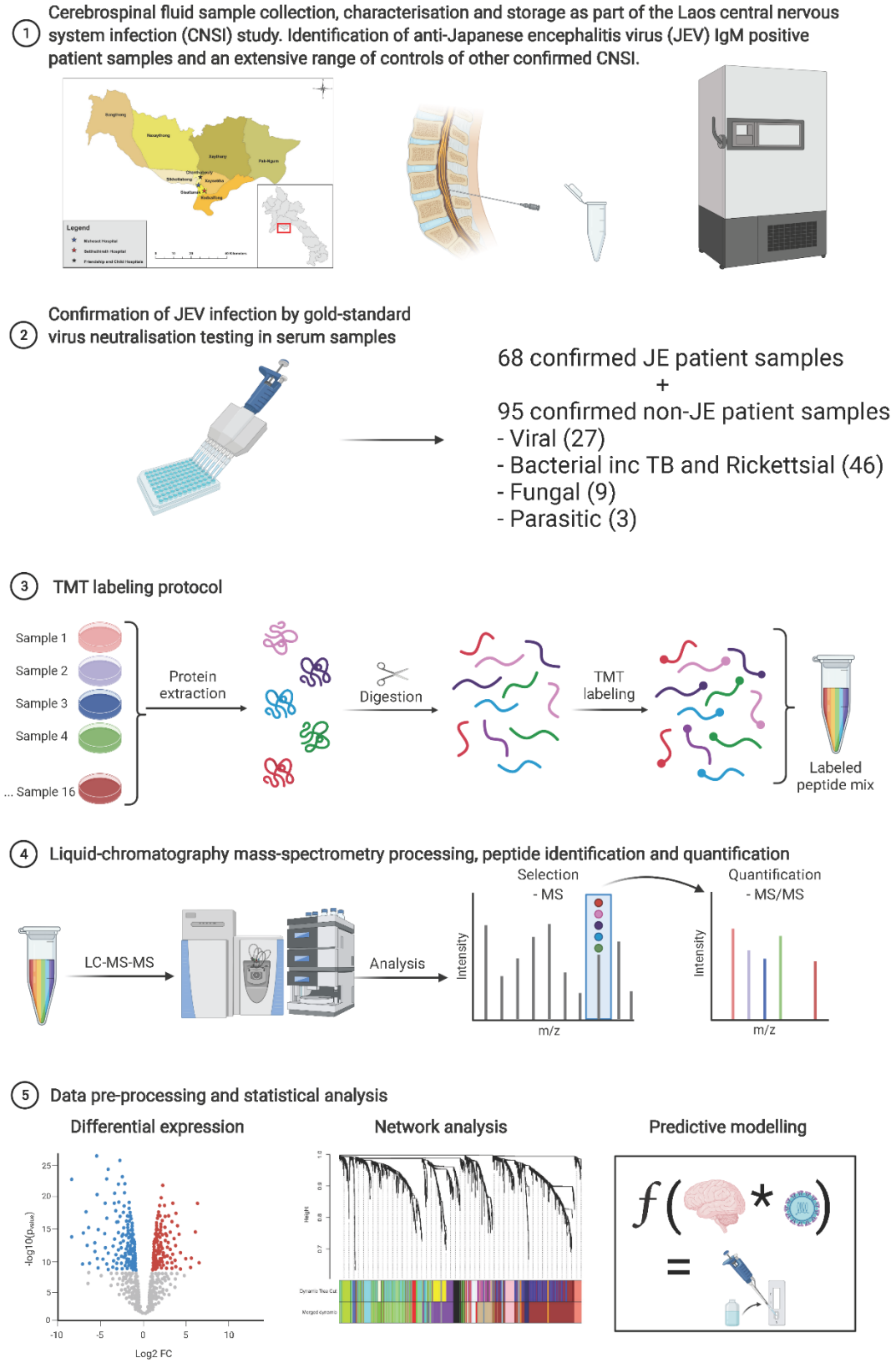
In this chapter, I aimed to test the hypothesis that there is a diagnostic protein signature of JE by performing LC-MS/MS analysis of samples from patients recruited as part of the Laos CNS infection study, incorporating differential expression, network and machine learning analysis. A subsidiary aim was to utilise the data in the same workflow to evaluate proteins associated with outcome of JE. I first performed a pilot feasibility study with TMT 11-plex (n=15) and then in a larger verification study with TMT 16-plex (n=148) including a sample size based on a power calculation. These data were combined in the final analysis. The results were verified by performing DIA LC-MS/MS in 16 (10%) of the samples.

Materials and methods

Patient samples

A prospective study of CNS infection has been conducted at Mahosot Hospital, Vientiane, Laos, since 2003. Methods and results from 2003-2011 have been described (293). Patients from 2014-2017 were part of the SEAe study (294). Inpatients of all ages were recruited for whom diagnostic lumbar puncture was indicated for suspicion of CNS infection because of altered consciousness or neurologic findings and for whom lumbar puncture was not contraindicated. There was no formal definition for CNS infection; patient recruitment was at the discretion of the responsible physician, reflecting local clinical practice. The laboratory also receives samples from patients from other hospitals in Vientiane City; Friendship, Children's and Setthathirat Hospitals. Written informed consent was obtained from patients or responsible guardians. Ethical clearance was granted by the Ethical Review Committee of the Former Faculty of Medical Sciences, National University of Laos and the Oxford University Tropical Ethics Research Committee. The confirmed aetiology was determined by the results of a panel of diagnostic tests which included tests for the direct detection of pathogens in CSF or blood, specific IgM in CSF, seroconversion, or a 4-fold rise in antibody titre between admission and follow-up serum samples (293). Pathogen detection was confirmed after critical analysis of test results to rule out possible contamination. Japanese encephalitis virus infection was confirmed, as recommended by WHO, by detection of anti-JEV IgM by MAC-ELISA in CSF or seroconversion in paired serum samples. All anti-JEV IgM positive samples were subsequently confirmed by the gold standard virus neutralisation assay as previously described in (303). Power analysis was performed to estimate the sample size that would be required using different values. A schematic representation of the study methods is illustrated in Figure 6.1.

Figure 6.1 Schematic representation of the study methods



LC-MS/MS sample preparation

CSF samples were diluted 1:5 in 9 M urea and vortexed intermittently at room temperature for 30 minutes, to solubilise and denature proteins, inactivating any pathogens and rendering the sample acellular. Protein concentration was assessed with a Nanodrop assay ND-1000 spectrophotometer (Thermo Scientific) by measuring the absorbance at 280 nm, normalised by aliquoting different volumes of each sample dependent on the protein concentration, and then the total volume equalised with 7.5 M urea (total volume = x µL). An equal volume (x µL) of 100 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate (AmBic) was added as a reducing agent, and the samples vortexed and incubated at 56˚C for 45 min. Twice the volume (2x µL) of 100 mM iodoacetamide (IAA) in 50 mM AmBic was added as an alkylating agent, vortexed and incubated at room temperature for 1 hr in the dark. 50 mM AmBic was added (3.5x µL) to each sample to reduce the urea concentration to below 1M. Digestion was performed with trypsin in a ratio of 1:20 m:m protein:trypsin (Promega, P/N V5072 for the pilot study; V5117 for the larger study); first 75% of the total amount of trypsin was added and incubated at 37°C for 18 hours overnight and then the remaining 25% was added and incubated at 37.5°C for 6 hours. The samples were frozen at -20˚C to quench the trypsin digestion reaction. A pooled aliquot of each sample was analysed by label-free LC-MS/MS to verify protein digestion.

RP C18 solid phase extraction (SPE) was used to desalt the digested proteins, as per the manufacturers' instructions (Waters P/N WAT023590 for the pilot study; Thermo Scientific P/N 60109-001 for the larger study). The total eluate was dried completely using a vacuum concentrator (Savant SpeedVac or Eppendorf concentrator) and for the samples to be labelled by TMT, resuspended in 100 mM triethylammonium bicarbonate (TEAB). The samples were vortexed, centrifuged, sonicated for 3 min, and then this was repeated. The Pierce Quantitative Colorimetric Peptide Assay (Thermo Scientific, UK) was performed as per the manufacturer's instructions. The samples were normalised for peptide concentration with TEAB to make up a final volume of 100 mL required for TMT labelling. TMT labelling was performed as per the

manufacturer's instructions, in two batches of TMT 11-plex (Thermo Scientific, P/N A37724) for the pilot study and ten batches of 16-plex (Thermo Scientific, P/N A44520) for the larger study. For the larger study, in order to examine technical variability and adjust for batch effects, each batch contained one reference pool and the batch 9 and 10 had two replicate samples. A pooled sample was analysed by LC-MS/MS to verify labelling efficiency.

Offline high pH reverse-phase fractionation

For the pilot study, offline high pH RP fractionation was performed using a Hypersil Gold column (Thermo Scientific, P/N 25002-202130). The mobile phase A was water adjusted with ammonium hydroxide to pH 10 and B was 10 mM AmBic in 80% acetonitrile (ACN) adjusted with ammonium hydroxide to pH 10 and a flow rate of 300 µL/min. The samples were separated into 91 fractions with each fraction collected every 60 seconds from the start of the run, using the gradient shown in Appendix 6.5. For the larger study, offline high pH RP fractionation was performed using an Xbridge BEH C18 column (Waters P/N 186006710). The mobile phase A was water adjusted to pH 10 with ammonium hydroxide and B was 90% ACN adjusted to pH 10 with ammonium hydroxide, at a flow rate of 200 µL/min. Fractions were collected every 60 seconds from the start of the run (100 fractions) and then concatenated into 44 fractions using the gradient shown in Appendix 6.5. The samples analysed by DIA LC-MS/MS were not processed by offline fractionation.

Liquid-chromatography mass-spectrometry

Online peptide desalting was performed with a Dionex Ultimate 3000 nano ultra-high-performance liquid chromatography (UHPLC) (Thermo Scientific) using 100% of loading mobile phase A = 0.05% trifluoroacetic acid (TFA) in water at a flow rate 10 µL/min for 4.6 min. The online desalting column (trap column) used was a C18 column (Thermo Scientific P/N 160454). At 4.6 min the flow from the nano pump was diverted to the trap column in a

backward flush direction. For online low-pH RP fractionation, as the trapped peptides were eluted from the column over the gradient time specified in Appendix 6.5. In view of the fact that TMTpro was relatively new, there was limited published data evaluating LC-MS/MS methods for TMTpro labelled peptides, and for this reason I performed optimisation work described in the Appendix 6.6. For the pilot study, Accucore C18 columns (Thermo Scientific P/N 16126-507569) were used with a nano source, at a flow rate of 250 µL/min. For the larger study, EASY-Spray PepMap C18 columns (Thermo Scientific P/N ES903) were used with an EASY-Spray source, and a flow rate of 300 µL/min. Mobile phase A was 0.1% FA and B was 0.1% FA in 80% ACN. MS was performed with a Q Exactive benchtop hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific), the settings are described in detail in Appendix 6.5. For the CSF samples processed by DIA LC-MS/MS, samples were analysed using a Dionex Ultimate 3000 nano UPLC (Thermo Scientific) coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). Briefly, peptides were trapped on a PepMap C18 trap column (Thermo) and separated on an EasySpray column (50cm, P/N ES803, Thermo) over a 60-minute linear gradient from 2% buffer B to 35% buffer B (A: 5 % DMSO, 0.1 % formic acid in water. B: 5% DMSO, 0.1% formic acid in ACN) at a flow rate of 250 nL/min. The instrument was operated in data-independent mode as previously described (526).

Data processing and statistical analysis

A flowchart summarising the data processing methods is presented in the results section in Figure 6.2 and the R code for the sample size calculation and data processing is included in Appendix 6.7.

Power calculation: The sample size was estimated using a power calculation based on a t test and multiple testing correction, with data from the pilot study and the R package 'FDRsampsize' (527).

Protein identification, quantification, missing value imputation and batch correction: Thermo raw files were imported into Proteome Discoverer v2.5 (Thermo Scientific, UK) for peptide identification using the SEQUEST algorithm (528) searching against the SwissProt *Homo sapiens* and pathogen databases according to the included samples with precursor mass tolerance 10ppm and fragment mass tolerance 0.02 Da. Carbamidomethylation of cysteine, TMT at N-termini and lysine residues were set as fixed modifications, and oxidation of methionine was set as a variable modification. False discovery rate (FDR) estimation was performed using the Percolator algorithm (529). The criteria for protein identification included FDR < 1%, ≥ 2 peptides per protein, ≥ 1 unique peptide per protein, ≤ 2 missed cleavages and ≥ 6 and ≤ 144 amino acids peptide length, coisolation threshold < 50%, average S/N threshold >10 and at least 2 channels with quantification data. Protein quantification was performed in R v 4.1.2 with the package 'MSstatsTMT' (530). Proteins with >50% missing data were removed and the data were imputed with the package 'DreamAI' (517). To incorporate peptide count per protein, jitter was added proportional to 1/median peptide count for each protein. The pilot and larger study data were merged, normalised with the package 'RobNorm' (518) and then batch correction was performed with the function ComBat (531) in the package 'sva' without modifiers as covariates (532). The protein list was filtered to remove potential contaminant proteins from the skin or red blood cells, see Appendix 6.8 for the list of proteins removed and Additional material 6.3 for the combined processed LC-MS/MS data.

Differential protein expression: Differential expression analysis between the protein abundance in the JE vs non-JE (confirmed CNS infections other than JE) patient samples was performed using a t test and Benjamini-Hochberg correction for multiple testing. FC cut-off of >1.2 or <0.8 was used, however the final selection of proteins for validation was based on machine-learning methods.

Network analysis: Weighted correlation network analysis (WGCNA) was performed using the package 'WGCNA': constructing a signed weighted co-expression network with a soft power threshold of 12 to produce a power distribution, that is, scale-free topology; applying

hierarchical clustering to detect modules of highly interconnected proteins with a minimum module size of five, deepSplit 4 and merge threshold 0.3; classifying intramodular hub proteins as the five proteins with the highest module membership for each module; and then correlating the modules with patient sample data (533).

Feature selection and predictive modelling: This was implemented with the Boruta algorithm (using the random forest classifier) using the package 'Boruta' (534) and with Lasso (least absolute shrinkage and selection operator) regression using the package 'glmnet' (521, 535). A final list of proteins based on the intersection between Boruta and Lasso were selected (536). Classification of JE vs non-JE was performed using machine learning with the entire data set, proteins selected by Boruta, Lasso, the intersect between Boruta and Lasso and with the top two proteins identified by feature selection. Four different machine learning models were used (random forest, support vector machine, logistic regression and naïve bayes) and then an ensemble model was built incorporating all the models and using the package 'caret' and 'caretEnsemble') (537). Models were trained using tenfold cross-validation repeated ten times evaluated on AUC-ROC.

An analysis of feature importance was performed to identify proteins that best predicted the outcome (alive/ died) in JE patients, however due to the small sample size this was considered an exploratory analysis. Feature selection was performed with Boruta and Lasso, and then five-fold cross-validation performed on the entire dataset using different machine learning models.

Protein involvement in biological, molecular and cellular processes was explored using gene ontology using the webserver STRING (538), the R package 'WebGestaltR' 0.4.4 (539), and tissue expression correlated with the Human Protein Atlas (HPA) (540, 541).

DIA data processing: For robustness, final verification was performed on 10% of the samples independently processed via a separate mass spectrometry pipeline using label-free DIA LC-MS/MS. Patient CSF samples were selected from the TMT labelled LC-MS/MS cohort that had remaining volume, were less than ten years old (collected from 2012), had no red cells, and

there was matching of the age and sex between JE cases and non-JE controls. DIA data were analysed using DIA-NN software (v0.8) with the library-free approach as previously described (542), using the default settings as recommended. Briefly, for the library-free processing, a library was created from human UniProt SwissProt database (downloaded 24/2/21 containing 20,381 sequences) using deep learning. Trypsin was selected as the enzyme (1 missed cleavage), with carbamidomethylation of C as a fixed modification, oxidation of methionine as a variable modification and N-term M excision. Identification and quantification of raw data were performed against the in-silico library applying 1% FDR at precursor level and match between runs (MBR). The DIA-NN 'report.proteingroup' matrix output was further analysed. Missing values were imputed with half the minimum value for each protein. These data were used as a test set in the predictive model for the diagnosis of JE. In view of the small numbers of JE patients included in the test set and missing outcome data for these patients, this was not used to test the predictive model for JE outcome.

Results

Patient data

Power analysis was performed to estimate the sample size that would be required to compare differential expression of proteins in JE vs non-JE using different values: with 1000-3000 biomarkers to be tested, 50-150 finally verified, effect size 0.8, power 90%, false discovery rate <5%, the total sample size with an equal number of JE cases and non-JE controls was 122. Overall, including the pilot and larger study, 163 patients were included – 68 JE and 95 non-JE, see Table 6.1, Additional material 6.1 and Appendix 6.9.

JE patients were confirmed by the assays with the highest diagnostic confidence; detection of JEV RNA, or detection of JEV IgM in CSF or by seroconversion and confirmed by VNT. Non-

JE patients included a range of different categories of infection that are common in the region. None of the patients had known dual infections. Details of patient demographics, clinical presentations, laboratory investigations and outcome are reported in Table 6.1, Additional material 6.1 and Appendix 6.9.

Table 6.1 Summary of patients' demographics, clinical presentations and details of diagnosis

		Virus_ JE (N=68)	Virus_ NonJE (N=27)	Bacteria_ main (N=32)	Bacteria_ TB (N=7)	Bacteria_ Rickettsia (N=5)	Bacteria_ OT (N=12)	Fungi (N=9)	Parasite (N=3)	Total (N=163)
Age (yrs)	Median	16	22	38	53	48	20	33	25	22
	IQR	10.0, 23.2	6.0, 35.0	20.0, 55.0	32.5, 55.5	32.0, 53.0	11.8, 24.0	27.0, 47.0	24.5, 42.0	12.0, 41.5
Sex	Female	24 (35.3%)	10 (37.0%)	9 (28.1%)	1 (14.3%)	1 (20.0%)	4 (33.3%)	0 (0.0%)	0 (0.0%)	49 (30.1%)
Ethnicity	Lao loum	35 (51.5%)	25 (92.6%)	23 (71.9%)	7 (100.0%)	5 (100.0%)	11 (91.7%)	8 (88.9%)	3 (100.0%)	117 (71.8%)
	Lao sung	16 (23.5%)	1 (3.7%)	3 (9.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	20 (12.3%)
	Lao theung	1 (1.5%)	0 (0.0%)	1 (3.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (1.2%)
	Lao unspecified	16 (23.5%)	1 (3.7%)	5 (15.6%)	0 (0.0%)	0 (0.0%)	1 (8.3%)	1 (11.1%)	0 (0.0%)	24 (14.7%)
Comorbidities	N-Miss	0	0	0	0	0	1	0	0	1
	Yes	3 (4.4%)	5 (18.5%)	3 (9.4%)	4 (57.1%)	1 (20.0%)	0 (0.0%)	5 (55.6%)	0 (0.0%)	21 (12.9%)
	HIV	0 (0.0%)	3 (11.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	5 (55.6%)	0 (0.0%)	8 (4.9%)
Duration of illness (days)	Median	4	3	3	6	2	5.5	5	1	4
	IQR	3.8, 5.2	1.0, 5.5	2.0, 5.2	3.0, 7.0	2.0, 4.0	2.8, 8.2	2.0, 7.0	0.5, 4.0	2.0, 6.0
Seizures	Yes	29 (42.6%)	9 (33.3%)	6 (18.8%)	0 (0.0%)	2 (40.0%)	3 (25.0%)	1 (11.1%)	0 (0.0%)	50 (30.7%)
Antibiotics prior to LP	N-Miss	13	1	3	2	0	3	3	0	25
	Yes	44 (80.0%)	15 (57.7%)	12 (41.4%)	4 (80.0%)	2 (40.0%)	8 (88.9%)	1 (16.7%)	1 (33.3%)	87 (63.0%)
GCS	N-Miss	7	0	1	0	0	1	0	0	9
	Median	11	14	12	10	10	15	15	15	12
	IQR	8.0, 13.0	9.5, 15.0	10.0, 15.0	8.5, 12.5	7.0, 14.0	14.0, 15.0	12.0, 15.0	15.0, 15.0	9.0, 15.0
Blood WCC (10^6/uL)	N-Miss	9	1	5	0	0	1	0	0	16
	Median	12.8	11.3	12.8	11.5	8.3	13	8.4	7.5	11.7
	IQR	8.4, 17.4	7.8, 12.9	9.4, 17.5	8.6, 12.1	7.0, 13.0	10.0, 13.6	4.8, 14.6	7.4, 8.9	8.1, 15.1
Blood CRP (mg/L)	N-Miss	50	11	18	5	1	5	3	1	94
	Median	45	45.1	184.5	20.3	41.6	102.5	33.5	9.8	46.7
	IQR	24.7, 73.1	4.5, 93.1	106.3, 196.5	16.5, 24.0	3.9, 103.4	33.6, 131.3	10.6, 45.5	5.1, 14.6	19.4, 112.6
CSF opening pressure (cm of H_20)	N-Miss	11	3	0	0	0	1	0	0	15
	Median	23	20	23.5	24	15	23	25.5	27	22
	IQR	18.0, 26.0	16.2, 27.4	11.4, 32.8	18.8, 28.0	11.5, 20.0	20.0, 25.0	12.0, 38.0	22.5, 28.0	16.8, 28.2
CSF colour	N-Miss	4	1	3	0	0	4	0	1	13

	Clear	50 (78.1%)	18 (69.2%)	6 (20.7%)	6 (85.7%)	3 (60.0%)	7 (87.5%)	6 (66.7%)	1 (50.0%)	97 (64.7%)
	Red	1 (1.6%)	3 (11.5%)	1 (3.4%)	0 (0.0%)	1 (20.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	6 (4.0%)
	Turbid	13 (20.3%)	4 (15.4%)	20 (69.0%)	1 (14.3%)	0 (0.0%)	0 (0.0%)	3 (33.3%)	1 (50.0%)	42 (28.0%)
	Yellow	0 (0.0%)	1 (3.8%)	2 (6.9%)	0 (0.0%)	1 (20.0%)	1 (12.5%)	0 (0.0%)	0 (0.0%)	5 (3.3%)
CSF WCC (10^6/L)	N-Miss	3	3	2	1	2	1	1	0	13
	Median	110	147.5	655	192.5	25	60	140	500	150
	IQR	50.0, 215.0	36.2, 255.0	206.2, 1573.8	98.8, 316.2	17.5, 35.0	47.5, 132.5	32.5, 260.0	492.5, 1115.0	51.2, 340.0
CSF % neutrophils	N-Miss	3	3	3	1	2	2	1	0	15
	Median	25	29	92.7	30.2	89	67	93.6	72	45.5
	IQR	9.3, 48.4	12.4, 62.8	74.0, 97.2	21.8, 76.6	69.5, 94.5	58.9, 78.0	75.2, 100.0	66.5, 85.0	15.0, 89.6
CSF % lymphocytes	N-Miss	3	3	3	1	2	2	1	0	15
	Median	75	71	7.3	69.8	11	33	6.4	28	54.5
	IQR	51.6, 93.3	37.2, 87.6	2.8, 26.0	23.4, 78.2	5.5, 30.5	22.0, 41.1	0.0, 24.8	15.0, 33.5	10.4, 85.0
CSF RCC (10^6/L)	N-Miss	2	0	3	0	0	0	0	0	5
	Median	0	0	0	0	75	14.5	5	75	0
	IQR	0.0, 0.0	0.0, 27.5	0.0, 135.0	0.0, 12.5	5.0, 1005.0	0.0, 37.5	0.0, 18.0	37.5, 85.0	0.0, 19.5
CSF total protein (mg/dL)	N-Miss	6	1	1	0	1	2	0	0	11
	Median	60	58.5	111	128	72.5	93.5	39	96	76.5
	IQR	38.2, 98.8	30.5, 117.5	57.5, 259.5	97.5, 270.0	60.0, 75.2	57.8, 139.5	20.0, 88.0	59.0, 108.0	38.8, 120.0
CSF glucose (mmol/L)	N-Miss	4	2	2	0	1	2	0	0	11
	Median	3.7	3.3	3	2	5.1	3.4	2.6	2.9	3.4
	IQR	2.9, 4.7	1.9, 4.9	1.7, 5.3	1.4, 4.6	3.7, 5.8	3.1, 6.5	1.8, 2.9	2.6, 4.2	2.3, 4.8
Duration of admission (days)	N-Miss	34	3	5	0	1	3	3	0	49
	Median	13.5	8.5	11	20	9.5	5	19.5	8	11
	IQR	10.2, 17.0	7.0, 12.2	6.0, 16.0	8.0, 21.5	6.5, 12.0	5.0, 8.0	14.2, 27.8	7.5, 10.0	7.0, 16.0
Outcome	N-Miss	26	3	7	0	1	2	5	0	44
	Died	9 (21.4%)	2 (8.3%)	7 (28.0%)	1 (14.3%)	0 (0.0%)	1 (10.0%)	0 (0.0%)	0 (0.0%)	20 (16.8%)

Proteins

5,069 proteins were identified, including 4,805 human proteins and 265 pathogen proteins, see Additional material 6.2 for MSstatsTMT output for the pilot and larger studies. The pathogen proteins were bacterial or parasitic proteins. 2,244 human proteins were identified in more than half of the samples included in both the pilot and larger studies. 68 proteins deemed to be contaminants were removed from the list, see Appendix 6.8, resulting in a filtered list of 2,176 proteins. The number of proteins identified by the final workflow, used for the pilot and verification TMT-labelled studies described in this chapter, in comparison to preliminary experiments, is summarised in Table 6.2.

Twelve proteins were deemed not to have been previously detected by proteomics methods in neXtProt, a comprehensive online knowledge platform on human proteins developed by the Swiss Institute of Bioinformatics (543-545). Six proteins had only been identified by transcriptomics data and the presence of the other six proteins were deemed to be uncertain. However, the peptides associated with the proteins identified in the study did not fulfil the stringent criteria of neXtProt (two unique peptides over nine aa), using their online peptide checker tool, to conclude that they had been identified.

Differential expression

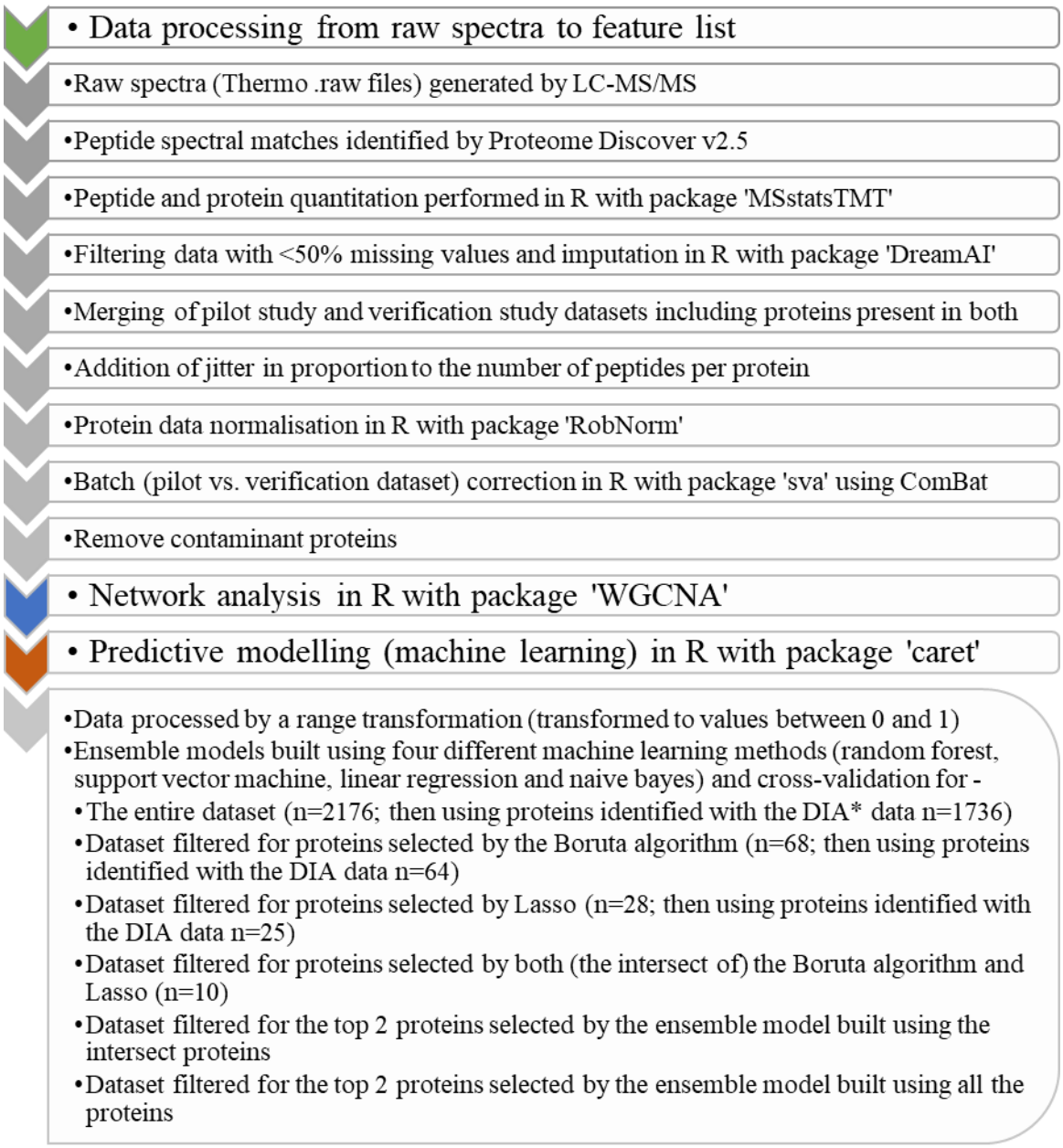
268 proteins showed differential expression (167 > 1.2 fold change, FC, and 101 <0.8 FC) based on the performance of a t test and Benjamini Hochberg multiple testing correction with p value <0.05, illustrated by the volcano plots in Figure 6.3.

Table 6.2 Summary of preliminary LC-MS/MS analysis of CSF samples used to establish the final methods

Date	Samples	Volume - CSF-urea 1:5 - CSF	Label-led	Separation	Number of proteins identified (MaxQuant[1])	No. of potential biomarkers[2]	Details
Aug 2017[3]	10; 6 JEV RNA samples vs 4 healthy CSF samples[4] 771, 782, 913, 917, 1326, MC 3604 BC531CSF-1 to 4	8 µL 1.3 µl CSF	No	1D; online low pH LC	549	N/A (only healthy CSF tested as controls)	Appendix 6.1
Nov 2018	10; 4 JEV RNA, 4 JEV IgM and 2 other confirmed CNS infections (*Streptococcus pneumococcus* and DENV) 771, 782, 913, 917, 181, 642, 1044, 1126 819 and 600	50 µL 8.3 µL CSF	TMT	3D; HILAC, high pH-RP and online low pH LC	710[5]	43	Appendix 6.2
Jan 2019	3; 3 healthy CSF samples[4] BC531CSF-1 to 3	500 µl 83.3 µL; gel electrophoresis and 2 bands cut out per sample	No	2D; 10 kDa MWCO[6] filter, in-gel and online low pH LC	162 Concentration increased the number of proteins identified– Median number of proteins identified for neat, ultrafiltrate and concentrate 25, 22 and 94.	N/A (only healthy CSF tested)	Appendix 6.3
Jan 2019	15; 4 JEV RNA, 4 JEV IgM, Ot, *Rickettsia typhi*, *Cryptococcus neoformans* (HIV negative), *Leptospira* spp., DENV, *Leptospira* spp., *Enterovirus* spp. 771, 782, 913, 917, 181, 642, 1044, 1126, 524, 553, 667, 796, 819, 820 and 1027	12 µL 2 µL CSF	No	1D; online low pH LC	762	Relative quantitation; none clearly seen/not in JE	Appendix 6.4
Mar 2019	15; 4 JEV RNA, 4 JEV IgM, Ot, Ot, *Cryptococcus neoformans* & Cytomegalovirus, *Leptospira* spp., DENV, Cytomegalovirus & *Leptospira* spp., *Enterovirus* spp. 771, 782, 913, 917, 181, 642, 1044, 1126, 305, 688, 704, 796, 819, 848 and 1027	100 – 400 µL ~75 µL CSF normalised by protein content	TMT	2D; High pH RP and online low pH LC	1808	137	Pilot TMT labelled study, Chapter 6

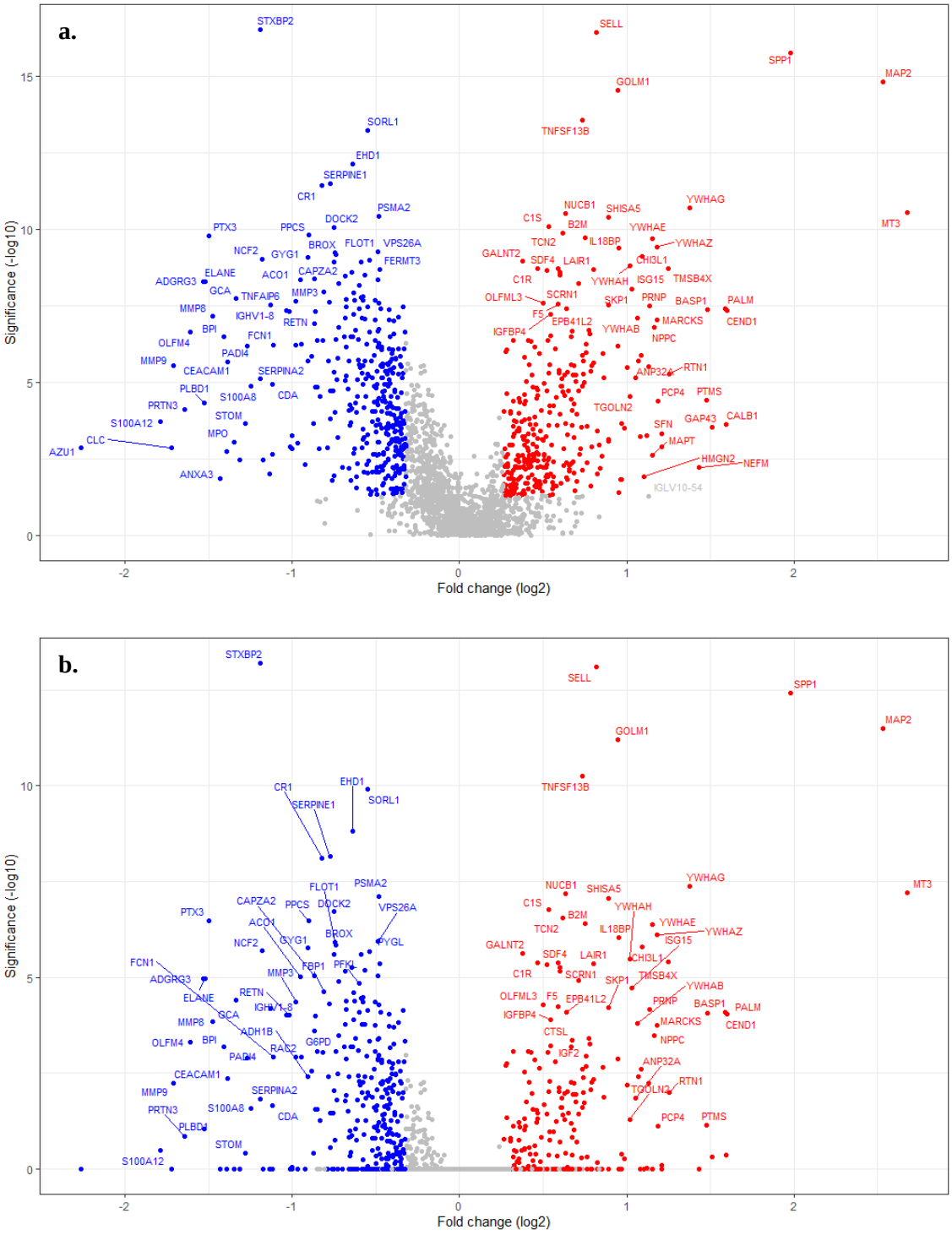
1. MaxQuant is an open-source software that was used in this comparison for consistency. Search criteria for identification included FDR <0.01, at least one unique peptide, at least 2 peptides, less than 3 missed cleavages; Peptide length (amino acids) ≥ 6 and ≤ 144. Subsequently, I was able to access Proteome Discoverer that has additional specifications for TMT-labelled data.
2. Potential biomarkers defined as differentially expressed in JE vs non-JE confirmed CNS infections (significant difference in the median normalised protein abundance with Mann Whitney p value < 0.05)
3. Performed prior to the commencement of my DPhil in the Zitzmann laboratory by Bevin Gangadharan and Abhinav Kumar.
4. Five healthy CSF samples were purchased commercially from the U.S.A. with minimal details of characteristics of the people sampled or the process of obtaining the sample.
5. HILIC = Hydrophilic interaction liquid chromatography. 3D fractionation was performed including HILIC, high pH RP and online low pH RP separation. This would be expected to increase the number of identified proteins, however there were poor peak shapes suggesting that the method would require additional optimisation. TMT labels add to the hydrophobicity of peptides, and HILIC separates by hydrophobicity.
6. MWCO= Molecular weight cut-off.

Figure 6.2 Flowchart for LC-MS/MS data analysis



*DIA data was processed using a pipeline developed in the Fischer laboratory DIA data were analysed using DIA-NN software (v0.8) with the library-free approach as previously described (42), using the default settings as recommended. Briefly, for the library-free processing, a library was created from human UniProt SwissProt database (downloaded 24/2/21 containing 20,381 sequences) using deep learning. Identification and quantification of raw data were performed against the in-silico library applying 1% FDR at precursor level and match between runs (MBR). The DIA-NN 'report.proteingroup' matrix output was further analysed. Missing values were imputed with half the minimum value for each protein.

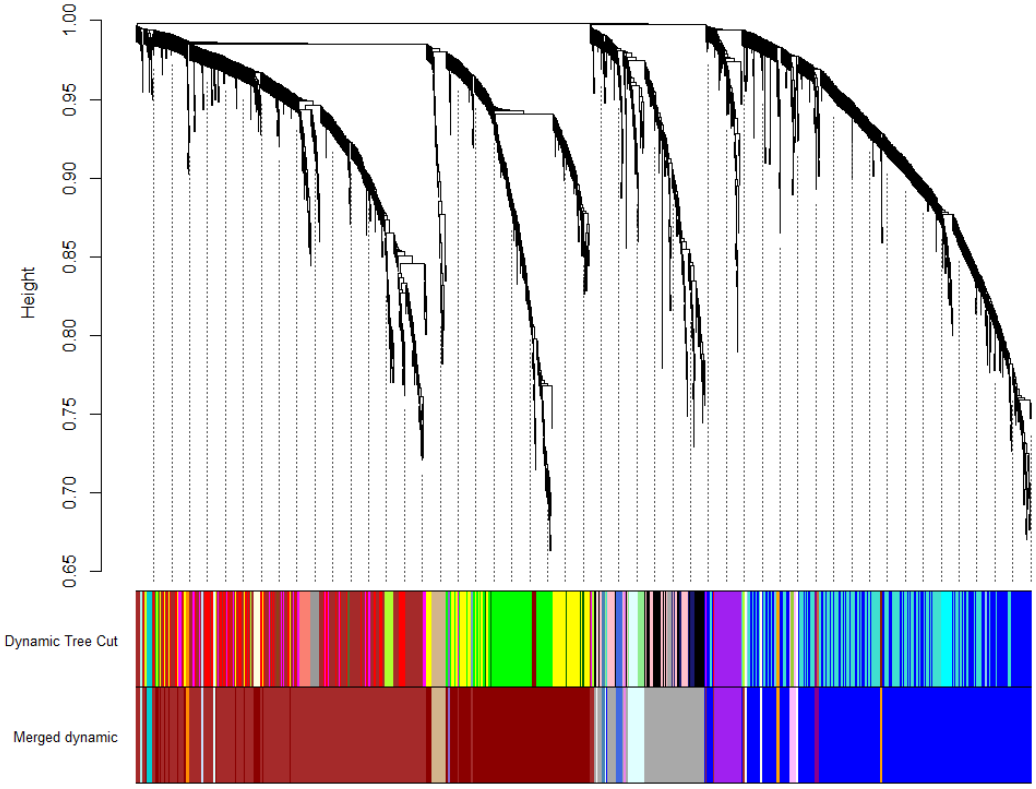
Figure 6.3 Volcano plots of the identified proteins illustrating the statistical significance (t test p values, a. uncorrected and b. corrected) against the magnitude of change (fold change) for JE vs non-JE neurological infections.



Network analysis

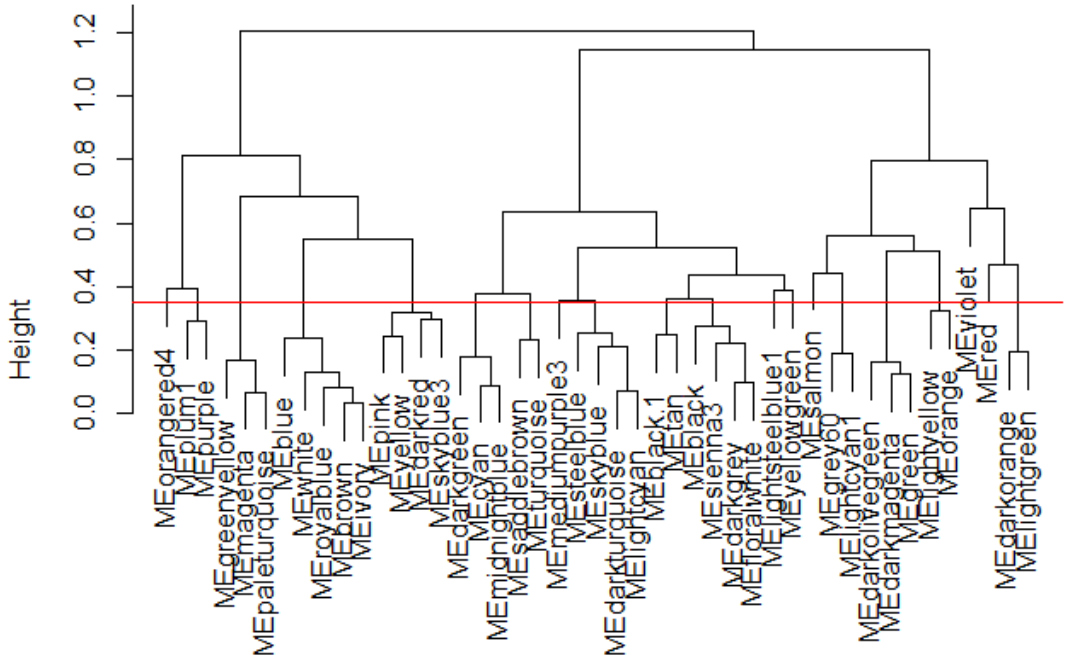
2176 proteins from 163 patient samples were used to build a weighted correlation network. A single outlier was identified, see Appendix 6.10, and removed. Further analysis revealed that this sample had higher overall protein abundances, in spite of peptide normalisation prior to TMT labelling and downstream normalisation in MSstatsTMT and RobNorm during data processing. 44 modules were identified, and then closely related modules merged into 20 modules, see the tree diagram illustrating the cluster dendrogram in Figure 6.4, modules in Figure 6.5 and WGCNA output in Additional material 6.4. Module-trait relationships are shown in Figure 6.6 and Additional material 6.5; suggesting that 15 modules were associated with JE (p value < 0.05), 9 upregulated (red) and 6 downregulated (green). 10 of the modules included proteins in the top five intramodular proteins, that is proteins with the highest modular membership, with significant differences in abundance between the JE and non-JE group.

Figure 6.4 Weighted correlation network analysis cluster dendrogram



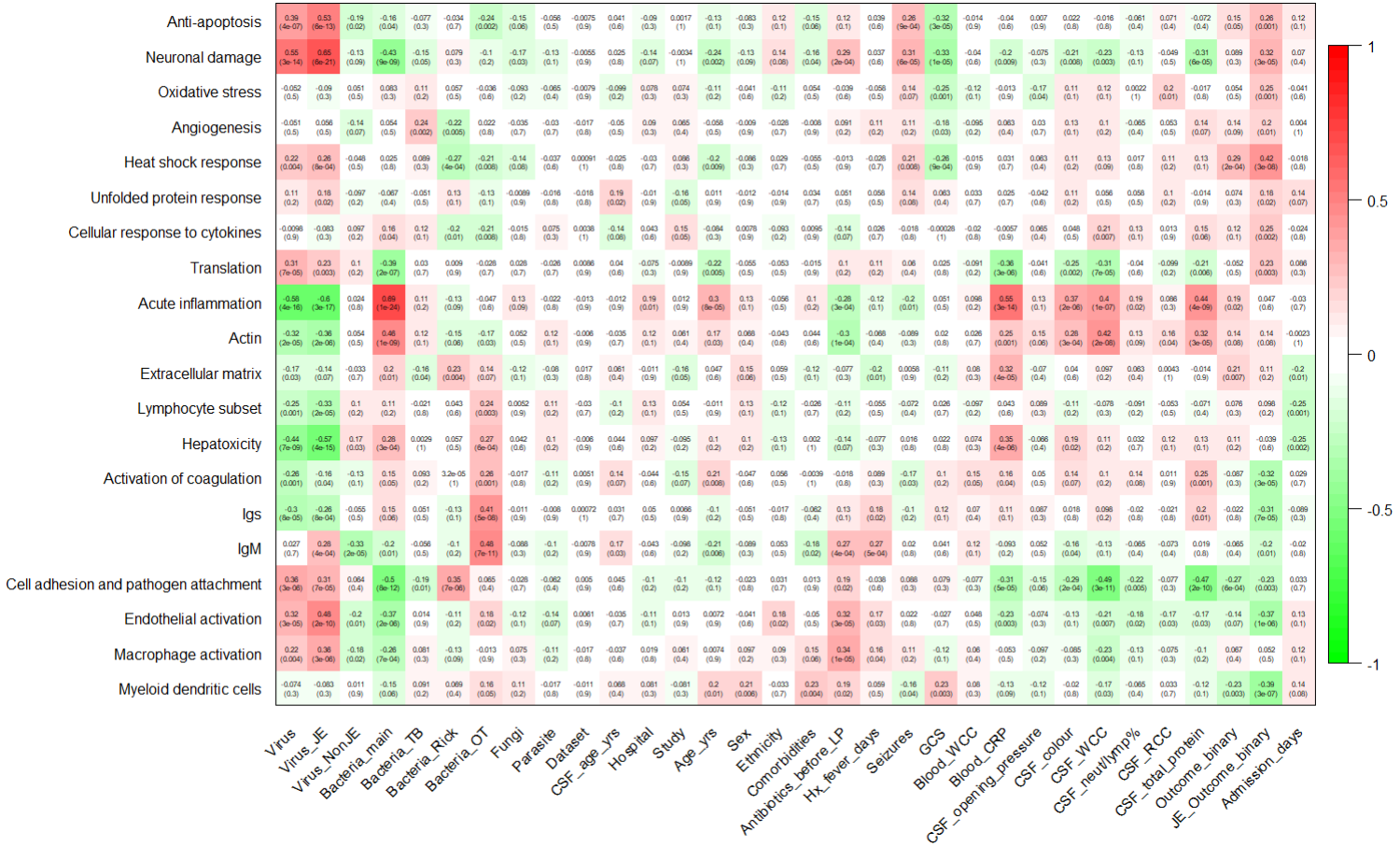
Hierarchical cluster tree illustrating modules identified by WGCNA. Leaves in the tree represent proteins and the major tree branches form 20 modules, each identified by a unique colour.

Figure 6.5 Weighted correlation network analysis clustering of module eigengenes.



Dendrogram of clustering of the modules. The red line in the figure indicates the threshold for merging modules together, here the threshold was 0.3.

Figure 6.6 Weighted correlation network analysis module-trait relationships.



Heatmap of the correlation of modules with patient tratis. darkred=anti-apoptosis, red=neuronal damage, sienna3=oxidative stress, orangered4=angiogenesis, yellowgreen=heat shock response, yellow=unfolded protein response, darkgreen=cellular response to cytokine, floralwhite=translation, darkolivegreen=acute inflammation, paleturquoise=actin, salmon=extracellular matrix, mediumpurple3=lymphocyte subset, plum1=hepatotoxicity, darkorange=activation of coagulation, greenyellow=Igs, skyblue3=IgM, brown=cell adhesion and pathogen attachment, magenta=endothelial activation, pink=macrophages, royalblue=myeloid dendritic cells.

Feature selection and predictive modelling

Diagnostic protein signature of JE

Feature selection: In total, 86 proteins were identified by at least one of the feature selection procedures as important in classifying JE vs non-JE; 68 proteins identified with the Boruta algorithm and 28 with Lasso, see Additional material 6.6. The proteins were associated with 11 different WGCNA modules, all of which had been identified as associated with JE through WGCNA. 48 were upregulated and 38 downregulated in comparison to other neurological infections. Functional enrichment analysis in STRING demonstrated interactions between the proteins,

Figure 6.7. Gene ontology analysis highlighted overexpression of proteins related to apoptosis and downregulation of proteins related to neutrophil degranulation, Appendix 6.11. 22 proteins were secreted proteins: Immunoglobulin lambda variable 3-9 (IGLV3-9), Immunoglobulin heavy variable 3-74 (IGHV3-74), Golgi membrane protein 1 (GOLM1), Cathepsin L (CTSL), CEA cell adhesion molecule 8 (CEACAM8), Phospholipase B domain containing 1 (PLBD1), Cerebellin 1 precursor (CBLN1), Secreted phosphoprotein 1 (SPP1), Natriuretic peptide C (NPPC), tau/MAPT, Chitinase 3 like 1 (CHI3L1), ISG15 ubiquitin like modifier (ISG15), Interleukin 18 binding protein (IL18BP), Beta-2-microglobulin (B2M), TNF superfamily member 13b (TNFSF13B), Bactericidal permeability increasing protein (BPI), Pentraxin 3 (PTX3), Matrix metallopeptidase 9 (MMP9), S100 calcium binding protein A12 (S100A12), Azurocidin 1 (AZU1), Olfactomedin 4 (OLFM4) and Matrix metallopeptidase 8 (MMP8). 15 proteins were associated with increased expression in the brain: Brain abundant membrane attached signal protein 1 (BASP1), Aldolase, fructose-bisphosphate C (ALDOC), CBLN1, Metallothionein 3 (MTX3), MAP2, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (YWHAG), Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein eta (YWHAH), MARCKS like 1 (MARCKSL1), Secernin 1 (SCRN1), SPP1, tau/MAPT, CHI3L1, Paralemmin (PALM), Reticulon 1 (RTN1), Purkinje cell protein 4 (PCP4), Cytidine/uridine monophosphate kinase 2 (CMPK2), NPPC, Glial fibrillary acidic protein (GFAP), Cell cycle exit and neuronal differentiation 1 (CEND1). Thus, three proteins were secreted and showed an increased expression in the brain: SPP1, tau/MAPT, CHIL3, NPPC and CBLN1.

JEV has a predilection for the thalamus and substantia nigra of the basal ganglia (303). One of the proteins was ‘group enriched’ in the thalamus, MMP9, from the HPA database. Four proteins were associated with the GO term ‘substantia nigra development’, associated with BASP1, Glucose-6-phosphate dehydrogenase (G6PD), YWHAH and 14-3-3 protein epsilon (14-3-3epsilon). The HPA database includes mRNA expression data from 13 brain regions,

including the basal ganglia and thalamus; substantia nigra expression on its own is not reported (https://www.proteinatlas.org/humanproteome/brain).

Nine of the 86 proteins are FDA approved drug targets: MAP2, tau/MAPT, MMP9, Proteasome 20S subunit beta 1 (PSMB1), Mitogen-activated protein kinase 1 (MAPK1), AXL receptor tyrosine kinase (AXL), Cytidine deaminase (CDA), Thioredoxin reductase 1 (TXNRD1), TNF superfamily member 13b (TNFSF13B).

Figure 6.7 STRING functional protein association network

STRING network functional clustering analysis of proteins identified by feature selection of important in the prediction of JE vs. other confirmed neurological infections.

Predictive modelling: Feature selection identified a final set of 10 proteins which together exhibited high predictive performance (Figure 6.8). When examined using the ensemble model, using ten-fold cross validation, JE classification demonstrated an AUC-ROC of 97.4 (96.2-98.5), in addition to high sensitivity and specificity – metrics in Table 6.3, Table 6.4, Table 6.5 and Appendix 6.12.

Figure 6.8 Differential expression across samples in ten proteins as a diagnostic signature of Japanese encephalitis virus infection

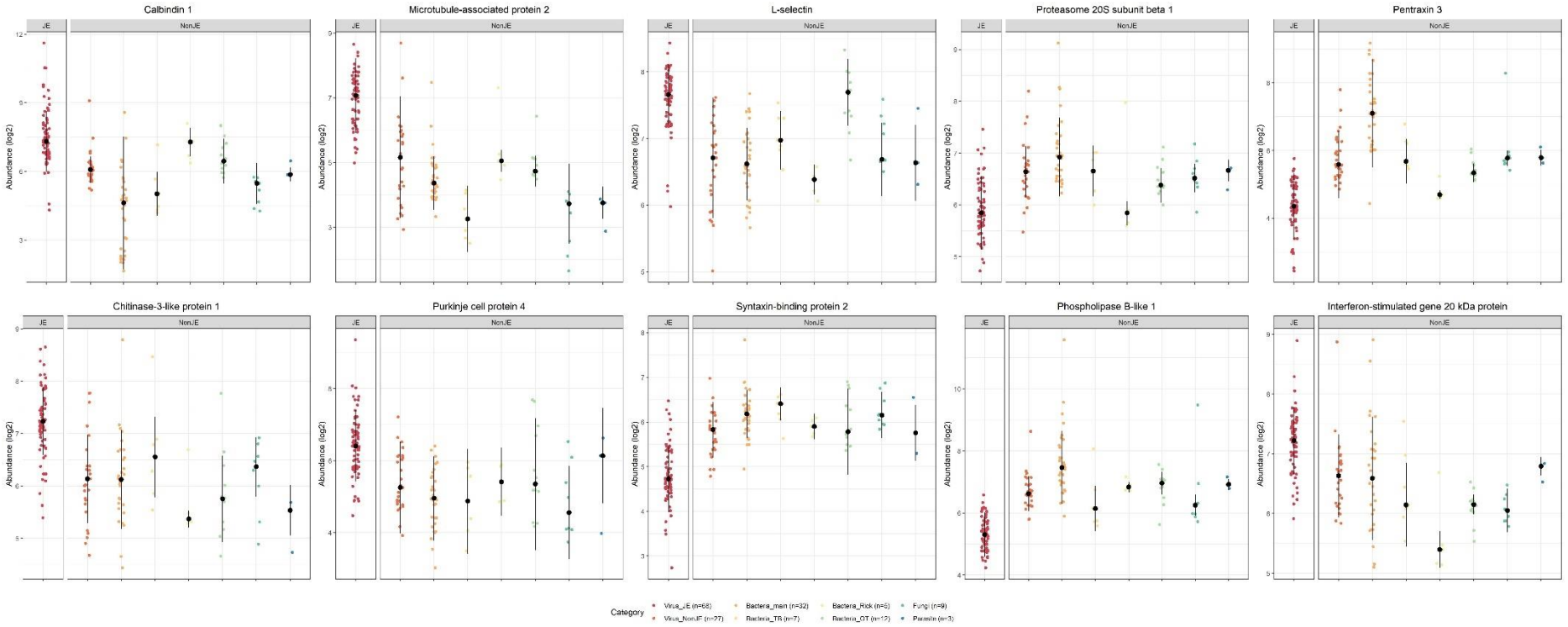


Table 6.3 Predictive modelling scores with 95% confidence intervals for the TMT-labelled study as a training set and the DIA study as a test set[1]

Features selected	Number of proteins	Data	AUC-ROC	Accuracy	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Top 2 proteins
All[2]	2176	Training set (n=163)	100 (100-100)	100 (99.7-100)	100 (99.4-100)	100 (99.4-100)	100 (99.4-100)	100 (99.4-100)	P11137, Q6P4A8
	1736	Training set (n=163)	100 (100-100)	100 (99.7-100)	100 (99.4-100)	100 (99.4-100)	100 (99.4-100)	100 (99.4-100)	P11137, Q6P4A8
		Test set (n=16)	81.8 (66.9-96.7)	62.5 (35.4-84.8)	100 (47.8-100)	45.5 (16.7-76.6)	45.5 (16.7-76.6)	100 (47.8-100)	-
Boruta[3]	68	Training set (n=163)	99.9 (99.9-100)	97.8 (96.7-98.5)	97.5 (95.9-98.6)	98.0 (96.5-99.0)	98.0 (96.5-99.0)	97.5 (95.9-98.6)	P11137, Q6P4A8
	64	Training set (n=163)	100 (99.9-100)	98.3 (97.3-98.9)	98.5 (97.2-99.3)	98.0 (96.5-99.0)	98.0 (96.5-99.0)	98.5 (97.2-99.3)	P11137, Q6P4A8
		Test set (n=16)	86.4 (72.6-100)	81.3 (54.4-96.0)	100 (47.8-100)	72.7 (39.0-94.0)	62.5 (24.5-91.5)	100 (63.1-100)	-
Lasso[4]	28	Training set (n=163)	100 (100-100)	100 (99.7-100)	100 (99.4-100)	100 (99.4-100)	100 (99.4-100)	100 (99.4-100)	P11137, Q6P4A8
	25	Training set (n=163)	100 (100-100)	99.3 (98.6-99.7)	100 (99.4-100)	98.5 (97.2-99.3)	98.5 (97.2-99.3)	100 (99.4-100)	P11137, Q6P4A8
		Test set (n=16)	81.8 (60.9-100)	81.3 (54.4-96.0)	100 (47.8-100)	72.7 (39.0-94.0)	62.5 (24.5-91.5)	100 (63.1-100)	-
Intersect	10	Training set (n=163)	97.4 (96.2-98.5)	94.1 (92.6-95.4)	95.3 (93.3-96.9)	92.8 (90.5-94.8)	93.0 (90.7-94.9)	95.2 (93.2-96.8)	Q15833, P11137
		Test set (n=16)	90.9 (79.0-100)	81.3 (54.4-96.0)	100 (47.8-100)	72.7 (39.0-94.0)	62.5 (24.5-91.5)	100 (63.1-100)	-
Top 2 proteins Intersect	2	Training set (n=163)	97.6 (96.9-98.3)	92.3 (90.6-93.7)	92.7 (90.3-94.6)	91.8 (89.3-93.9)	91.9 (89.4-93.9)	92.6 (90.2-94.6)	Q15833, P11137
		Test set (n=16)	97.9 (79.0-100)	93.8 (69.8-99.8)	100 (47.8-100)	90.9 (58.7-99.8)	83.3 (35.9-99.6)	100 (69.2-100)	-
Top 2 proteins all	2	Training set (n=163)	96.5 (95.1-97.8)	97.1 (96.0-98.0)	97.3 (95.7-98.5)	96.8 (95.1-98.1)	96.8 (95.1-98.1)	97.3 (95.7-98.5)	P11137, Q6P4A8
		Test set (n=16)	72.7 (46.7-98.8)	37.5 (15.2-64.6)	100 (47.8-100)	9.1 (0.2-41.4)	33.3 (11.8-61.6)	100 (2.5-100)	-

1. An ensemble model was built using random forest, support vector machine, generalised linear model and naïve bayes, except for the modelling with all the data in which the naïve bayes model failed and the ensemble model was built the other three methods. The training set included patient samples (68 JE and 95 non-JE confirmed neurological infections) processed by TMT LC-MS/MS. 4. The test set included 10% of the patient samples from the TMT LC-MS/MS analysis processed separately by label-free DIA LC-MS/MS.
2. 2176 proteins were included in the final processed TMT LC-MS/MS dataset, however 440 were not present in the DIA data, and a model was built consisting of 1736 proteins.
3. 68 proteins were identified by the Boruta algorithm, however 4 of these were not present in the DIA data, and that a model was built consisting of 64 proteins.
4. 28 proteins were identified by Lasso, however 3 of these were not present in the DIA data, and that a model was built consisting of 25 proteins.

Table 6.4 Predictive modelling scores with 95% confidence intervals for the TMT-labelled study with the samples included in the DIA study removed as a training set and the DIA study as a test set[1]

Features selected	Number of proteins	Data	AUC-ROC	Accuracy	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Top 2 proteins in the model
All[2]	2176	Training set (n=147)	100 (100-100)	100 (99.6-100)	100 (99.3-100)	100 (99.3-100)	100 (99.3-100)	100 (99.3-100)	P11137, Q6P4A8
	1736	Training set (n=147)	100 (100-100)	100 (99.6-100)	100 (99.3-100)	100 (99.3-100)	100 (99.3-100)	100 (99.3-100)	Q6P4A8, Q15833
		Test set (n=16)	77.3 (61.8-92.7)	62.5 (35.4-84.8)	100 (47.8-100)	45.5 (16.7-76.6)	45.5 (16.7-76.6)	100 (47.8-100.0)	-
Boruta[3]	68	Training set (n=147)	97.4 (96.4-98.4)	95.4 (93.9-9661)	96.0 (93.9-97.5)	94.8 (92.5-96.6)	94.9 (92.6-96.6)	96.0 (93.8-97.5)	Q6P4A8, Q15833
	64	Training set (n=147)	99,7 (.9942-0.999	95.2 (93.4-96.4)	96.0 (93.9-97.5)	94.4 (92.0-96.2)	94.5 (92.1-96.3)	95.9 (93.8-97.5)	Q6P4A8, Q15833
		Test set (n=16)	32.7 (11.4-54.1)	62.5 (35.4-84.8)	100 (47.8-100)	45.5 (16.7-76.6)	45.5 (16.7-76.6)	100 (47.8-100)	-
Lasso[4]	24	Training set (n=147)	100 (100-100)	100 (99.6-100)	100 (99.3-100)	100 (99.3-100)	100 (99.3-100)	100 (99.3-100)	Q6P4A8, Q15833
	20	Training set (n=147)	100 (100-100)	100 (99.6-100)	100 (99.3-100)	100 (99.3-100)	100 (99.3-100)	100 (99.3-100)	Q6P4A8, Q15833
		Test set (n=16)	74.5 (48.4-100)	81.3 (54.4-96.0)	100 (47.8-100)	72.7 (39.0-94.0)	62.5 (24.5-91.5)	100 (63.1-100)	-
Intersect	9	Training set (n=147)	98.7 (98.0-99.4	97.0 (95.7-98.0)	99.8 (98.9-100)	94.2 (91.8-96.1)	94.5 (92.2-96.3)	99.8 (98.8-100)	Q15833, P11137
		Test set (n=16)	95.5 (86.6-100)	81.3 (54.4-96.0)	100 (47.8-100)	72.7 (39.0-94.0)	62.5 (24.5-91.5)	100 (63.1-100)	-
Top 2 proteins Intersect	2	Training set (n=147)	97.4 (96.2-98.6)	95.6 (94.1-97.0)	95.6 (93.4-97.2)	95.6 (93.4-97.2)	95.6 (93.4-97.2)	95.6 (93.4-97.2)	Q15833, P11137
		Test set (n=16)	96.4 (88.0-100)	68.8 (41.3-89.0)	100 (47.8-100)	54.5 (23.4-83.3)	50.0 (18.7-81.3)	100 (54.1-100)	-
Top 2 proteins all	2	Training set (n=147)	95.0 (93.3-96.8)	96.6 (95.3-97.6)	97.6 (95.8-98.8)	95.6 (93.4-97.2)	95.7 (93.5-97.3)	97.6 (95.8-98.7)	P11137, Q6P4A8
		Test set (n=16)	79 (43.0-98.9)	37.5 (15.2-64.6)	100 (47.8-100)	9.1 (0.2-41.3)	33.3 (11.8-61.6)	100 (2.5-100)	-

1. An ensemble model was built using random forest, support vector machine, generalised linear model and naïve bayes, except for the modelling with all the data in which the naïve bayes model failed and the ensemble model was built the other three methods. The training set included patient samples (63 JE and 84 non-JE confirmed neurological infections) processed by TMT LC-MS/MS. 4. The test set included 10% of the patient samples from the TMT LC-MS/MS analysis processed separately by label-free DIA LC-MS/MS.
2. 2176 proteins were included in the final processed TMT LC-MS/MS dataset, however 440 were not present in the DIA data, and a model was built consisting of 1736 proteins.
3. 68 proteins were identified by the Boruta algorithm, however 4 of these were not present in the DIA data, and that a model was built consisting of 64 proteins.
4. 24 proteins were identified by Lasso, however 4 of these were not present in the DIA data, and that a model was built consisting of 20 proteins.

Data acquired by DIA LC-MS/MS of 16 (10%) of the samples was used to verify the ten-protein JE diagnostic predictive model. The metrics using the DIA data to test the model built using the TMT data are reported in Table 6.3 and Table 6.5. Additionally, the model was rebuilt training on a smaller dataset without the patient samples included in the DIA study (n=147), producing a nine-protein JE diagnostic predictive model, and the metrics using the DIA data to test the model built using the TMT data are reported in Table 6.4.

Table 6.5 Summary of the key predictive modelling scores with 95% confidence intervals

Classification task	Data	AUC-ROC	Accuracy	Sensitivity	Specificity	Positive predictive value	Negative predictive value
JE diagnosis (JE vs. non-JE)	Training set[1] (n=163)	97.4 (96.2-98.5)	94.1 (92.6-95.4)	95.3 (93.3-96.9)	92.8 (90.5-94.8)	93.0 (90.7-94.9)	95.2 (93.2-96.8)
	Test set[2] (n=16)	90.9 (79.0-100)	81.3 (54.4-96.0)	100 (47.8-100)	72.7 (39.0-94.0)	62.5 (24.5-91.5)	100 (63.1-100)
JE outcome (dead vs alive)	Training set[3] (n=42)	88.5 (84.7-92.2)	86.3 (83.5-88.8)	42.0 (32.2-52.3)	93.7 (91.4, 95.5)	52.5 (41.0-63.8)	90.6 (88.1-92.8)

1. The training set included patient samples (68 JE and 95 non-JE confirmed neurological infections) processed by TMT LC-MS/MS. 2. The test set included 10% of the patient samples from the TMT LC-MS/MS analysis processed separately by label-free DIA LC-MS/MS. 3. The training set included all the JE patients included in the TMT LC-MS/MS analysis for which outcome data was available.

Predictors of JE outcome

Feature selection: Subgroup analysis was performed using 42 JE samples for which outcome data at hospital discharge (died vs alive) were available. Seven proteins were identified as important in predicting outcome using the Boruta algorithm and 2 proteins using Lasso, such that 2 proteins were identified by both Boruta and Lasso, see Appendix 6.13. In view of the small sample size, the data were not split into a training and test set. These proteins were used to train different models with five-fold cross-validation repeated ten times evaluated on ROC, and then combined in an ensemble model with cross-validation scores reported in Data acquired by DIA LC-MS/MS of 16 (10%) of the samples was used to verify the ten-protein JE diagnostic predictive model. The metrics using the DIA data to test the model built using the TMT data are reported in Table 6.3 and Table 6.5. Additionally, the model was rebuilt training on a smaller dataset without the patient samples included in the DIA study (n=147), producing a nine-protein

JE diagnostic predictive model, and the metrics using the DIA data to test the model built using the TMT data are reported in Table 6.4.

Table 6.5, see the list of proteins in Appendix 6.13 and ROC in Appendix 6.14 There were five JE patients in the DIA LC-MS/MS analysis of which 3 had outcome data, and this was considered too small to report test metrics.

Discussions

I performed deep untargeted analysis of well-characterised patient CSF samples from a large number of different confirmed neurological infections. To my knowledge, the highest number of proteins in CSF identified in any study to date has been 3,174 (546); thus this research represents a notable improvement in terms of the numbers of proteins identified (discussed in Chapter 5) and this serves as a marker of the depth of analysis and prospects for biomarker identification (54). The identification of pathogen proteins is also an indicator of the analytical sensitivity that the methods achieved. Offline fractionation into 90 fractions in the pilot study, and 100 fractions concatenated into 44 in the larger study, with two-hour online LC gradients and multiplexing with TMT-16plex contributed to the depth of analysis. Furthermore, the diverse range of neurological infections also augmented the variety of proteins identified. WGCNA analysis identified 20 clusters of proteins with highly correlated expression, and provided insight into the proteins and how they associate with disease mechanisms. The modules were allocated a descriptor, according to functional enrichment analysis of the proteins. For example, one module was associated with IgM (proteins in the module included Immunoglobulin heavy constant mu and Immunoglobulin J chain) and correlated with JE and Ot, as well as the duration of illness. Other important modules associated with upregulation in JE included neuronal damage, anti-apoptosis, heat shock response, unfolded protein response, cell adhesion, macrophage and dendritic cell activation. In contrast, in comparison to other non-JE neurological infections, there was an association with downregulated acute inflammatory response, hepatotoxicity, activation of coagulation, extracellular matrix and actin regulation.
A primary aim was to evaluate the hypothesis that there is a diagnostic protein signature of JE, and, if present, to explore the constituent proteins and produce a list of 10-20 proteins for further investigation. Ultimately, the goal would be to identify 2-3 proteins that could be used in an RDT, however the rationale for honing in on 10-20 proteins was this would be a pragmatic subset for validation by targeted proteomics method. I used predictive modelling to test the hypothesis, using the entire dataset (2176 proteins) as well as different subsets of proteins

identified by feature selection (n=68 to n=2). Selecting proteins that were identified as important using two contrasting feature selection methods, the Boruta algorithm and Lasso, provided greater confidence in the proteins, and produced the desired subset of proteins. Predictive modelling using the 10 protein ensemble model enabled classification of JE and non-JE samples with a cross-validation accuracy of 99.1 (95% CI 92.6-95.4) across all the samples using the TMT labelled DDA data, and 81.3 (95% CI 54.4-96.0) in verification with 16 (10%) of the samples by DIA. The results are summarised in Table 6.3 and in the Appendix 6.7. It is notable that three proteins selected as the best disease classifiers were not "significant" i.e. p value < 0.05 with t-test and adjustment for multiple testing, highlighting the limitations of univariate analysis in biomarker identification (547). Biomarker discovery is a lengthy process, akin to the pharmaceutical pipeline (367). The work demonstrates important CSF proteins in classifying JE vs non-JE.

DIA is a label-free method of analysis, with ongoing improvements in depth and throughput; in this case providing a complementary method to verify the TMT data rather than performing traditional targeted LC-MS/MS proteomics such as PRM. The DIA study was performed with a small number of patient samples, the results included considerable missing data (38%) and DIA is not an entirely orthogonal method of analysis to TMT-labelled methods. Nonetheless, in my perspective, the DIA data served an important role in substantiating the TMT data that may be associated with batch effects and false positives (36). In recognition of potential criticism that the DIA samples that constituted the test set should have been excluded from the training set during predictive modelling, I reanalysed the data to this effect. The results for predictive modelling including (Table 6.3) and excluding (Table 6.4) the DIA data were very similar. There is no doubt that the protein signature needs to be validated with orthogonal antibody-based methods in additional patient groups. It will also be useful to compare this with protein profiling in other body fluids. This will inform the use of a smaller subset of proteins in an ELISA or RDT to be tested alongside the existing anti-JEV IgM assay.

To date, to our knowledge, two studies have utilised unbiased techniques to examine the CSF proteome in human patients with confirmed JEV infection; while they demonstrated the

feasibility of the methods, the patients were not confirmed by VNT and the studies included relatively small numbers of patients (10 and 26 JE patients) (348, 548). There have been a handful of studies utilizing ELISA methods to target specific proteins, however these rarely used power calculations in their experimental design, nor did they include adequate controls (549-554). Analysis of the transcriptome and proteome in animal models (555-559) and cell culture (478, 549, 555, 560-564) have been performed, however the comparability to human CSF and comparison with other neurological infections is limited. Furthermore, mRNA expression does not directly correlate with that of the corresponding protein (565).

As expected, while I included JEV proteins in the search database, I did not identify any JEV pathogen proteins. This is compatible with previous publications; non-structural protein 1 is the major secreted protein during flavivirus infections and is harnessed widely as a diagnostic biomarker for DENV infection, but it is not a useful diagnostic biomarker for JE (279). The data provide useful interrogation of the host response to JEV infection *in vivo*. The identified proteins fit well into the existing literature on the host response in JEV and other closely-associated flavivirus infections, most importantly WNV infection (342, 566). Tau/MAPT and MAP2 are both closely associated microtubule stabilising proteins specific to neuronal cells (567). Both proteins were identified in this study as being biomarkers of JE in CSF, and the high levels in comparison to other neurological infections is striking. The association of the former has previously been demonstrated by ELISA, in one of the only studies of this type (427). The role of actin, microtubule and intermediate filament cytoskeletal re-organisation in flavivirus infection has been described (456) and upregulation of tau/MAPT and MAP2 may represent neuronal damage following transneural spread of JEV. Other proteins that were associated with JE in this study, all within the red WGCNA module, that may reflect neuronal damage include Paralemmin, Calbindin 1, MAP2, Parvalbumin, Secernin 1 and Cell cycle exit and neuronal differentiation. The upregulation of ISG15 and ISG20 fit in with the known upregulation of a host of ISGs as part of the innate immune response to a viral infection (568, 569). Additional functional enrichments reflecting different WGCNA modules have previously been described including anti-apoptosis (570), heat shock response (571, 572), unfolded protein

response (573), translation (574), IgM (575), cell adhesion and pathogen attachment (576), endothelial activation (577) and macrophage activation (578, 579). In comparison to other neurological infections, there was a downregulation in acute phase response proteins and neutrophil enriched proteins, as has been seen by other studies (514, 580-582). In these, however, the sample size for the analysis of proteins predictive of outcome was less substantial and not supported by an a priori power calculation.

Improved understanding of the host response *in vivo* provides insight into potential proteins and pathways to be targeted in host-directed antiviral therapy. Honing in on specific therapeutic targets would require considerable further research, but it encouraging that nine of the 86 proteins identified by one of the feature selection methods are FDA approved drug targets. In particular, one of the proteins, AXL, has already been identified as a potential therapeutic target. AXL is a key member of receptor tyrosine kinase TAM family, and has been shown to alleviate neuroinflammation in mice models of JE (583).

Incomplete coverage and missing data between LC-MS/MS runs is an ongoing issue in the field (517). It is notable that comparing with other similar studies in the literature, the important proteins may not be exactly the same but are closely related. These issues are now being improved by DIA methods. In this analysis, coverage could have been improved by having technical LC-MS/MS replicates of each sample, however this was not possible due to the increased run time that this would have involved. Further limitations are that I did not include CSF from healthy people in Laos on ethical grounds, or from cohorts from elsewhere on the basis that samples that have undergone different storage conditions may not be comparable. The latter is also the reason for there being no samples from neurological flaviviruses occurring in other geographical areas, such as WNV and ZIKV. Furthermore, for the purposes of the objective of finding a diagnostic protein signature of JE, of utmost importance was comparing JE with controls of a wide range of other neurological infections. The analysis of proteins predictive of different categories of infectious aetiologies was not sufficiently powered in this study, and has not been reported. It is important to keep in mind that the comparison is between different neurological infections in the analysis of proteins that are up and down-regulated.

An RDT to detect JE in 182ccesssible areas is urgently needed. The experiments described in this chapter demonstrates the feasibility of an unbiased LC-MS/MS approach for the identification of novel protein biomarkers of neurological infections. Additional data using antibody-based methods will allow the 10-protein signature to be refined. This will require identifying commercial ELISAs or developing them in-house and comparing the specific protein abundance in JE and non-JE patients. These data will need to be validated in a larger group of patients, in different locations and in field settings. Ultimately, this will enable the selection of 2-3 proteins for the development of an RDT.

Publications arising from this chapter

This substance of this chapter has been submitted for publication, and uploaded online as a preprint to biorxiv (doi:10.1101/2022.06.19.496758). The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD034789 (doi:10.6019/PXD034789).

Chapter 7

LC-MS/MS studies to identify protein biomarkers of JE and scrub typhus in other body fluids

In this chapter, I present the background and aims of collaborative work between the University of Oxford and the Mahidol Oxford Tropical Medicine Research Unit (MORU) for which I co-ordinated a research proposal that was awarded a Collaborations to Catalyse Translation (CCT) grant in September 2020. Unfortunately, the work has been postponed on multiple occasions related to the COVID-19 pandemic, and the samples have not yet arrived in Oxford. The research involves LC-MS/MS studies to identify protein biomarkers of JE and scrub typhus (Ot infection) by deep probing in other body fluids - throat, serum and urine samples.

Background and experimental plans

CSF is a precious fluid; the LP procedure of taking it requires technical skills and equipment, and even then, may be contraindicated, not possible, or even if it is performed, may not lead to sufficient sample volumes for adequate testing or be contaminated by blood. It is clear that a diagnostic test that uses a non-invasive (urine or throat swabs) or minimally (venous blood) invasive sample would represent a significant improvement for patients and health workers (584). CSF is the proximal body fluid for a neurological disease as it is in direct contact with the brain, such that it is enriched in brain-derived proteins. Other body fluids, such as blood, have been studied in neurological diseases, however these samples represent more of a challenge due to the low concentration of brain-derived protein biomarkers (585). Dementia research is at the forefront of neurological disease biomarker research, and it is apparent that discovery work is typically initiated in CSF and then translated to targeted assays in blood (584). Nonetheless, a considerable body of work in this field remains to be completed to allow clinical implementation (586).

Most infectious diseases that infect the CNS involve a systemic host response that could be detectable in blood. Furthermore, blood may also contain protein biomarkers associated with BBB dysfunction and brain damage (585). It has also been shown that testing other body fluids increases detection of pathogen nucleic acid, and potentially pathogen proteins in CNS infections. For JE, a few studies have demonstrated detection of JEV RNA in throat and urine (587-591).

As discussed in Chapter 5, scrub typhus is a leading cause of febrile illness in Asia and an important differential diagnosis for JE in Asia (592). Effective treatment is widely available and inexpensive with medicines such as doxycycline (593). However, empirical treatment is not without risks of side-effects and antimicrobial resistance (594, 595), and a key unmet challenge is the reliable and rapid diagnosis of the disease via an RDT that is suitable for use in low resource settings. None of the tests currently available offer sufficient clinical utility for diagnosing scrub typhus, either due to lack of accuracy or ease of implementation (see Table 7.1) (417, 418, 422, 595-597).

Table 7.1 Diagnostic tests for Scrub Typhus or *Orientia tsutsugamushi* (Ot) infection

Assay	**Detection mechanism**	**Accuracy (%)**	**Rapid? (Y/N)**	**Resources/expertise required**	**Other limitations/ details**
Presence of eschar	Visual inspection during clinical examination	Inadequate sensitivity (7-80%); high specificity 98.9% where other eschar causing rickettsiosis are rare	Y	Clinical expertise	-
Polymerase chain reaction (PCR)	Molecular (target genes include the 47 kDa, 56 kDa, 16S rRNA and *groEL* genes)	Inadequate sensitivity (15-75%); high specificity (>90%)	N (3 hrs)	Laboratory capabilities and resources	Sensitivity is limited due to low bacteraemia, and depends on sample timing and type (i.e. buffy coat and eschar are better)
Cell culture	*In vitro* isolation	Lower sensitivity than PCR (20-30%); high specificity (>90%)	N (7-60 days)	Specialist laboratory expertise and infrastructure (BSL3)	As above

Weil-Felix test	Serological (based on detection of antibodies cross-reactive to antigens of the OX-K strain of the unrelated bacteria *Proteus mirabilis*)	Low sensitivity (30-50%) and specificity (40-60%)	N (6 hrs)	Laboratory capabilities and resources	-
Immunofluorescent assay (IFA) and indirect immunoperoxidase test (IIP)	Serological (based on cell-culture-derived *Ot* antigens)	Sensitivity (50-80%) and specificity (70-90%)	N (2-3 hrs)	Laboratory capabilities and resources	Paired acute and convalescent samples; absence of consensus on cut-off titres, subjectivity in end-point determination
IgM +/- IgG ELISA	Serological e.g. InBios and in-house methods such as the US Naval Medical Research Centre	Sensitivity (50-90%) and specificity (70-90%)	N (3-4 hrs)	Laboratory capabilities and resources	-
Loop-mediated isothermal amplification (LAMP)	Molecular	Limitations in sensitivity, as for PCR	Y (30 mins)	Resources	Not readily available
Immunochromatographic assays (ICTs/ lateral flow assays) to detect antibody	Serological e.g. Panbio IgM, the Standard Diagnostics IgM, the AccessBio IgM, and the AccessBio total antibody	Inadequate sensitivity (37-86%) and specificity (83-97%)	Y (5 mins)	Variable cost	-

Winterberg *et al.* at MORU generated preliminary data to suggest that Ot pathogen peptides could be detected in patient urine samples by LC-MS/MS (unpublished data). I hypothesised that an MS approach can identify antigen(s) present in clinical samples, and that an antigen-based RDT could provide sufficient accuracy, low-cost, rapidity and ease of use to both significantly impact on the burden of disease and yield valuable epidemiological data to help inform public health policy.

I aimed to perform a pilot discovery study in minimally/non-invasive clinical samples of patients with JE vs scrub typhus; testing 3 serum, 3 buffy coat (fraction of an anticoagulated blood sample that contains most of the white blood cells and platelets following centrifugation), 3 urine and 3 throat swabs samples of each type of infection. For the serum, I would perform immunodepletion of 12 highly abundant proteins. I would then perform high-pH RP fractionation into 60 fractions and run these by LC-MS/MS. The results would inform a

verification study in the urine of 30 patients with Ot infection and 30 controls with non-Ot febrile illness from Laos.

I was not able to travel to Thailand due to the COVID-19 pandemic, and the project was revised such that the first phase (sample preparation and offline fractionation) would be performed by a post-doctoral researcher in Thailand, Dr Phornpimon Tipthara Wong, supervised by Professor Joel Tarning. I have had weekly meetings to explain the experimental plan and co-ordinate the project. There has been numerous obstacles related to issues with equipment, and at the moment the samples have been processed but await offline fractionation and transport to Oxford. I plan to complete this after submission of my DPhil.

Chapter 8

Vaccine Identity and Evaluation (VIE) transdisciplinary research collaboration

In this chapter, I introduce the VIE transdisciplinary research collaboration, and present the methodology and results of experimental work developing methods for the identification of SF JEV vaccines. The work was initiated as a response to the COVID-19 pandemic, but is relevant to my thesis as it represents an important aspect of JEV control, my thesis and my training. It involves the analysis of vaccines rather than clinical samples, the application of an alternative MS method from that described in previous chapters, Chapter 6 and Chapter 7, MALDI-ToF MS, and the analysis of metabolomics in addition to proteomics.

Introduction

The COVID-19 pandemic has highlighted the neglected public health problem of substandard (due to incorrect storage) or falsified (counterfeit) vaccines in supply chains, officially referred to as SF vaccines by WHO (598, 599). There have been multiple pre-pandemic reports of SF vaccines and 178 reports of diverted and SF COVID-19 vaccines from 45 countries until December 2021 (600). There were numerous examples of SF vaccines pre-pandemic but meagre research on this issue (601, 602). This significant public health concern risks global vaccination implementation, that is of ever-increasing importance. JEV vaccines are arguably the only effective measure for the public health response to reduce the JEV clinical and socioeconomic burden. The greatest burden of JE is in India and China, and these countries are also recognised to have issues with SF vaccines (603-607). The emergence of SF vaccines threatens control of the JE, risking the exacerbation of existing problems of vaccine distribution and hesitancy (608).

There are no devices for inspectors to screen for SF vaccines in supply chains, nor is there adequate recognition of the full scale of the problem (598). Therefore, there is a vital need for research into innovative, accurate, affordable, rapid and user-friendly screening devices that can be effectively implemented to yield a step change in our ability to rapidly detect SF vaccines, at different levels in diverse supply chains, to protect vaccine-related global healthcare (609). A network of validated screening device methodologies, of known advantages and disadvantages, at different vaccine supply chain levels, with high sensitivity and specificity to detect SF vaccines would greatly enhance SF vaccine detection. One device will not cover the needs of all levels.

The screening devices hold promise of being integrated into the supply chain risk-based post-market surveillance and reference laboratories. For effective application, devices are needed at three main supply chain levels, 'proximal', 'provincial' and 'distal'. Devices must be adapted to the circumstances encountered and must be accurate, affordable and effective for that level. As vaccine supply chains and risk assessments vary internationally, the position of devices in the supply chain should be adaptable for different networks. Devices will be linked via cloud-computing to reference laboratories/regulators so that failing samples can be rapidly investigated, verified and reported.

A consortium of scientists in the Nuffield Department of Medicine, Biochemistry Department and Chemistry Department of Oxford University and the Rutherford-Appleton Laboratory of UKRI set up the VIE collaboration early in the COVID-19 pandemic to perform urgent research to evaluate the repurposing of devices for detecting SF vaccines. The VIE interdisciplinary team includes virologists, biochemists, chemists, physicists, pharmacists and clinicians, from academia and industry. Since July 2020, we have been evaluating innovative techniques for detecting SF vaccines, focussing on rapid and accessible methods, including, amongst others, spatially offset Raman spectroscopy (SORS), MALDI-ToF MS and repurposing of RDTs.

MALDI-TOF is a widely used mass spectrometry technique, providing rapid characterisation of molecular components – the sample is mixed or overlayed with matrix on a plate and inserted

into an instrument with an overall turnaround time of approximately 30 minutes. MALDI-ToF detects the mass-to-charge ratio (*m/z*) of biomolecules and the resulting protein and other analyte mass spectra are then compared with reference spectra. This is used extensively in the routine identification of bacterial, and to a lesser extent fungal, pathogens from patient samples in clinical microbiology laboratories (610). MS has been used, in combination with other techniques such as LC, to distinguish different influenza vaccines and identify some of their constituents (611, 612). LC-MS/MS spectra have also been used to characterise diphtheria vaccine (613). Although not portable and vials/syringes containing vaccines need to be opened, the technique is highly sensitive and specific (614). The simplicity of the sample inlet means that potentially more complex sample compositions, including those containing both proteins, peptides, lipids and small molecules can be analysed without having to pre-fractionate samples (e.g. milks, soils and blood samples) (610, 615). To date, we are not aware of the use of MALDI-MS for vaccine characterisation and authentication studies. In comparison to LC-MS/MS that has been the crux of my DPhil, MALDI-ToF is relatively accessible, quick and high-throughput. In the application as a device for the identification of SF vaccines, MALDI-ToF would be introduced at the proximal and provincial supply chain level.

I have been working in the MALDI-ToF and RDT arms of the VIE project. I set up a data analysis pipeline for MALDI-ToF raw spectra obtained from various vaccines and falsified vaccine surrogates using R; performing spectra processing and generating peak lists with the package 'MALDIquant' (616) and predictive modelling with the package 'caret' (617) to differentiate between different vaccines and falsified vaccine surrogates. I have also been co-ordinating work on repurposing RDTs to detect SF vaccines, partnering with Global Access Diagnostics (formerly Mologic Ltd).

There are more than 15 licenced JEV vaccines in use worldwide (618). These can be classified into four classes: inactivated mouse brain-derived, inactivated Vero cell-derived, live-attenuated and live-chimeric vaccines. Mouse brain-derived vaccines are the oldest class of vaccines in use, but they have gradually been replaced by the newer classes of vaccines due to the safety

concerns, high cost and multiple dose regimes required. An assortment of JEV vaccines are included in country-specific immunisation programmes, and these are summarised in Table 2.2.

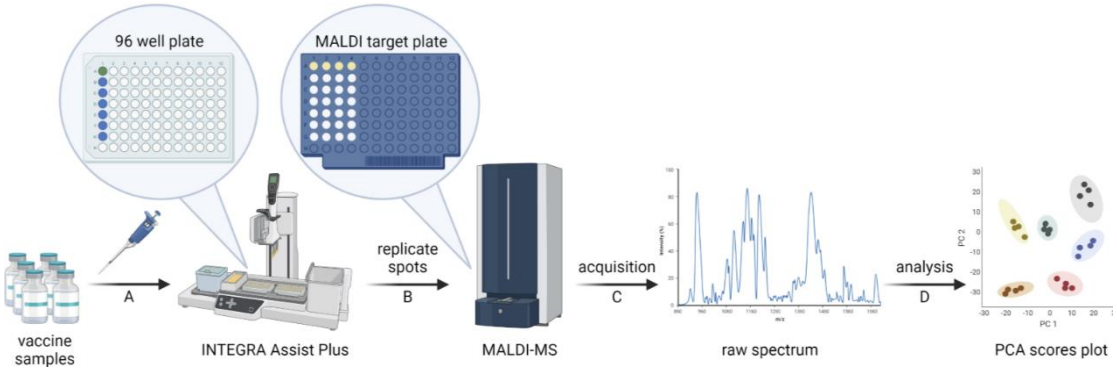
I aimed to evaluate the potential of MALDI-ToF to authenticate vaccines including the only JEV vaccine that is licenced in the UK, Ixiaro, using a variety of exemplar falsified/counterfeit vaccines. I also performed JEV vaccine degradation studies, to investigate the potential of MALDI-ToF to identify substandard vaccine.

Methods

Samples

The samples listed below were stored after receipt at 2-8℃ prior to analyses in accordance with manufacturers' storage guidance. The term falsified vaccine surrogates refers to samples that could be used as falsified vaccines, detailed below. There were four vaccines and eight falsified vaccine surrogates (12 sample types), with at least six vials replicates of each sample type, permitting the analysis of six vials of each for the falsification experiments. In order to assess reproducibility on different days, three vials were analysed on one day and three vials were analysed on another day. There were ten vials of Ixiaro vaccine, all ten vials were compared for the degradation experiments (see below). All the vials were analysed on one day, and then re-analysed on another day. Figure 8.1 illustrates the MALDI-MS workflow of the sample preparation and analysis.

Figure 8.1 MALDI-ToF sample preparation and analysis workflow



Step A, vaccine samples to be analysed and a pooled quality control sample (QC) were pipetted into a 96 well plate positioned in the INTEGRA Assist Plus. Step B, replicate spots of 1:1 premixed sample/QC and CHCA matrix were pipetted onto the MALDI target plate using the Assist Plus robot. Step C, raw MS spectra were acquired using the MALDI-MS instruments. Step D, data processing of the raw spectra and statistical analysis were performed.

1. Vaccines: Ixiaro (Valneva UK Limited; PC 0506045314000; Lot JEV19F11A), Flucelvax Tetra (Seqirus UK Limited; PC EMEA/H/C/004814; Lot 3079661A), Nimenrix meningococcal group ACW$_{135}$Y conjugate vaccine (Pfizer; PC: 05013457022032; Lot FE7696, ET9885 and FE7696), and Engerix B Hepatitis B vaccine (GlaxoSmithKline UK; PC: 05000483111083; Lot AHBVC999AL). Ingredients of the vaccines are presented in Table 8.1 The recommended storage temperature for all four vaccines is 2-8°C.
2. Falsified vaccine surrogates: These were identified from reports available in the public domain, both scientific and lay literature (601); hyaluronic acid ("Amazon" Guangzhou Ailian Cosmetic Co Ltd. P/N QB/T 2660; Lot A10.20), antibiotics gentamicin (40 mg/ml Wockhardt P/N 29831/0660; Lot WK2108680 and 2200396) and amikacin (250 mg/ml MA Holder Tillomed Laboratories Limited P/N 11311/0604; Lot ES200079B and FM9809AA), D-glucose (5.0% w/v; B/Braun P/N 03551/0059; Lot 22041405), sterile water (for injection Ph. Eur Demo S.A Pharmaceutical Industry P/N 24598/001; Lot 2101723), Milli-Q water from a Milli-Q® Direct 8 water purification system (Merck Millipore, Merck, Darmstadt, Germany), tap water from the Biochemistry and Chemistry Research Laboratories, Oxford University and saline (0.9% w/v sodium chloride, NaCl; Injection BP Demo S.A Pharmaceutical Industry P/N 24598/0002; Lot 2102386).

Degradation analysis

1. 450 µl of each vial of Ixiaro vaccine was pipetted into glass Total Recovery Vials (Waters Corporation P/N 186000384C and P/N 186000385C) in 50 µl aliquots and sealed with screw-top lids.
2. The following temperature conditions were investigated: samples stored at 2-8℃ (as per manufacturers guidance of storage) and additionally to produce degraded samples at an elevated temperature of 45℃. Additionally, some samples were also freeze-thawed, in total 3 times. For each of these storage conditions, 10 vials of each vaccine were used. The 2-8℃ aliquots were refrigerated for 7 days and the 45℃ aliquots were left in an oven at this temperature for 7 days. The aliquots undergoing freeze-thaw cycles were frozen at –80℃ for 24 hours and then thawed at 2-8℃ for 1 hour. The freeze-thaw cycle was repeated twice more.
3. All samples were stored at 2-8℃ after undergoing the above temperature conditions.

Table 8.1 Ingredients of vaccines analysed in the study as per the manufacturers' summary of product characteristics accessed from the electronic medicines compendium*

Sample	Organism	Type of vaccine	Active ingredients	Adjuvant or conjugate	Excipient
Ixiaro	Japanese encephalitis virus (JEV)	Inactivated virus vaccine	JEV strain SA14-14-2 produced in Vero cells and inactivated with formaldehyde 6 antigen units corresponding to a potency of ≤ 460 ng ED50	Aluminium hydroxide hydrated (approximately 0.25 milligrams Al3+)	Phosphate buffered saline (0.0067 M) consisting of: Sodium chloride (9 mg/mL), potassium dihydrogen phosphate (0.144 mg/mL), disodium hydrogen phosphate (0.795 mg/mL), water for injections.
Engerix B 20	Hepatitis B virus	Recombinant protein vaccine	Hepatitis B surface antigen produced in yeast cells (Saccharomyces cerevisiae) by recombinant DNA technology 20 µg per dose	Aluminium hydroxide, hydrated (approximately 0.50 milligrams Al3+)	Sodium chloride, disodium phosphate dihydrate, sodium dihydrogen phosphate, water for injections
Nimenrix	Neisseria meningitidis serogroups A, C, W-135 and Y	Conjugate vaccine	Neisseria meningitidis group A polysaccharide (5 µg) Neisseria meningitidis group C polysaccharide (5 µg) Neisseria meningitidis group W-135 polysaccharide (5 µg) Neisseria meningitidis group Y polysaccharide (5 µg)	Conjugated to tetanus toxoid carrier protein (44 µg)	Sucrose, trometamol, sodium chloride, water for injections
Flucelvax tetra	Influenza virus	Inactivated virus vaccine	Influenza virus produced in Madin Darby Canine Kidney (MDCK) cells, inactivated ß-propiolactone and disrupted by the detergent cetyltrimethylammonium bromide of the following strains: A/Wisconsin/588/2019 (H1N1)pdm09-like strain (A/Delaware/55/2019 CVR-45) 15 µg HA A/Darwin/6/2021 (H3N2)-like strain (A/Darwin/11/2021, wild type) 15 µg HA B/Austria/1359417/2021-like strain (B/Singapore/WUH4618/2021, wild type) 15 µg HA B/Phuket/3073/2013-like strain (B/Singapore/INFTT-16-0610/2016, wild type) 15 µg HA	-	Sodium chloride, potassium chloride, magnesium chloride hexahydrate, disodium phosphate dihydrate, potassium dihydrogen phosphate, water for injections. May contain traces of beta-propiolactone, cetyltrimethylammonium bromide, and polysorbate 80.

* (https://www.medicines.org.uk/emc)

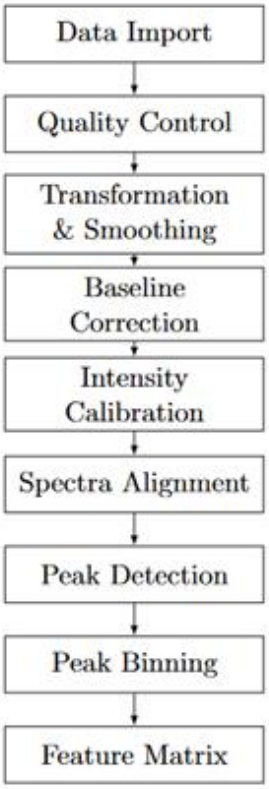
MALDI-TOF analysis

1. Sample preparation: The most widely used routine sample preparation and MALDI-TOF analysis settings in microbiology laboratories involve the use of α-cyano-4-hydroxycinnamic acid (CHCA or HCCA) matrix and positive ionisation mode analysis with a mass range of approximately 2,000-20,000 Da. It would be easier to implement methods that adhere to standard practice, and for this reason we first assessed these standard conditions.

   4 replicates of each vaccine aliquot or falsified vaccine surrogate aliquot (12 replicates in total per sample per condition per day) were spotted onto disposable MALDI target plates (VITEK P/N 410893 P/N 1840375) compatible with VITEK® mass spectrometer (bioMerieux) MALDI-TOF instruments. A quality control (QC) was prepared as a mixture of all the samples in equal volumes, and eight replicates spotted on each plate. Each replicate spot was produced using the INTEGRA Assist Plus by pipetting 1 µL sample onto the target plate and overlaying immediately with 1 µL of α-cyano-4-hydroxycinnamic acid (CHCA/HCCA, VITEK MS-CHCA; P/N 411071 and matrix P/N 8255344) matrix. The sample spots were allowed to air dry at room temperature.
2. Mass spectrometry measurements were acquired using the VITEK® mass spectrometer instrument in positive ionisation mode. The mass ranges analysed by the VITEK® mass spectrometer were 0-900, 700-2,500 and 2,000-20,000 *m/z*.
3. Data analysis: Raw spectra were visually analysed and plotted using Shimadzu LaunchPad. Raw data files were either acquired in mzXML format using Shimadzu LaunchPad software or converted to mzXML format using Shimadzu Batch Processor. Data exploration and visualisation was performed in Metaboanalyst v5.0 (619) and R v4.1.1 using the package 'MALDIQuant' (616) (see workflow in Figure 8.2). Hierarchical clustering was performed using euclidean and ward methods. Machine learning algorithms were used to model the data using ten-fold cross-validation, including partial least squares-discriminant analysis

(PLS-DA), random forest, k-Nearest Neighbours (kNN) and support vector machine (SVM) using the R package 'caret' (617). The R code is presented in Appendix 8.1 and 8.2.

Figure 8.2 MALDI-ToF data processing pipeline using the R package 'MALDIquant'



Results

Quality control

An initial evaluation of the spectra demonstrated the 0-900 *m/z* range provided the majority of mass spectral peaks and further analysis focussed on this range. Amikacin and Gentamicin proved to be difficult sample types to analyse, demonstrating poor crystallisation with the CHCA matrix that was associated with weak adherence to the target plate and difficulty obtaining spectra. After multiple attempts, a few spectra were obtained, but these were low quality with insufficient replicates. These spectra were removed from further analyses.

Falsification

Visual inspection: There were clear differences between the spectra from different types of samples stored according to manufacturer's instructions at 2-8°C, see Figure 8.3. There were differences between the peaks present at specific *m/z* values and their abundances. The spectra obtained for MilliQ water and water for injection appeared identical. The spectra obtained for all four vaccines was similar to saline; in particular, Engerix B appeared identical to saline but with higher intensity overall.

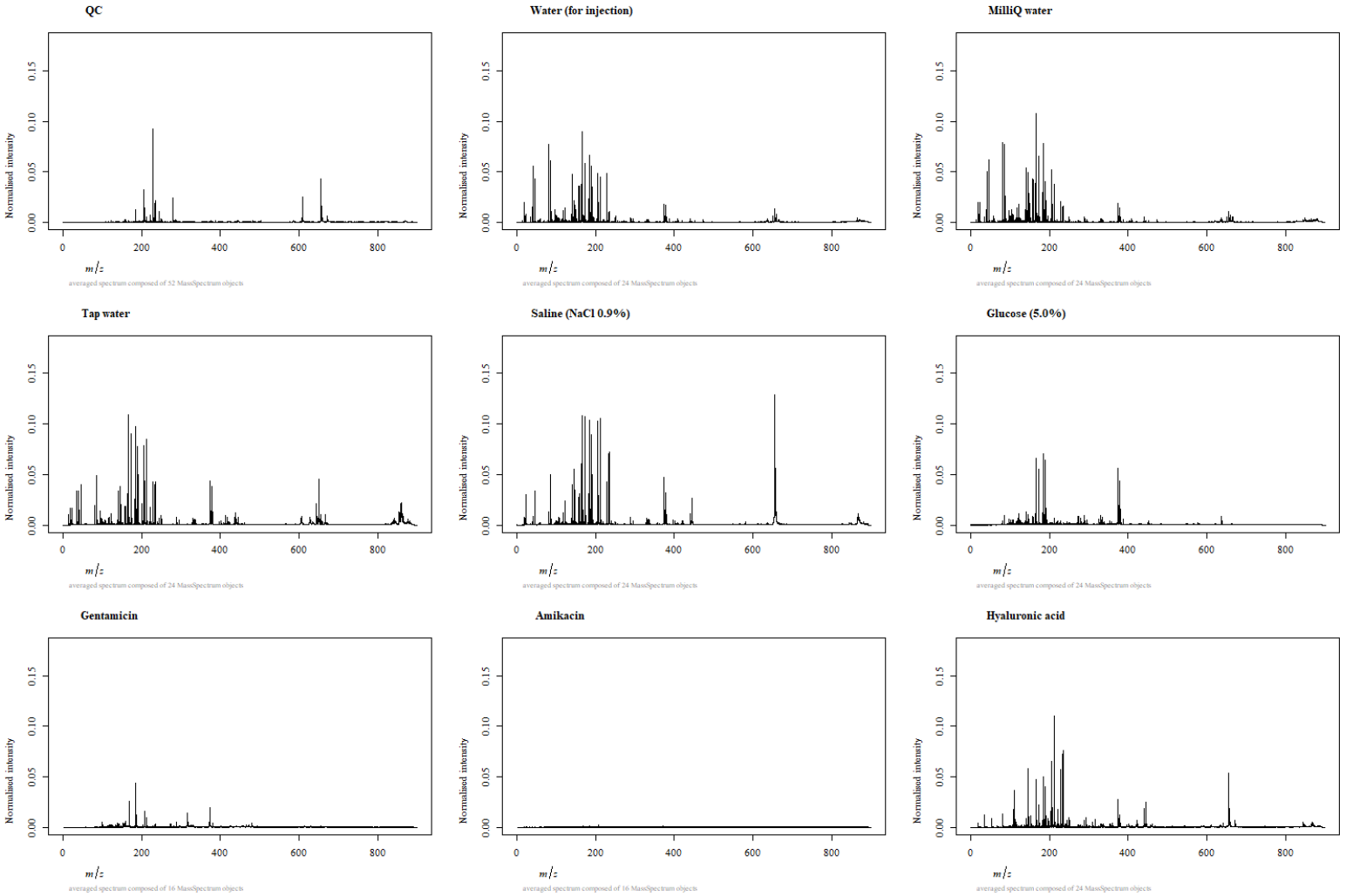
Peak alignment, normalisation, batch correction and hierarchical clustering: Initial hierarchical clustering analysis of the whole dataset illustrated clustering of spectra obtained on different days, see Figure 8.4. Analysis of the peak lists suggested that the spectra on the two days were not perfectly aligned even though a spectral alignment had been performed in MALDIQuant. This meant that a given peak was recorded with a slightly different m/z on different days in spite of daily calibration. After various attempts, the issue was largely remedied by performing a peak alignment in MALDIQuant (in addition to the previously performed spectral alignment). Furthermore, systematic differences in the peak intensities on the two days was corrected by performing data normalisation and batch adjustment. Normalisation was implemented using the R package 'caret' preProcesss function to 'center' by subtracting the mean, 'scale' by dividing by the standard deviation and then perform a 'Box Cox' transformation) (620). Batch effects were corrected using the R package 'sva' function ComBat (532). Hierarchical clustering of the whole dataset after peak alignment, normalisation and batch correction demonstrated that pre-processing unmasked differences between the types of samples, see Figure 8.4.

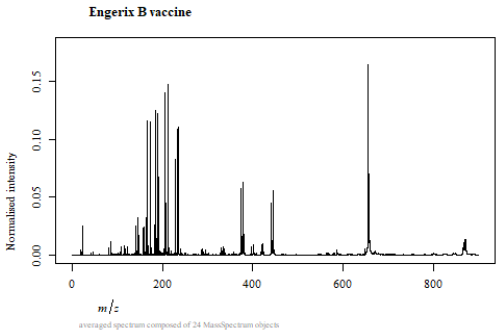
Machine learning: Predictive modelling was performed on the entire data set using ten-fold cross-validation with a number of different methods including PLS-DA see Figure 8.5, random forest, k-NN and SVM. The peaks with the best predictive performance in differentiating the different sample types are shown in the heat map in Figure 8.6. The data were then split to train a model with the day 1 data (n=120; 4 replicates of 3 vaccine vials of 10 samples) using ten-fold cross-validation together with the aforementioned methods. The performance of the model was assessed using the day 2 data (n=120; 4 replicates of 3 vaccine vials of 10 samples) as a test

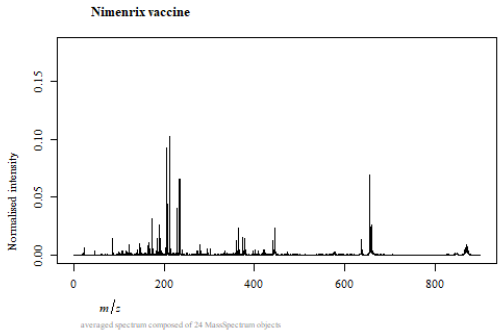
(hold-out) set. The performance of the model in discriminating between the four vaccine samples and the falsified vaccine surrogates is presented in Table 8.2.

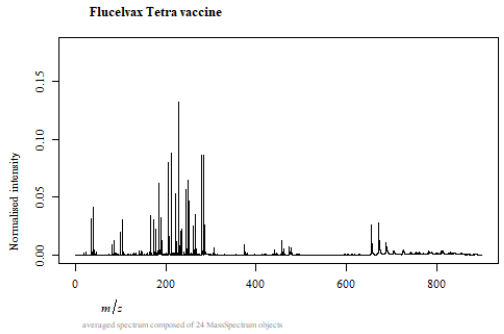
In view of the similarities between MilliQ water and water for injection, and Engerix B and Saline (0.9%), highlighted in Figure 8.4, MilliQ water and Saline (0.9%) were removed from the dataset. The filtered dataset were reanalysed as before; splitting to train a model with the day 1 data (n=120; 4 replicates of 3 vaccine vials of 10 samples) using ten-fold cross-validation together with the aforementioned methods. The performance of the model was assessed using the day 2 data (n=120; 4 replicates of 3 vaccine vials of 10 samples) as a test (hold-out) set. The performance of the model in discriminating between the four vaccine samples and the falsified vaccine surrogates improved, presented in Table 8.2.

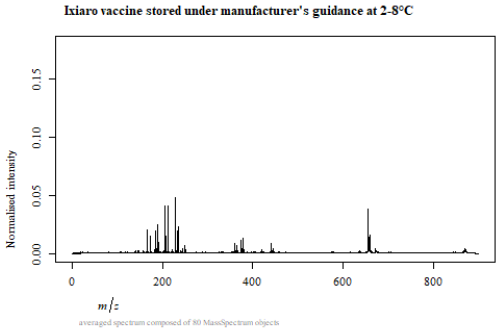
Figure 8.3 MALDI-TOF average spectra in the 0-900 *m/z* range for vaccines and common falsified vaccine surrogates.

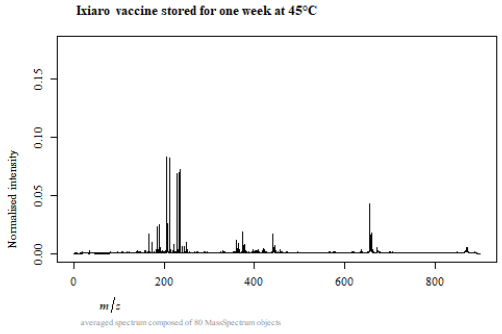



Engerix B vaccine
Normalised intensity
0.00
0.05
0.10
0.15
0
200
400
600
800
m/z
averaged spectrum composed of 24 MassSpectrum objects


Nimenrix vaccine
Normalised intensity
0.00
0.05
0.10
0.15
0
200
400
600
800
m/z
averaged spectrum composed of 24 MassSpectrum objects


Flucelvax Tetra vaccine
Normalised intensity
0.00
0.05
0.10
0.15
0
200
400
600
800
m/z
averaged spectrum composed of 24 MassSpectrum objects


Ixiaro vaccine stored under manufacturer's guidance at 2-8°C
Normalised intensity
0.00
0.05
0.10
0.15
0
200
400
600
800
m/z
averaged spectrum composed of 80 MassSpectrum objects


Ixiaro vaccine stored for one week at 45°C
Normalised intensity
0.00
0.05
0.10
0.15
0
200
400
600
800
m/z
averaged spectrum composed of 80 MassSpectrum objects

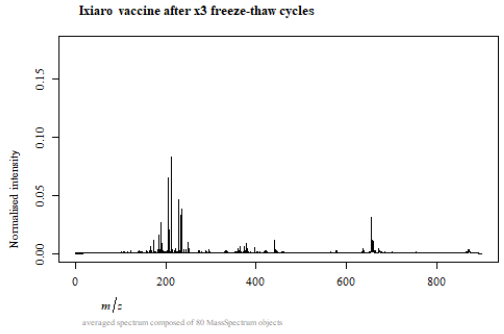

Ixiaro vaccine after x3 freeze-thaw cycles
Normalised intensity
0.00
0.05
0.10
0.15
0
200
400
600
800
m/z
averaged spectrum composed of 80 MassSpectrum objects

Figure 8.4 Dendrogram of peak lists obtained from MALDI-ToF spectra in the 0-900 *m/z* range of vaccines as compared to common falsified vaccine surrogates (n=240) – a. raw data, b. normalised and batch corrected and c. peaks aligned.

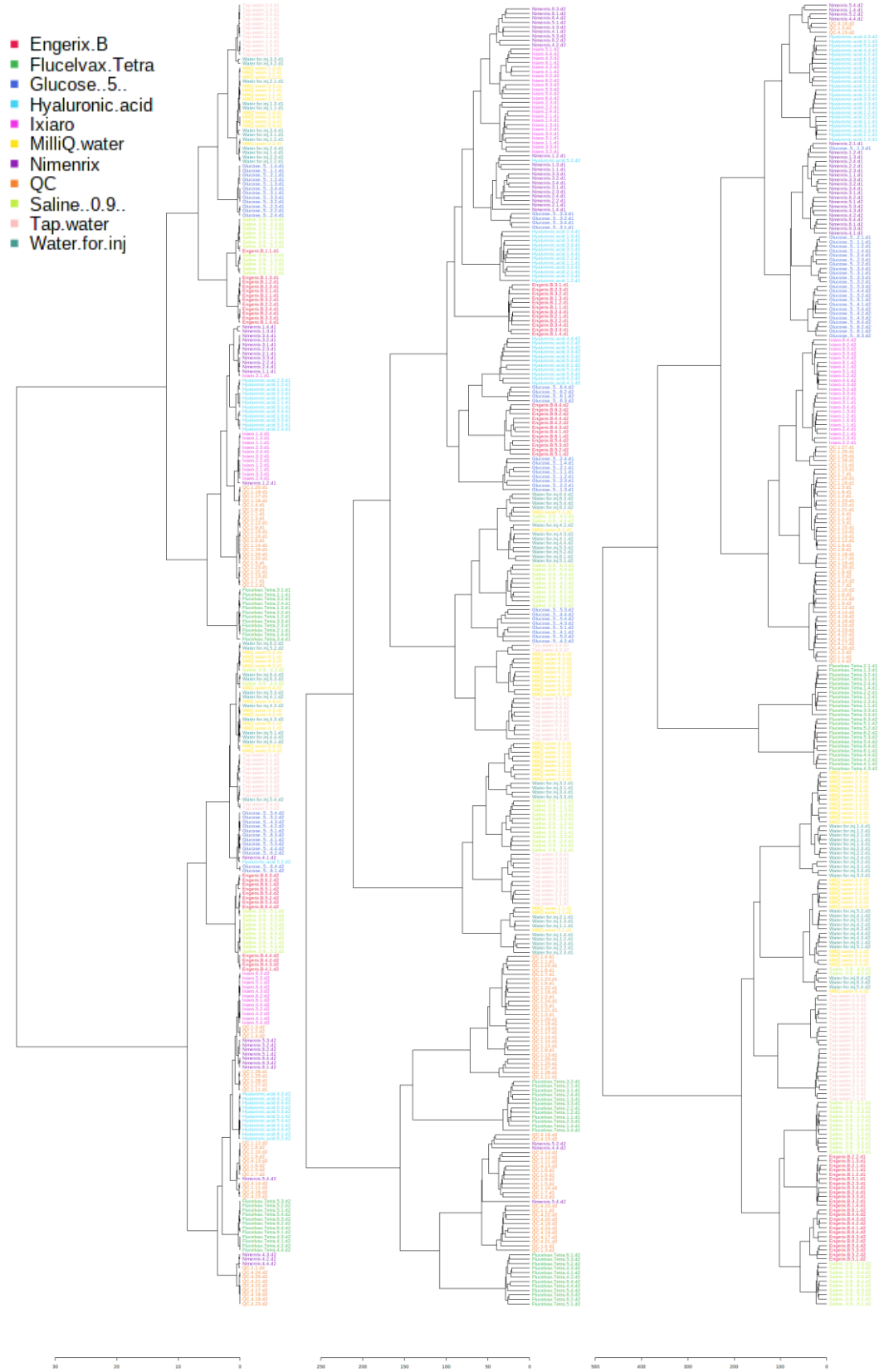
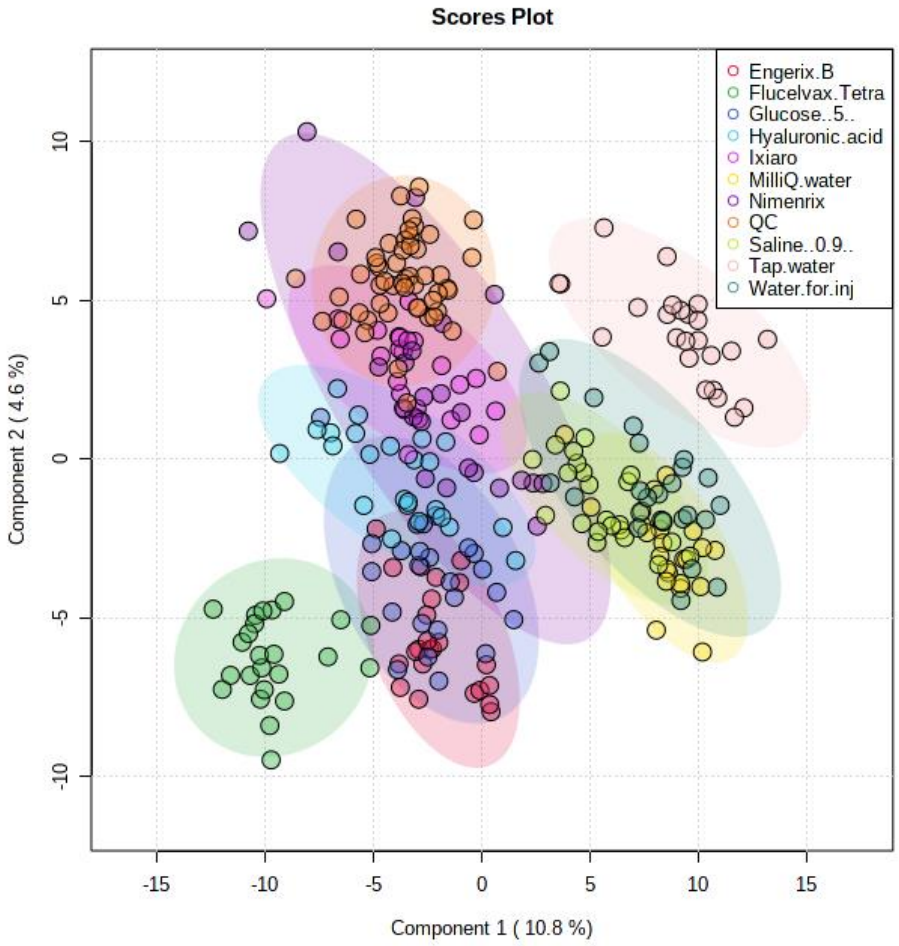
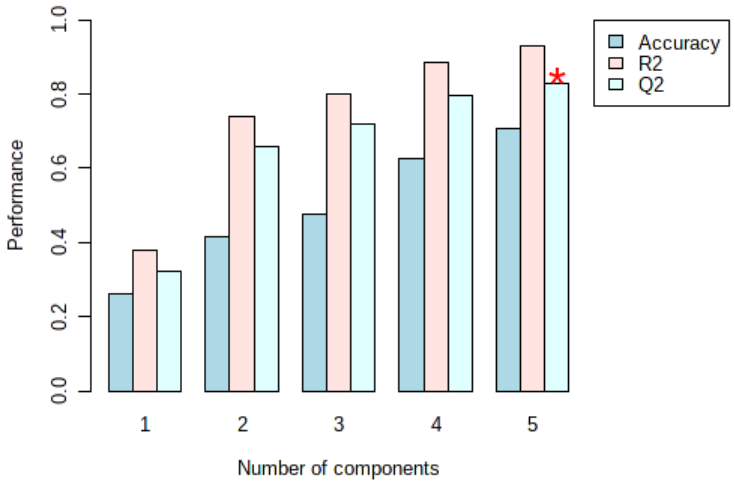


Figure 8.5 Partial least squares-discriminant analysis (PLS-DA) score plots and cross-validation results[#] of the MALDI-TOF spectra in the 0-900 *m/z* range for vaccines as compared to common falsified vaccine surrogates (n=240).





[#]Q2 is an estimate of the predictive ability of the model, and is calculated via cross-validation (CV). In each CV, the predicted data are compared with the original data, and the sum of squared errors is calculated. The prediction error is then summed over all samples (Predicted Residual Sum of Squares or PRESS). For convenience, the PRESS is divided by the initial sum of squares and subtracted from 1 to resemble the scale of the R2. Results are presented for the model with the number of components with the highest predictive ability (Q2), with a maximum of 5 components.

Figure 8.6: The best performing features (ions) using partial least squares-discriminant analysis (PLS-DA) of the MALDI-TOF spectra in the 0-900 *m/z* range for vaccines as compared to common falsified vaccine surrogates (n=240).

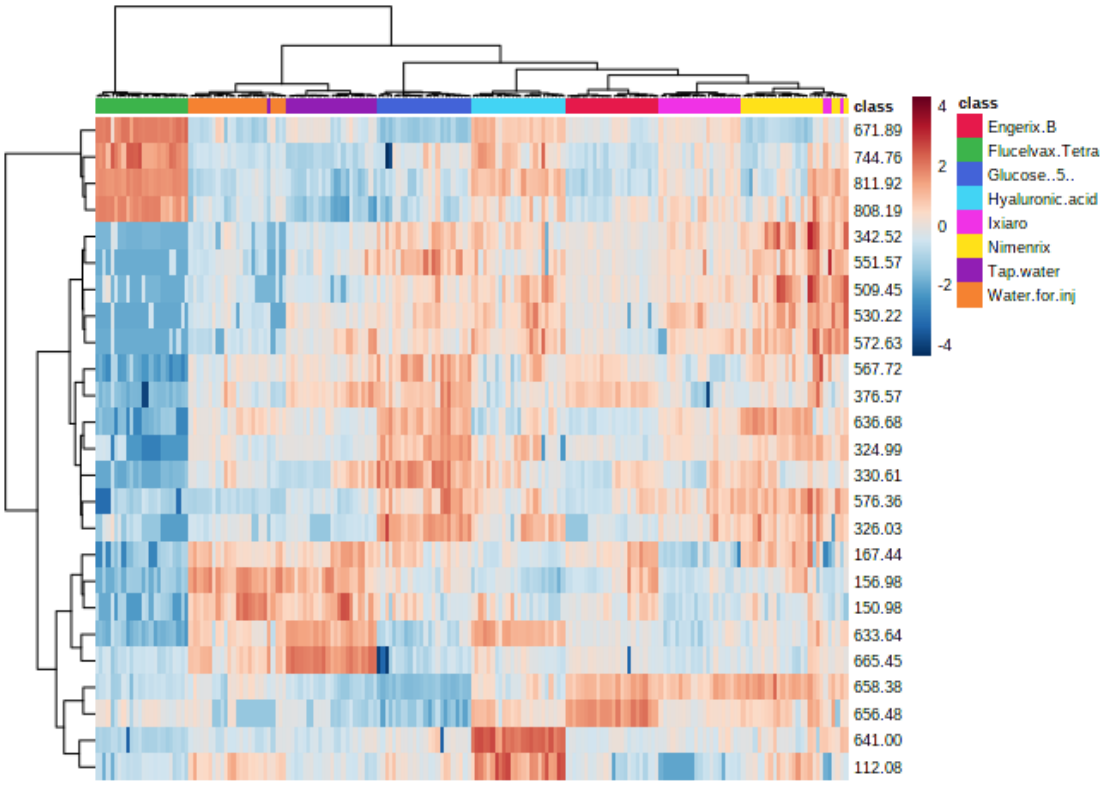


Table 8.2 Results of the best performing model, partial least square-discriminant analysis (PLS-DA), to differentiate vaccines and common vaccine surrogates.

The data were split into a training set (day 1 n=120; 4 replicates of 3 vials each of 10 sample types, 4 vaccines and 6 falsified vaccine surrogates) and testing set (day 2 n=120; 4 replicates of 3 vials each of 10 sample types, 4 vaccines and 6 falsified vaccine surrogates). Overall accuracy 80.0% (95% confidence intervals 71.7-86.8).

Confusion matrix		Reference				
		Engerix B	Falsified vaccine*	Flucelvax Tetra	Ixiaro	Nimenrix
Prediction	Engerix B	8	2	0	0	0
	Falsified vaccine*	3	59	0	0	5
	Flucelvax Tetra	0	0	12	0	0
	Ixiaro	1	2	0	11	1
	Nimenrix	0	9	0	1	6

	Engerix B	Falsified vaccine	Flucelvax Tetra	Ixiaro	Nimenrix
Sensitivity	66.7%	81.9%	100.0%	91.7%	50.0%
Specificity	98.1%	83.3%	100.0%	96.3%	90.7%
Positive Predictive Value	80.0%	88.1%	100.0%	73.3%	37.5%
Negative Predictive Value	96.4%	75.5%	100.0%	99.0%	94.2%
Prevalence	10.0%	60.0%	10.0%	10.0%	10.0%
Detection Rate	6.7%	49.2%	10.0%	9.2%	5.0%
Detection Prevalence	8.3%	55.8%	10.0%	12.5%	13.3%
Balanced Accuracy	82.4%	82.6%	100.0%	94.0%	70.4%

After removing MilliQ water and saline (0.9%); overall accuracy 81.3% (95% confidence intervals 72.0-88.5)

Confusion matrix		Reference				
		Engerix B	Falsified vaccine*	Flucelvax Tetra	Ixiaro	Nimenrix
Prediction	Engerix B	11	0	0	0	0
	Falsified vaccine*	0	38	3	0	2
	Flucelvax Tetra	0	0	9	0	0
	Ixiaro	1	1	0	12	2
	Nimenrix	0	9	0	0	8

	Engerix B	Falsified vaccine*	Flucelvax Tetra	Ixiaro	Nimenrix
Sensitivity	91.7%	79.2%	75.0%	100.0%	66.7%
Specificity	100.0%	89.6%	100.0%	95.2%	89.3%
Positive Predictive Value	100.0%	88.4%	100.0%	75.0%	47.1%
Negative Predictive Value	98.8%	81.1%	96.6%	100.0%	94.9%
Prevalence	12.5%	50.0%	12.5%	12.5%	12.5%
Detection Rate	11.5%	39.6%	9.4%	12.5%	8.3%
Detection Prevalence	11.5%	44.8%	9.4%	16.7%	17.7%
Balanced Accuracy	95.8%	84.4%	87.5%	97.6%	78.0%

*Amikacin and Gentamicin crystallised poorly with CHCA matrix leading to difficulties obtaining spectra and these samples were not included in the analysis.

Degradation

The spectra obtained after storing samples of the Ixiaro vaccine at 45°C for one week and another set of Ixiaro samples after performing three freeze-thaw cycles, were compared to the vaccine stored under manufacturers' instructions at 2-8°C. More subtle changes were observed for the three different conditions compared to those seen in the analysis of vaccine samples vs falsified vaccine surrogates, Figure 8.3 shows average spectra. Hierarchical clustering was performed after data processing as per the previous analysis, including peak alignment, normalisation and batch correction, see Figure 8.7. This demonstrated that there appeared to be differences between the samples that had undergone three freeze-thaw cycles and those stored at 2-8°C or at 45°C.

Prediction models did not perform well. PLS-DA scores plots show partial overlap but some separation of each experimental group (Figure 8.8). The same data was also used to train models with cross-validation, with five vials analysed on day 1 used for training and five different vials from day 2 used for testing; a random forest model demonstrated the highest accuracy of 56.7% (95% confidence intervals 43.2-69.4), see Table 8.3. Notably, the model demonstrated good sensitivity, 80.0%, for detecting samples that had undergone three freeze-thaw cycles.

Figure 8.7 Dendrogram of peak lists obtained from MALDI-ToF spectra in the 0-900 *m/z* range of Ixiaro vaccine samples stored under different conditions

- at 2-8°C (4C - blue), 45°C (45C - green) and x3 freeze-thaw cycles (3FT - red) (n=240).

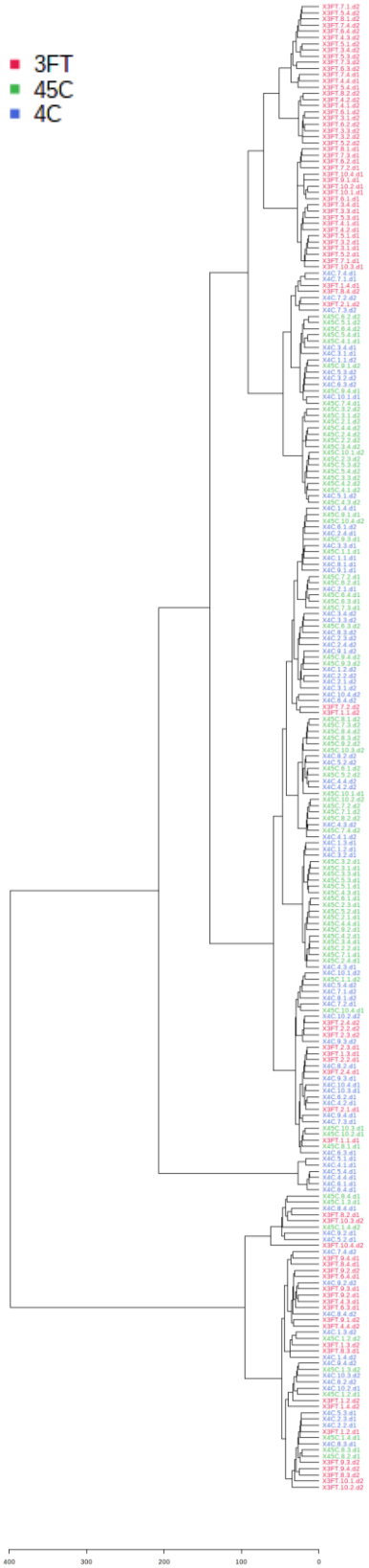
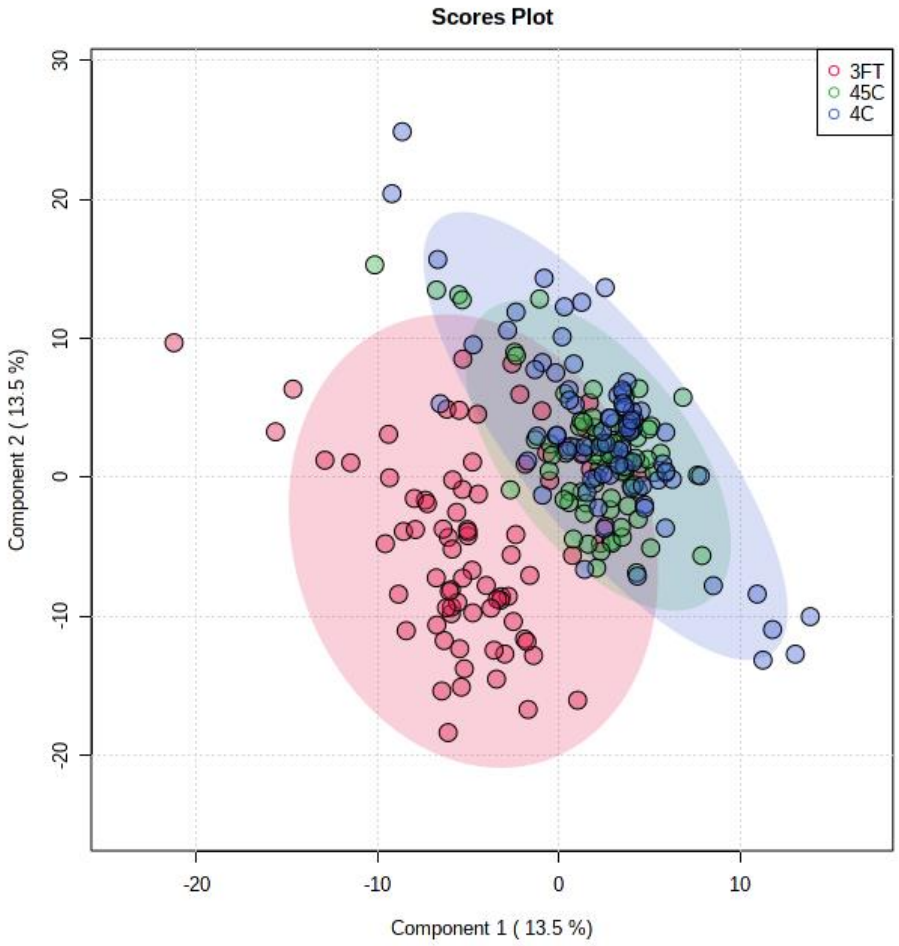
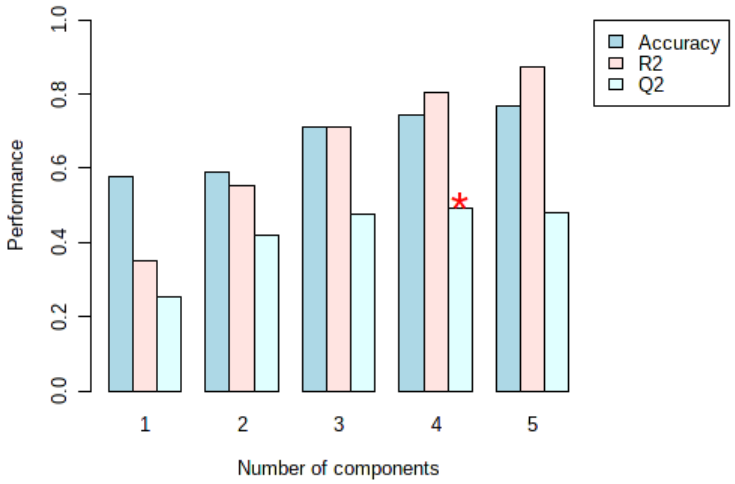


Figure 8.8 Partial least squares-discriminant analysis (PLS-DA) score plots and cross-validation results[#] of the MALDI-TOF spectra in the 0-900 *m/z* range for Ixiaro vaccine samples stored under different conditions





[#]Q2 is an estimate of the predictive ability of the model, and is calculated via cross-validation (CV). In each CV, the predicted data are compared with the original data, and the sum of squared errors is calculated. The prediction error is then summed over all samples (Predicted Residual Sum of Squares or PRESS). For convenience, the PRESS is divided by the initial sum of squares and subtracted from 1 to resemble the scale of the R2. Results are presented for the model with the number of components with the highest predictive ability (Q2), with a maximum of 5 components.

Figure 8.9 The best performing features (ions) using partial least squares-discriminant analysis (PLS-DA) of the MALDI-TOF spectra in the 0-900 *m/z* range for Ixiaro vaccine samples stored under different conditions

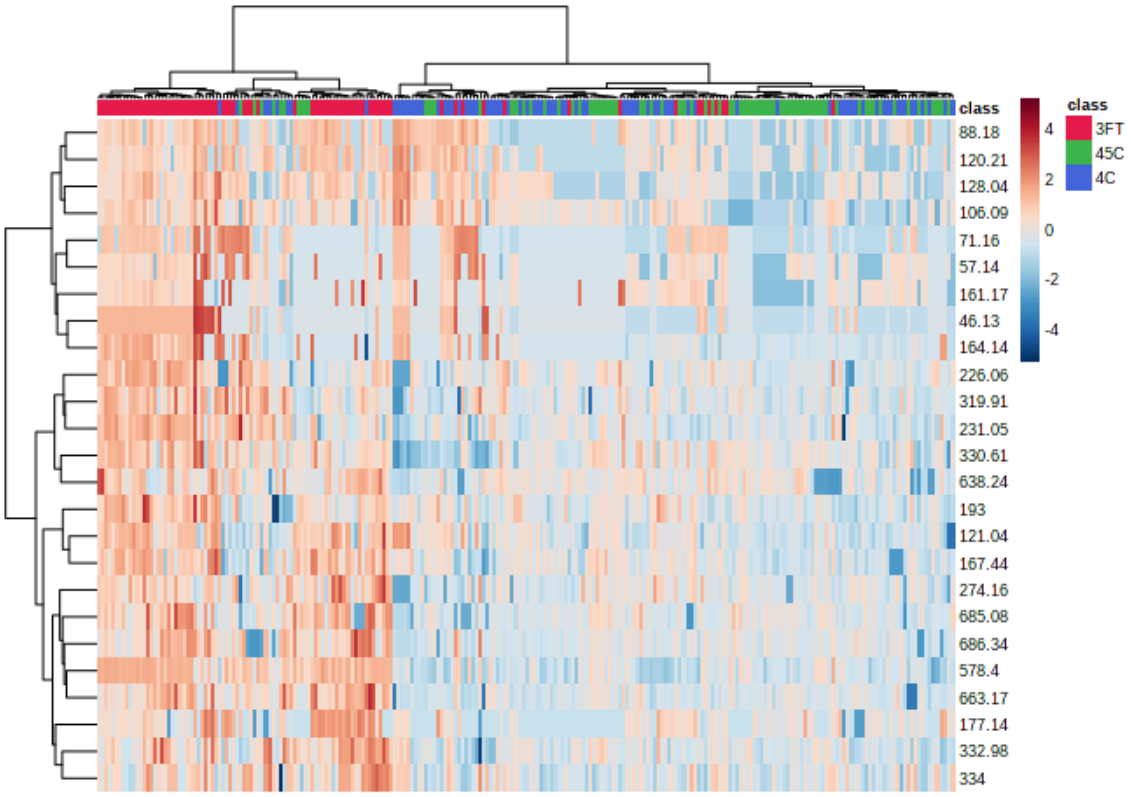


Table 8.3 Results of the best performing model, random forest, to differentiate Ixiaro vaccine samples stored under different conditions

Confusion matrix		Reference		
		x3 freeze-thaw cycles	45°C one week	2-8°C as per manufacturer
Prediction	x3 freeze-thaw cycles	16	1	11
	45°C one week	0	9	0
	2-8°C as per manufacturer	4	10	9

	x3 freeze-thaw cycles	45°C one week	2-8°C as per manufacturer
Sensitivity	80.0%	45.0%	45.0%
Specificity	70.0%	100.0%	65.0%
Positive Predictive Value	57.1%	100.0%	39.1%
Negative Predictive Value	87.5%	78.4%	70.3%
Prevalence	33.3%	33.3%	33.3%
Detection Rate	26.7%	15.0%	15.0%
Detection Prevalence	46.7%	15.0%	38.3%
Balanced Accuracy	75.0%	72.5%	55.0%

The data were split into a training set (day 1 n=60; 4 replicates of 5 vials of 3 storage conditions) and testing set (day 2 n=60; 4 replicates of 5 vials of 3 storage conditions); accuracy 56.7% (95% CI 43.2-69.4).

Discussions

MALDI-ToF was selected for utilisation in SF vaccine screening in light of its high prevalence in microbiology laboratories worldwide. The bench-top mass-spectrometer provides a rapid and accurate method for the identification of microorganisms, harnessing its capacity for protein profiling in relation to a reference database. We aimed to follow standard methods that are implemented in microbiology laboratories, utilising CHCA matrix, with positive ionisation mode and 2,000-20,000 *m/z*. However, poor spectra were obtained in the standard mass range, and we also investigated 0-900 and 700-2,500 *m/z*. The most consistent spectra, with characterizable peaks, were seen in the 0-900 *m/z* range, and the analysis focussed on this range. We inferred from this that the analysis focussed on small (0-900 Da) molecules, mainly metabolites; for the vaccine samples it is likely that these represent excipients. Table 8.1 lists the ingredients of the included vaccines.

Visual inspection of the spectra demonstrated the potential of MALDI-TOF to identify both falsified (counterfeit) and substandard (degraded) vaccines. MALDI-TOF provided characteristic spectra for the vaccines and falsified vaccine surrogates tested. Similarly, modelling the data looking at both vaccines vs falsified vaccine surrogates, and different storage conditions of Ixiaro vaccine using machine learning methods and cross-validation demonstrated very high predictive performance. However, there is a risk of over-fitting, and the real test involved splitting the data into a training set from day 1 and a test set from day 2. The instrument is calibrated each day, and this has been associated with minor differences in the spectra. Results highlighted issues of batch effects in the data, both due to misalignment of the peaks, and systematic differences in their intensities. This was largely remedied by an additional alignment, normalisation and batch correction.

For the falsification experiment, a predictive model was built to discriminate between the four vaccines and the falsified vaccine surrogates with an overall accuracy of 81.3%. The model demonstrated over 95% sensitivity and specificity in identifying Ixiaro vaccine samples. Clearly, some of the samples contain the same ingredients at similar concentrations and the

spectra are identical or very similar, such that classification is not an easy task and the accuracy would not be expected to reach 100%. However it is possible that misclassification could be related to residual issues with alignment and batch effects. To this effect, more complex machine learning methods such as incorporation of time series classification, that is to take into account the position (*m/z*) of peaks in the spectrum could improve the performance. Equally, additional replicates on different days would be expected to assist a model in focussing on prioritising real peaks over noise.

The best performing model in discriminating Ixiaro samples stored under different conditions demonstrated an overall accuracy of 52.5%. However, a higher performance (accuracy 73%) was achieved in identifying samples that had undergone x3 freeze-thaw cycles. This is in line with manufacturers guidance that it is imperative that Ixiaro vaccine vials are not frozen (621). The reproducibility and clinical implications (in terms of vaccine efficacy, of the observed spectra for degraded vaccines) remain to be determined. This would require further work, potentially involving animal models to test for correlates of immune protection.

This work represents a preliminary analysis of the role of MALDI-ToF for detection of SF vaccines. There are a number of limitations that will need to be addressed in subsequent studies. Importantly, it appears that the ions detected are not active ingredients in the vaccine, for example nucleic acid or proteins. The low *m/z* suggests that the detected ions are excipients. These could also represent matrix adducts, ions that are formed by the addition of matrix ions, and this could be an issue if the matrix formulation changes (622). Several vaccines have very similar excipients, such that the quantitative accuracy of detection is important. To this end, an alternative method and potentially more accurate method than MALDI-ToF could be the use of routine clinical chemistry instruments used in hospital laboratories to detect common clinical biomarkers. Most hospital laboratories are able to detect sodium, potassium, magnesium, chloride, phosphate, calcium, glucose, protein and lipids. Nonetheless, these instruments would not detect rarer excipients such as organic compounds that are present in some vaccines are would produce unique MALDI-ToF peaks. The number of vial ‘biological’ replicates was relatively small, and different batches were not tested.

Further work will involve investigating sample cleaning and enrichment (e.g. C3 ziptips, molecular weight cut-off filters), alternative matrices and negative ionisation mode, inter- and intra-batch variability, exploring different machine learning models and developing quantitative methods for evaluating the observed spectral peaks. We are currently submitting a three-year grant application to support this work. In the proposal, we aim to analyse ten non-COVID vaccines and six COVID vaccines. We will evaluate additional falsified vaccine surrogates, degraded vaccines and non-invasive methods of detection, such as SORS. We are also working on strategies for the implementation of these methods.

Overall, these results are encouraging and support the idea that a wider range of SF vaccines will be amenable to being identified by MALDI-TOF analysis. We will build on this work to allow us to use the information to create a database for field authentication building on the global MALDI-TOF infrastructure for analysis in proximal and provincial vaccine supply chains. For JEV, ensuring the quality of vaccines utilised in immunisation programmes represents an integral aspect control of the disease.

Publications arising from this chapter

In view of the sensitive nature of this work, we have been cautious about putting the data in the public domain. The results could be used by criminals to develop their falsification methods, and raising awareness of the issues could heighten vaccine hesitancy. We have organised an in-person meeting in July 2022 to discuss the issues with a variety of stakeholders including sociologists, members of Interpol, the Medicines and Healthcare products Regulatory Agency (MHRA), WHO and vaccine manufacturers. Shortly after this, we will submit several manuscripts for publication.

Chapter 9

Conclusions and further work

In this final chapter, I report my conclusions and suggestions for further work.

The primary aim of this thesis was to answer the question of whether there is a diagnostic host protein signature of JE in human CSF that could be harnessed for the development of an RDT. I approached this with rigorous methodology, starting with reviewing the literature on existing diagnostic tests for JEV (623) and existing proteomics methods that have been applied (367). I was fortunate to have access to a relatively large cohort of well-characterised patient samples from the Laos CNS infection study (19). I confirmed suspected JE patients, positive by JE MAC-ELISA, by the gold-standard method of VNT (303). A pilot LC-MS/MS study involving 15 patient CSF samples (8 JE and 7 non-JE), demonstrated feasibility and informed a power calculation. I subsequently performed a larger LC-MS/MS study involving 148 additional patients (60 JE and 88 non-JE, enabling network and machine learning analysis. The main outcome of the research is the identification of a unique ten-protein signature of JE in CSF. This was verified in 10% of patient samples using an alternative LC-MS/MS platform with label-free DIA. In summary, my thesis research provides evidence to suggest that there is a diagnostic host protein signature of JE in CSF. Re-analysis of a previous study performed in Laos (427) on the same patient cohort (15/61 were included in the LC-MS/MS work) provides preliminary antibody-based ELISA data to support the development of an RDT to diagnose JE using host biomarkers.

In addition to the primary research question, I have contributed to understanding of issues related to diagnosing neurological infections, most importantly elucidating those for JE. The review of existing diagnostics for JE suggests that in spite of evidence that there is poor specificity of the

current standard diagnostic test (JE MAC-ELISA), particularly for serum, there is increasing reliance on detection of serum IgM by ELISA for confirmation (623). The experimental work performed to confirm the JE patients by virus neutralisation incorporated a novel adaptation of IgG depletion such that there is detection of IgM NAb (303). This method enables confirmation of JE in a single serum sample, in contrast to requiring paired serum demonstrating increasing total NAb titres. Furthermore, a pilot study suggested that the IgM VNT method could be performed from serum stored on filter paper. Introduction of serum spotted on filter paper for storage and transport to reference centres for confirmation of JE patients in endemic area could improve our understanding of JE epidemiology, and has potential to transform the implementation of the JEV vaccine programme (292).

Nonetheless, IgM VNT performed at reference centres on serum stored on filter paper does not solve the problem. At an individual patient level, improving access to an RDT is essential but no such test exists (608, 624). JE remains a leading cause of neurological infection in Asia, and an RDT with high specificity would allow clinicians to stop unnecessary antibiotics in patients with JE, or to explore alternative treatable causes in potential non-JE cases, for example scrub typhus. It was for these reasons that the findings from addressing the primary research question remain crucial. The LC-MS/MS proteomics work also strengthens understanding of the host response to JEV *in vivo* and has highlighted potential novel targets for therapeutics. Lastly, the research provides an exemplar to the approach to the diagnosis of other neurological infections which are hitherto poorly characterised.

In response to the COVID-19 pandemic, I also worked on the VIE project, expanding the scope of my DPhil, investigating a related neglected public health problem that could help to ensure the efficacy of JEV vaccination. This improved my understanding of a range of complementary proteomics methods to those described for my other DPhil work, such as MALDI-ToF, RDTs and metabolomics.

I acknowledge limitations of this work, as described in individual chapters. There may have been sampling bias, in that more severe infections presented to hospital and were included in the study. The CSF samples had been stored for up to sixteen years, with the possibility of having undergone multiple freeze-thaw cycles, limited details on centrifugation, there was missing patient data, and some of the samples contained red cells. The LC-MS/MS processing pipelines involved multiple steps that were unavoidable but could have introduced errors in protein quantification. TMT labelling may be associated with bias due to 'ratio distortion' (discussed in Chapter 6) (516), and relies on protein normalisation between samples, that may not translate to ELISA testing with a standard sample volume. While the 163 patients represents an impressive and diverse collection of precious CSF samples, from the perspective of host biomarker research and machine learning analysis, this is a relatively small number and needs to be validated by high-throughput targeted proteomics methods in a larger group of patient samples. Ideally, each patient sample 'biological replicate' would have been tested under the same conditions at least three times. Equally, the biomarkers need to be evaluated in different settings. I appreciate that ten proteins will not be incorporated in an RDT in remote areas, however the rationale was to include a larger number of proteins at this stage that will then be evaluated further. In this thesis, I had intended to perform testing of other body fluids (Chapter 7) and antibody-based confirmation, but this was not possible due to the COVID-19 pandemic and equipment problems.

Further work will involve validating the use of filter paper for storage and transport of blood and CSF from remote areas to reference centres for gold-standard confirmation of JE. The ten proteins will need to be evaluated by ELISA in a larger number of patient samples and from other locations, ideally testing patient samples prospectively. A lack of validated commercial ELISAs for these proteins for testing CSF suggest that this may require the in-house development of ELISA assays. Importantly, it will be vital to test non-invasive patient samples. Moreover, a longer-term aim will be to develop serum host biomarkers to categorise patients

with suspected CNS infections, to inform downstream diagnostic testing and management (625).

My thesis work offers support to the notion that the study of proteins in CSF does provide a 'window into the brain' of patients with JE (361). I have gained experience of a variety of proteomics and MS techniques. My impression of the field of clinical proteomics is that the scientific methodology still needs to develop; to improve the sensitivity, reproducibility, throughput and cost. The human proteomics project was set up in 2010 (626), a decade after the human genomics project, and proteomics research has lagged behind that of genomics (374). Admittedly, proteomics research is much more complicated than current genomics research; it needs to take into account isoforms, three-dimensional conformation and PTMs. In the field of LC-MS/MS, the increasing application of DIA (580, 627-629) offers improved depth (2800 proteins identified in this study) and coverage (90-100%), but remains low-throughput (1 hour) and expensive (£170/sample). Alternative technologies for discovery work include SomaScan (aptamers) and O-link (proximal extension assays) but these are not truly untargeted and are prohibitively expensive (630). It is difficult raising the funds for research for a neglected disease such as JE (631) to achieve the full potential of proteomics technology, and the research presented here represents an important step in this direction, and one that I hope to follow.

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