THE TRANSLATIONAL POTENTIAL OF NEXT-GENERATION SEQUENCING AND BIOLOGICAL MASS SPECTROMETRY IN PATIENTS WITH CENTRAL NERVOUS SYSTEM INFECTIONS IN VIETNAM

by

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Abstract

Central nervous system (CNS) infections cause significant mortality and morbidity worldwide. Novel diagnostic approaches are therefore urgently needed to improve patient outcomes. My study was conducted at a tertiary referral hospital for Southern Vietnam between September 2017 and September 2020, aiming at improving the diagnosis in adult patients with CNS infection syndromes using a combination of metagenomics- and proteomics-based approaches. I set out to study significant insights into the epidemiology, causes, clinical features, and outcomes of 581 patients. Despite extensive laboratory investigations, the causes were established in 58.7% of the study patients. Diverse infectious agents encompassing bacteria, viruses, fungi, and parasites were detected with *Mycobacterium tuberculosis*, *Streptococcus suis*, HSV and VZV as the major causes. My research also revealed for the first-time that anti-NMDAR encephalitis is a common cause of CNS infection syndromes, accounting for 21.3% of 221 patients with suspected encephalitis, and disability documented up to 12 months post-discharge. CNS infection syndromes in our setting are associated with high morbidity and mortality (2397/581, 68.3% and 29/581, 5.0%, respectively) and with overlapped clinical and laboratory findings. To address diagnostic challenges of CNS infection syndromes, I developed and prospectively evaluated a metagenomic pipeline for simultaneous detection of both bacterial and viral causes. The results emphasised that metagenomics could provide a complementary approach to improve pathogen identification alongside conventional diagnostic assays. Additionally, I evaluated the diagnostic performance of two novel CSF biomarkers, Lipocalin 2 (LCN2) and a disintegrin and metalloprotease like decysin (ADAMDEC1). The results showed that LCN2 outperformed existing CSF parameters (leukocytes, protein, lactate, and glucose) in discriminating bacterial meningitis from other causes. In contrast, ADAMDEC1 did not offer any extra diagnostic values as compared to current CSF biomarkers. Collectively, my findings open new research opportunties to improve the diagnosis of patients with CNS infections in Vietnam and other regions where pathogen exposure is prevalent.

Co-Authorship

This thesis is composed of my original work under the supervisions of Associate Professor Le Van Tan, Associate Professor Catherine Louise Thwaites, Professor Benedikt Kessler and Dr Nguyen Van Vinh Chau. The work presented in this thesis contains no material previously published or written by other investigators unless otherwise stated herein.

Patient recruitment and follow up were done in collaboration with Dr Vu Thi Ty Hang, Emerging Infection group of Oxford University Clinical Research Unit, and study doctors and nurses at Viet Anh Department of the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam.

Mass spectrometry-based proteomics for biomarker discovery was carried out in collaboration with Dr Climent Casals-Pascual and Professor Benedikt Kessler at the Target Discovery Institute, Centre for Medicines Discovery Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom. For verification and validation of novel biomarkers, I based my analysis on results of those original mass-spectrometry findings (outlined in Chapters 5 and 6).

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I am deeply grateful to the Hosital for Tropical Diseases, especially Department of Clinical Laboratories to give me opportunities to apply for this programme. I especially wish to thank my colleagues at Molecular Diagnostic Laboratory, especially Ms Pham Thi Ngoc Thoa, who shared my responsibilities and duties during the time I enrolled in the progamme.

I would like to take this opportunity to say thank you to Dr Leigh Jones and Ms Le Thi Kim Yen from OUCRU Training Committee for their valuable support and guidance.

Lastly, I would like to give my sincere thanks to my dad, my aunt, my husband and my kids for their endless support to me and sharing with me sorrows and happiness.

Publications

A. Manuscripts as parts of the thesis

1. **Nghiem My Ngoc**, Ho Dang Trung Nghia, Nguyen Thi Hoang Mai, Nguyen Thi Thu Hong, Vu Thi Ty Hang, Dinh Nguyen Huy Man, Tran Tan Thanh, Nguyen Thi Han Ny, Tran Ba Thien, Nguyen Phu Huong Lan, Le Nguyen Truc Nhu, Lam Minh Yen, Le Kim Thanh, Lam Anh Nguyet, Nguyen To Anh, Du Trong Duc, Le Thi My Chau, Bui Thi Hong Hanh, Van Xuan Quynh, Nguyen Ho Hong Hanh, Le Thi Diem, Bui Thi Bich Hanh, Tran Bao Nhu, Pham Kieu Nguyet Oanh, Nguyen Hoan Phu, Nguyen Van Vinh Chau, Louise Thwaites, Benedikt M. Kessler, Guy Thwaites, and Le Van Tan. Development and prospective evaluation of an internally controlled metagenomic workflow for simultaneous detection of bacterial and viral causes of central nervous system infections. (Submitted to Heliyon, June 2023: currently under review).
2. **Nghiem My Ngoc**, Ho Dang Trung Nghia, Vu Thi Ty Hang, Nguyen Thi Hoang Mai, Nguyen Thi Thu Hong, Dinh Nguyen Huy Man, Tran Tan Thanh, Nguyen Thi Han Ny, Tran Ba Thien, Nguyen Phu Huong Lan, Le Nguyen Truc Nhu, Lam Minh Yen, Le Kim Thanh, Lam Anh Nguyet, Nguyen To Anh, Du Trong Duc, Le Thi My Chau, Bui Thi Hong Hanh, Van Xuan Quynh, Nguyen Ho Hong Hanh, Le Thi Diem, Bui Thi Bich Hanh, Tran Bao Nhu, Pham Kieu Nguyet Oanh, Nguyen Hoan Phu, Nguyen Van Vinh Chau, Louise Thwaites, Benedikt M. Kessler, Guy Thwaites, and Le Van Tan. Frequency, clinical features, and long-term outcomes of autoimmune N-Methyl-D-Aspartate Receptor (NMDAR) encephalitis in a tertiary hospital in Ho Chi Minh City, Vietnam. (Manuscript in preparation).
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B. Other publications during candidature

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Impact of COVID-19

I am head of the Molecular Diagnostics Laboratory of the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City Vietnam. HTD is a 550-bed tertiary referral hospital for patients with (emerging) infectious diseases in southern Vietnam, with a population of over 40 million. HTD was one of the first to qualify for COVID-19 diagnostics by the Ministry of Health in early 2020. Therefore my lab was responsible for conducting the majority of COVID-19 testing in Ho Chi Minh City in 2020 and 2021. During the early phase of the pandemic, we also trained another four laboratories in Ho Chi Minh City to conduct COVID-19 diagnostics. Apart from being a frontline healthcare worker, contributing to the pandemic responses in Vietnam, I also took part in several studies which led to publications listed above which were related to COVID-19. These outputs were possible in part because of the established research capacities, especially the sequencing pipelines that were also set up as part of my PhD research.

Abbreviations

ADAMDEC1	A disintegrin and metalloprotease like decysin
ADEM	Acute disseminated encephalomyelitis
AF	Acid-fast
AFB	Acid-fast bacillus
AIDS	Acquired immunodeficiency syndrome
AIE	Autoimmune encephalitis
ASDR	Aged-standardized DALY rate
AUROC	Area under the receiver operating characteristic curve
AV	Alphaviruses
BM	Bacterial meningitis
BV	Bunyaviruses
CZID	Chan Zerkerberg ID
CM	Cryptococcal meningitis
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Cerebral toxoplasmosis
DALYs	Disability-adjusted life-years
ddNTP	Dideoxynucleoside triphosphate
DENV	Dengue virus
DNA	Deoxyribonucleic acid
DOR	Diagnostic odds ratio
dsDNA	Double-stranded DNA
EAPC	Estimated annual percentage change
EAV	Equine Arteritis Virus
EBV	Epstein-Barr virus
EI	Emerging Infections
ELISA	Enzyme-linked immunosorbent assay
EM	Eosinophilic meningitis
ESI	Electrospray ionization

FTICR	Fourier-transform ion cyclotron resonance
g	Gram
GBS	Group B *Streptococcus*
GCS	Glasgow Coma Score
HHV-6	Human herpesvirus 6
HiB	*Haemophilus influenza* serotype B
HIV	Human immunodeficiency virus
HP adapter	Hairpin adapter
HSV	Herpes simplex virus
HTD	Hospital for Tropical Diseases
HEVs	Human enterovirus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IQR	Interquartile range
IVIG	Intravenous immunoglobulins
JCV	John Cunningham virus
JEV	Japanese encephalitis virus
L	Liter
LCMV	Lymphocytic choriomeningitis virus
LCN2	Lipocalin- 2
LFA	Lateral flow assay
LIT	Linear ion trap
LMICs	Low- and middle-income countries
MAC	IgM antibody capture
MALDI	Matrix-assisted laser desorption/ionization
MeV	Measles virus
MGIT	Mycobacteria Growth Indicator Tube
ml	Milliliter
mm^3	Cubic millimeter
mmol	Millimolar

mNGS	Metagenomic next-generation sequencing
MRI	Magnetic resonance imaging
mRS	Modified Rankin scale
MS	Mass spectrometry
MTB	*Mycobacterium tuberculosis*
Mumps	Mumps virus
NA	Nucleic Acid
Nipah	Nipah virus
NMDAR	N-Methyl-D-Aspartate Receptor
OLC	Overlap-layout-consensus
ONT	Oxford Nanopore Technologies
OxTREC	Oxford Tropical Research Ethics Committee
PARV4	Human parvovirus 4
PCR	Polymerase chain reaction
PhHV	Phocid Herpes Virus
PV	Poliovirus
Q	Quadrupole
QIT	Quadrupole ion trap
RIF	Rifampicin
RNA	Ribonucleic acid
rPCR	Random PCR
RV	Rabies virus
SARS-CoV-2	Severe acute respiratory coronavirus 2
SBS	Sequencing by synthesis
SGS	Second-generation sequencing
SLEV	St. Louis encephalitis virus
TBEV	Tick-borne encephalitis virus
TBM	Tuberculosis meningitis
TGS	Third-generation sequencing
TOF	Time-of-flight
UI	Uncertainty interval

VDRL	Venereal disease research laboratory
VME	Viral meningoencephalitis
VZV	Varicella zoster virus
WCC	White cell count
WHO	World Health Organization
WNV	West Nile virus
YLDs	Years of life lived with disability
ZIKV	Zika virus
ZN	Ziehl-Neelsen
USA	United States of America

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Chapter 1

Central Nervous System Infection Syndromes and Diagnosis

1.1 Central nervous system infection syndromes

1.1.1 Introduction

Central nervous system (CNS) infections are major causes of mortality and morbidity throughout the world, but especially in low- and middle-income countries (LMICs) (1–3). According to the anatomic localization, CNS infections are classified as meningitis, encephalitis and brain abscess (4). Meningitis is defined as an inflammation of the brain membranes when pathogens penetrate the subarachnoid space of the meninges structure. The injury can be caused by both the causative agents and the host immune response. Encephalitis denotes inflammation of the brain parenchyma whereas myelitis refers to inflammation of the spinal cord. More diffuse processes of infection are referred to combinations of terms, including “meningoencephalitis” and “encephalomyelitis”. Collections of infective and purulent material may coalesce within the CNS as abscesses.

CNS infections can be caused by a wide variety of pathogenic organisms, encompassing bacteria, viruses, fungi, and parasites. But occasionally, non-infectious causes (e.g., autoimmune encephalitis) can be associated with clinical signs and symptoms that mimic those of infectious causes. Herein, the term CNS infection syndromes is used to refer to clinical entities that can be associated with infectious or non-infectious causes.

1.1.2 Global burden of CNS infection syndromes

Accurate global estimate of the volume and burden of CNS infection syndromes has been difficult because i) population-wide data are limited, ii) the heterogeneity in CNS infection types and locations is tremendous, and iii) underdiagnosis and underreporting in resource-limited settings are suspected (5). Nevertheless, available data show that the burden of CNS infection syndromes is unequally distributed in terms of geographic areas, disease categories and causative pathogens (3,5,6). The number of bacterial

meningitis (BM) cases reported to global surveillance increased from 2.50 million (95% uncertainty interval (UI), 2.19-2.91) in 1990 to 2.82 million (95% UI, 2.46-3.31) in 2016 whereas the global deaths to be due to meningitis decreased by 21% during this period (6). In 2016, globally 1.48 million (95% UI, 1.04-1.96) years of life lived with disability (YLDs) were due to meningitis compared with 21.87 million (95% UI, 18.20-28.28) disability-adjusted life-years (DALYs). The “meningitis belt” of Africa is composed of 26 countries in the sub-Saharan region and so-called as it has the highest burden of meningitis with 152,813 cases and 15,783 deaths reported to the World Health Organization (WHO) in 2016 (Figure 1.1) (7). Across this region, the incidence rate of seasonal epidemics during the dry season is 10-100 cases per 100,000 population with periodic outbreaks occurring every 8-12 years where there are more than 1,000 cases per 100,000 population (8).

Globally, 1,44 million cases, 89,900 deaths, and 4.8 million disability-adjusted life years (DALYs) related to encephalitis were estimated in 2019 (3). The current global incidence of encephalitis is estimated between 3.5 and 7.4 cases/100,000 inhabitants/year, excluding outbreaks (9,10). Of these, the incidence of pediatric encephalitis is more than 16 cases per 100,000 patient/year (11). The fatality of viral encephalitis ranges from 4.6% to 29% (12). Lower socio-demographic index (SDI) regions in South Asia, Western and Eastern Sub-Saharan Africa had the highest burden of encephalitis reported by the Global Burden of Disease Study 2019 (Figure 1.2) (3).

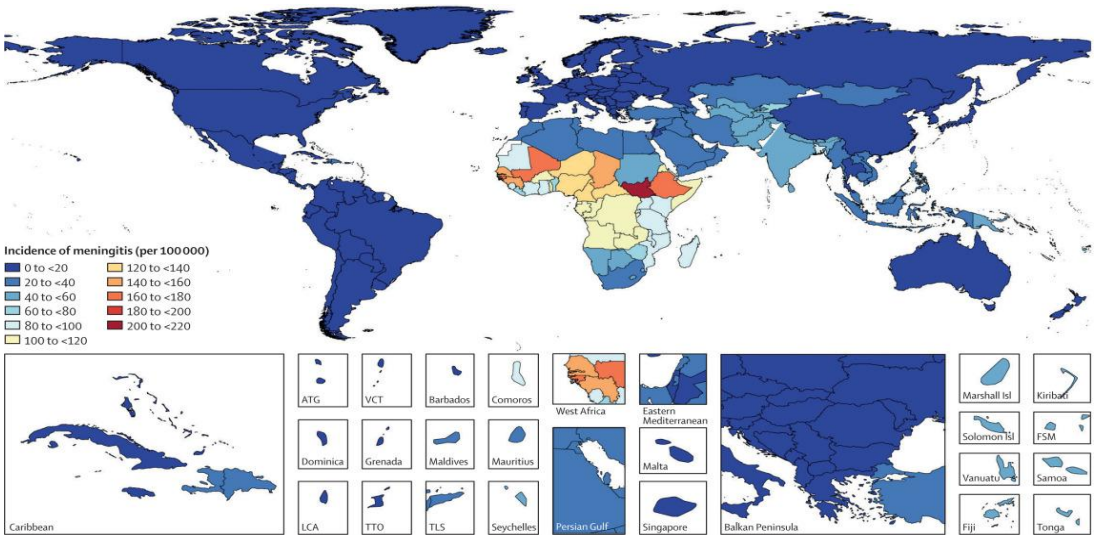


Figure 1.1: Incidence of meningitis per 100,000 population by age and location for both sexes, 2016 (Global Burden of Disease Study).

The figure was adapted from “Global, regional, and national burden of meningitis, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016” by Junt, J. E. et al, 2018 (6).

Note to figure 1.1:

ATG=Antigua and Barbuda. Isl=Islands. LCA=Saint Lucia. VCT=Saint Vincent and the Grenadines. TTO=Trinidad and Tobago. TLS=Timor-Leste. FSM=Federated States of Micornesia

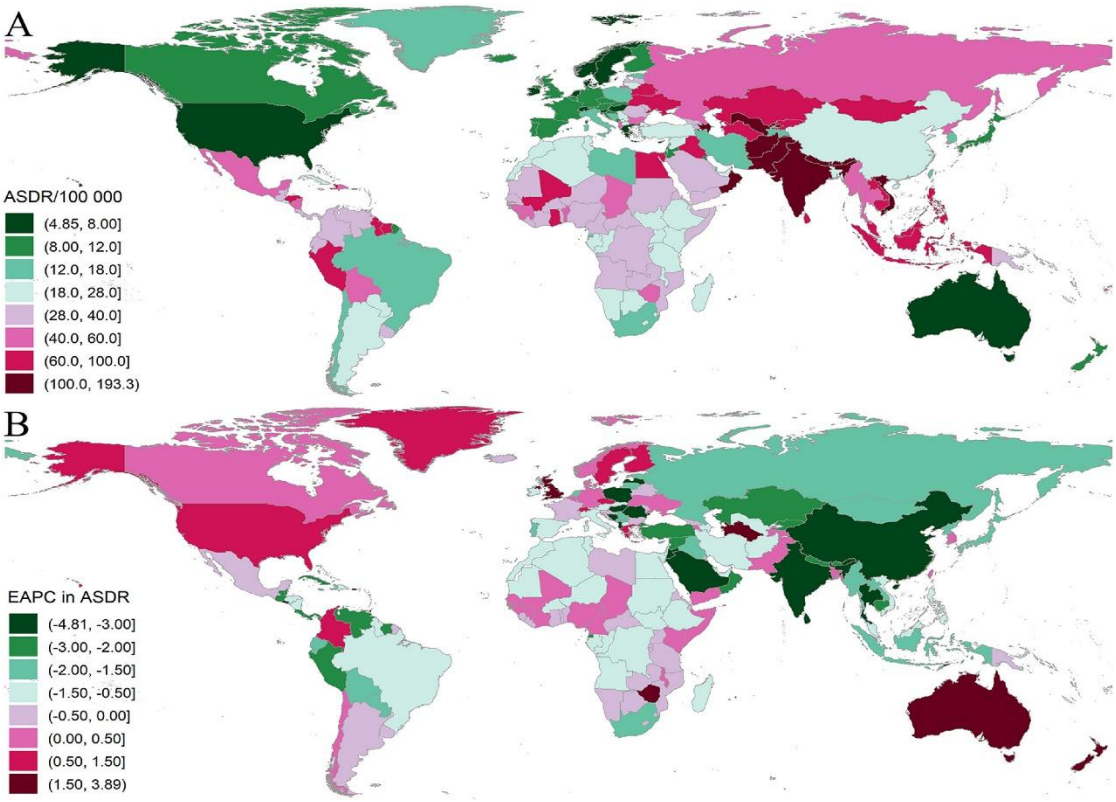


Figure 1.2: Map of global encephalitis DALYs (disability-adjusted life years) for both sexes in 204 countries and territories.

The figure was adapted from “Global magnitude of encephalitis burden and its evolving pattern over the past 30 years” by Wang, H. et al, 2022 (3)**.** (A) ASDR (aged-standardized DALY rate) in 2019; (B) EAPC (estimated annual percentage change) in the ASDR from 1990 to 2109.

1.1.3 Diverse aetiologies of CNS infection syndromes

The causes of CNS infection syndromes are diverse, consisting of bacteria, viruses, fungi, parasites (Table 1.1). There are hundreds of different pathogens reported to be responsible for CNS infection syndromes and the spectrum of causative agents varies by geographic location, age group, vulnerable population and seasonality (Figure 1.3) (13,14). Southeast Asia countries have been identified at high-risk for emergence and re-emergence of zoonotic diseases due to frequent exposure to a wide range of animals and animal products (15–18). Recent studies on the clinical epidemiology of CNS infections in Laos revealed that orientia, rickettsia, and leptospira pathogens are important causes of CNS infections in Laos (15,18). In Vietnam, the most common zoonotic pathogens causing bacterial meningitis in adults were *S. suis* (16,19–21). Leptospirosis and rickettsial infections are neglected infections in Vietnam and their circulation in Vietnam has not been well documented (22,23) . Across Asia, Japanese encephalitis virus causes around 50,000 infections despite the availability of an effective vaccine (17,24).

Differential diagnosis of non-infectious causes includes autoimmune N-Methyl-D-Aspartate Receptor (NMDAR) encephalitis, a newly identified form of autoimmune encephalitis (AIE) that was first described in 2007 (25). The first series of anti-NMDAR encephalitis cases was reported in Vietnamese adolescents and adults in 2017 (26). It is important to distinguish between AIE from infectious (basically viral) encephalitis as the treatment strategies of AIE are specific involving immunomodulation (27).

1.1.4 Diagnosis of CNS infection syndromes: an overview

Diagnosis of CNS infection syndromes requires a combination of clinical and laboratory findings. Definitive diagnosis of CNS infections requires the detection of the presence of specific pathogen markers (antigens, nucleic acids (NA), and/or antibodies) in the CSF samples (28). CSF culture is the “gold standard” for laboratory diagnosis of BM. Lumbar puncture should be undertaken promptly before antibiotic treatment (29). It is obligatory to obtain

the *in vitro* susceptibility of the causative microorganisms to rationalize treatment (28). Additional diagnostic tests including Gram staining, latex agglutination testing, PCR and sequencing might aid in aetiological investigations, especially for patients after antibiotic treatment (29). A confirmed encephalitis case requires the evidence of the presence of an infectious agent in brain biopsy or the detection of the pathogen genetic material or specific antibodies in the CSF. The definitive test for cryptococcal meningitis requires the detection of *Cryptococcus* antigen in the spinal fluid, visualization of *Cryptococcus* using India ink staining, polymerase chain reaction, enzyme immunoassay and latex agglutination (30,31). Recently, a lateral flow assay (LFA) was introduced into the diagnosis of Cryptococcosis as a point-of-care and low- cost test in resource-limited settings (31). Diagnosis of *A. cantonensis* is mostly based on clinical symptoms together with CSF eosinophilic pleocytosis, and a history of exposure to a potential source in an endemic area (32). Definitive diagnosis includes finding the intact larvae during microscopic examination of the CSF, detection of NA and/or antibodies in CSF. A definitive diagnosis of cerebral toxoplasmosis (CT) requires compatible clinical presentations, identification of ring-enhancing mass lesion(s) by neuroimaging, and detection of the organism in a biopsy specimen (33).

Diagnosis of AIE is based on a combination of a clinical history and supportive diagnostic testing, which includes but is not dependent on antibody testing (34). CSF investigations show moderate pleocytosis in 80% of patients, normal or mildly increased protein concentration in 30% of patients and oligoclonal bands in 60% (35). The detection of IgG-type antibodies against the GluN1 subunit of NMDAR provides a definitive diagnosis for anti-NMDAR encephalitis. The sensitivity of anti-NMDA receptor antibody testing is higher in CSF than in serum (36). Neuroimaging is complementary testing for the diagnosis of anti-NMDAR encephalitis. Systematic screening for ovarian teratomas is recommended through abdominopelvic magnetic resonance imaging (MRI) or computed tomography studies or transvaginal ultrasound in young women (37).

1.1.5 Challenges in the diagnosis of CNS infection syndromes

Rapid identification of causative agents benefits both public health, society, and individual patients. For example, early recognition and prompt treatment is crucial for patient survival and reduction of long-term sequelae (38). For some infections, rapid identification of pathogens would also enable local outbreaks to be quickly contained (39). Diagnosis of CNS infection syndromes based on clinical symptoms is unpractical as they are often nonspecific and overlapped with many other infectious and non-infectious syndromes (40). Laboratory investigations of causative agents are highly challenging because there are over 100 pathogens (including drug-resistant pathogens) known to cause CNS infections (41–44). Additionally, Asia is highly susceptible to emerging infectious diseases as illustrated by the emergence of (novel neurotropic) viruses such as Hendra virus, Nipah virus, influenza A virus subtype H5N1, enterovirus A71, Zika virus, and most recently severe acute respiratory coronavirus 2 (SARS-CoV-2) (45,46). This challenges routine diagnosis and illustrates the unprecedented threats of emerging pathogens. There have been several studies describing the aetiological findings of CNS infections in Vietnam recently (47–50). The evidence of aetiologies was identified between 27% and 52% of the patients using molecular-, serological-, or culture-based diagnostic methods. Similar studies in Nepal, India and England described the etiological findings established in 38% to 42% (51–53). In a recent study conducted in four referral hospitals in Cambodia, Vietnam, Lao and Myanmar among of 664 children with encephalitis during 2014 to 2017, the diagnostic yield was 64%, with the application of intensive diagnostic approaches including next-generation sequencing (54). Collectively, despite extensive laboratory work-up, the aetiology remains unidentified in 50-80% of patients presenting with meningitis or meningoencephalitis (47,49,51,52).

Table 1.1: Common causes of CNS infection syndromes.

Organism	Main risk groups	Geographical areas	References
Bacteria pathogens			
Group B *Streptococcus*	Neonates	Worldwide	(7,55)
Listeria monocytogenes	Neonates, elderly people, immunocompromised patients, pregnant women, and alcoholics	Worldwide	(7,28,56,57)
Escherichia coli	Neonates and elderly people	Worldwide	(7,55,58,59)
Haemophilus influenzae	Children	Worldwide	(55)
Streptococcus pneumoniae	Children, adults, and elderly people Those living in close quarters (such as military barracks or dormitory settings)	Worldwide	(60–63)
Neisseria meningitidis	Children and adults Those living in close quarters (such as military barracks or dormitory settings)	Worldwide	(60,64)
Streptococcus suis	Adults, professional exposure to pigs and pig meat (such as butchers and farmers)	Sporadic in European countries and America but cause large outbreaks in Vietnam, Thailand, and China	(65–70)
Leptospira spp.	Human infections are acquired by direct contact with infected urine or tissue or indirectly by contact with contaminated water or soil. Major affected groups are farmers, slaughterhouses, animal	Worldwide, common in Asia.	(15,18,22,71,72)

	raisers.		
Rickettsia spp.	Transmitted to humans by arthropod vectors such as mites, ticks, fleas and lice. Geographic distribution of the reservoir hosts determines the infections of rickettsial species.	Many parts of the world (the United States, Russia, South Africa), especially in Asia	(23,73–75)
Mycobacterium tuberculosis	Immunocompromised hosts, especially HIV patients	Worldwide	(1,55,76)
Viral pathogens			
Herpes simplex virus (HSV)	Children and adults (HSV-1) Neonates (HSV-2)	Worldwide	(13,77)
Varicella zoster virus (VZV)	Children and adults Those with immunocompromised status, and those on immunosuppressant drugs.	The second most common pathogen after HSV in the United States (US)	(27,78)
Enteroviruses (EVs)	Children	Outbreaks are restricted in the Asia-Pacific region (Vietnam, Taiwan)	(4,27,43,79–81)
Other herpes viruses, Epstein-Barr virus (EBV), Cytomegalovirus (CMV) and Human herpesvirus 6 (HHV-6)	Immunocompromised patients (usually HIV patients with a CD4 count of less than 100 cells/μL) (EBV and CMV), patients undergoing hematopoietic stem cell transplantation (HHV-6).	Rare	(27)
Rabies	Adults	Most cases are reported in Southeast Asia, Africa, and Latin America.	(13,82)

Flaviviruses (Dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and Zila virus (ZIKV)	Children and adults	Worldwide distribution but high prevalence in South, Central American and Southeast Asia (DENV). Most of the JEV cases occur in Asia, including Vietnam. WNV was established as a widespread epidemic across the US. ZIKV has become endemic in sub-Saharan Africa, most of Central American, South American, the Caribbean and the US.	(11,83–90)
Measles, Mumps and Rubella	Children and adults	Rare Outbreak of measles in Vietnam in 2014	(91–96)
Nipah and Hendra viruses	Humans and livestock	Serious outbreak in Australia, Malaysia Singapore, and Bangladesh.	(97–100).
Fungal pathogens			
Cryptococcus neoformans	Immunocompromised patients, especially HIV-infected patients with CD4 count of less than 100 cells/mL	Occurs mostly in sub-Sahara Africa, followed by Southeast Asia.	(13,101)
Parasitic pathogens			
Angiostrongylus cantonensis	People who ingest uncooked/undercooked food containing larvae of *A.cantonensis*	Mainly in tropical areas. An important emerging infectious disease.	(102–104)

Toxoplasma gondii	Patients with acquired immunodeficiency syndrome (AIDS) when the CD4 cell counts drop below 100 cells/µL, patients with hereditary immunologic disorders, on systemic chemotherapy	Worldwide	(1,55,105,106)
Autoimmune causes			
Autoimmune N-Methyl-D-Aspartate Receptor (NMDAR) encephalitis	More likely to impact women and young individuals, often with associated teratoma.	Increasing reports worldwide	(25,35,107,108)

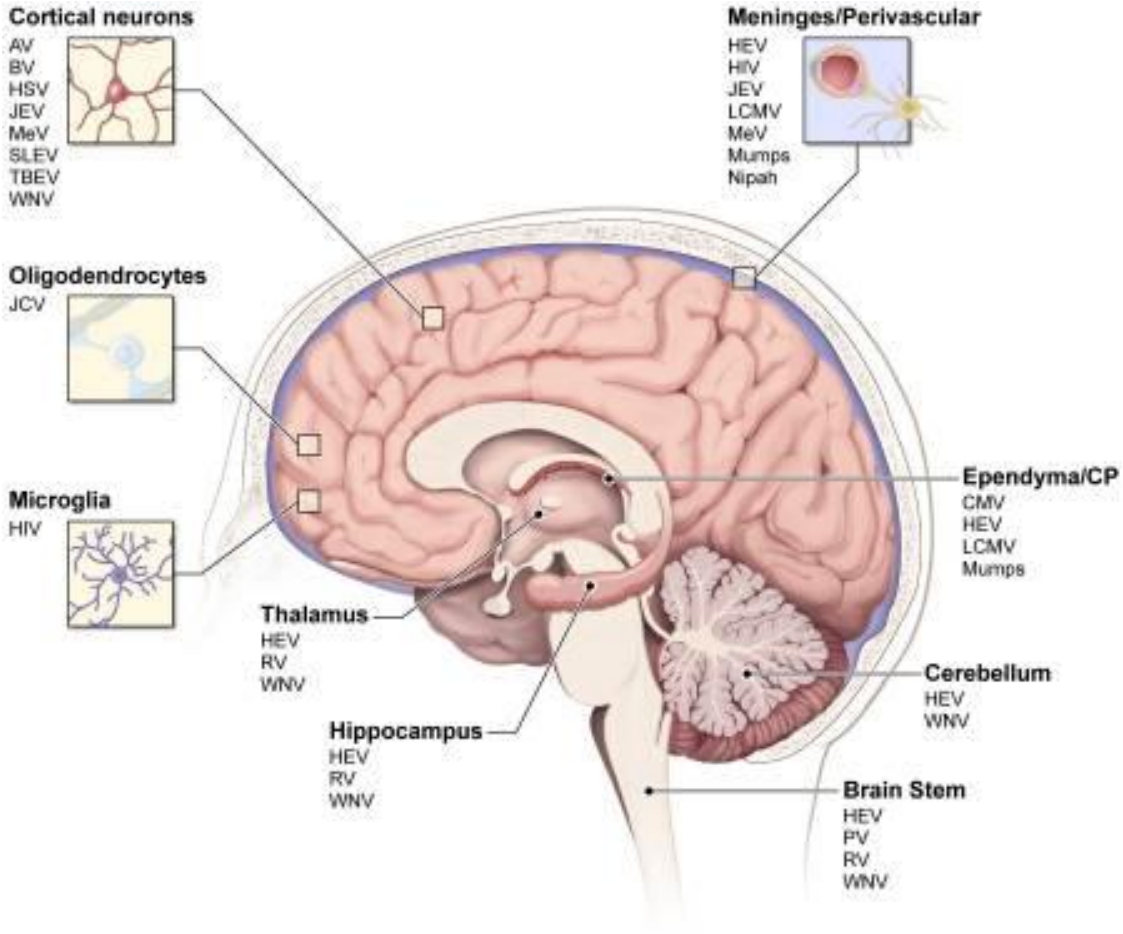


Figure 1.3: Aetiologies of meningitis and encephalitis by anatomical brain regions.

The figure was adopted from "Viral diseases of the central nervous system" by Swanson, P.A. et al, 2015 (43).

Note to figure 1.3:

AV, alphaviruses, BV, bunyaviruses, CMV, cytomegalovirus, HEV, human enteroviruses, HIV, human immunodeficiency virus, HSV, herpes simplex virus, JCV, John Cunningham virus, JEV, Japanese encephalitis virus, LCMV, lymphocytic choriomeningitis virus, MeV, measles virus, Mumps, Mumps virus, Nipah, Nipah virus, PV, poliovirus, RV, rabies virus, SLEV, St. Louis encephalitis virus, TBEV, tick-borne encephalitis virus, WNV, West Nile virus.

1.2 Laboratory diagnostic methods for CNS infection syndromes

There are multiple approaches to the diagnosis of CNS infection syndromes currently in use. Results of routine CSF analysis (WBC counts, glucose, lactate, and protein levels) can rapidly provide suggestive evidence of possible causes (bacterial, viral, or fungal) to inform timely initiation of empiric therapies. However, routine CSF parameters lack specificity (49,109,110). Definitive diagnosis of CNS infection syndromes is based on laboratory investigations to identify the evidence of infectious and non-infectious causes in CSF. These methods include microscopic examination, culture, nucleic acid-based methods, antigen, and antibody detection assays. The sensitivity and specificity of these methods vary according to organisms under investigation and can be dependent on the time interval from illness onset to CSF samples taken.

1.2.1 Microscopic examination

The Gram stain method was first described as a method of microscopic identification of bacteria by the Danish scientist, Hans Christian Gram, in 1884 and continues to be commonly used in clinical microbiology laboratories around the world. The method is simple, inexpensive and is based on structural characteristics of bacterial cell walls. Gram-positive microorganisms have higher peptidoglycan content, whereas gram-negative organisms have higher lipid content. The basic principle of Gram staining involves the ability of the bacterial cell wall to retain the crystal violet dye during solvent treatment (111). “Gram positive” bacteria are stained purple with a crystal violet-iodine complex and a safranin counterstain where as “gram-negative” bacteria appears in pink. Despite recent advances in clinical laboratory techniques, Gram stain still plays an important role in providing rapid information to guide empirical therapies (112). Previous studies have reported that the sensitivity of this technique ranges from 60 to 90% and the specificity reaches 100% (113–117). The yield of CSF Gram staining is dependent on the patient population, type of bacteria, and antimicrobial treatment prior to lumbar puncture (117–120). However, poor specimen

quality, smear preparation, and interpretation of the smears are the factors that contribute to Gram stain error rate. In a previous study, the discrepancy between Gram stain and bacterial culture was 5%. Of these, the error rate accounted for 24% (112).

The Ziehl-Neelsen (ZN) stain was developed in 1882 by Franz Ziehl and Friedrich Neelsen from the previous work of the scientists Koch, Ehrlich and Rindfleisch. It is also known as the acid-fast (AF) stain, a rapid, simple and cost-effective method for detecting *M. tuberculosis* (121). The method is based on specific characteristics of *M. tuberculosis* cell wall, which consists of a thick, lipid-rich outer layer made up of mycolic acids. This thick waxy coat attributes to the resistance of the bacteria to Gram stain and gives it the property of acid-fastness (122). Despite many efforts to improve the microbiological diagnosis, the sensitivity of ZN test is still low. In a recent multi-country study conducted in Vietnam, South Africa and Indonesia, the sensitivity of the modified ZN stain incorporating cytosine was 34.5% as compared to 33.9% of conventional ZN (123). In a comparative study of ZN stain and culture of respiratory samples from patients with suspected pulmonary tuberculosis, the overall sensitivity of ZN stain was 22.2% (124). The reasons for low detection rate of ZN stain could be the hard stain of AF dyes when the *M. tuberculosis* enter the cells and low volume of CSF samples (121). Modification on ZN stain methods and development of new techniques including GeneXpert are continuing to improve the sensitivity and effectiveness of *M. tuberculosis* diagnosis (121).

India ink stain is a rapid, cheap and reliable technique that directly examines the Cryptococcus in body fluids via microscopy (125). Different from the Gram stain, the fungus polysaccharide capsule does not absorb the India ink, resulting in an appearance of a halo around the cell against the dark background (126). Although culture remains the gold standard for Cryptococcus diagnosis and antigen detection has higher sensitivity and specificity, the India ink stain is still popular for microscopic examination of Cryptococci in CSF, especially in low-resource settings. Its detection rate was recorded in 75% of cryptococcal meningitis patients co-infected with

AIDS while the number of positive cases was much lower (≤ 50%) in individuals without AIDS infections (126). The disadvantages of India ink stain include the difficulty in distinguishing Cryptococci from lymphocytes, mostly attributable to experience of laboratory technicians and/or capsule change of Cryptococci identified from AIDS patients (126). In practical procedure, complementary methods including culture and antigen test should be included to increase the diagnosis sensitivity.

The Giemsa stain method carries the name of the German chemist, Gustav Giemsa. The method was originally designed for the identification of parasites in malaria (127). Currently, it is the standard diagnostic method for malaria *Plasmodium* species around the world. Wright-Giemsa, the modified stain, is a routine procedure in hematology laboratories to stain peripheral blood and bone marrow aspirate smears (128). It allows differentiation of various types of granulocytes and other blood cells by identifying basophilic and eosinophilic cytoplasmic properties of lymphoid and myeloid cells. Many scientists have made efforts to improve the Giemsa stain methods in terms of simplicity and better result achievement. However, there are crucial disadvantages that make the method less common. They includes impermanent slides due to quick evaporation when exposed to sunlight and inconsistent staining quality between different technicians and laboratories, leading to inaccurate interpretation of cellular characteristics (127).

1.2.2 Culture of CSF

Microbiological culture of CSF is considered the reference standard for the diagnosis of bacterial, mycobacterial, and fungal meningitis (129). However, its implementation requires sophisticated clinical laboratories and well-trained staff to ensure the quality of the results. The reported sensitivity values of CSF culture ranges from 67-90% (130–132), which are dependent on inclusion criteria, patient characteristics, laboratory practices, and pathogen spectrum (133). A positive bacterial culture also allows subsequent phenotypic drug susceptibility testing of the causative agents (134,135). The disadvantages of CSF culture methods include long turnaround time required

to recover the organisms from the original samples, which can be up to 72 hours, or in the case of *M. tuberculosis* can be longer (40). Additionally, the sensitivity of the assay can be significantly affected by the prior administration of antibiotics. The yield of CSF culture decreased in patients who received antibiotic pretreatment before lumbar puncture, ranging from 9-70% (113,136).

Viral culture can be performed using different cell lines (African green monkey cells, Vero cells, human amniotic epithelial cells, and human embryonic skin fibroblast). The use of different cell lines is to maximize the chance of recovering a viral pathogen from clinical samples (137). After inoculation with clinical samples, culture cell lines are evaluated using microscope to examine the morphological changes of the cells, known as cytopathic effect (CPE). The appearance of CPE is suggestive of viral replication, although not all viruses can cause CPE. Subsequently, identification of viral specific species can be achieved using viral specific monoclonal antibody based staining approach, PCR and/or sequencing. However, the major disadvantages of viral culture are low sensitivity and the time required to complete a culture experiment (138,139). The time required to detect CPE varies from 1–2 days after inoculation for HSV to 1–3 weeks for CMV (140). Many viruses are difficult to culture or even cannot be cultured. For example, JEV, WNV, HSV and VZV require specific cell lines and by the time the patients present to the hospital, the viruses might have been cleared out by the immune responses (141–144). Because of these reasons, viral culture is no longer a diagnostic tool in clinical laboratories, although it remains an important approach in biological research. Successful recovery of the viral pathogens would also allow for various downstream analyses, including vaccine development and development of non-culture based diagnostic assays.

1.2.3 Rapid antigen detection

Antigen tests are available for many agents causing CNS infections and have the advantage of a very short turnaround time. Among these tests,

Cryptococcal antigen detection is the most widely used assay. The test relies on the enzyme immunoassay to detect Cryptococcal capsular polysaccharide antigens in CSF samples. The sensitivity and specificity of the Cryptococcal antigen test are relatively high (greater than 90%) (145); however, they may reduce in patients with human immunodeficiency virus (HIV)/AIDS (40). For the diagnosis of acute BM, rapid antigen assays are used to detect pneumococcal capsular antigen, latex agglutination of *H. influenza* type B and *N. meningitidis* polysaccharide antigen (146–148). Antigen levels are also associated with clinical severity and may be used for the prognosis of tuberculosis meningitis and monitoring response to antifungal therapy (149,150). DENV rapid diagnostic tests (RDTs), especially those targeting NS1 antigen, have increasingly being used to diagnose DENV infection (151). However, the performance of dengue RDTs varied among manufacturers, with the sensitivity ranging from 58%-87% and the specificity ranging from 96.7% to 100% (152,153).

1.2.4 Serological assays

Antibody titers in CSF can be measured for definitive diagnosis of specific causes of CNS infection syndromes (135). For serological diagnosis, serum, plasma, or CSF samples may be investigated (154). Diagnosis relies on detection of immunoglobulin M (IgM) antibodies or demonstration of at least a four-fold increase in antibodies titers between acute and convalescent phases (155,156). Serological tests of DENV infections can be performed by detection of specific viral antigens and/or antibodies, produced by the host in response to DENV infection (enzyme-linked immunosorbent assay (ELISA) to detect IgM (MAC-ELISA) and Immunoglobulin G (IgG)-ELISA) (154,157). Additionally, detection of JEV-specific IgM by IgM-capture ELISA is a reliable serological method for confirmation of JEV (158). However, the presence of cross-reactive IgM responses of DENV and JEV may complicate the diagnosis and need further considerations, especially in areas where DENV and JEV co-circulate (159). Additionally, CSF IgM is the most widely used test for WNV (160) as antibodies may appear as early as 3 days after infection and are persistent for up to 3 months. However, cross-reactivity

represents a major limitation of serological assays in diagnosing flavivirus infections (161). Neutralizing antibody titers also have an important role for the diagnosis of neurosyphilis (162). Subsequently, neurosyphilis can be confirmed by a positive CSF venereal disease research laboratory (VDRL) test. Additionally, albeit less commonly applied, detection of antibodies to VZV IgG in CSF can be useful methods for the diagnosis of VZV related CNS syndrome (154,157–159,163). Investigation of CSF antibodies is also important for definitive diagnosis of AIE (164). The drawback of serological testing includes the lack sensitivity in immunocompromised hosts, and the delay of antibody response after the onset of symptoms (40,140). An initial negative antibody response might need to be re-tested.

1.2.5 Nucleic acid-based detection methods

Polymerase chain reaction (PCR) technique, developed by Karry Mullis in 1983, enables in vitro synthesis of a specific gene segment of a pathogen under investigation, thereby allowing for accurate detection of even a small amount of targeted nucleic acids in the original sample (40,165). PCR with its higher sensitivity and specificity has become a commonly used method for several microorganisms causing CNS infections that are difficult to detect and/or identify by culture, antigen and/or antibody-based methods (135,165). The use of PCR has greatly been expanded for the diagnosis of many infections, including CNS infections worldwide, especially with the introduction of automatic systems that have reduced the turnaround time, and increased reliability. However, PCR could only be reliably performed in well-designed laboratories with dedicated spaces for nucleic isolation, reagent preparation and amplification steps (135).

Several mono/multiplex PCR assays have been developed for the diagnosis of causative pathogens (bacteria, viruses, fungi, and parasites) of CNS infections (103,133,166–173). Of these, PCR is widely used to detect herpes virus, especially HSV, in clinical settings because HSV encephalitis outcome is exceptionally good if timely treated by acyclovir. HSV PCR sensitivity and specificity are >95% and >94%, respectively (40,129,174,175).

The GeneXpert MTB/RIF assay is an automated, cartridge-based system and has been recommended by WHO for the molecular diagnosis of TBM since 2004 (176). The technology allows detection of *M. tuberculosis* and rifampicin resistance conferring mutation within 2 hours and has been shown to greatly improve the sensitivity of TBM diagnosis (177). Xpert MTB/RIF Ultra assay is a new generation of GeneXpert MTB/RIF assay and was first released in 2017. It offers a larger reaction chamber, and has two extra molecular targets for *M. tuberculosis*, aiming to improve analytical sensitivity of the assay. However, according to a recent report from Vietnam, the sensitivity of Xpert Ultra was not statistically higher than that of the original Xpert assay for the diagnosis of TBM in HIV-uninfected and HIV-infected adults (178).

Despite the above-mentioned advantages, the requirement of well-designed laboratory and well-trained personnel, and the high cost represent some of the main barriers preventing PCR from being widely used in resource limited settings. In fact, the costs of PCR testing can be high in settings where potential pathogens implicated in CNS infections are diverse that would require testing for a wide range of pathogens per patients (40,179). As PCR is very sensitive, the risk of carryover contamination is high, leading to false positives. To minimise this, well-designed laboratories with dedicated spaces for NA isolation, PCR reagent preparation and amplification steps are needed (40). False negatives can also occur in the early phase of the disease or can be attributed to mutations occurring in the primer binding regions of the PCR, and/or the presence of inhibitors in the tested specimen (180,181). More importantly, molecular diagnostic methods could only detect genetic material of pathogens without knowing their viability. Because of these collective factors, interpretation of PCR results should be based on the clinical presentations and/or epidemiological variables attached to specific settings (150).

1.3 Novel technologies with high potential to improve CNS infections diagnosis.

1.3.1 Next-generation sequencing technologies

1.3.1.1 Introduction

Next-generation sequencing (NGS) technologies were first commercially available in 2005. Since then, they have revolutionised the DNA sequencing approaches, and thus have opened up new opportunities for biomedical research and clinical diagnostics (182,183). NGS enables deep sequencing of NA (DNA or RNA) in clinical specimens. Apart from prion disease, all infectious disease-causing pathogens are made from NA. Therefore, when performing deep sequencing of a patient sample using NGS (metagenomics), NA originating from both the hosts and pathogens (viruses, bacteria, fungi, and parasites) can be simultaneously sequenced in a single experiment. Metagenomics represents a pan-pathogen detection approach that enables accurate detection of any pathogen genetic materials presenting in a clinical sample under investigation without the need of using pathogen specific primers. Like other NA based detection assays such as PCR, metagenomics is less impacted by prior antibiotic use as compared to Gram stain and bacterial culture methods (184).

NGS systems developed by Illumina, Ion Torrent, Pacific Biosciences and Oxford Nanopore Technologies (ONT) are commonly used in the field of infectious diseases, especially for pathogen identification and novel virus discovery. Because of the focus of my PhD research, herein I will provide an overview about the Illumina and ONT.

1.3.1.2 Illumina sequencing

Illumina sequencing principles

Illumina sequencing adopts the technology of sequencing by synthesis (SBS) using removable fluorescently labeled chain-terminating nucleotides (185). The method allows massive parallel sequencing that detects single bases as they are incorporated into growing DNA strands. In each growing chain, a single labeled dideoxynucleoside triphosphate (ddNTP) is added and imaged

to identify the incorporation. DNA polymerization then terminates, and the fluorophore and terminator are cleaved to allow the incorporation of the next base.

The Illumina sequencing platforms and reagents

Illumina purchased the Solexa Genome Analyser in 2006 and was first commercialized in 2007. Until now, Illumina sequencing is the most commonly used technology worldwide (186). Illumina offers 5 benchtop sequencing platforms (iSeq 100, MiniSeq, MiSeq series, NextSeq 550 series, NextSeq 1000 & 2000), producing a wide range of output (1.2G-330G) (Table 1.2). These platforms have high throughput, generating short length reads with low error rates. Of these, MiSeq platform with a turnaround time of 2-3 days is commonly used for sequencing of viral and bacterial genomes (183,187,188).

Illumina offers a library preparation kit that is compatible with small and large genomes, PCR amplicons, and plasmids, with a low DNA input requirement (189). During library preparation, double unique indexes are used to flagged individual samples, therefore sequencing reads can be identified and sorted according to the original samples before final data analysis. The use of multiple double unique indexes allows for multiple samples to be pooled and simultaneously sequenced in a single sequencing run, thus reducing the costs.

Table 1.2: Summary of Illumina sequencing platforms.

The table was adapted from https://sapac.illumina.com/systems/sequencing-platforms.html (190).

Sequencer platforms	Maximum output (Gb)	Maximum read length (bp)	Run time (hours)	Applications
 iSeq 100	1.2	2 x 150	9.5-19	• Small Whole-Genome Sequencing • Targeted Gene Sequencing • Targeted Gene Expression Profiling • miRNA & Small RNA Analysis
 MiniSeq	7.5	2 x 150	4-24	• Similar to iSeq 100 • 16S Metagenomic Sequencing
 MiSeq	15	2 x 300	4-55	• Similar to MiniSeq • DNA-Protein Interaction Analysis
 NextSeq 550 series	120	2 x 150	12-30	• Similar to MiSeq • Exome & Large Panel Sequencing • Single-Cell Profiling • Transcriptome Sequencing • Methylation Sequencing • Metagenomic Profiling • Cell-Free Sequencing & Liquid Biopsy Analysis
 NextSeq 1000 & 2000	330	2 x 150	11-48	• Similar to NextSeq

Illumina sequencing workflow

The flow cells of Illumina sequencing kits consist of optically transparent slides with individual lanes. On the surface of each lane, small oligonucleotide anchors are immobilized. There are four basic steps in the Illumina NGS workflow (Figure 1.4) (189). First, in the library preparation, the target template DNA is fragmented, phosphorylated at the 5' end and adenylated at the 3' end and ligated to adaptors. Second, in the cluster generation, the adaptor-ligated oligonucleotides are attached to flow cell anchors by complementary linkage. Next, DNA fragments flip over and form a bridge by hybridizing to adjacent and complementary anchors. During the sequencing reactions, bound libraries are then extended by polymerase and double-strand molecules are separated on denaturation. While the original template is washed away, the newly synthesized strand is covalently attached to the flow cell surface. As a result, a single DNA template is amplified to thousands of clonal molecules and millions of clusters of different template molecules can be generated per flow cell.

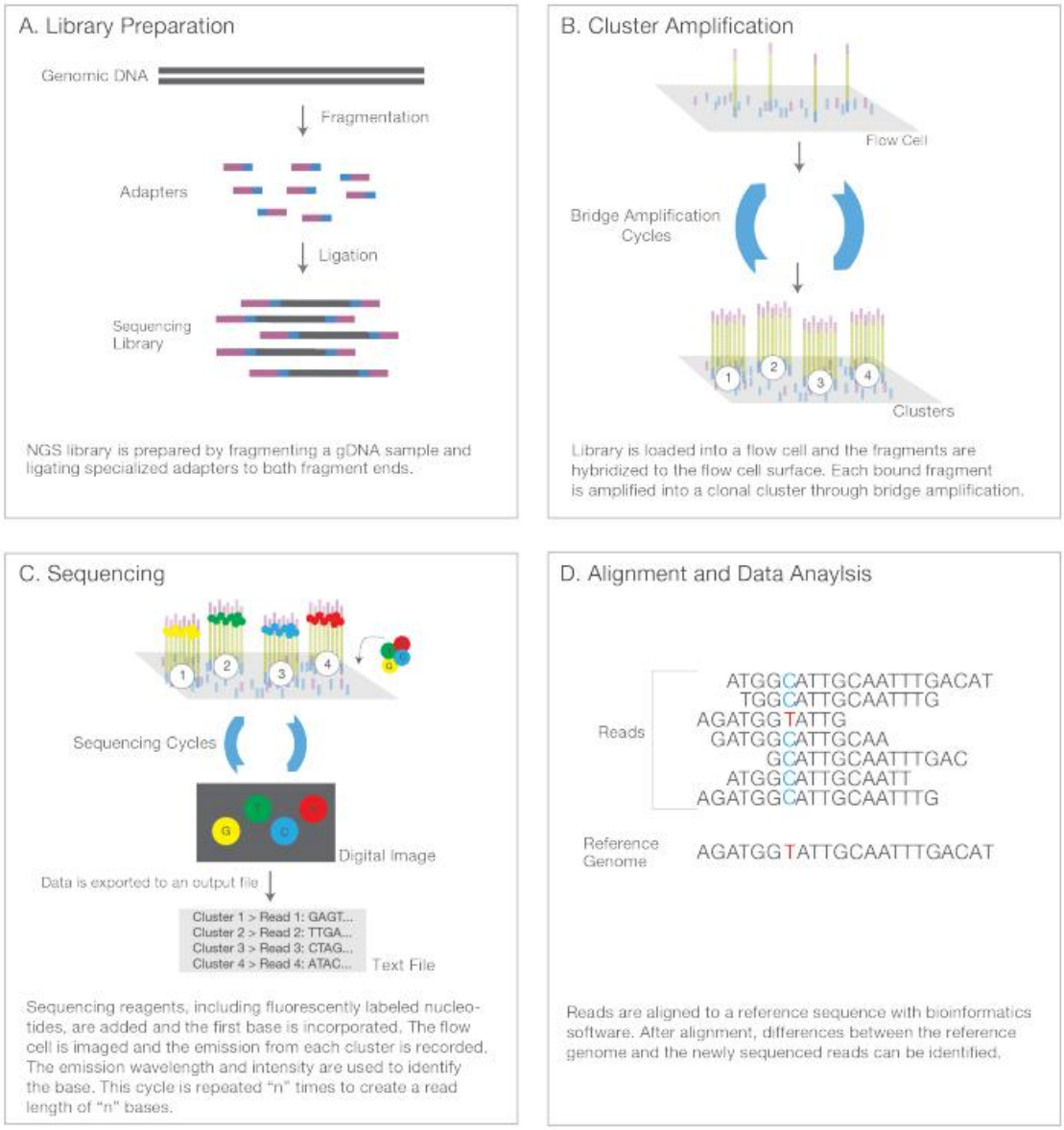


Figure 1.4: Next-generation sequencing workflow.

The figure was adapted from www.illumina.com/technology/next-generation sequencing.html (189). Illumina NGS includes four steps (A) library preparation, (B) cluster generation, (C) sequencing and (D) alignment and data analysis**.**

1.3.1.3 Oxford Nanopore sequencing

Founded in 2015, Oxford Nanopore Technologies (ONT) iprovides third-generation sequencing technologies (TGS) that can generate long reads (up to 950 kb) (191). Currently, there are severall sequening platforms offered by ONT. These are Flongle, MinION, GridION and PromethION. They are different in the number of the flow cells incoporated in the corresponding platfoms, ranging from 1 (Flongle and MinION) to 48 (PromethION), but the principle and the read length are no different. In this thesis, I will focus my literature review on MinION.

ONT sequencing principles

All ONT sequencing devices use flow cells which contain an array of tiny holes - nanopore - embedded in an electro-resistant membrane (192). A nanopore is a tiny hole with an internal diameter of 1 nm. It is made up of certain transmembrane cellular pore-forming proteins. Each nanopore corresponds to its own electrode connected to a channel and sensor chip, which measures the electric current that flows through the nanopores. Application of a voltage across the membrane with nanopores incorporated drives DNA through a pore creating an ionic current which can be measured. DNA sequencing is performed by adding the sample to the flow cell. When DNA/RNA molecules pass through the nanopores, there will be a change in the magnitude of the current in the nanopores, which is measured by a sensor to identify bases (191). The MinION sequencer provides long-read generations (up to tens of thousands of bases per reads) and real-time data collection (reducing sequencing time from days to hours (191,193).

The MinION device and reagents

The MinION sequencer provides a portable platform, and together with the Flongle, they are the two smallest sequencing device currently available in the market (Figure 1.5). The device can be directly connected to a laptop/computer installed with the MinKNOW software (191). The software enables multiple core tasks including data acquisition, real-time analysis and feedback, data streaming while providing device control, as well as sample

identification and tracking. Currently, the MinION flow cell has 512 channels and each channel is connected to 4 cells, allowing sequencing of up to 512 independent DNA molecules simultaneously (194).

ONT provides a range of sequencing kits for whole genome sequencing (WGS), targeted DNA sequencing and RNA sequencing (195). ONT has also released several versions of flow cells to improve the data quality and throughput. The version R10.4.1 provides the average base accuracy of 99% when aligned to the reference (196).

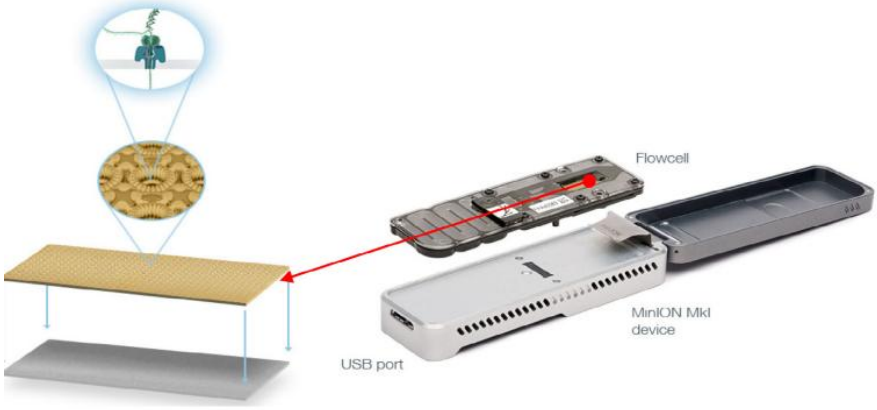


Figure 1.5: The MinION sequencing device (229).

ONT sequencing procedure

The library generation procedure consists of several steps including genomic DNA fragmentation, repairing damaged DNA, creating blunt ends in sheared DNA and PCR fragment, dA-tailing to the end of the fragments, adapter ligation and a final purification step to remove unwanted nucleotides and enzymes. The prepared library usually contains the dsDNA ligated with the leader adapter (Y adapter) in one end and the hairpin adapter (the HP adapter) in the other end. Sequencing begins when the motor protein starts to unzip the ds DNA at the turning point of the Y adapter complementary region, followed by passing of the first DNA strand into the nanopore with the speed controlled by the motor protein. When the sequencing reaches the HP adapter, the “hairpin protein” allows the continuity for the complementary

passing through the nanopores. Base calling is subsequently performed from information of either one strand (1-directional) or both strands (2-directional).

Base calling of ONT sequencing reads

Base calling of ONT reads can be performed in real time (i.e., as the sequencing is progressing) or offline (i.e., after the sequencing procedure is completed). The output of an ONT run is in FAST5 format, that has a hierarchical structure storing both the metadata associated with a read and the events processed by the sequencing device (197). Base calling is currently achieved using an Amazon cloud-based system developed by ONT. The procedure of base calling basically consists of the following steps. Firstly, template and associated events are separately processed, and the information is used for 1D base calling. Secondly, the ratio of template sequence length to complement sequence length is calculated. If the ratio is between 0.5 and 2.0, 2D base calling is processed and a 2D read is generated. Additionally, a quality score (Q-score) is calculated to evaluate the 2D read quality. If mean Q-score of the 2D reads > 9, the FAST5 is placed into a “pass” directory. Otherwise, all other FAST5 files are stored in a “failed” directory.

1.3.1.4 Comparison of the ONT and Illumina NGS platforms

Collectilvey, both ONT and Illunina are high-throughput sequencing technologies. They both can produce many DNA sequences per run at low cost within a short window of time. Therefore they can be applied to whole genome sequence any orgamism under investigation, from small to large genomes. However, compared to Illumina technologies, the ONT platforms have both advantages and disadvantages. They are small devices, some of which are portable, and therefore can be deployed in remote settings (198–200), whereas Illumina platforms require complex infrastructure to operate. While ONT produces ultra-long reads but with highh error rate, Illumina produces short reads with higher sequencing accuracy (201,202). The high error rate produced by ONT platforms hinders the application of ONT reads

in single-nucleotide variant analysis (203–205), which can be overcome by using Illumina sequencing technologies.

1.3.1.5 Analysis of metagenomic data

Both Illumina and ONT platforms can be used for metagenomics associated research. Accurate analysis of metagenomics data is essential to ensure that the presence of pathogen NA in the tested samples is precisely identified (206). This step involves the removal of human DNA sequences and mapping of non-human DNA sequences to a prepared NA database deriving from known pathogens (viruses, bacteria, parasites and fungi) (206). Recently, several bioinformatics platforms have been developed to translate metagenomic results into interpretable reports. EPI2ME developed by ONT is specifically designed for ONT read analysis. Otherwise, most of the current publically-available platforms have been developed and optimized for Illumina reads. Taxonomer is an interactive web-tool for comprehensive metagenomics analysis for universal pathogen detection and host mRNA expression profiling (207). Kraken provides a fast and accurate program for metagenomic sequence classification using exact alignment of *k*-mers (208). CosmosID offers a fast and reliable bacterial detection and antibiotic resistance gene detection (209). Chan Zuckerberg ID (CZID, formerly known as IDSeq ID) is an open- source cloud-based pipeline for metagenomics data analysis (210). In this thesis, I will apply the CZID platform for the analysis of metagenomics data generated by Illumina MiSeq platform. EPI2ME will be used for the analysis of ONT reads.

1.3.1.6 Metagenomics for the diagnosis of CNS infections

As outlined above, metagenomics is a promising approach for the diagnosis of CNS infections as it could simultaneously detect a wide range of pathogens, including bacteria, viruses and fungi, in a single assay (211,212). This has been illustrated in several case studies in different settings. Metagenomics successfully detected St. Louis encephalitis virus (SLEV) responsible for a fatal case of meningoencephalitis in California in September 2016 (213). Using metagenomics, my research group has

previously identified a novel Cyclovirus in ~4% of CSF samples of Vietnamese patients with CNS infections of unknown causes (214). Most recently, my research group employed metagenomics to detect Japanese encephalitis virus for the first time in urine of a 16-year-old child presenting with acute encephalitis of unknown cause (215). Because metagenomics offers an un-targeted approach, it could also help identify unexpected pathogens in CSF of undiagnosed encephalitis patients. These include the detections of human parvovirus 4 (PARV4), coronavirus OC-43, and astrovirus MLB1, although the contribution of these viruses to the ongoing CNS infections is subject to further investigations (206,211,216–219). Additionally, studies assessing the diagnostic performance of metagenomics in clinical settings showed that the analytic performance of the assay were 51% to 73% in terms of sensitivity and 86% to 99% in terms of specificity compared to conventional gold microbiologic testing (staining, culture and PCR) (184,187,212). These results demonstrate the potential applications of metagenomics in the diagnosis of diseases that can be caused by a wide range of pathogens such as CNS infection syndromes.

In additional, Illumina and ONT have so far played critical roles in outbreak and pandemic resposes. During the Ebola outbreak in Liberia and Guinea, West Africa, the MinION device provided a rapid diagnostic tool for genomic surveillance and outbreak management (220,221). The whole workflow was reported to take less than 24 hours (including amplification, library preparation and MinION run). MinION sequencing has also been used for real-time strain typing and predicting of antibiotic resistance by sequencing of bacteria genomes (222–224). Recently, MinION sequencing has been utilised in case studies on rapid detection of monkeypox virus (225). Since the beginning of the pandemics, over 15 million SARS-CoV-2 genomes have been sequenced. These data have provided significant insights into the pandemic dynamics, and have helped to inform vaccine design and the development of effective non-pharmaceutical intervention strategies that are critial to the pandemic control.

1.3.1.7 Challenges for applying metagenomics to diagnose CNS infections

There are several challenges that might prevent the integration of metagenomics into clinical microbiology laboratories, particularly in resource-limited settings. These include the current high costs of the instruments and the reagents, turnaround time, and the lack of local bioinformatics skills and computational resources (207,226,227).

Importantly, there is a lack of established guidelines for metagenomics validation and requirement for compliance with regulations in the context of clinical diagnostic testing (228,229). As metagenomics could sequence NA of any origins in a clinical sample, sequences related to commensal viruses and/contaminants represent a challenge in result interpretation (187). It should also be noted that the detection of NA of a novel virus by metagenomics in clinical samples represents the beginning of a new research story. The reason is that extensive investigations are needed to demonstrate pathogenic potential of the new virus, which might involve testing of biopsy samples to assess the tropism, the study of seroconversion and/or the use of animal models and their associated experiments (183).

1.3.2 Mass spectrometry-based proteomics

1.3.2.1 Introduction

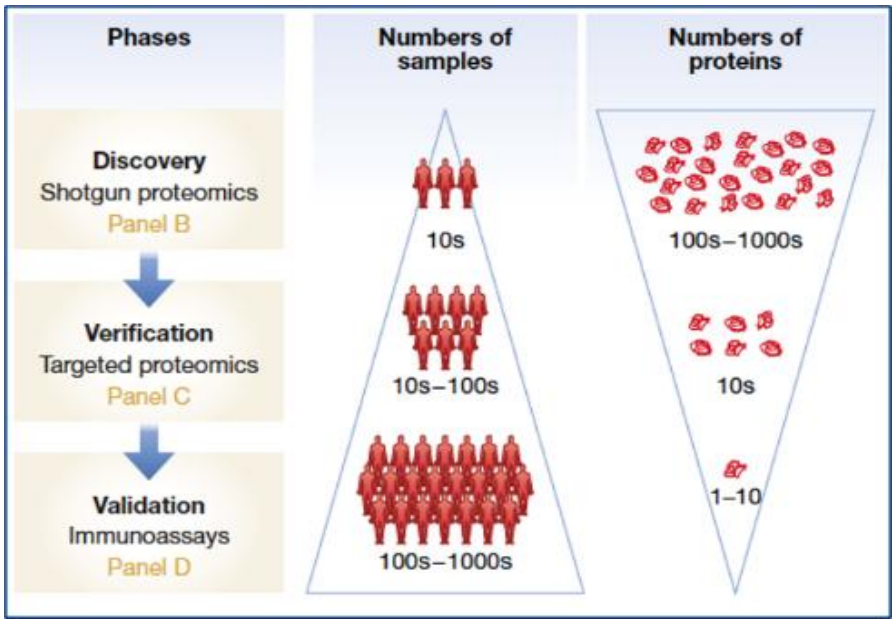
In the last two decades, the application of multi-omics (transcriptomics, proteomics and metabolomics) into research has revealed novel insights into the pathophysiology of infectious diseases (230). Particularly, mass spectrometry (MS)-based proteomics has emerged as uniquely unbiased, sensitive, and quantitative approach for analysis of body fluids and tissues to identify pathogen- and host derived markers (231). MS provides the ability to identify and to precisely quantify thousands of proteins from complex samples (232). Advances in MS instruments and workflows have transformed proteomics applications in accelerating the discovery of novel molecular signatures for diagnostics and therapies (230). Enormous

investigations in proteomics biomarker discovery for neurodegeneration diseases and cancer have also been reported (233–236).

1.3.2.2 MS-based proteomics principles

MS-based proteomics is a powerful tool that can be used to identify and quantify proteins in biological samples. There are two major MS proteomics approaches: “top-down” (intact protein analysis, and “bottom-up” (protein digestion and sequencing) (Figure 1.6). The key steps include sample preparation, protein separation, protein digestion, peptide separation, mass spectrometry analysis, and data analysis (235). Briefly, during sample preparation, the sample is typically lysed, and the protein is extracted and purified using techniques such as gel electrophoresis and liquid chromatography. Proteins are separated based on their size, charge, or other physical properties. Next, the proteins are digested into smaller peptides using enzymes such as trypsin, Lys-C and labeled to multiplex samples. The digested proteins are ionized by the mass spectrometer, then the gaseous charged peptides go through acceleration and deflection and are separated by mass and charge. These peptides are then detected by an ion detector by multiple microprocessors and recorded as peaks in an MS spectrum. Finally, the data generated by the mass spectrometer are analysed using bioinformatics tools to identify the proteins and peptides and to quantify their abundance.

A



B

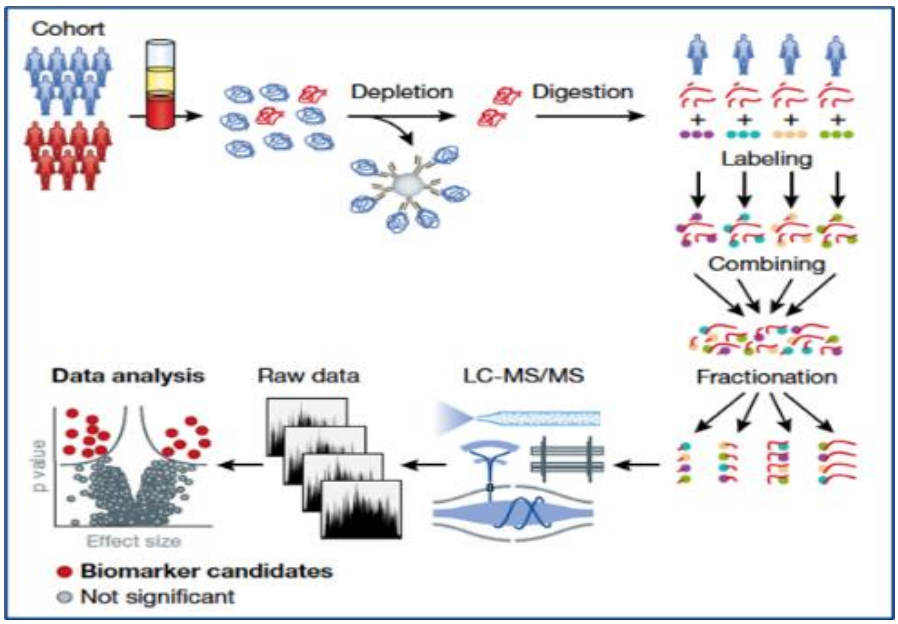


Figure 1.6: Current paradigms in mass spectrometry-based proteomics.

The figure was adapted from “Revisiting biomarker discovery by plasma proteomics” by Geyer et al., 2017 (237).

(A) “Top-down” approach in biomarker research

(B) Workflow for hypothesis-free discovery proteomics

1.3.2.3 MS instrumentation

A basic mass spectrometer consists of an ion source that convert proteins/peptides into gas-phase ions, a mass analyser that measures the mass-to-charge ratio *(m/z)* of gas-phase ions and a detector that records the number of ions at each *m/z* value (Figure 1.8) (232,238).

Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are two commonly used techniques to volatize and ionize the proteins/peptides for mass spectrometry analysis (239,240). ESI can be coupled to liquid-based (for example, chromatographic and electrophoretic) separation tools. Integrated liquid-chromatography ESI-MS systems (LC-MS) are compatible with the analysis of complex samples whereas MALDI-MS is basically used to analyse relatively simple peptide mixtures.

The mass analyser is the central component of a mass spectrometer. The mass analyser is responsible for separating and detecting ions based on their mass-to-charge ratio (*m/z*) to generate information-rich ion mass spectra from peptide fragments (tandem mass of MS/MS spectra). There are four types of mass analysers commonly used for proteomics research: quadrupole (Q), ion trap (quadrupole ion trap, QIT; linear ion trap, LIT or LTQ), time-of-flight (TOF) and Fourier-transform ion cyclotron resonance (FTICR). They vary in physical principles and analytic performance and can work independently or put together in tandem to take advantage of the strengths of each. The combined instruments include the Q-Q-Q, Q-Q-LIT, Q-TOF, TOF-TOF and LTQ-FTICR (238).

1.3.2.4 MS data analysis

Peptide mapping, also called peptide-mass mapping or peptide-mass fingerprinting, is commonly used to identify proteins in Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) instruments (232). The principle of this method is that proteins are defined by matching a list of experimental peptide masses with a list of peptide masses in a comprehensive protein database. Protein identifications using peptide CID (collision-induced) spectra is a more decisive method than peptide mapping

that provides information about peptide sequence. Basically, the CID spectra are scanned against comprehensive protein sequence database using specific algorithms. There are three main approaches to identify proteins. The “peptide sequence tag” method extracts a short, unambiguous amino acid sequence from the peak pattern and then uses it as a specific probe to determine the origin of the peptide (241). The cross-correlation and the probability- based matching methods identify peptides from tandem mass spectrometry (MS/MS) data by comparing the measured mass spectra to theoretical spectra constructed by peptide sequences in the database, but they differ in approaches to scoring and ranking peptide-spectrum matches (PSMs) (241,242). The method of choice is based on the nature of the data and the goals of the analysis. For example, the cross-correlation method may be preferred for identifying rare peptides, while probability-based matching may be preferred for more stringent identifications with a lower false discovery rate.

1.3.2.5 Applications of proteomics to identify CSF biomarkers for diagnosis of CNS infections.

Advances in mass-spectrometry instruments and workflow has facilitated the applications of proteomics in infectious disease research (233,243,244) Proteomics using mass spectrometry provides a sensitive and unbiased method for the discovery of novel biomarkers in the diagnosis of CNS infections (231,235). Recently, several candidate diagnostic biomarkers have been identified to distinguish specific causes of CNS infections (243,245,246). For example, Myeloperoxidase and lactotransferin were identified as the host CSF proteome response to acute bacterial meningitis (*H. influenza* and *S. pneumoniae*) in comparison with cerebral malaria (243). A quantitative proteomic study was performed to identify differential proteins in the CSF obtained from TBM patients and healthy controls. CSF samples were labelled with iTRAQ™ and analysed by LC-MS/MS. As a result, neural epidermal growth factor-like like 2 (NELL2) was identified to be able to distinguish TBM subjects from healthy controls (245). Additionally, mass spectrometry based quantitative proteomics was used to

identify protein changes in CSF from children with *S. pneumoniae* infection, compared with children admitted to hospital with bacterial meningitis symptoms but negative diagnosis. Proteins involved in the immune response and exosome signaling were significantly expressed in the infected samples, including Myeloperoxidase, S100 calcium binding protein A9, Cathelicidin antimicrobial peptide, Ceruloplasmin and Cystatin C (246). Most recently, the MS proteomics data provided JEV protein signatures in human CSFs that could be harnessed in a rapid diagnostic test (247).

1.3.2.6 Challenges of applying proteomics in clinical research.

Despite promising applications, MS-based proteomics still has several limitations attributable to the low concentrations of potential biomarkers and variations of protein levels in clinical samples (248). Consequently, not all protein makers can be detected by MS and likewise not all the original findings can be replicated in subsequent verification and validation phases of MS studies (231,249). Additionally, the major barriers preventing the application of mass-spectrometry from clinical practice in LMICs include current high costs of the instruments and the reagents. The requirement of expertise to analyse MS data represents another challenge.

1.4 Aims of the thesis

Collectively, CNS infection syndromes are devastating clinical problems worldwide, but especially in LMICs, with overlapping clinical features. There are over 100 pathogens that can cause CNS infection syndromes, while autoimmune encephalitis has been recognised with increased frequency. Consequently, the cause remains unidentified in 50-80% of patients presenting with CNS infection syndromes. Additionally, the emergence of (novel) neurotropic viruses such as West Nile virus, Hendra virus, Nipah virus, enterovirus A71, and Zika virus further challenges routine diagnostics, and illustrates the unprecedented threats of emerging infectious diseases. Novel diagnostic approaches are therefore urgently needed to improve patient diagnostic and management, and to support outbreak response. Over the last decade, metagenomics and MS-based proteomics have emerged as

sensitive, hypothesis-free approaches for infectious disease diagnostics and pathogen surveillance, and the discovery of novel diagnostic biomarkers, respectively. Therefore, I hypothesise that metagenomics and MS-based proteomics could improve upon current standard laboratory assays routinely used for the diagnosis of CNS infection syndromes. Accordingly, my PhD research aims are to:

1. Investigate the aetiology and epidemiology of both infectious and non-infectious causes of CNS infections syndromes in Vietnamese adults.
2. Explore the clinical and laboratory features, and (long-term) outcomes in Vietnamese adults with CNS infection syndromes.
3. Develop and prospectively evaluate an internally controlled metagenomic pipeline for simultaneous detection of both bacterial and viral causes of CNS infections.
4. Evaluate the diagnostic performance of two novel CSF biomarkers, Lipocalin-2 (LCN2) and disintegrin and metalloprotease like decysin (ADAMDEC1), in discriminating major clinical groups of CNS infection syndromes.

Chapter 2

Epidemiology and Causes of Central Nervous System Infection Syndromes in a Tertiary Hospital in Ho Chi Minh City, Vietnam during 2017 and 2020

2.1 Introduction

As described in Chapter 1, CNS infection syndromes are important causes of morbidity and mortality worldwide. The overall hypothesis of my PhD research thesis is that new technologies can improve the diagnosis of CNS infection syndromes, thereby potentially improving the patient outcomes. To lay the foundation for my PhD research, I conducted an observational study of CNS infection syndromes in Vietnamese adults admitted to the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City, a tertiary referral hospital for Southern Vietnam with a population of over 40 million between September 2017 and September 2020. In this chapter, I investigated the infectious and non-infectious causes, and epidemiology of CNS infection syndromes. The associated clinical features, laboratory findings, management, and (long-term) outcomes of the study participants will be explored in Chapter 3.

2.2 Materials and Methods

2.2.1 Study Design and Setting

A 3-year prospective observational study was conducted at Viet-Anh ward of HTD in Ho Chi Minh City. HTD provides secondary and tertiary care for adults and children with infectious diseases in Southern Vietnam with a population of over 40 million. Viet-Anh ward is a dedicated department for patients with CNS infection syndromes.

2.2.2 Ethics

The clinical study was approved by the Scientific and Ethics Committee of the HTD and the University of Oxford Tropical Research Ethics Committee (OxTREC).

2.2.3 Patient inclusion criteria and enrolment procedure

All adult patients (age ≥ 16 years) admitted to the Viet-Anh ward of HTD who had an indication for lumbar puncture as a part of routine care were eligible for enrolment into the study. Study staff identified potential patients and the patients or their legal relatives (if the patients were unconscious) were then given information about the study. Patients/legal relatives were then invited to sign an informed consent form to participate in the study, which included permission to store and test the leftover of their CSFs taken as part of routine care, and to record their in-hospital management and outcome.

2.2.4 Clinical data and specimen collection

Demographics, baseline clinical data, management and results of standard laboratory tests, final diagnosis, and in-hospital outcome were collected at enrolment or during hospitalisation. CSF samples were collected for routine diagnosis from each patient when suspected with CNS infections. The remainder of the whole CSF after routine diagnosis was retained for the study purpose.

2.2.5 First-line aetiological investigations

As part of routine care at HTD, blood and CSF samples were examined for a wide range of infectious pathogens (Figure 2.1 and Appendix A, Table S2.1). For bacterial pathogens, routine assays included Gram stain, standard culture, and antibiotic susceptibility testing. Routine CSF analysis also covered India ink and Cryptococcal antigen test for Cryptococcus**,** Ziehl-Neelson stain, Mycobacteria growth indicator tube (MGIT) and GeneXpert MTB/RIF assay or Expert_Ultra MTB/RIF for *M. tuberculosis*, and real-time PCR for Herpes Simplex virus (HSV) (250), and Varicella Zoster virus (VZV) (251). Additionally, serological testing for specific IgM against JEV and DENV was conducted when clinically indicated (215).

2.2.6 Enhanced diagnosis

If the first-line diagnostics outlined above was negative, the samples were subjected to enhanced diagnostic testing to establish the responsible cause as per the requests of treating physicians based on clinical progression and history of illness. These methods included real-time PCR assays for a wide range of bacterial and viral pathogens (*S. pneumoniae*, *S. suis*, *N. meningitidis,* DENV, JEV, enteroviruses, and Zika virus) (252–255). Patients with suspected eosinophilic meningitis (defined of more than 10% eosinophils of the total CSF leucocyte count) were tested for *A. cantonensis* using real-time PCR (256). For patients presenting with clinically suspected anti-NMDAR encephalitis, an indirect immunofluorescence assay (EUROIMMUN, Luebeck, Germany) was used to detect antibodies against NMDAR, following the manufacturer's instructions, and the results were read by fluorescence microscopy (Nikon).

2.2.7 Diagnostic interpretation

Case definitions for CNS infections diagnosis are displayed in Table 2.1. Patients were assigned as confirmed or suspected bacterial meningitis (BM) and viral meningoencephalitis (VME), based on the modified WHO case definitions (49,257). Tuberculosis meningitis (TBM) was defined according to the case definition of TBM developed for use in clinical research (258). Suspected eosinophilic meningitis (EM) was defined by the presence of more than 10% eosinophils of the total CSF white cell count (259). A definitive diagnosis of cerebral toxoplasmosis (CT) was supported by brain imaging and serological testing (33,260). Anti-NMDAR encephalitis cases were confirmed when IgG against NMDAR was detected in the tested CSF samples (26).

Table 2.1: Case definitions of diagnostic interpretation of CNS infection syndromes

Bacterial meningitis (Modified from case definition of WHO) (49), (257)	
Suspected	***Confirmed***
1. Sudden onset of fever (>38^{o}C) less than 7 days 2. AND at least one of the following signs ✓ Meningeal signs (neck stiffness, Kernig sign and Brudzinski sign) ✓ Altered consciousness 3. AND CSF investigations showing at least one of the following ✓ Leukocytosis (≥10 cells/µl) AND at least 2 of the following criteria • An elevated protein (>1 g/l) • Decreased glucose (<2.2 mmol/l or <50% of blood glucose) • Lactate ≥4 mmol/l ✓ Turbid appearance 4. AND no aetiological agents identified	- Positive culture, Gram stain or Real-time PCR/sequencing of CSF samples - Positive bacterial blood culture and clinical syndrome consistent with BM
Viral meningoencephalitis (modified from case definition of acute encephalitis syndrome of WHO (257)	
Suspected	***Confirmed***
1. Acute onset of fever (less than 7 days) 2. AND at least one of the following: ✓ Meningeal signs (neck stiffness, Kernig sign, and Brudzinski sign) ✓ Change in mental status (confusion, disorientation, coma or inability to talk) ✓ New onset of seizures (excluding simple febrile seizures) 3. AND CSF examination showing at least one of the following: ✓ Leukocytosis (≥ 10 cells/µl) AND at least 2 of these criteria • Protein ≤ 1g/l • Normal glucose (≥ 2.2 mmol/l or ≥ 50% of blood glucose) • Lactate < 4 mmol/l ✓ Clear appearance 4. AND no aetiological agents identified	- Positive Real-time PCR (Enterovirus, VZV, HSV, JEV, Mumps) in CSF - Detection of JEV specific IgM in CSF - Detection of Dengue virus specific IgM in CSF
Tuberculous meningitis (258)	
Suspected	***Confirmed***
Probable - Total diagnostic score ≥10 (when cerebral imaging is not available) or ≥12 (when cerebral imaging is available) - At least 2 points should either come from CSF or	Positive Ziehl-Neelsen stain, and/or GeneXpert and/or MGIT*

cerebral imaging criteria. **Possible** - Total diagnostic score of 6–9 points (when cerebral imaging is not available) or 6– 11 points (when cerebral imaging is available) - Possible tuberculosis cannot be diagnosed or excluded without doing a lumbar puncture or cerebral imaging	
Eosinophilic meningitis (259)	
Suspected	***Confirmed***
- Percentage of eosinophilic cells in blood >10% - AND meningitis manifestations - AND no pathogen confirmed in CSF by culture, PCR or ELISA methods	- Meningitis and a percentage of eosinophils in CSF greater than 10% - OR positive *A. cantonensis* PCR
Anti-NMDAR encephalitis (26)	
Suspected	***Confirmed***
- With presumed encephalitis - AND exhibited at least one of abnormal movements (orofacial, limb or trunk dyskinesia), seizures, autonomic dysfunction and/or personality change or psychosis. - AND CSF tested negative on all microbiological investigations.	Positive with IgG against NMDA receptor
Cryptococcal meningitis (49)	
Suspected	***Confirmed***
- Immunosuppressed patients - Clinical features including headache, neurological symptoms, and fever	- India ink stain of CSF positive showing encapsulated yeasts. - AND/OR *Cryptococcus neoformans* cultured from CSF
Cerebral toxoplasmosis (33)**,**(260)	
Suspected	***Confirmed***
- Immunosuppressed patients - Clinical features including headache, neurological symptoms, and fever	- Identification of ring-enhancing mass lesion(s) by brain imaging - AND/OR detection of toxoplasmosis-specific IgG and IgM antibodies in the CSF

Note to Table 2.1: *Mycobacteria Growth Indicator Tube

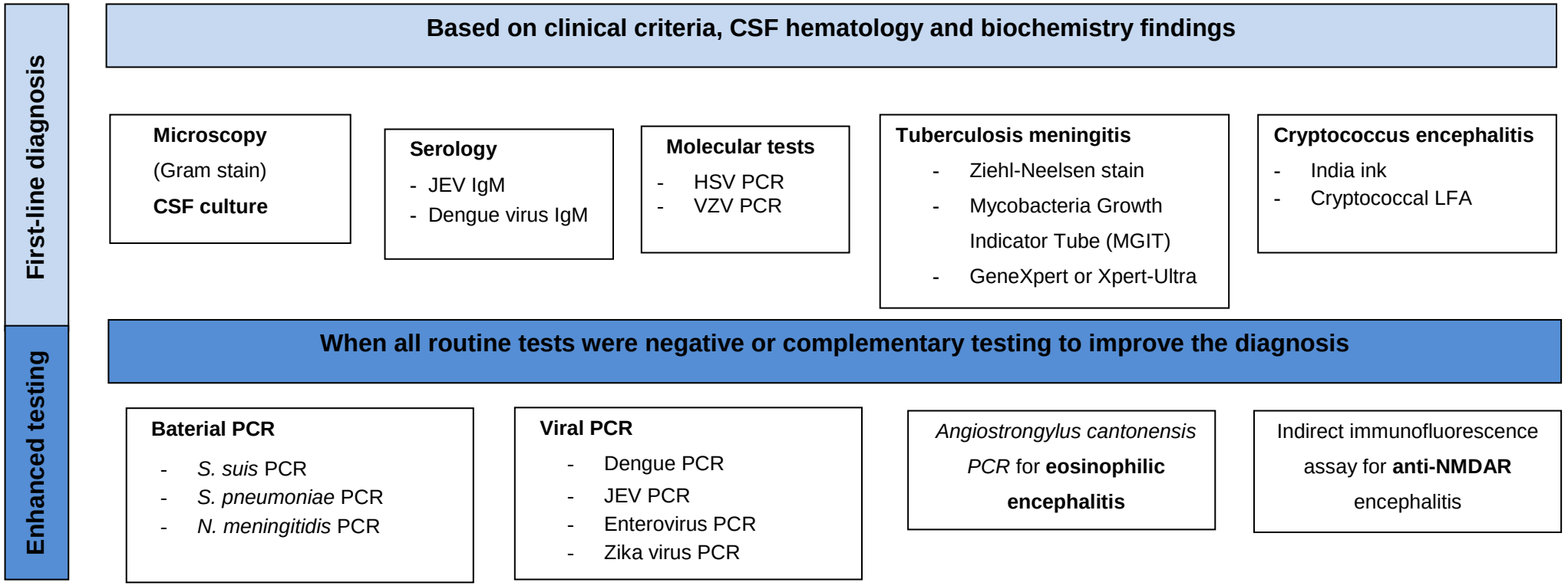


Figure 2.1: Diagnostic testing algorithms for cases with CNS infection syndromes.

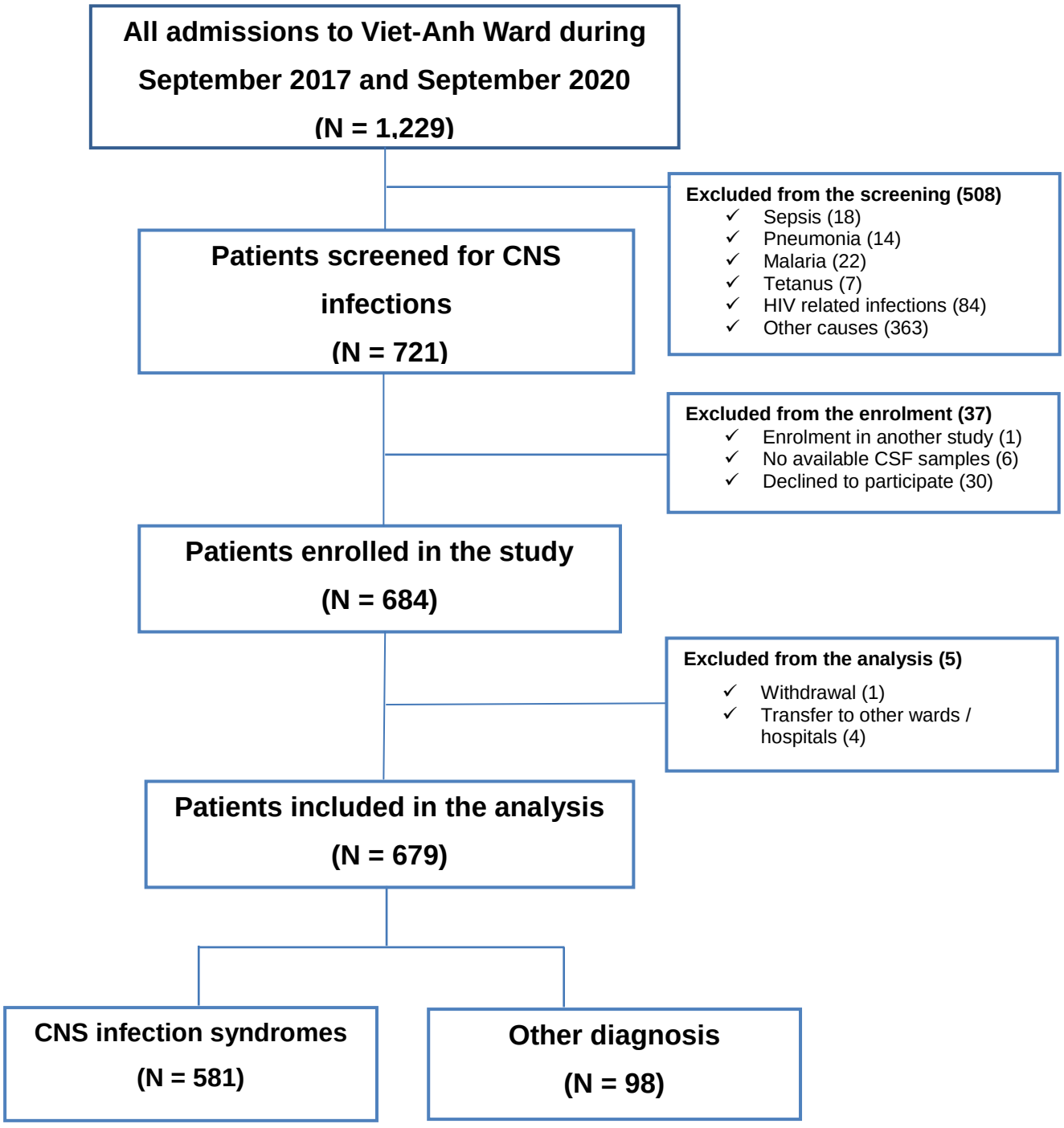


Figure 2.2: Flowchart showing an overview about patient admission and enrolment during the study period.

2.3 Results

2.3.1 Overview of the study participants

Between September 2017 and September 2020, a total of 1,229 patients with CNS infection syndromes were admitted to Viet-Anh Ward (Figure 2.2). Of these, 721 patients were screened for eligible criteria. Subsequently, 37 patients were excluded due to: i) enrolment in another study (n=1), ii) CSF unavailability (n=6) and iii) unwilling to participate (n=30). In total, 684 patients agreed to participate in the clinical study. During in-hospital follow-up, one patient withdrew, and 4 were transferred to other wards or hospitals, leaving 679 patients with clinical and laboratory data available for analysis. Of these, 581 patients (85.6%) were diagnosed with CNS infection syndromes and 98 patients (14.4 %) had conditions other than CNS infection syndromes (herein referred to other diagnosis group).

The study participants enrolled in our study came from 33/63 provinces in Southern Vietnam and two provinces in the North and Central of Vietnam. Majority of the patients came from Ho Chi Minh City 134/679 (19.7%) and Dong Nai (63/679, 9.3%), (Figure 2.3).

2.3.2 Clinical groups of CNS infection syndromes

According to case definitions, results of laboratory testing and microbiological investigations, 581 cases with CNS infection syndromes were assigned to 7 clinical groups (Figure 2.4). TBM was the most common group, accounting for 32.4% (188/581) of cases, followed by VME (29.6%, 172/581) and BM (22.9%, 133/581). CM, EM, and CT were diagnosed in 3.4% (20/581), 2.9% (17/581) and 0.3% (2/581), respectively. During the study period, anti-NMDAR encephalitis was clinically diagnosed in 49 out of the total 581 cases with CNS infection syndromes (8.4%), representing the 4th most common group after TBM, VM and BM. The frequency of clinical conditions of the 98 patients with other diagnosis is presented in Table S2.2.

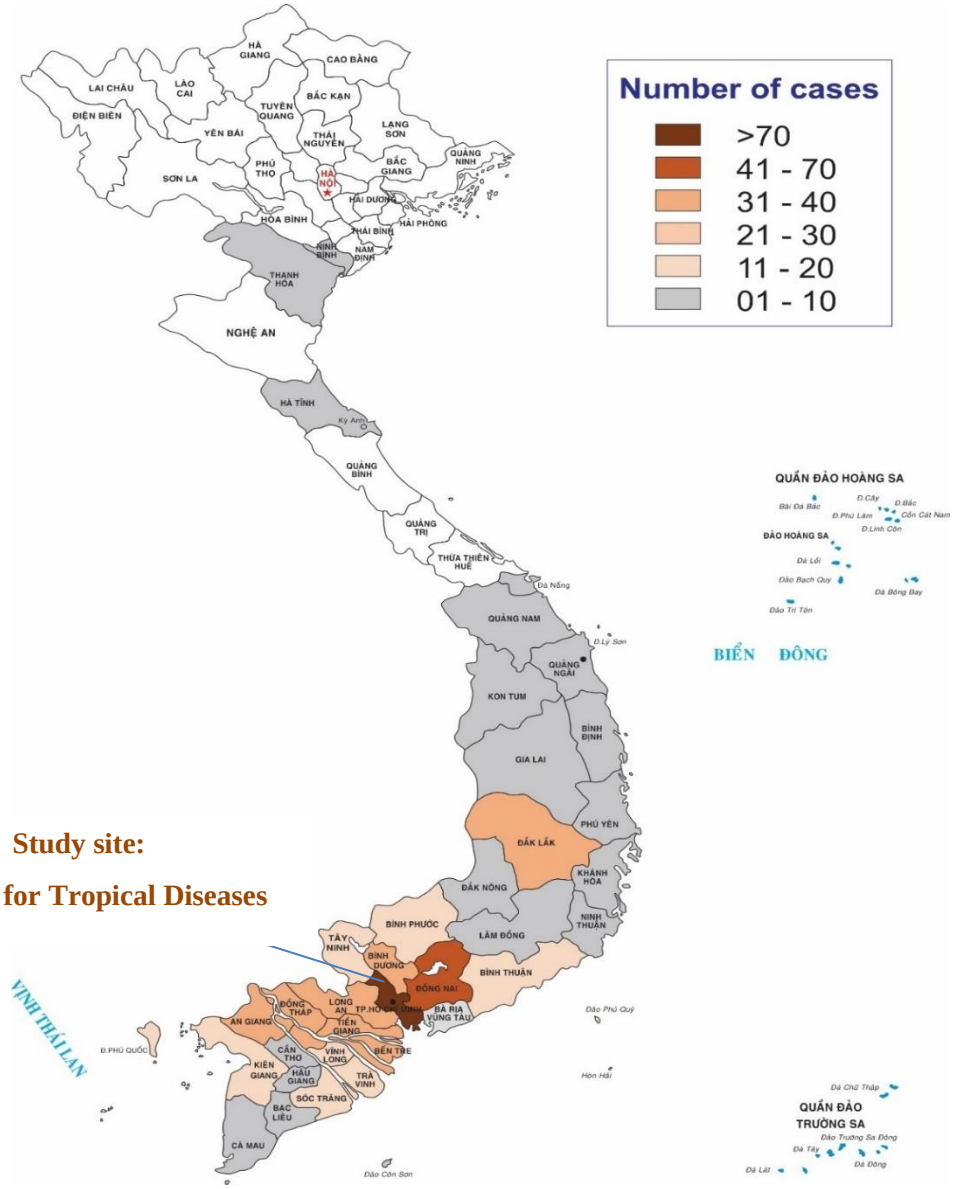


Figure 2.3: Geography map of Vietnam showing distribution of cases with CNS infection syndromes per provinces admitted to HTD during the study period.

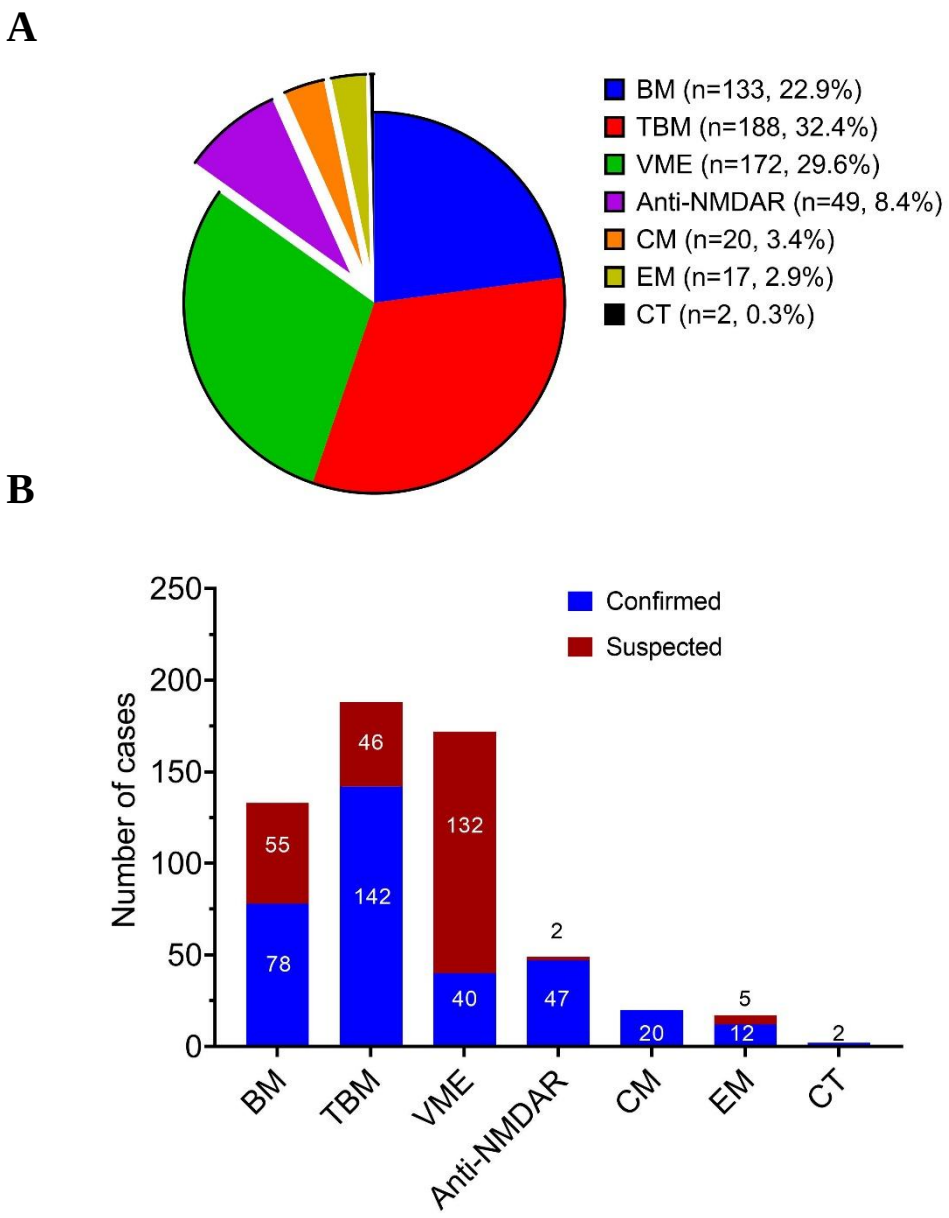


Figure 2.4: Spectrum of CNS infection syndromes of the study participants (N=581)

A: The proportion of clinical entities of patients with CNS infection syndromes

B: Clinical groups of CNS infection syndromes including suspected and confirmed cases

Note to Figure 2.4: TBM: Tuberculous meningitis, BM: Bacterial meningitis, VME: Viral meningoencephalitis, Anti-NMDAR: Anti-N-Methyl-D-Aspartate Receptor encephalitis, CM: Cryptococcal meningitis, EM: Eosinophilic meningitis, CT: Cerebral toxoplasmosis.

2.3.3 Diagnostic assays and results

The frequency and results of diagnostic tests performed on CSF samples of the study participants are displayed in Table 2.2. Gram stain and CSF culture were routinely performed for most of the patients (665/679, 97.9% and 643/679, 94.7%); respectively). Additionally, routine HSV PCR was performed in 286/679 (42.1%) study participants.

Here, I focused my diagnostic yield analysis on the 581 patients fulfilling the criteria of CNS infection syndromes. Of these, an aetiological agent was identified in 341/581 (58.7%) patients, including 270/581 (46.5%) diagnosed by routine assays and 71/581 (12.2%) by enhanced diagnostic assays (Figure 2.5). More details about the frequency of pathogens and causes detected are presented in Table 2.3.

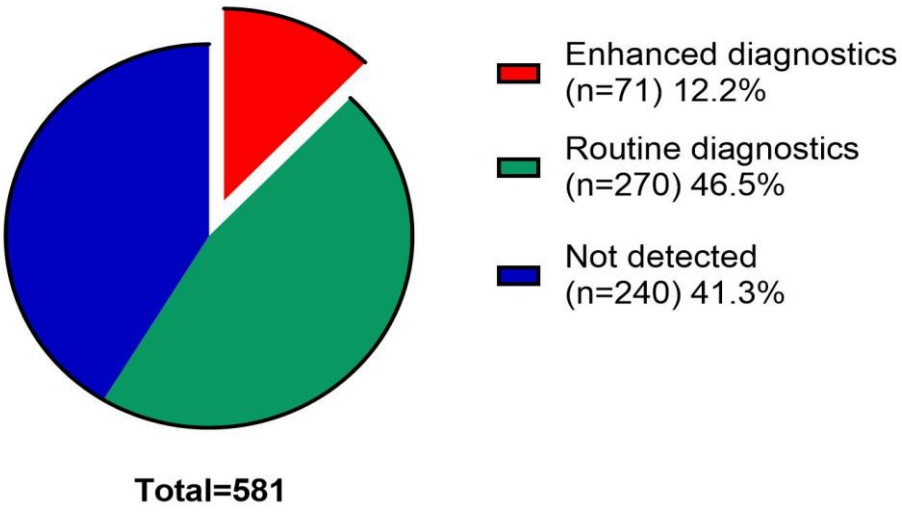


Figure 2.5: Diagnostic yields by routine diagnostic and with combined enhanced testing

Table 2.2: Summary of first-line diagnosis and enhanced testing for CNS infection syndromes (N=679).

	Number of patients tested	Percentage of patients tested (%)	Number of tests positive	Percentage of positive test (%)
First-line diagnosis				
Microscopy				
Gram stain	665	97.9	47	7.1
Zn stain	417	61.4	131	31.4
India ink	572	84.2	17	3.0
CSF culture	643	94.7	79	12.3
Cryptococcal LFA	383	56.4	20	5.2
Serology				
JEV IgM	35	5.2	4	11.4
Dengue IgM	18	2.7	5	27.8
Molecular tests				
HSV PCR	286	42.1	20	7.0
VZV PCR	NA*	NA	11	NA
GeneXpert or Xpert_Ultra	154	22.7	66	21.3
Enhanced testing				
Bacterial PCR				
S. suis PCR	29	4.3	7	24.1
S. pneumoniae PCR	27	4.0	3	11.1
N. meningitidis PCR	8	1.2	2	25.0
Viral PCR				
Dengue PCR	15	2.2	3	20.0
JEV PCR	10	1.5	1	10.0
Enterovirus PCR	8	1.2	0	0
Zika virus PCR	4	0.6	0	0
Parasitic PCR				
A. cantonensis PCR	17	2.5	12	70.6
Indirect immunofluorescence assay for anti-NMDAR encephalitis	78	11.5	47	60.3

Note to table 2.2*:* NA: not applicable. CSF HSV PCR was done as a first-line diagnosis to initiate acyclovir therapy in all patients with suspected viral meningoencephalitis. *Information about all VZV PCR tests conducted as part of routine diagnosis was not captured as part of the observational study.

Table 2.3: Results of laboratory diagnosis in patients with CNS infection syndromes.

Pathogen**	Routine diagnostic n (%)					Enhanced diagnostics, n (%)	Combined results, n (%)
	Microscopy	Culture	Crypto LFA	IgM ELISA	PCR		
Tuberculosis meningitis (N = 188)							
Mycobacterium tuberculosis	137 (72.9)	68 (36.2)	-	-	66 (35.1) *		142 (75.1)
Bacterial meningitis (N = 133)							78 (58.6)
S. suis	29 (21.8)	33 (24.8)	-	-	-	8 (6.0) [#]	39 (29.3)
E. coli	5 (3.8)	10 (7.5)	-	-	-	-	10 (7.5)
S. pneumoniae	5 (3.8)	6 (4.5)	-	-	-	3 (2.3) [#]	8 (6.0)
K. pneumoniae	1 (0.8)	2 (1.5)	-	-	-	-	2 (1.5)
N. meningitidis	0 (0.0)	1 (0.8)	-	-	-	2 (1.5) [#]	2 (1.5)
S. agalactiae	1 (0.8)	1 (0.8)	-	-	-	1 (0.8) [@]	2 (1.5)
S. gallolyticus	0 (0.0)	2 (1.5)	-	-	-	-	2 (1.5)
E. gallinarum	0 (0.0)	1 (0.8)	-	-	-	-	1 (0.8)
L. monocytogenes	1 (0.8)	1 (0.8)	-	-	-	-	1 (0.8)
B. pseudomallei	0 (0.0)	1 (0.8)	-	-	-	-	1 (0.8)
S. constellatus	1 (0.8)	1 (0.8)	-	-	-	-	1 (0.8)
S.aureus	0 (0.0)	1 (0.8)	-	-	-	-	1 (0.8)
P. mirabilis+ E. faecalis	0 (0.0)	1 (0.8)	-	-	-	-	1 (0.8)
S. anginosus+ E. coli	1 (0.8)	1 (0.8)	-	-	-	-	1 (0.8)
Unidentified	6 (4.5)	0 (0.0)	-	-	-	-	6 (4.5)
Cryptococcal meningitis (N = 20)							
Cryptococcus neoformans	18 (85.7)	17 (81.0)	20 (100.0)		-		20 (100.0)
Eosinophilic meningitis (N=17)							
A. cantonensis	-	-	-	-	-	12 (70.6) [#]	12 (70.6)
Viral meningoencephalitis(N=172)							40 (23.3)
HSV	-	-	-	-	20 (15.4)	-	20 (11.6)
VZV	-	-	-	-	11 (8.4)	-	11 (6.4)
Dengue virus	-	-	-	3 (2.3)	-	3 (2.3) [#]	5 (2.9)
JEV	-	-	-	4 (3.1)	-	1 (0.8) [#]	4 (2.3)
Anti-NMDAR (N=49)	-	-	-		-	47 (95.7) [$]	47 (95.7)

Note to table 2.3:

-Not applicable

* Diagnosed by GeneXpert or Xpert_Ultra

[#]Diagnosed by PCR

[@] Diagnosed by MinION sequencing of 16S rRNA (261)

[$] Diagnosed by immunofluorescence assay.

**In interpretation of the results, when an uncommon cause was detected, discussions were held between the laboratory scientists and the treating physicians.

2.3.4 Causes of CNS infection syndromes

2.3.4.1 Bacterial meningitis

A bacterial pathogen was detected in 78/133 (58.6%) patients presenting with clinically suspected BM (Table 2.3). Of these, *S. suis* was the leading cause, accounting for 29.3% (39/133), followed by *S. pneumoniae* 7.5% (10/133) and *E. coli* 6.0% (8/133). Dual infections were detected in 2/133 (1.5%), including *P. mirabilis* and *E. faecalis,* and *S. anginosus* and *E. coli.*

2.3.4.2 Tuberculosis meningitis

Of the 188 patients presenting with clinically suspected TBM*,* evidence of *M. tuberculosis* was established in 142/188 (75.1%). Of these, 137 (72.9%), 68 (36.2%) and 66 (35.1%) were diagnosed by ZN, culture and GeneXpert/Xpert_Ultra, respectively, including 63 by both ZN and culture, and 54 by all (Zn stain, culture and Xpert).

2.3.4.3 Meningoencephalitis

A viral pathogen was established in 40/172 (23.3%) of patients presenting with clinically suspected VME. HSV was the major viral pathogen, accounting for 11.6 % (20/172), followed by VZV (6.4%, 11/ 172). Other detected viral pathogens included Dengue virus (5/172, 2.9%) and JEV (4/172, 2.3%).

2.3.4.4 Fungal and parasitic encephalitis

Cryptococcus neoformans was detected in 20 cases. No cases of *C.gatii* were detected. Cerebral toxoplasmosis was diagnosed in 2. Of the 17 patients with clinically suspected EM, *A. cantonensis* was detected in 12 cases (70.6%).

2.3.4.5 Anti-NMDAR encephalitis

Anti-NMDAR encephalitis was clinically diagnosed in 49/221 (22.2%) patients with suspected meingoencephalitis. Of these, 47/221 (21.3%) patients had IgG against NMDAR detected in CSF, surpassing the combined frequency of HSV, VZV, DENV and JEV patients (Figure 2.6).

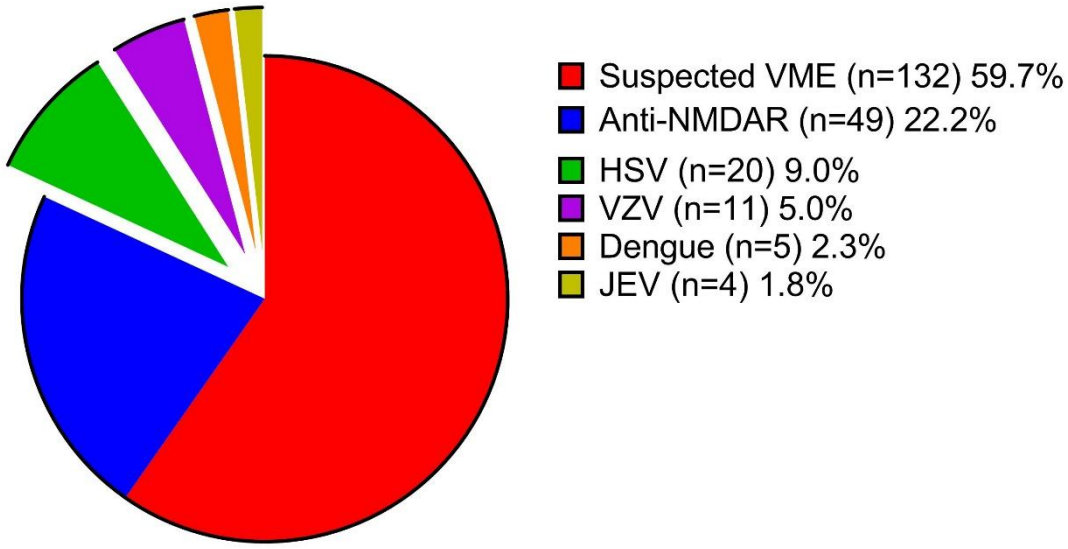


Figure 2.6: Infectious and non-infectious causes in 221 patients with clinically suspected meningoencephalitis.

2.3.5 Seasonality of CNS infection syndromes

CNS infection syndromes occurred throughout the year during the study period with a drop in enrolment in 2020, which was attributed to COVID-19 disruption (Figure 2.7). TBM patients were predominantly admitted around February-March and October-November each year. Otherwise, there was no clear seasonal trend observed for other causes (Figure 2.7 and 2.8).

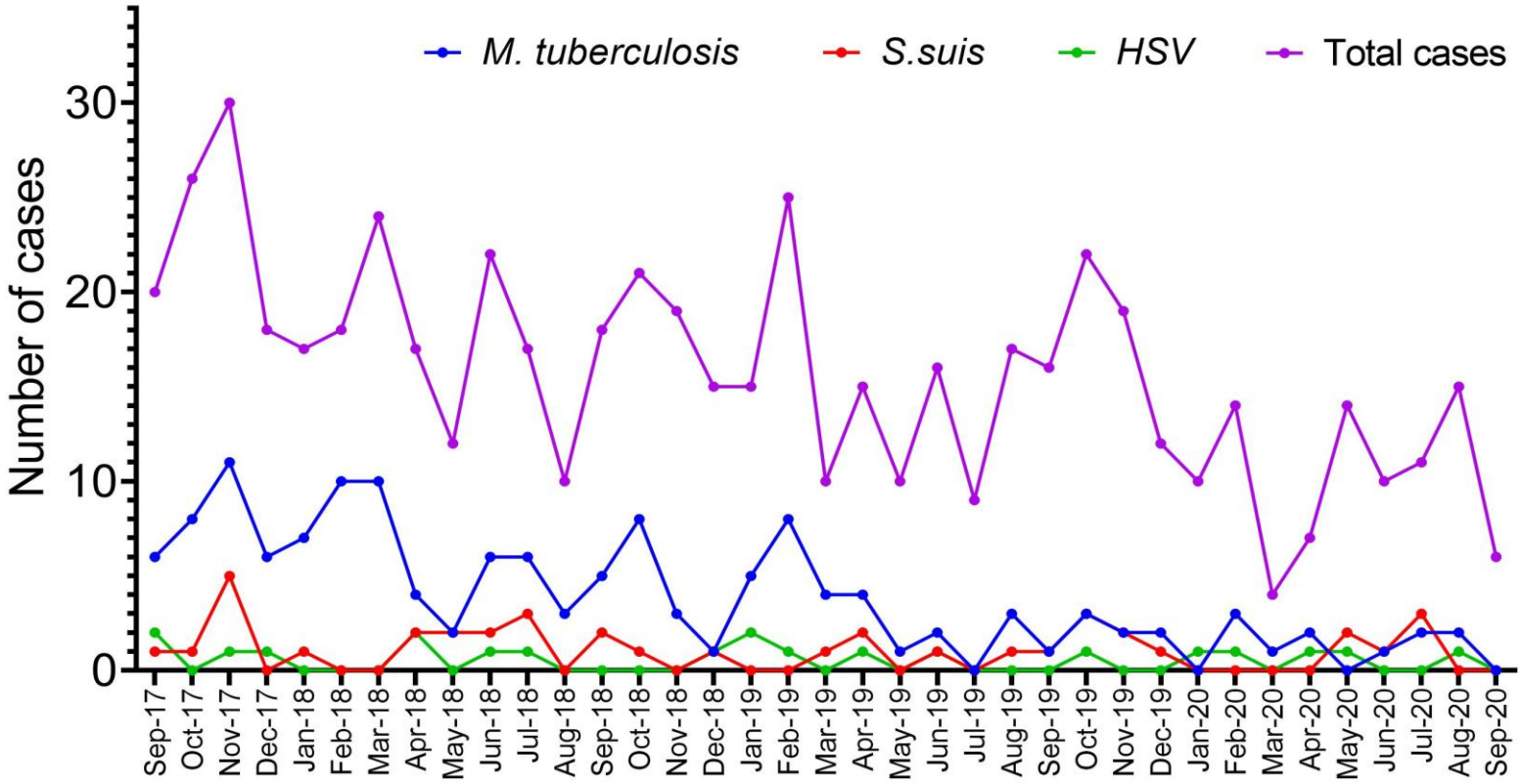


Figure 2.7: Monthly distribution of major pathogens of CNS infection syndromes during September 2017 and September 2020.

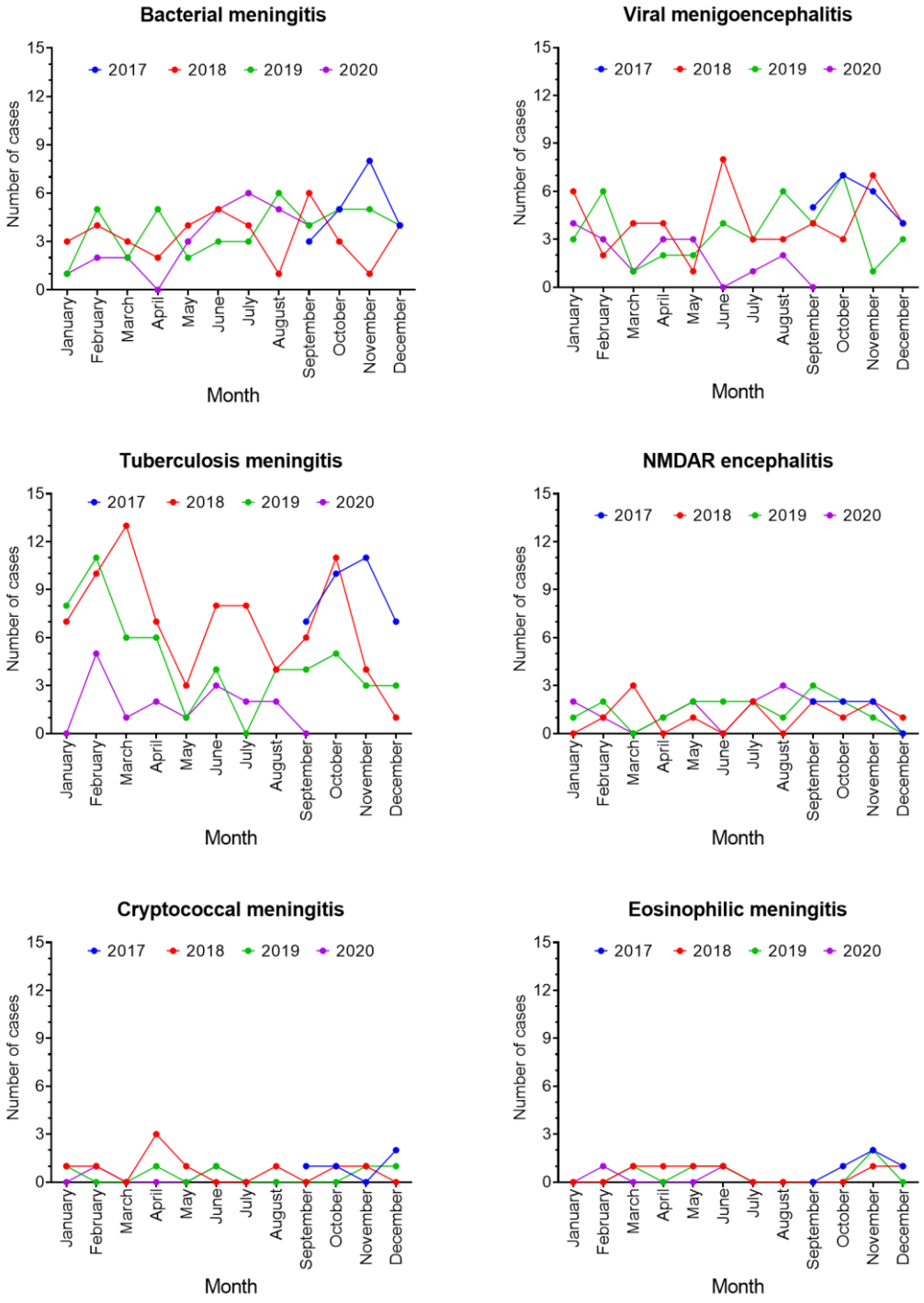


Figure 2.8: Monthly distribution of major entities of CNS infection syndromes during September 2017 and September 2020.

2.4 Discussion and conclusion

In this chapter, I set out to study the infectious and non-infectious causes in patients presenting with CNS infection syndromes admitted to my hospital, HTD. Using a combination of diagnostic assays, encompassing culture, PCR and ELISA, I demonstrated that a wide range of pathogens (bacteria, viruses, fungi, parasites) can cause CNS infections in Vietnamese adults, supporting previous reports (47–50,54,262–265). Additionally, my research revealed for the first-time anti-NMDAR encephalitis is a major clinical problem in Vietnamese adults. Despite extensive diagnostic workup, 41.3% of the patients remained undiagnosed, paralleling previous reports (47–53). Therefore, it remains a challenge to establish the causes in patients with CNS infection syndromes. However, testing for a wide range of infectious and non-infectious causes are critical to improve the diagnostic yield as illustrated by the contribution of the enhanced diagnostics to the overall yield of 58.7%, corresponding to an improvement of 12.2%, in the present study.

Data on the prevalence of NMDAR encephalitis in Vietnam is limited (26,266,267). In this study, I reported a proportion of 21.3% patients with confirmed NMDAR encephalitis of 221 patients with clinically suspected meningoencephalitis, as compared to a detection rate of 9.1% of NMDAR encephalitis in 99 patients with encephalitis admitted to my hospital Between January 2015 and February 2016 (26). Remarkably, the number of NMDAR encephalitis patients surpassed the number of patients HSV, VZV, Dengue and JEV encephalitis combined. This figure is in line with data from the California Encephalitis project (107). Collectively, our data emphasise that NMDAR encephalitis should be considered as an important differential diagnosis in adults presenting with CNS infection syndromes in Vietnam.

M. tuberculosis was the leading cause of CNS infection syndromes in the present study, much higher than findings from a previous report from Vietnam (24% vs 6%) (49). Although TBM is a major clinical problem in Vietnam and worldwide, the predominance of patients with TBM in the present study should be interpreted with caution. One of the reasons could

be that the diagnostic capacity for TBM in provincial hospitals, where the previous study from Vietnam was conducted, was limited. My study was conducted at HTD, a referral hospital in Southern Vietnam where TBM diagnostic capacity has been well established (178,268,269). More importantly, during the study period there was an ongoing TBM trial conducted at Viet-Anh ward. Therefore, there was a potential admission bias favoring TBM patients.

Similar to previous reports from Vietnam, including Ho Chi Minh City (12,49,103,263,264,270), my data revealed that many patients were infected with bacterial, viral and parasitic pathogens that can be prevented through an effective vaccination program (*S. pneumoniae* and JEV vaccines are available but not part of the national immunization programme) or by raising public awareness (A. cantonensis, and S. suis). Additionally, humans are most frequently infected with *A cantonensis* and *S. suis* by direct ingestion of molluscan intermediate hosts (e.g., raw snails) and consuming raw pig-blood pudding, respectively (103,271). *H. influenzae* PCR was not done in our study as it is a frequent cause of meningitis in children under the age of 5 years while our study patients were adults. Additionally, the number of *H. influenza* infections was reduced by expanding vaccine coverage into developing countries.

Most cases with CNS infection syndromes in my study were from provinces outside Ho Chi Minh City, reflecting the functions of HTD as a tertiary referral hospital for infectious diseases in Southern Vietnam. The drop in recruitment and TBM patients during the last 9 months of the study period (e.g., Jan-September 2020) was likely attributable to the COVID-19 disruption. Notably, between June 2021 and December 2022, HTD was deployed for COVID-19 patients during the pandemic. Consequently, CNS infection patients (including TBM) were referred to other hospitals, especially Pham Ngoc Thach hospital (a specialized hospital for *M. tuberculosis* associated diseases in Ho Chi Minh City, Vietnam).

There are still several limitations in my study. First, patient recruitment was undertaken at only a referral hospital in Ho Chi Minh, and the study population was restricted to adults. Second, I only tested *A cantonensis*, leaving other parasites such as *Baylisascaris procyonis* and *Gnathostoma spinigerum* for testing. Finally, I only tested the most common cause of autoimmune encephalitis (anti-NMDAR encephalitis). Additional testing for antibodies against other receptor types (such as anti-glutamate receptor type AMPA, anti-LGI1, anti-GAPA, anti-DPPX, anti-CASPR2) should be included.

In summary, CNS infection syndromes are associated with diverse infectious causes, and a substantial proportion of patients with meningoencephalitis can be caused by host-produced antibodies against NMDAR. Thus, while my findings have greatly contributed to the growing body of knowledge about the epidemiology and causes of CNS infection syndromes in Vietnam. The data also point to the diagnostic challenges presented by patients with neurological manifestations. Additionally, Southeast Asia is a recognised hotspot of emerging infectious diseases, including those caused by neurotropic viruses (Nipah virus, Zika virus and enterovirus A71). Novel diagnostic approaches are therefore urgently needed to improve patient diagnostics and pandemic preparedness.

Chapter 3

Clinical and Laboratory Features of Patients with Central Nervous Infection Syndromes at a Tertiary Referral Hospital in Ho Chi Minh City, Vietnam during 2017 and 2020

3.1 Introduction

In chapter 2, I described the results of laboratory investigation for infectious and non-infectious causes of CNS infection syndromes in Vietnamese adults. In this chapter, I associated the diagnostic results with clinical and laboratory findings, and patient outcomes. Additionally, as anti-NMDAR encephalitis represents a newly recognised cause of CNS infection syndromes in Vietnam, I conducted 6 and 12-month follow-up to shed light on its long-term outcome.

3.2 Materials and Methods

3.2.1 Patients and meta-data

The study participants included for analysis in this chapter were derived from the clinical study described in Chapter 2. Accordingly, in additional to the results of laboratory investigations described in Chapter 2, meta-data, including demographics, baseline characteristics, results of antimicrobial susceptibility testing, treatment, and in-hospital outcome, collected as part of the clinical study were used for analysis. Long-term outcomes collected at 6- and 12 months post discharge from patients with anti-NMDAR encephalitis were also used for analysis.

3.2.2 Outcome assessment and follow up

Discharge and long-term outcomes were assessed using the modified Rankin scale (mRS) ranging from 0 (without any disability) to 6 (death) (Table 3.1) (272). The mRS was previously validated (273–275), commonly used and has been shown to work well in the Vietnamese population (50), and can be administered by telephone (276). In addition to patients who died during hospitalisation, patients who were discharged for palliative care

at home as per the request of the relatives were also considered to have an in-hospital fatal outcome.

Table 3.1: Modified Rankin Scale (mRS) for neurologic disability (272)

0	No symptoms
1	No significant disability despite symptoms; able to carry out all usual duties and activities
2	Slight disability, unable to carry out all previous activities, but able to look after own affairs without assistance
3	Moderate disability, requiring some help, but able to walk without assistance
4	Moderately severe disability; unable to walk and attend to bodily needs without assistance
5	Severe disability; bedridden, incontinent, and requiring constant nursing care and attention
6	Death

3.2.3 Statistical analysis

Statistical analyses were performed using SPSS 23.0 (IBM, White Plains, NY, USA). Prism 9.2.0 (GraphPad Software, La Jolla, CA, USA) and Alluvial Plots in ggplot2 (R package version 4.3, Vienna, Austria) were used to generate figures. Categorical variables were presented as numbers and percentages (%). Continuous variables were reported as median and interquartile range (IQR). For comparison between two groups, Chi-square or Fisher's exact test was used for categorical variables and Man-Whitney test was used for continuous data. Statistical significance was defined by a two-sided *p*-value of <0.05.

3.3 Results

3.3.1 Baseline characteristics of all the study patients

The demographics, clinical presentations, CSF findings, treatment, and discharge outcomes of the 679 study participants enrolled in the clinical study are presented in Table 3.2. The median age was 40 years (IQR: 27-56). Males were dominant (432/679, 63.5%), and most of the patients (522/679, 76.9%) came from provinces other than Ho Chi Minh City. Among 679 study patients, 257 were tested for HIV, and 53 were HIV positive. Of these, 40 (75.5%) patients had TBM, and 7 (13.2%) patients had CM. At admission, a Glasgow Coma Score (GCS) of ≤9 was recorded in 20.6% (135/679). In terms of outcome, death, severe neurological deficit, moderate neurological deficit, and slight neurological deficit were recorded in 4.7% (n=32), 17.7% (n=120), 23.8% (n=161), and 27.6% (n=187) of the 679 patients, respectively. And only 177 (26.1%) made full recovery at discharge (Table 3.3).

There were some similarities in terms of clinical presentations (e.g. fever, consciousness reduction, seizure, psychosis and language change) at admission between patients with other diagnosis (including psychiatric disorder, alcoholic, etc.) and those with CNS infection syndromes (Table 3.2).

Table 3.2: Baseline characteristics of 679 patients enrolled in the study.

Characteristics	Whole group (N =679)	CNS infection syndromes (N=581)	Non-CNS infections (N=98)	*p*- value CNS infections vs Non-CNS infections
Demographics				
Median age in years (IQR)	40 (27-56)	39 (27-55)	43 (32-62)	0.012
Male, n (%)	432 (63.5)	382 (65.7)	50 (51.0)	0.006
HCMC origin, n (%)	157 (23.1)	134 (23.1)	23 (23.5)	0.898
Clinical findings				
Median illness day on admission, n (IQR)	7 (4-14)	7 (4-14)	5 (3-8)	<0.001
HIV positive status*, n (%)	53 (7.8)	50 (8.6)	3 (3.1)	0.066
Fever, n (%)	617 (90.9)	532 (91.6)	85 (86.7)	0.164
Weight loss, n (%)	97 (14.3)	93 (16.0)	4 (4.1)	0.005
Sweat, n (%)	32 (4.7)	31 (5.3)	1 (1.0)	0.005
Cough > 2 weeks, n (%)	38 (5.6)	33 (5.7)	5 (5.1)	0.171
Headache, n (%)	519 (76.4)	472 (81.2)	47 (48.0)	<0.001
Consciousness reduction, n (%)	567 (83.5)	482 (83.0)	85 (86.7)	0.580
Local seizure, n (%)	35 (5.2)	29 (5.0)	6 (6.1)	0.679
General seizure, n (%)	124 (18.3)	99 (17.0)	25 (25.5)	0.239
Psychosis, n (%)	104 (15.3)	83 (14.3)	21 (21.4)	0.297
Language change, n (%)	102 (15.0)	77 (13.3)	25 (25.5)	0.013
Hemiplegia, n (%)	40 (5.9)	35 (6.0)	5 (5.1)	0.733
Movement disorder, n (%)	87 (12.8)	73 (12.6)	14 (14.3)	0.932
Limb weakness (Paraplegia/ Tetraplegia), n (%)	54 (7.9)	37 (6.4)	9 (9.2)	0.390
Convulsion, n (%)	59 (8.7)	41 (7.1)	10 (10.2)	0.515
Stiff neck, n (%)	352 (51.8)	318 (54.7)	34 (34.7)	0.005
Abnormal movement				
Face-mouth-tongue	54 (8.0)	45 (7.7)	9 (9.2)	0.825
Trunk	25 (3.7)	23 (4.0)	2 (2.0)	0.562
Extremity	35 (5.2)	32 (5.50	3 (3.1)	0.570
Glasgow Coma Scale ≤ 9, n (%)	135 (20.6)	126 (21.7)	9 (9.2)	0.008
Blood and CSF findings				
Opening pressure cmCSF, median (IQR)	18 (13-24)	18 (14-25)	15 (12-18)	<0.001
CSF Hematology				
WCC/mm^3, median (IQR)	162 (22-529)	204 (44-675)	4 (1-17)	<0.001
Neutrophil %, median (IQR)	26 (13-66)	26 (13-69)	25 (12-50)	0.209
Lymphocyte %, median (IQR)	68 (30-86)	69 (29-86)	57 (35-83)	0.261
Eosinophil%, median (IQR)	0 (0-0)	0 (0-0)	0 (0-0)	
Biochemistry				
Protein g/L, median (IQR)	1.0 (0.5-2.1)	1.2 (0.6-2.2)	0.4 (0.3-0.7)	<0.001
Lactate nmol/L, median (IQR)	3.5 (2.4-6.1)	3.9 (2.6-6.8	2.4 (2.0-3.3)	<0.001
CSF: blood glucose ratio, median (IQR)	0.50 (0.31-0.67)	0.5 (0.3-0.6)	0.7 (0.6-0.7)	<0.001

Note to Table 3.2: Patients with history of HIV and patients with suspected TBM, Cryptococcus and Toxoplasmosis were tested HIV

Table 3.3: Interventions and outcomes of 679 study patients

Characteristics	Whole group (N =679)	CNS infection syndromes (N=581)	Non-CNS infections (N=98)	*p*- value CNS infections vs Non-CNS infections
Interventions				
Ventilation, n (%)	104 (15.3)	91 (15.7)	13 (13.3)	0.486
Sedative drug administration, n (%)	142 (20.9)	115 (19.8)	27 (27.6)	0.122
TB treatment, n (%)	187 (27.5)	186 (32.0)	1 (1.0)	<0.001
Acyclovir, n (%)	181 (26.7)	156 (26.9)	25 (25.5)	0.694
Antibiotics, n (%)	369 (54.3)	307 (52.8)	62 (63.9)	0.062
Outcomes				
Days of hospitalisation, median (IQR)	15 (7-30)	17 (8-32)	8 (6-18)	<0.001
Rankin scale, n (%)				0.849
0	177 (26.1)	153 (26.4)	24 (24.5)	
1-2	187 (27.6)	162 (28.0)	25 (25.5)	
3-4	161 (23.8)	136 (23.5)	25 (25.5)	
5	120 (17.7)	99 (17.1)	21 (21.4)	
6	32 (4.7)	29 (5.0)	3 (3.1)	

3.3.2 Comparison between major CNS infection syndrome groups

Comparison between patient groups of CNS infection syndromes revealed considerable heterogeneity in demographic, clinical and laboratory findings, and outcomes (Table 3.4). Patients with confirmed and suspected diagnoses of BM, TBM and VME did not present significant difference in demographic, clinical presentations, laboratory findings, treatment, and outcome, except for CSF findings between confirmed and suspected BM (Appendix B, Table S3.1). Herein, I focused my analysis on patients with a confirmed diagnosis.

3.3.2.1 Demographics

Patients with anti-NMDAR encephalitis were younger than those with CNS infections (BM, TBM, VME, EM and CM) (Table 3.4). Males were predominant in patients with BM, TBM and VME, but not in those with anti-NMDAR encephalitis or EM (Table 3.4).

3.3.2.2 Clinical Findings

While fever, headache, neck stiffness and consciousness reduction were common features observed in all patient groups, psychosis, language dysfunction and movement disorders were predominantly found in those with anti-NMDAR encephalitis (Table 3.4). Meanwhile, weight loss (53/142, 37.3%), sweating (16/142, 11.3%) and cough more than 2 weeks before admission (16/142, 11.3%) were the common features in TBM patients.

Late hospital admission was documented in patients with TBM, EM, CM and anti-NMDAR encephalitis, with a median illness day of 13 (IQR: 9-20), 18 (IQR: 15-30), 18 (IQR: 10-30) and 20 (IQR: 9-30), respectively, as compared to 3 (IQR: 2-5) and 5 (IQR: 4-7) in those with BM and VME. The median duration of hospital stay ranged from 15 days (IQR: 11-24) in BM patients to 43 days (IQR: 31-54) in anti-NMDAR patients (Table 3.4).

3.3.2.3 Laboratory and brain imaging findings

There were significant differences in CSF findings between patient groups. Anti-NMDAR encephalitis patients had CSF findings within normal range (Figure 3.1). BM patients had the highest level of white cell count, median: 3,806 cells/mm^3 (IQR: 942-7,666) with a predominance of neutrophil, median: 87% (IQR: 77-94).

As for the CSF protein and lactate profiles, the highest and lowest levels were documented in patients with BM and anti-NMDAR encephalitis, respectively (Figure 3.1). Meanwhile, CSF/blood glucose ratio of the BM patients was lower than that of the other groups (VME, TBM, CM and EM) (Figure 3.1).

Even though all patients underwent screening, trans-abdominal computed tomography scan/MRI results were available in 62.5% (15/24) of female patients with anti-NMDAR encephalitis. Subsequently, ovarian teratoma was detected in 6.7% (1/15).

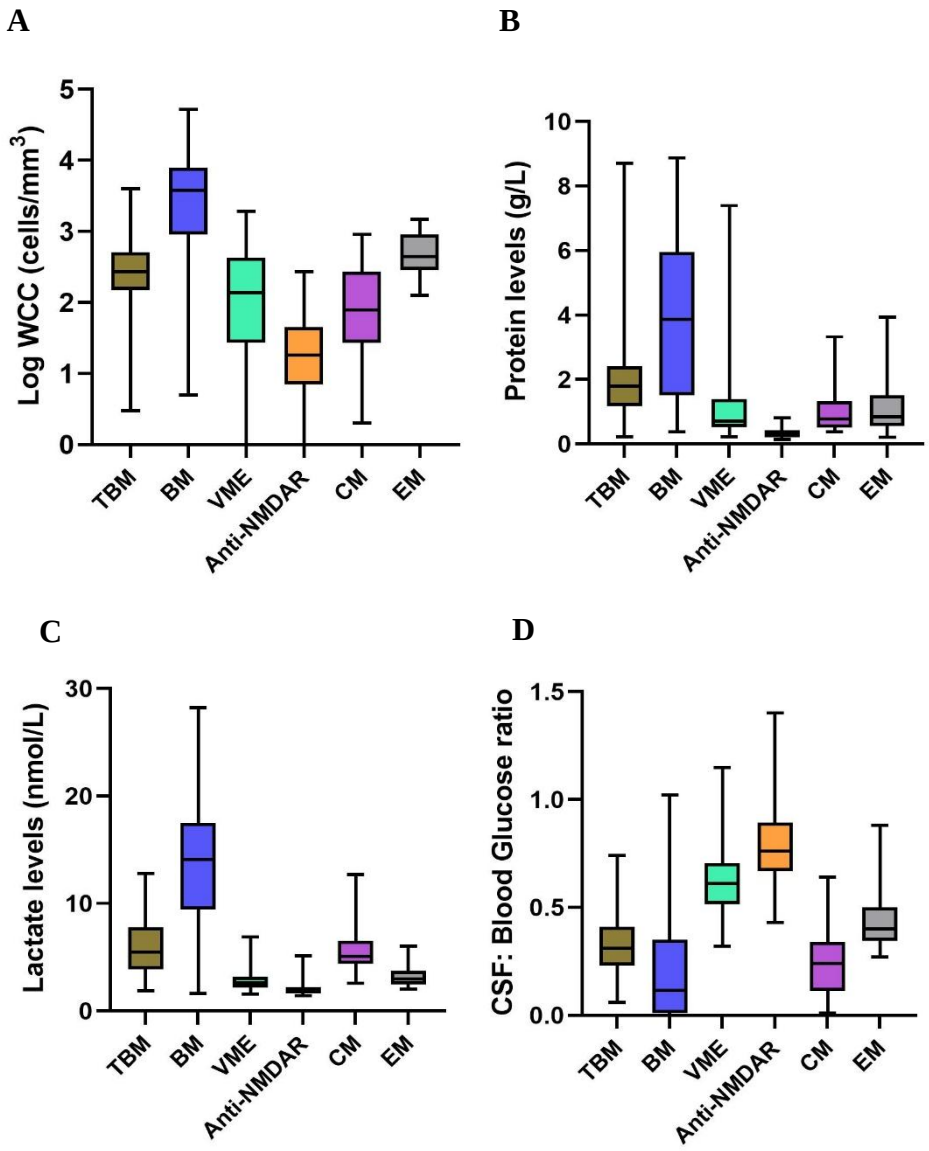


Figure 3.1: Comparison of CSF hematology and biochemistry laboratory findings between major groups of CNS infection syndromes.

(A) White cell counts (WCC), (B) Protein levels,

(C) Lactate levels, (D) CSF: Blood glucose ratio

Table 3.4: Comparison of demographics, clinical characteristics, laboratory findings, treatment, and outcome between major groups of CNS infection syndromes.

Characteristics	Confirmed BM (N=78)	Confirmed TBM (N=142)	Confirmed VME (N=40)	Confirmed anti-NMDAR (N=47)	CM (N=20)	EM (N=17)
Demographics						
Median age in years (IQR)	55 (42-63)	39 (29-52)	35 (25-53)	25 (19-31)	41 (30-59)	32 (30-47)
Male, n (%)	51 (65.4)	106 (74.6)	30 (75.0)	24 (51.1)	14 (70.0)	9 (52.9)
HCMC origin, n (%)	13 (16.7)	34 (23.9)	10 (25.0)	9 (19.1)	4 (20)	3 (17.6)
Clinical findings						
Median illness day on admission (IQR)	3 (2-5)	13 (9-20)	5 (4-7)	20 (9-30)	18 (10-30)	18 (15-30)
Duration of hospitalisation in days, median (IQR)	15 (11-24)	27 (3-40)	19 (13-25)	43 (31-54)	24 (11-39)	22 (11-27)
HIV positive status, n (%)	0 (0.0)	37 (26.1)	1 (2.5)	0 (0.0)	7 (35.0)	1 (5.9)
Fever, n (%)	72 (92.3)	137 (96.5)	36 (90.0)	36 (76.6)	17 (85.0)	13 (76.5)
Weight loss, n (%)	2 (2.6)	53 (37.3)	1 (2.5)	1 (2.1)	6 (30.0)	3 (17.6)
Sweat, n (%)	2 (2.6)	16 (11.3)	0 (0.0)	1 (2.1)	0 (0.0)	1 (5.9)
Cough > 2 weeks, n (%)	1 (1.3)	16 (11.3)	2 (5.0)	1 (2.1)	1 (5.0)	1 (5.9)
Headache, n (%)	69 (88.5)	132 (93)	24 (60.0)	26 (55.3)	19 (95.0)	17 (100.0)
Consciousness reduction, n (%)	72 (92.3)	108 (76.1)	37 (92.5)	46 (97.9)	12 (80.0)	11 (64.7)
Local seizure, n (%)	3 (3.8)	1 (0.7)	4 (10.0)	6 (12.8)	0 (0.0)	0 (0.0)
General seizure, n (%)	3 (3.8)	4 (2.8)	16 (40.0)	18 (38.3)	4 (20.0)	0 (0.0)
Psychosis, n (%)	6 (7.7)	3 (2.1)	7 (17.5)	36 (76.6)	1 (5.0)	1 (5.9)
Language change, n (%)	8 (10.3)	11 (7.7)	4 (10.0)	25 (53.2)	1 (5.0)	0 (0.0)
Movement disorder, n (%)	2 (2.6)	8 (5.6)	6 (15.0)	30 (63.8)	0 (0.0)	1 (5.9)
Hemiplegia, n (%)	5 (6.4)	16 (11.3)	5 (12.5)	1 (2.1)	2 (10.0)	1 (5.9)

Characteristics	Confirmed BM (N=78)	Confirmed TBM (N=142)	Confirmed VME (N=40)	Confirmed anti-NMDAR (N=47)	CM (N=20)	EM (N=17)
Limb weakness (Paraplegia/ Tetraplegia), n (%)	2 (2.6)	14 (9.9)	2 (5.0)	2 (4.3)	2 (10.0)	4 (23.5)
Convulsion, n (%)	2 (2.6)	2 (1.4)	5 (12.5)	5 (10.6)	0 (0.0)	0 (0.0)
Stiff neck, n (%)	65 (83.3)	73 (54.1)	15 (37.5)	12 (25.5)	13 (65.0)	12 (70.6)
Abnormal movement, n (%)						
Face-mouth-tongue	0 (0.0)	0 (0.0)	4 (10.0)	26 (55.3)	0 (0.0)	0 (0.0)
Trunk	0 (0.0)	1 (0.7)	2 (5.0)	11 (23.4)	0 (0.0)	0 (0.0)
Extremity	2 (2.6)	4 (2.8)	3 (7.5)	16 (34.0)	0 (0.0)	0 (0.0)
Glasgow Coma Scale ≤ 9, n (%)	20 (25.6)	28 (19.7)	10 (25.0)	20 (42.6)	4 (21.1)	1 (5.9)
Interventions						
TB treatment, n (%)	2/76 (2.6)	134 (94.4)	1/39 (2.6)	0	1 (5.3)	2 (11.8)
Acyclovir, n (%)	3/75 (4.0)	3/136 (2.2)	34 (85.0)	22/43 (51.2)	1 (5.3)	1 (5.9)
Antibiotics, n (%)	78 (100%)	45 (31.7)	18 (45.0)	32 (68.1)	6 (30.0)	5 (29.4)

3.3.3 Antimicrobial admission and resistance

All the BM patients with a confirmed diagnosis received antimicrobial therapy (Table 3.4). As per our standard treatment guidelines applied at HTD, those patients were prescribed Ceftriaxone with one dose of 100 mg/kg/day for 10-14 days with/without Vancomycin. Empiric antibiotics prescriptions were frequently given in patients with non-BM and non-TBM, ranging from 29% in EM to 68% in anti-NMDAR encephalitis group (Table 3.4).

The antibiotic resistance profiles of major bacterial meningitis pathogens are displayed in Table 3.5. Of the *S. suis* isolates, 100% were sensitive to Vancomycin (30/30), Ampicillin (32/32), Ceftriaxone (32/32) and Levofloxacin (24/24) but 100% (22/22) of the isolates were resistant to Tetracycline, and 45.5% (5/11) and 46.2% (6/13) were intermediately resistant to Erythromycin and Clindamycin, respectively. Notably, 15.6% (5/32) of *S. suis* isolates were associated with multi-drug resistance (i.e., resistant to 3 antibiotics: Clindamycin, Erythromycin and Tetracycline). All the *S. pneumoniae* isolates were sensitive to Vancomycin, Levofloxacin and Rifampin, but 75% (6/8) of them were multi-drug resistant, with 25% (2/8) resistant to 5 antibiotics (Penicillin, Oxacillin, Erythromycin, Clindamycin and Trimethoprim/ sulfamethoxazole), 50% (4/8) resistant to 4 antibiotics (Penicillin, Erythromycin, Clindamycin and Oxacillin or Trimethoprim/sulfamethoxazole). All the 10 *E. coli* isolates with available antimicrobial susceptibility testing were sensitive to Carbapenems (Meropenem, Ertapenem and Imipenem) but more than 50% of them were resistant to Cephalosporins (Cefepime and Cefotaxime) and Fluoroquinolones (Levofloxacin), with resistance to >=3 antibiotics recorded in 70% (7/10). Rifampicin resistance was detected in 9/66 (13.6%) of *M. tuberculosis* isolates (Table 3.6).

Table 3.5: Antibiotic resistance profile of major bacterial agents of CNS infections.

Antibiotics*	*S. suis* (n=39)		*S. pneumoniae* (n=8)		*E. coli* (n=10)	
	n	%	n	%	n	%
Penicillin	1/31**	3.2%	6/6	100%		
Ampicillin	0/32	0%				
Amoxicillin+ Clavulanic acid					2/7	28.6%
Piperacillin + Tazobactam					1/10	10.0%
Oxacillin			4/4	100%		
Vancomycin	0/30	0%	0/6	0%		
Erythromycin	5/11	45.5%	6/6	100%		
Clindamycin	6/13	46.2%	6/6	100%		
Amikacin					1/2	50%
Gentamycin					3/9	33.3%
Tetracycline	22/22	100%				
Ceftriaxone	0/32	0%	2/6**	33.3%	6/10	60.0%
Ceftazidime					0/1	0%
Cefepime					5/7	71.4%
Cefotaxime	0/3	0%			4/8	50.0%
Levofloxacin	0/24	0%	0/6	0%	5/9	55.6%
Ciprofloxacin					0/1	0%
Meropenem	0/5	0%			0/5	0%
Ertapenem					0/7	0%
Imipenem					0/6	0%
Linezolid	0/3	0%				
Rifampin			0/6	0%		
Trimethoprim-sulfamethoxazole			4/6	66.7%	2/4	50.0%
Colistin					0/1	0%

Notes to Table 3.5: *Antibiotic susceptibility testing include Kirby-Bauer disk diffusion test, Epsilometer test, testing in VITEK 2 Compact and MIC (Minimal Inhibitory Concentration) micro-dilution method, following CLSI guidelines.** Intermediate

Table 3.6: Results of Nucleic Acid amplification test (NAAT) of TBM cases

Nucleic Acid Amplification Test	Positive n (%)	Rifampicin resistance n (%)
GeneXpert	38/101 (37.6)	4/38 (10.5)
Xpert_Ultra	28/53 (47.2)	5/28 (17.9)
Total	**66/154 (42.9)**	**9/66 (13.6)**

3.3.4 Duration of hospitalization, treatment and clinical outcome

Patients with CNS infection syndromes had a long duration of hospital stay, with a median of 17 days (IQR: 8-32). The longest hospitalisation was documented in patients with anti-NMDAR encephalitis (median: 43 days, IQR: 31-54), and the shortest duration was recorded in BM patients (median: 15 days, IQR: 11-24). More details are presented in Table 3.4.

Immunotherapy was described in all patients with anti-NMDAR encephalitis. Methylprednisolone and/or prednisolone were prescribed for 14 of 47 (29.8%) patients. Nine patients (19.1%) were managed with a combination of steroids and intravenous immunoglobulin (IVIG). Another 9 patients (19.1%) received a combination of steroids with plasma exchange. The last 15 patients (31.9%) were intensively treated with a combination of steroids plus IVIG and albumin exchange.

The in-hospital mortality varied between patient groups (Figure 3.2). Of the 19 CM patients with available outcome at discharge, two (10.5%) had a fatal outcome during hosptalisation, while 6 (31.6%) had a mRS of 5 at discharge, which likely resulted a fatal outcome post discharge. Of the 142 TBM patients with a confirmed diagnosis, the mortality rates were recorded in 13 (9.2%). Three out of 47 anti-NMDAR patients (6.4%) died during hosptalisation while 6 patients (12.8%) were discharged for palliative care were also considered to have a fatal outcome. None of the patients with VME and EM had a fatal outcome. Among the surviving patients, over half of those with BM and VME had the best recovery with no symptoms or mild disability at discharge (42/77, 54.6% and 24/40, 60%; respectively). This figure was 15/47 (31.9%) patients with anti-NMDAR encephalitis.

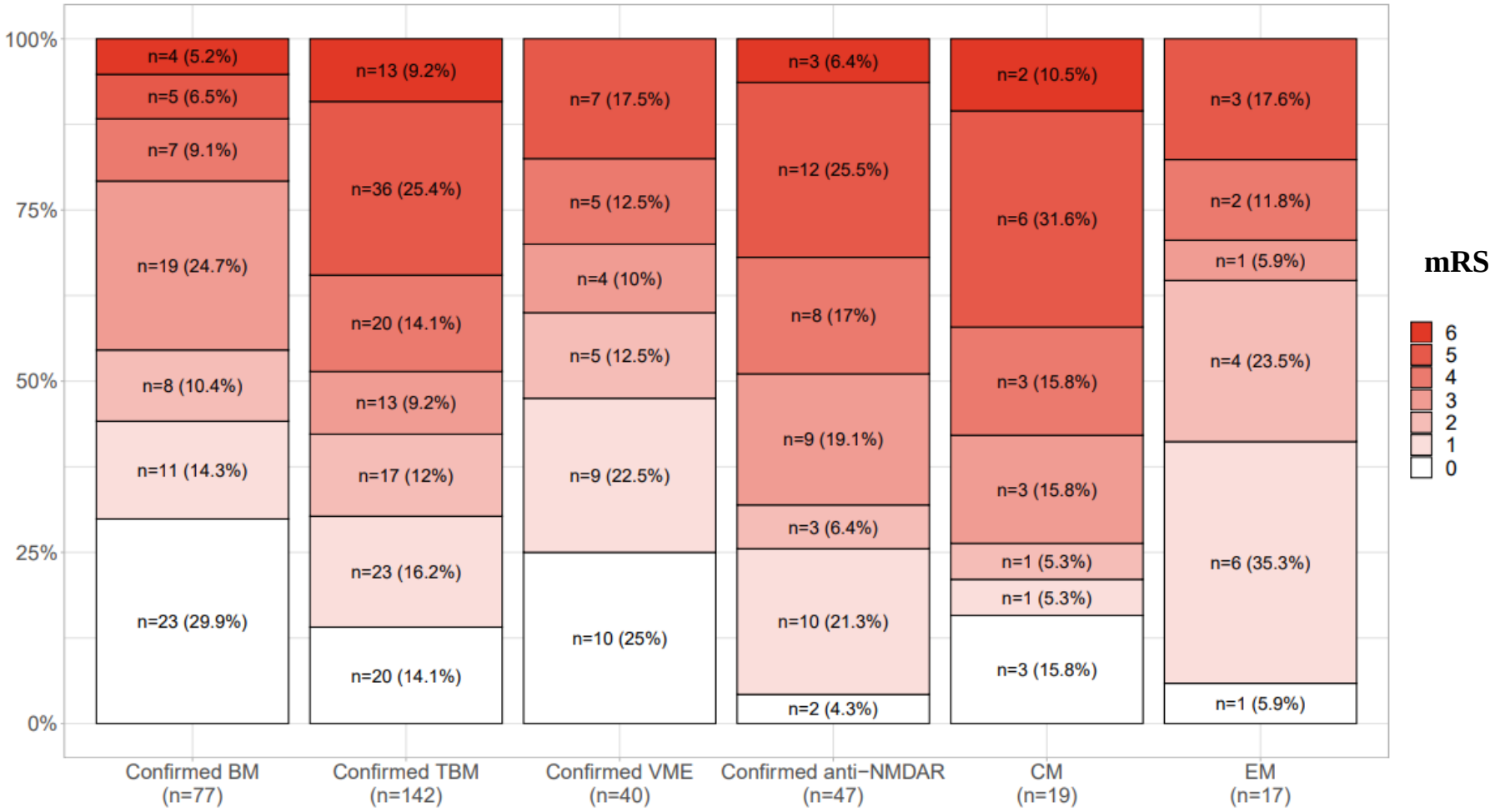


Figure 3.2: Modified Rankin scales recorded at discharge of different clinical groups of CNS infection syndromes.

3.3.5 Long-term outcomes of patients with anti-NMDAR encephalitis

Apart from the 3 fatal cases recorded while in hospital, 6 were discharged for palliative care at home prior to passing away as per the requests of their relatives. Thus 38 patients were included for 6 and 12-month follow-up. Subsequently, 25/38 (65.8%) and 15/38 (39.5%) patients were successfully followed up at 6 and 12 months, respectively (Figure 3.3). At 6 months, no symptoms or mild sequelae (mRS: 1 (n=7) and mRS: 2 (n=3)) was documented in 24/25 (96.0%) patients, while one patient (4.0%) had moderate sequelae with a mRS of 3, unchanged since discharge (Figure 3.3A). Of 15 patients successfully followed up at 12 months, 8 (53.3%) had a mRS of 0, and 7 patients (46.7%) remained suffering from mild disability (mRS: 1 (n=6) and mRS: 2 (n=1)) (Figure 3.3B).

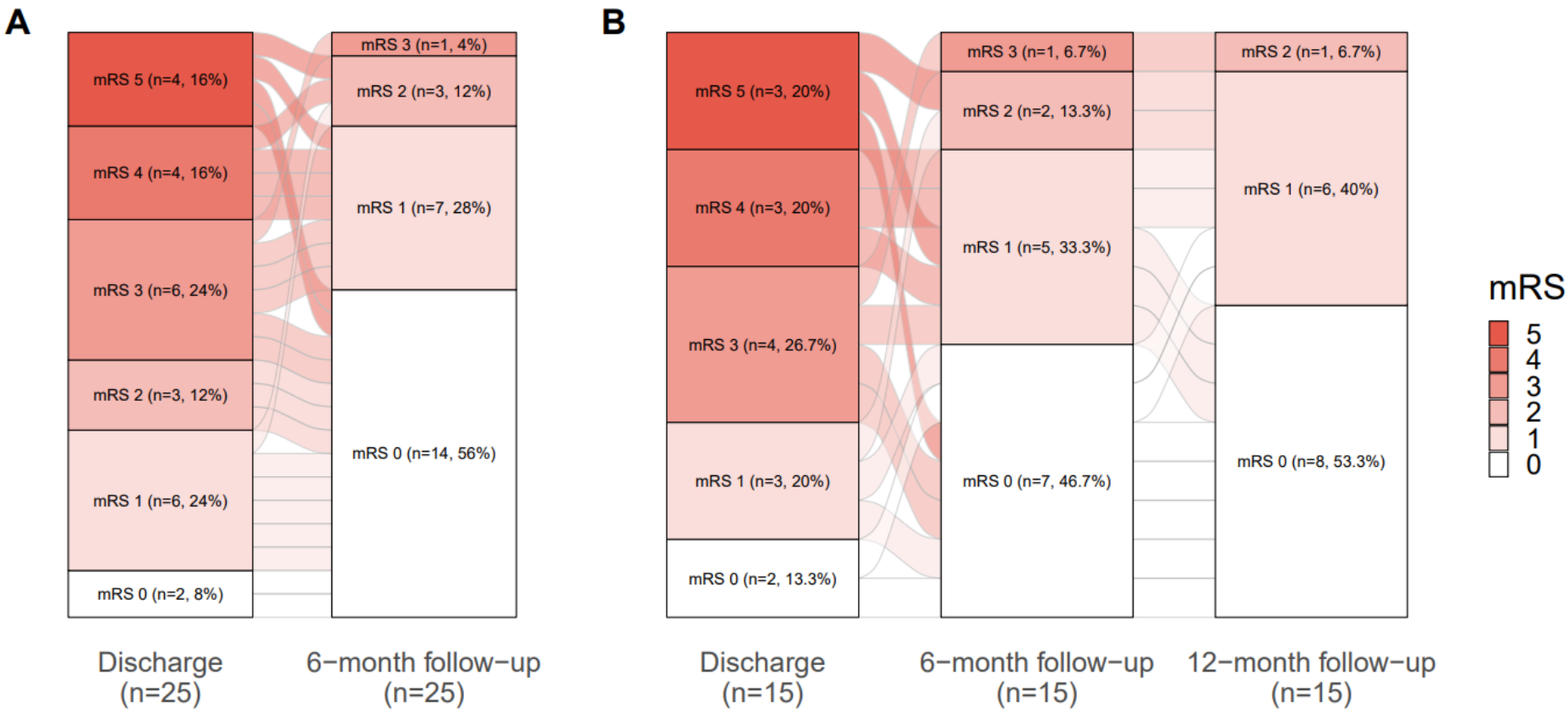


Figure 3.3: Long-term outcomes of patients with anti-NMDAR encephalitis

(A) Outcomes of patients completed 6-month follow-up.

(B) Outcomes of patients completed 12-month follow-up.

3.4 Discussion and conclusion

Here, I show that infectious and non-infectious causes of CNS infection syndromes in Vietnamese adults are associated with high morbidity and mortality, with overlapped clinical and laboratory findings. Additionally, these findings coupled with data in Chapter 2 demonstrate for the first time that anti-NMDAR encephalitis is a common cause of CNS infection syndromes alongside TBM, BM, VME, CM and EM in Vietnamese adults, with disability documented up to 12 months post discharge.

The diagnosis and management of CNS infection syndromes require specific approaches tailored the corresponding syndromes and infections. Yet, the high rates of (multi-)drug resistance of major causative agents of CNS infection syndromes represents new challenges for clinical management, and likely explain the observed poor outcomes in the study. The high prevalence and high levels of multi-drug resistance of *S. suis, S. pneumoniae*, *E. coli* and *M. tuberculosis* were also described in human and animals in recent studies in Vietnam (277–280). However, on several occasions, bacterial pathogens were diagnosed by PCR methods alone, and routine culture was negative. Thus, my findings may not fully account for a complete picture about antibiotic resistance profiles of bacterial causes of CNS infections in Vietnam.

Since the first case series of anti-NMDAR encephalitis reported in Vietnamese adolescents and adults (26), this is the first prospective study detailing the frequency, demographics, clinical features, management and long-term outcome of this newly recognised problem in Vietnam in the context of other CNS infection syndromes. Consistent with findings from previous reports (14,77–79) anti-NMDAR encephalitis in Vietnamese adults is associated with young age, psychosis, language dysfunction, movement disorder, prolonged illness duration, long hospital stays and poor outcome. Because of those clinical features, anti-NMDAR encephalitis can be confused with psychiatric disease. Therefore, patients are often referred to psychiatric hospitals before admitted to HTD. This explains the delay in

diagnosis and hence prolonged illness history prior to hospital admission, leading to poor clinical outcome. Notably, the total mortality rate of anti-NDMAR encephalitis in my study was 19.2% which is higher than reported data from previous studies in China and USA, ranging from 2.3% to 15% (35,108,281).

In the previous studies, anti-NMDAR encephalitis was more likely detected in young women, especially those with teratoma (25,35,107,108). However, in this study, I found female and male patients contributed equally, and teratoma was detected in only one patient. Differences in settings and recruitment approach might be the contributing factors. For example, other studies focused on patients with psychiatric disorder or those with ovarian teratoma (35,282) while my study enrolled a cohort of patients with CNS infection syndromes more broadly. Thus, my study more comprehensively captures the epidemiology of anti-NMDAR encephalitis in Vietnamese adults.

The proportion of anti-NMDAR encephalitis patients with paraneoplastic varied between studies. In my study, ovarian teratoma was confirmed in one (2.1%). This is considerably lower than previous reports. Wang et al reported a tumor rate of 7.0% (3/43) (283). Irani et al. reported 26.5% (9/34) adult patients had tumors (284). The frequency of patients with neoplasm was even higher in Dalmau et al. study (35), showing that 59% (58/98) of 98 patients had a tumor, most commonly ovarian teratoma. In a recent study from China, 19.5% of 220 study patients had an underlying neoplasm (108). Sample size, selection bias, ethnicity backgrounds and epidemiology are potentially the contributing factors, which warrants further research.

My study has some limitations. For assessment of discharge and long-term outcomes, I did not use a control group for comparison. Additionally, my team and I used the modified Rankin scale, a simple approach allowing for assessment over the phone. However, mRS only provides basic information about physical disability but not about emotional problems or ability to return to work. An alternative multidimensional assessment could be used for further evaluation of recovery.

In conclusion, CNS infection syndromes in Vietnamese adults are associated with high morbidity and mortality. Although both infectious and non-infectious causes can be responsible for this devastating clinical problem, there are considerable overlaps in clinical manifestations and laboratory findings, while the current diagnostic assays are inadequate. These factors render early diagnosis that would otherwise enable rapid initiation of appropriate therapies, thereby potentially improving patient outcome. Therefore, clinical trials to improve the current treatment pathways in patients with CNS infection syndromes are urgently needed. And of equal importance is to improve the current diagnostic approach, which will be the focus of the next chapters.

Chapter 4

Development and Prospective Evaluation of an Internally Controlled Metagenomics Workflow for Simultaneous Detection of Bacterial and Viral Causes of Central Nervous System Infections

4.1 Introduction

In the previous chapters, despite extensive diagnostic testing, the aetiology remains unidentified in >40% of patients presenting with CNS infection syndromes in our setting. Novel diagnostic approaches are therefore urgently needed to improve patient diagnostic and management, and to support outbreak response.

Metagenomics is a novel approach for infectious disease diagnosis and pathogen surveillance (183,285,286). However, few studies, in particular those applying real-time metagenomics for infectious disease diagnosis, have been conducted in LMICs (39,287,288). Many exisiting metagenomic workfows have been optimised for either viral or bacterial pathogens. Few have been developed for the simultaneous detection of both viruses and bacteria in a single experiment (80,289–291). Additionally, quality assessment of metagenomics is often based on non-template controls, without the inclusion of positive internal controls for reliable assessment of intra-assay variations (291–293).

In this chapter, I set out to achieve two aims. Firstly, I aimed to develop an internally controlled metagenomic workflow for simultaneous detection of both bacterial and viral causes of CNS infections (phase 1). And secondly, I prospectively evaluated the potential application of the established workflow in diagnosing CNS infections in patients without a cause identified by the conventional diagnostic assays of the clinical study (phase 2).

4.2 Materials and Methods

4.2.1 Setting and study design

The research described in this Chapter formed part of the observational study detailed in Chapters 2&3. The design of the present study consisted of two phases. Phase 1 was to develop an internally controlled metagenomic workflow for simultaneous detection of both bacteria other than *M. tuberculosis*, and viruses causing CNS infections in CSF samples. Phase 2 represented a pilot and was designed to prospectively evaluate the potential application of the pipeline established as part of phase 1 in identifying the causes of CNS infections in patients of unknown origin enrolled in the clinical study as described in Chapter 2. The collected CSF samples were immediately stored at -80^{o}C for the study purpose. The duration of storage before metagenomics analysis was 2-3 years for phase 1, and 1-2 weeks for phase 2, pending the results of routine diagnosis.

4.2.2 Patient groups and selection criteria

For phase 1, from the case series of the clinical study, I selected consecutive CSF samples from patients enrolled between September 2017 and June 2018, fulfilling the selection criteria of one of the three patient groups 1-3, specifically assigned for the purpose of assay evaluation. For group 1, the selection criteria consisted of 1) any patients with clinical presentation suggestive of meningitis or meningoencephalitis, and 2) a viral or bacterial pathogen identified by at least one of the assays of the diagnostic workup (Gram stain and/or bacterial culture and/or PCR in Appendix C (Table S4.1)). For group 2, the selection criteria included 1) any patients with a discharge diagnosis of non-CNS infections established on the basis of clinical assessment of treating physicians, and 2) no CNS infection pathogen identified by the diagnostic workup of the clinical study. For group 3, the selection criteria consisted of 1) any patients with clinically suspected meningitis or meningoencephalitis and 2) no aetiology identified by the diagnostic workup of the clinical study. In the absence of reference standards, groups 1 and 2 were used to assess the sensitivity and specificity

of the metagenomic workflow, respectively. Group 3 was included to assess the presence of new or rare pathogens causing CNS infections.

For phase 2, the inclusion criteria were any patients participating in the clinical study with 1) clinically suspected meningitis or meningoencephalitis, 2) no aetiology identified by conventional assays, and 3) a request for metagenomic testing decided by treating physicians based on clinical progression.

4.2.3 Metagenomics NGS workflows

Workflows and internal controls

To maximize the chance of detecting both DNA and RNA pathogens (bacteria and DNA/RNA viruses), we divided the tested CSF samples into two aliquots which were then subject to two separate workflows, DNA and RNA pathogens, prior to sequencing (Figure 4.1). Additionally, to monitor the performance of the metagenomic assay from nucleic acid isolation to sequencing, 10 µl of diluted viral culture supernatants of a DNA virus, Phocid Herpes Virus (PhHV) or an RNA virus, Equine Arteritis Virus (EAV) were added to each CSF aliquot of the DNA- and RNA- pathogen workflows, respectively. The concentrations of the internal controls were selected based on their titers used for the PCR assays implemented at the laboratory of OUCRU (7, 8). A non-template control was also included in each run.

Sample pretreatment and nucleic acid extraction

For the RNA pathogen workflow, the EAV-spiked CSF aliquot was first pretreated with DNase and RNase using 2 U/µL of turbo DNase (Ambion, Life Technology, Carlsbad, CA, USA) and 0.4 U/µL RNase 1 (Ambion) at 37°C for 30 minutes. Viral RNA was then isolated from the treated materials using the QIAamp viral RNA kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's instructions. The extracted RNA was finally recovered in 50ul of elution buffer provided with the extraction kit.

For the DNA pathogen workflow, the PhHV-spiked CSF aliquot was subject to the DNA extraction step without the nuclease pre-treatment step, using the

DNeasy blood and tissue kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was then recovered in 50 µL of elution buffer.

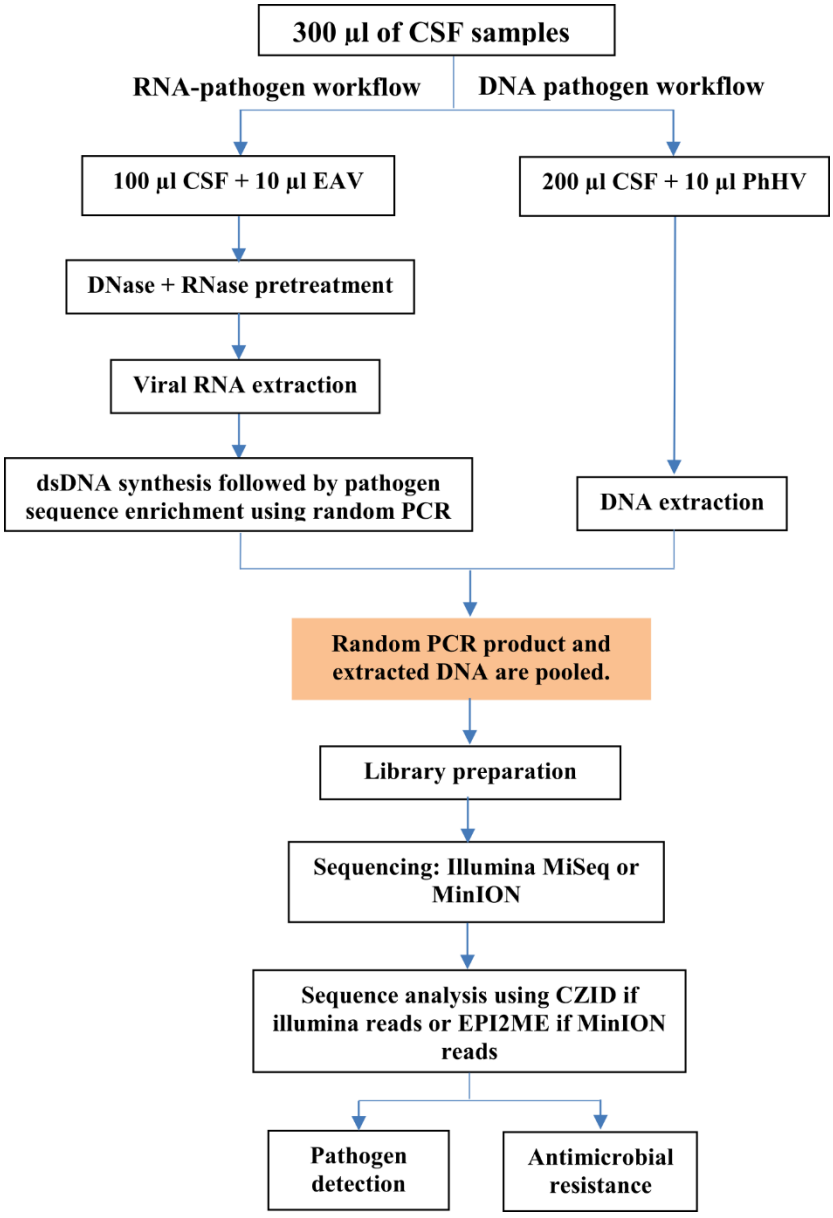


Figure 4.1: Flowchart showing an overview of the metagenomic workflows applied in the present study.

Double-stranded DNA synthesis and random amplification

Double-stranded DNA (dsDNA) synthesis was performed on the isolated RNA of the RNA pathogen workflow, using a set of 96 non-ribosomal random hexanucleotides which are specific for viral sequences, followed by a random amplification step to enrich for viral RNA prior to sequencing as previously described (295–298). Briefly, 10µl of extracted RNA was firstly mixed with 2µl of non-ribosomal random primer mixture and 1µl of dNTPs (10mM each) (Roche Diagnostics GmbH, Mannheim, Germany). The mixture was incubated at 65^{o}C for 5 min and was then immediately chilled on ice for 1 min. Secondly, 7µl of a reaction mix containing 200U of Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA, US), 40U of RNase OUT (Invitrogen), 0.1M DTT (Invitrogen) and 5X first strand buffer (Invitrogen) was added into the first reaction mixture. The reaction was then continued at 25^{o}C for 10 min, 37^{o}C for 1 min and 94^{o}C for 2 min, and immediately chilled on ice for 2 min. Next, 5U of exo-Klenow fragment (Ambion) and 10U of Ribonuclease H (Ambion) were then added into the reaction mixture. The mixture was subjected to a thermal condition consisting of 25^{o}C for 5 min, 37^{o}C for 1h and 94^{o}C for 2 min. For random PCR (rPCR) step, 5µl of the resulting dsDNA was pre-amplified using FR20RV primer (5'-GCCGGAGCTCTGCAGATATC-3'). The reaction was carried out in a total volume of 50µl consisting of 3µl of dsDNA, 2µl of primer FR20RV at a final concentration of 40nM and 45µl of Platinum PCR Supermix (Invitrogen). The thermal cycling condition consisted of 94^{o}C for 2 min and followed by 40 cycles of 94^{o}C for 30s, 55^{o}C for 30s and 72^{o}C for 3min and 1 cycle of 72^{o}C for 2min. Finally, the obtained random PCR product was purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified using the Qubit dsDNA HS kit (Invitrogen), following manufacturer's instruction.

Library pooling, and sequencing on an Illumina MiSeq platform

The extracted DNA of the DNA-pathogen workflow and purified random PCR product of the RNA-pathogen workflow from the same sample were pooled with the same ratio (Figure 4.1). The pooled materials were then subjected to the library preparation step using the Nextera XT sample preparation kit

(Illumina, San Diego, CA, USA), following the manufacturer's instructions. Prior to sequencing, the quality and quantity of prepared library was assessed using TapeStation 4150 system (D1000 SreenTap, Agilent) and Kappa qPCR (KAPA Library Quantification Kit, Roche). The resulting libraries were sequenced on a MiSeq platform available at the laboratory of OUCRU, using MiSeq Reagent Kits v2 (300 cycles; Illumina) for libraries containing 30 CSF samples, or MiSeq Reagent Nano Kit v 2 (300 cycles) for libraries containing ≤7 CSF samples.

Complementary analysis using Oxford Nanopore MinION flow cells

A subset of 18 consecutive CSF samples of phase 1 was also sequenced using the Oxford Nanopore technologies (OTN) MinION flow cells. The sample pretreatment steps (including internal control spiking, and nuclease digestion and random PCR) were carried out as described above. The pooled DNA products were then subjected to the library preparation using the 1D Native Barcoding Genomic DNA kit (ONT, Oxford, UK) with the use of unique barcoding systems, following the manufacturer's protocol. The prepared libraries were sequenced on R9.4 flow cells (ONT). Details are presented in Appendix C (Table S4.3)

Analysis of the obtained sequences

For sequences generated by the Illumina MiSeq platform, I applied a cloud-based metagenomics platform, namely Chan Zuckerberg ID (CZID), publically available at czid.org. Sequence analysis primarily aimed to identify sequences related to viral and/or bacterial pathogens in the tested samples. Additionally, in the case of bacterial pathogens, detection of antimicrobial-resistance conferring mutations in the obtained dataset was carried out using the CZID platform (Figure 4.1) For complementary analysis using MinION flow cells, I first conducted base-calling using MinKNOW (OTN), and then demultiplexing of the collected reads using Porechop (https://github.com/rrwick/Porechop). The obtained reads were analysed using EPI2ME (ONT).

For phase 1, the results were independently assessed by two individuals. When there was a discrepancy, the two assessors discussed the readout to resolve the discrepancy. For phase 2, the results were also discussed with the treating physicians to assess the likelihood whether the detected pathogen in the CSF samples was indeed responsible for the ongoing infection.

PCR confirmatory testing

For pathogens detected by metagenomic in CSF samples of group 3, specific PCRs were performed to verify the obtained results. The tested samples were considered to be positive if confirmed by specific PCR. When PCR assay was not available for confirmatory testing, mNGS was considered as positive, if 1) the likelihood of contamination was considered as unlikely; i.e. there was no ongoing culture or molecular research activitities conducted in the associated laborataries during the same period, and 2) metagenomics generated reads mapped to three or more genomic regions of the pathogen genome, pragmatically chosen based on previous studies (187,295).

Result interpretation

Specimens producing target signals (in this case pathogens causing CNS infections) regardless of the internal control signals, which could be atributed to the competition between the DNA targets and internal control DNA as experienced with PCR, were interpreted as positive. Specimens yielding no target signals but signals of at least one internal control (EAV or PhHV) were considered as negative. Specimens yielding no target signals and no internal control signals were considered as unsuccessfully sequenced.

Phylogenetic analysis

Sequence alignment and phylogenetic tree reconstructions of the obtained sequences were carried out using ClustalW alignment and maximum likelihood methods available within Geneious 8.1.5 (Biomatters) and IQ-TREE (299), respectively.

GenBank accession numbers

Metagenomics data were deposited at NCBI (GenBank) under SRA accession number PRJNA971352.
(https://www.ncbi.nlm.nih.gov/sra/PRJNA971352).

4.2.4 Statistical analysis

Categorical variables were presented as numbers and percentages (%). Continuous variables were reported as median and range. The sensitivity and specificity were calculated to assess the performance of the metagenomics workflow.

4.3 Results

4.3.1 Study phase 1 – retrospective assessment of metagenomics workflow

Patients and CSF samples

Between September 2017 and June 2018, a total of 207 patients were enrolled in a prospective observational study. Of these, 93 patients fulfilled the selection criteria of one of the three groups of the metagenomic study, group 1; n=30, group 2; n=25 and group 3; n= 38 (Figure 4.2 and Appendix C (Table S4.2)).

The baseline characteristics, clinical manifestations, laboratory findings and outcome of the study patients are described in Table 4.1. Males were predominant (65%). Most patients were presented with fever (90.3%), headache (67.7%) and altered consciousness (89.2%). The study patients had a median length of hospital stay of 12 days. Most of the patients suffered from moderate to moderately severe disability at discharge (61.3%) with modified Rankin scale (mRS) of 3-4. A proportion of 29.0% had no significant or slight disability (mRS of 1-2), 7.5% with severe disability (mRS of 5) and 2.2% died during hospitalisation. Of the 30 patients of group 1, bacterial pathogens accounted for 21/30 (70%) with the predominance of *Streptococcus suis*, followed by Herpes simplex virus (HSV) (Figure 4.5).

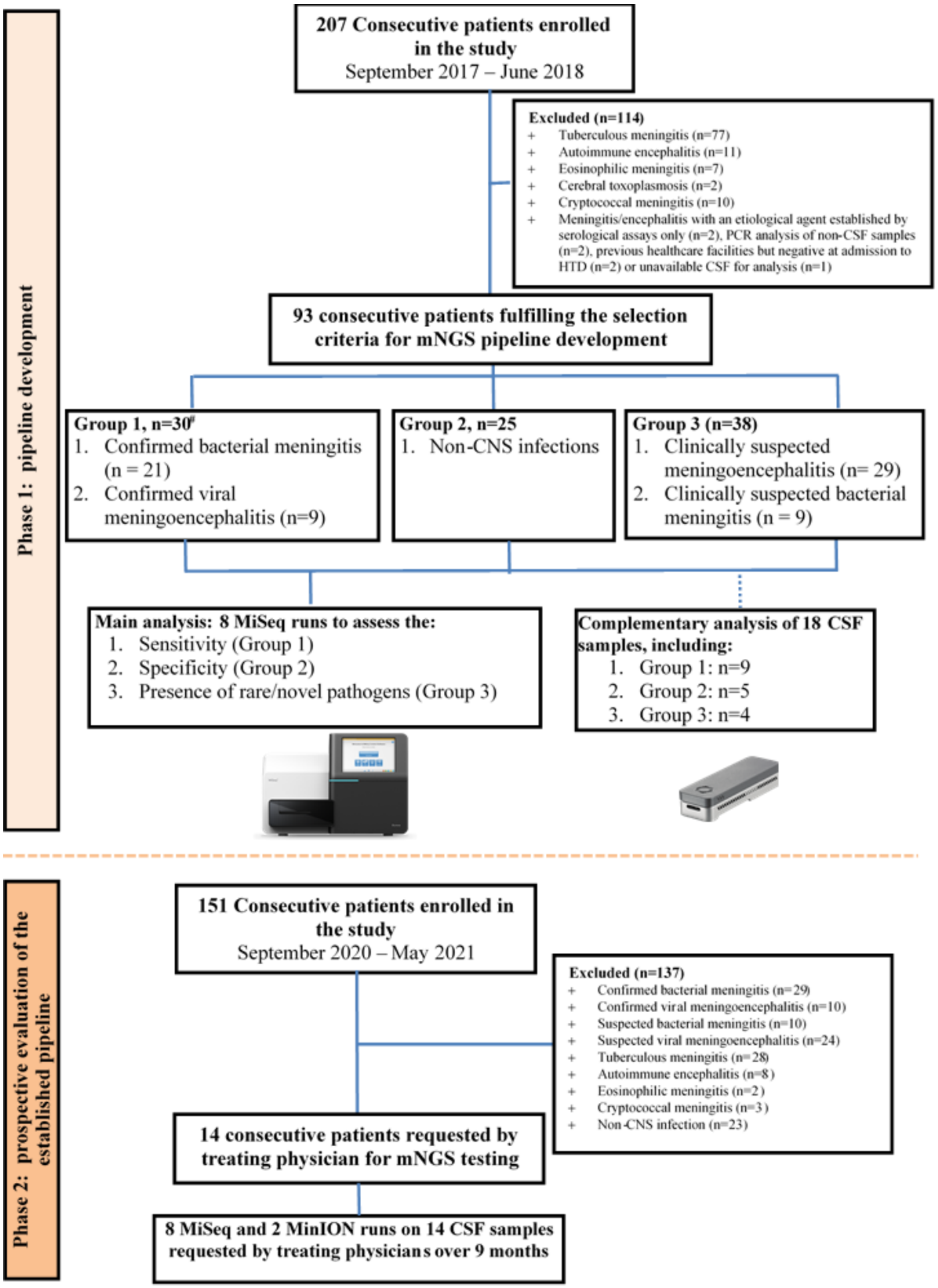


Figure 4.2: Flowchart illustrating the selection of CSF samples for phase 1 and 2 of the metagenomic study.

Table 4.1: Baseline characteristics and outcome of 93 patients included for phase 1 of the study

Characteristics	All (n=93)	Group 1		Group 2	Group 3	
		Confirmed BM (n = 21)	Confirmed VM (n = 9)	Non-CNS infection (n = 25)	Suspected BM (n = 9)	Suspected VM (n = 29)
Demographics						
Median age, years (Range)	46 (16-92)	59 (21-87)	44 (17-78)	46 (20-92)	68 (37-77)	26 (16-63)
Male, n (%)	61 (65.6)	16 (76.2)	6 (66.7)	16 (64.0)	7 (77.8)	16 (55.2)
Clinical symptoms before admission						
Day of illness (Range)	5 (1-90)	3 (1-8)	6 (4-10)	5 (1-60)	4 (1-20)	7 (1-90)
Fever, n (%)	84 (90.3)	21 (100.0)	8 (88.9)	22 (88.0)	8 (88.9)	25 (86.2)
Headache, n (%)	63 (67.7)	19 (90.5)	7 (77.8)	8 (32.0)	8 (88.9)	21 (72.4)
Altered consciousness, n (%)	83 (89.2)	20 (95.2)	9 (100.0)	20 (80.0)	9 (100.0)	25 (86.2)
Seizure, n (%)	29 (31.5)	2 (9.5)	4 (44.4)	9 (37.5)	1 (11.1)	13 (44.8)
Psychosis, n (%)	12 (12.9)	0 (0.0)	1 (11.1)	4 (16.0)	0 (0.0)	7 (24.1)
Language dysfunction, n (%)	8 (9.0)	1 (5.0)	0 (0.0)	3 (12.0)	0 (0.0)	4 (14.8)
Movement disorder, n (%)	7 (7.8)	0 (0.0)	0 (0.0)	1 (4.0)	0 (0.0)	6 (21.4)
Baseline neurological Symptoms						
Hemiplegia, n (%)	7 (7.6)	2 (9.5)	0 (0.0)	2 (8.3)	1 (11.1)	2 (6.9)
Limb weakness (Paraplegia/ Tetraplegia), n (%)	9 (9.7)	1 (4.8)	0 (0.0)	5 (20.0)	0 (0.0)	3 (10.3)
Convulsion, n (%)	9 (9.8)	0 (0.0)	2 (22.2)	2 (8.3)	0 (0.0)	5 (17.2)
Abnormal movement, n (%)	2 (2.2)	0 (0.0)	0 (0.0)	1 (4.0)	0 (0.0)	1 (3.6)
Stiff neck n (%)	29 (31.2)	13 (61.9)	2 (22.2)	3 (12.0)	2 (22.2)	9 (31.0)
Glasgow coma score ≤ 9, n (%)	15 (16.1)	7 (33.3)	1 (11.1)	1 (4.0)	0 (0.0)	6 (20.7)
CSF investigations						
WCC/mm^3 (Range)	54 (1-51810)	2958 (50-51810)	140 (27-752)	2 (1-611)	818 (24-30843)	46 (1-909)
Neutrophil % (Range)	35 (0-97)	83 (18-97)	11 (11-18)	25 (0-93)	76 (12-93)	17 (0-88)
Lymphocyte % (Range)	35 (0-99)	17 (3-82)	89 (82-89)	50 (0-99)	24 (7-88)	78 (0-90)
Protein g/L (Range)	0.7 (0.1-7.1)	3.2 (0.6-7.1)	0.8 (0.3-1.7)	0.4 (0.1-3.1)	1.3 (0.5-3.7)	0.5 (0.1-1.2)
Lactate nmol/L (Range)	2.7 (1.4-20.5)	12.4 (4.6-20.5)	2.3 (1.7-3.2)	2.3 (1.4-6.4)	4.7 (2.3-14.6)	2.3 (1.6-4.4)
CSF: blood glucose ratio (Range)	0.6 (0.0-1.2)	0.2 (0.0-1.0)	0.6 (0.3-1.1)	0.7 (0.5-1.2)	0.5 (0.3-0.6)	0.7 (0.4-1.0)
Management						
Ventilation, n (%)	9 (9.7)	5 (23.8)	1 (11.1)	1 (4.0)	0 (0.0)	2 (6.9)
Sedative drug administration, n (%)	13 (14.0)	4 (19.0)	1 (11.1)	3 (12.0)	1 (11.1)	4 (13.8)
Acyclovir, n (%)	25 (27.5)	0 (0.0)	8 (88.9)	1 (4.3)	0 (0.0)	16 (55.2)
Antibiotics, n (%)	42 (45.2)	21 (100.0)	1 (11.1)	9 (36.0)	8 (88.9)	3 (10.3)
Outcome						
Day of hospitalisation (Range)	12 (1-62)	14 (1-62)	25 (5-52)	7 (0-30)	12 (5-27)	8 (1-45)
Rankin scale, n (%)						
1-2	27 (29.0)	5 (23.8)	1 (11.1)	7 (28.0)	2 (22.2)	12 (41.4)
3-4	57 (61.3)	13 (61.9)	6 (66.7)	16 (64.0)	6 (66.7)	16 (55.2)
5	7 (7.5)	2 (9.5)	2 (22.2)	2 (8.0)	0 (0.0)	1 (3.4)
6	2 (2.2)	1 (4.8)	0 (0.0)	0 (0.0)	1 (11.1)	0 (0.0)

Metagenomic results: general description

The selected CSF samples (n=93) were sequenced in 8 MiSeq runs, generating 41,898 - 2,736,388 reads per sample (median: 857,286 reads), with a comparable number of reads between CSF samples with and without a pathogen causing CNS infections detected by metagenomics (Figure 4.3). Seven common causes of CNS infections were detected in the tested CSF samples, with none found in the 25 CSF samples of group 2. The detected pathogens consisted of 4 bacteria (*S. pneumoniae*, *S. suis*, *S. agalactiae* and *N. meningitidis*) and 3 viruses (HSV, DENV, mumps virus). Sequences related to HIV and HBV genomes were also detected in 3 CSF samples of group 2. Additionally, sequences related to commensal viruses (including Torque teno virus and Torque teno mini virus), or contaminants commonly found in our metagenomic datasets (*E. coli*, feline leukemia virus, Bluetongue virus), likely derived from laboratory reagents (295,298) were also detected (data not shown). SARS-CoV-2 sequences were detected in one CSF sample. This case was a result of carry-over contamination from ongoing SARS-CoV-2 genomic surveillance at the time because subsequent SARS-CoV-2 testing of the original CSF was negative.

Sequences related to internal controls (either PhHV or EAV) were detected in 93/93 (100%) of the samples included for analysis (Figure 4.4). Thus according to my predefined criteria based on internal control signals, all the 93 CSF samples were successfully sequenced.

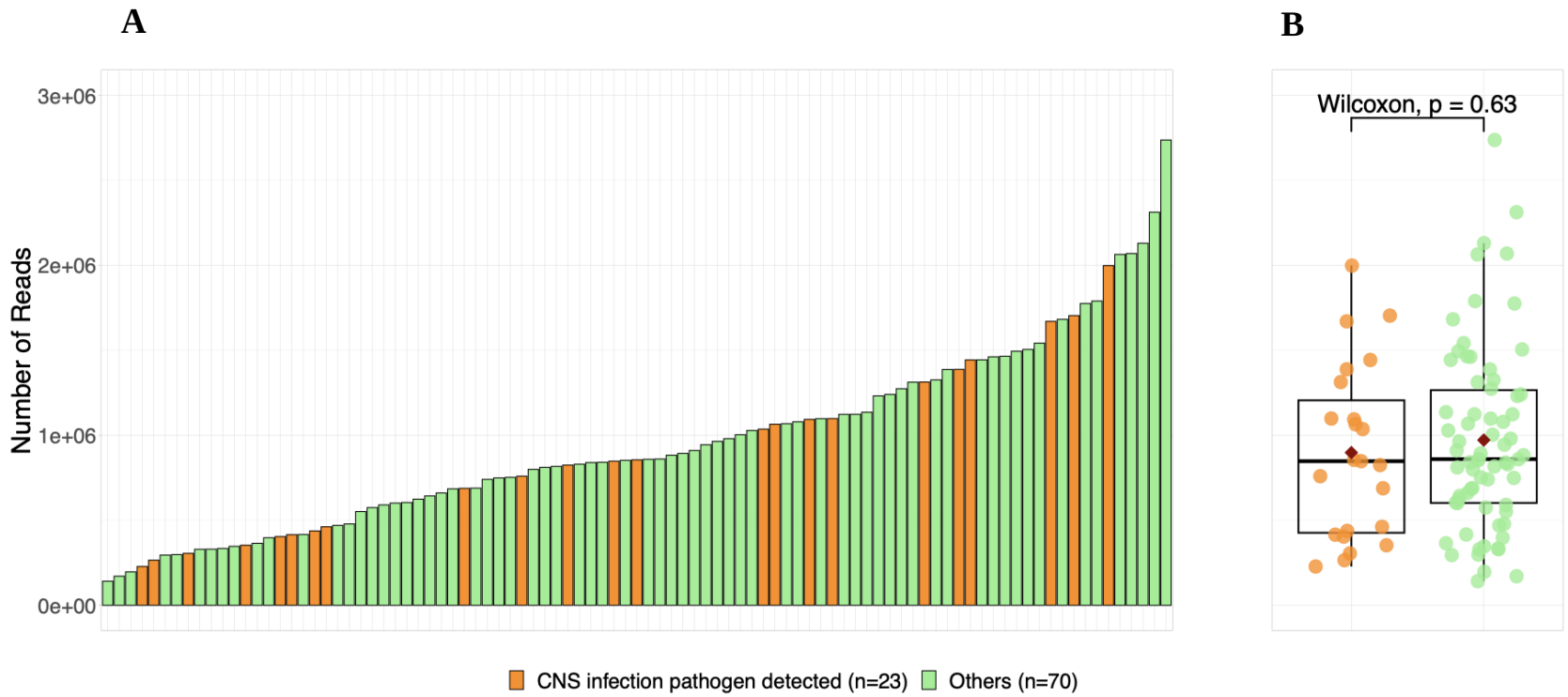


Figure 4.3: Results of MiSeq runs of development phase.

A) Bar chart showing the distribution of the number of reads per sample of phase 1

B) Box-plot comparing the number of reads between samples with and without a CNS infection pathogen detected by MiSeq-based metagenomic workflow.

Note to Figure 4.3A: Each bar represents one sample. Others: samples in which a pathogen causing CNS infections were not detected by metagenomics.

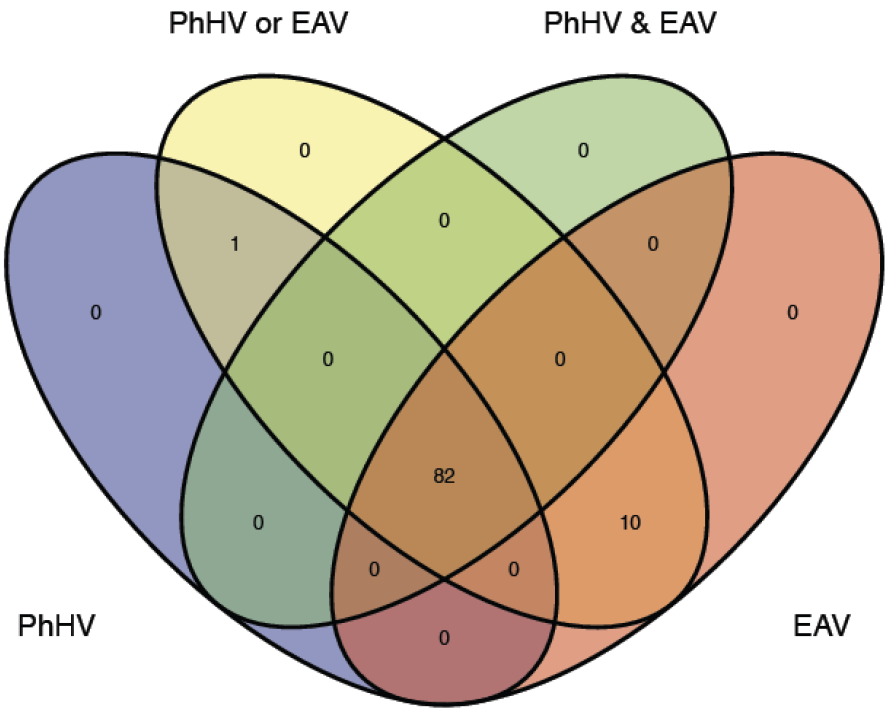


Figure 4.4: Venn diagram showing the frequency of internal controls (PhHV and EAV) detected in the 93 CSF samples included in phase 1 of the study.

Detection of pathogens in CSF samples positive by conventional assays

Of the 21 CSF samples positive for a bacterial pathogen by conventional diagnostic assays, metagenomics successfully detected a bacterial pathogen in 17 (81%), including S. *suis* (n=11), *S. pneumoniae* (n=3) and *N. meningitidis* (n=2) and *S. agalactiae* (n=1). Of these, in one case, metagenomics returned *S. pneumoniae*, while routine diagnostics only yielded evidence of Gram-positive bacteria (Figure 4.5). Of the 9 CSF samples in which a viral pathogen was detected by PCR, metagenomics successfully detected the corresponding viruses in 4 patients (44%); all were HSV (Figure 4.5). Of the 9 CSF samples that were positive by conventional diagnostic tests but negative by metagenomics, 4 were positive for uncommon causes of meningitis in Vietnam and 5 had low viral loads (Table 4.2).

Complementary analysis of 18 CSF samples using MinION flow cells also replicated the findings of the MiSeq-based metagenomic protocol. Details are presented in Figure 4.6 and Appendix C (Table S4.3). Thus, my established workflow can be carried out on both Illumina MiSeq and MinION platforms, producing comparable results in terms of pathogen detection.

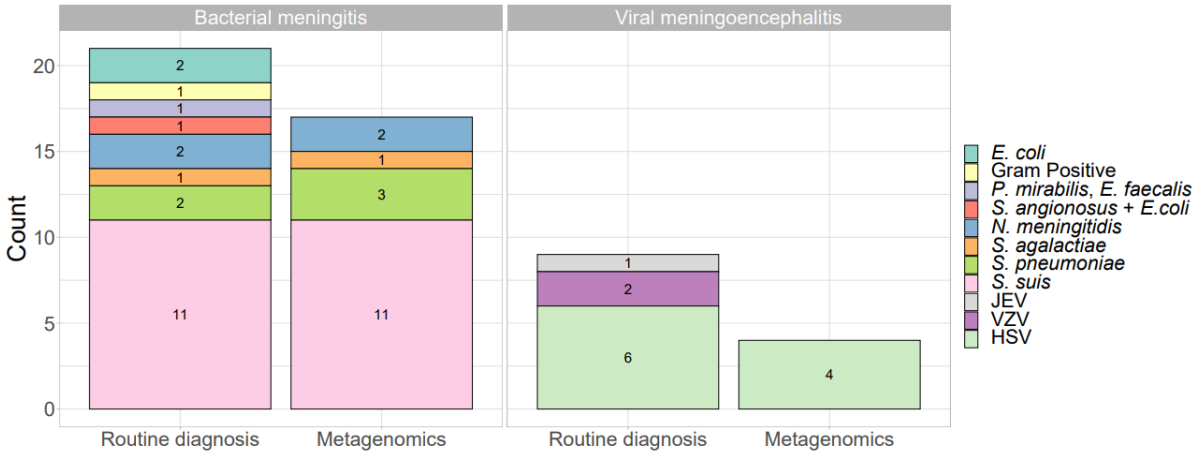


Figure 4.5: Detection of CNS infection pathogens by metagenomics in CSF samples of the reference standard established by conventional diagnostic assays (Gram stain, PCR and/or culture).

Table 4.2: Bacterial and viral pathogens detected by conventional assays but negative by mNGS

Sample	Clinical presentation	Aetiology	Routine diagnosis	Ct value
1	Meningitis	*Streptococcus angionosus* and *Escherichia coli*	Gram stain and Culture	NA
2	Meningitis	*Escherichia coli*	Culture	NA
3	Meningitis	*Proteus mirabilis* and *Enterococus faecalis*	Culture	NA
4	Meningitis	*Escherichia coli*	Culture	NA
5	Encephalitis	Herpes simplex virus	PCR	34
6	Encephalitis	Herpes simplex virus	PCR	29
7	Encephalitis	Varicella zoster virus	PCR	34
8	Encephalitis	Varicella zoster virus	PCR	35
9	Encephalitis	Japanese encephalitis virus	PCR and serology	40

Note to Table 4.2: NA: not applicable.

PCR methods can be found in Appendix C (Table S4.1)

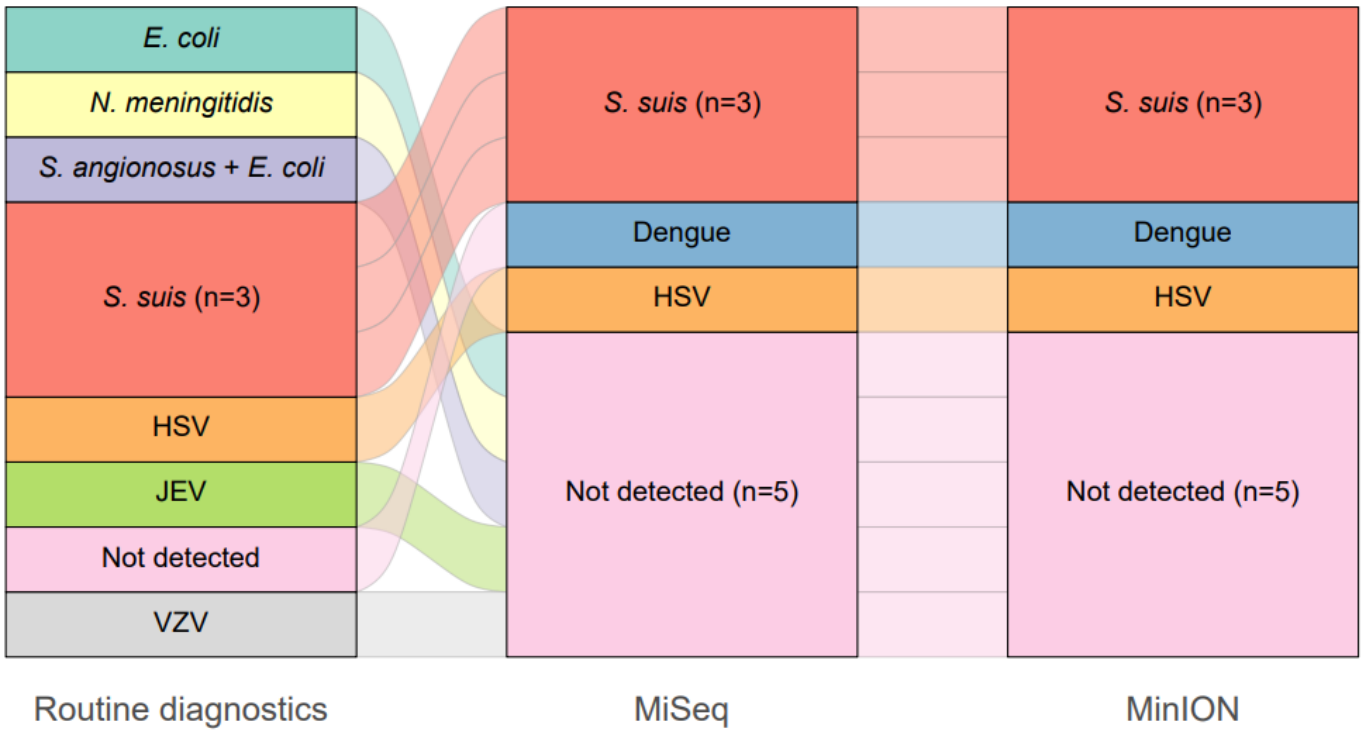
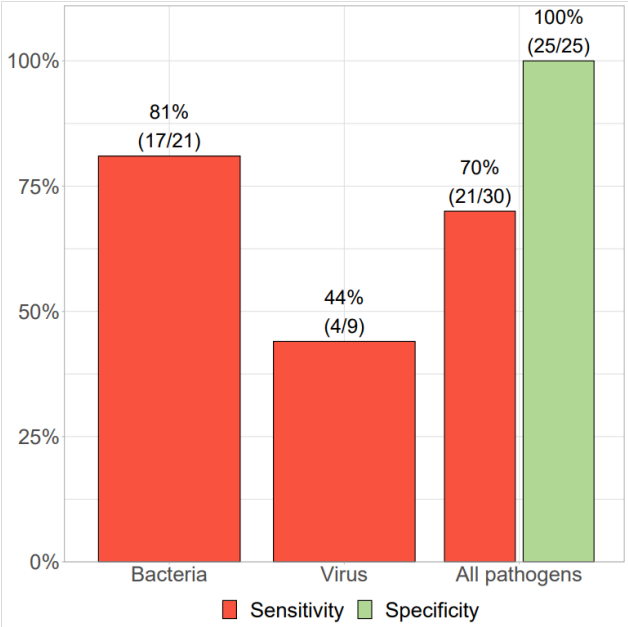


Figure 4.6: Diagram showing the agreement between Illumina-MiSeq and MinION based metagenomic workflows in a subset of patients analysed by both methods.

Note to Figure 4.6: Each box represents one CSF sample, unless otherwise specified.

Diagnostic performance of metagenomics

The diagnostic performance of metagenomics in relative to conventional testing is presented in Figure 4.7. Because metagenomics did not yield any evidence of an infectious cause of CNS infections in the 25 CSF samples of group 2, the specificity of mNGS assay was thus 100%. Using CSF samples of group 1 as a reference standard, the sensitivity of the mNGS pipeline in detecting bacterial and viral pathogens in CSF samples was accordingly 21/30 (70%), with a better assay performance for bacterial pathogens [sensitivity: 17/21 (81%)] than viral pathogens [sensitivity, 4/9 (44%)].



	Conventional Testing (+)		Conventional Testing (-)	Total
	Bacteria	**Virus**	**All**	
mNGS (+)	17	4	0	21
mNGS (-)	4	5	25	34
Total	21	9	25	55

Figure 4.7: Sensitivity and specificity of mNGS assay in relative to conventional testing.

Antimicrobial resistance conferring mutations detection

Of the 17 CSF samples from bacterial meningitis patients that were successfully sequenced by metagenomics, antimicrobial resistance conferring mutation detection was successfully established in 12 (71%) (Table 4.3). Of these, two (one *S. pneumoniae* and one *S. suis*) had no resistant profiles identified by routine diagnosis because bacterial culture was unsuccessful. In the remaining 10 cases, evidence of resistance conferring mutations were in line with the results generated by culture-based phenotyping approach. Additionally, on several occasions, resistance-conferring mutations for antimicrobials that were not covered by routine diagnosis were also detected, e.g., Ant6-Ia mutation associated aminoglycosides resistance in case of *S. suis*.

Table 4.3: Antimicrobial resistance conferring mutations detected by sequences generated by metagenomics in comparison with routine culture-based method.

ID	Aetiology	Routine diagnostics			IDSeq
		S	I	R	Antibiotic class (gene)
003-018	*S. pneumoniae*	Ceftriaxone Levofloxacin Rifampin Trimethoprim-sulfa methoxazole Vancomycin		Penicillin Erythromycin Oxacillin Clindamycin	Beta-lactam (PBP1b) Tetracycline (tetM, tetS)
003-024	*S. pneumoniae*	NA	NA	NA	Tetracycline (tetM, tetS) Beta-lactam (PBP1b) Macrolides (MsrD, MrfA)
003-045	*S. pneumoniae*	Ceftriaxone Levofloxacin Rifampin Trimethoprim-sulfa methoxazole Vancomycin		Clindamycin Erythromycin Penicillin Oxacillin	Tetracycline (TetS, TetO, TetM) Macrolides (ErmB) Beta-lactam (PBP1b, PBP1a)
003-052	*S. suis*	NA	NA	NA	Tetracycline (TetM, TetS)
003-070	*S. suis*	Ampicillin Clindamycin Ceftriaxone Erythromycin Levofloxacin Meropenem Penicillin Vancomycin		Tetracycline	Tetracycline (TetM, TetS) Aminoglycosides (Ant6-Ia)
003-080	*S. suis*	Ampicillin Clindamycin Ceftriaxone Erythromycin Levofloxacin Penicillin Vancomycin		Tetracycline	Tetracycline (TetM)
003-084	*S. suis*	Ampicillin Clindamycin Ceftriaxone Erythromycin Levofloxacin Penicillin Vancomycin Meropenem		Tetracycline	Tetracycline (TetM)
003-085	*S. suis*	Ampicillin Ceftriaxone Levofloxacin Penicillin Vancomycin Meropenem	Penicillin	Tetracycline Erythromycin Clindamycin	Tetracycline (TetM, TetS) Macrolides (ErmB) Aminoglycosides (Aph3-III, Sat4A, Ant6-Ia) Oxazolidinones (OptrA)
003-125	*S. suis*	Ampicillin Clindamycin Ceftriaxone Erythromycin Levofloxacin Meropenem Penicillin Vancomycin		Tetracycline	Tetracycline (TetS, TetO, TetM) Aminoglycosides (Ant6-Ia)
003-197	*S. suis*	Ampicillin Clindamycin Ceftriaxone Erythromycin Levofloxacin Meropenem Penicillin Vancomycin		Tetracycline	Tetracycline (TetM)
003-198	*S. suis*	Ampicillin Ceftriaxone Levofloxacin Penicillin Vancomycin		Tetracycline Erythromycin Clindamycin	Tetracycline (TetM, TetS) Macrolides (ErmB) Aminoglycosides (Aph3-III, Sat4A, Ant6-Ia, Aac6-Aph2)
003-200	*S. agalactiae*	Ampicillin Clindamycin Ceftriaxone Erythromycin Penicillin Vancomycin		Tetracycline Levofloxacin	Tetracycline (TetM)

Metagenomic detection of pathogens in CSF samples negative by routine diagnostic assays

Of the 38 CSF of group 3, metagenomics could detect a pathogen in 3 (5%), including two DENV and one mumps virus. Of the two DENV cases, PCR confirmatory testing using a previously published assay was successful in one (Ct value: 40) (300). This sample had reads mapped to various regions of DENV genomes (Figure 4.7A). Phylogenetic analysis suggested that the DENV belonged to serotype 4 (Figure 4.8). In the remaining case, metagenomics reads were mapped to only one region of the DENV genome (Figure 4.7B), and subsequent confirmatory DENV PCR testing was negative.

Because mumps virus PCR was not available, we were not able to verify the mNGS result. However, this case was considered positive for mumps virus because metagenomic reads were mapped to several regions of the genome (Figure 4.7C) and contamination was unlikely since there has been no ongoing mumps virus related work in our laboratory.

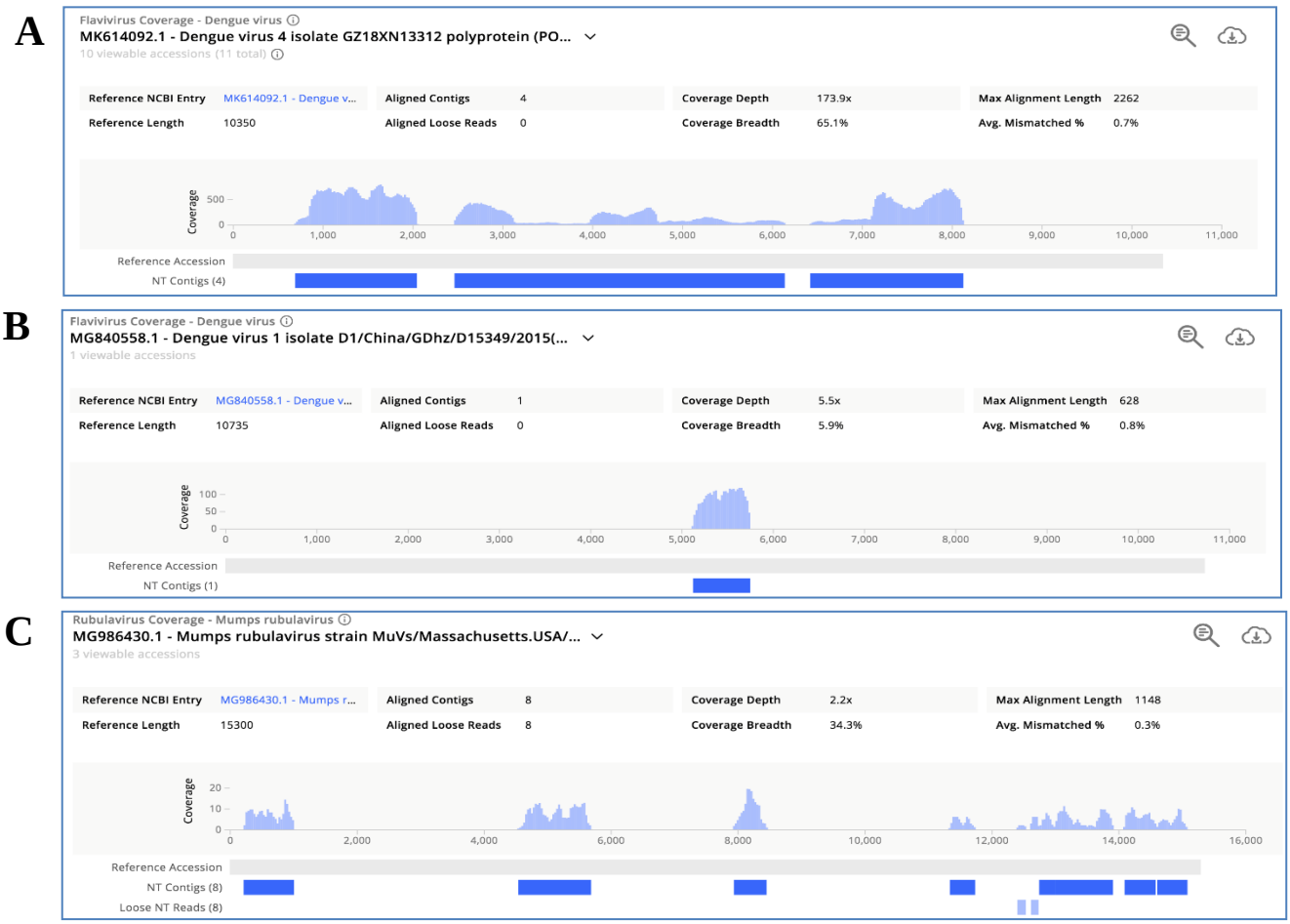


Figure 4.8: Results of reference-based mapping approach implemented in CZID platform.

(A&B) Metagenomics detection of DENV in two CSF samples from patients with clinically suspected meningoencephalitis. The first case (A) was then confirmed by DENV PCR (Ct=40) while the second one (B) was negative by subsequent PCR testing

(C) Metagenomic detection of mumps virus in CSF a patient with clinically suspected meningoencephalitis. PCR was not available for confirmatory testing.

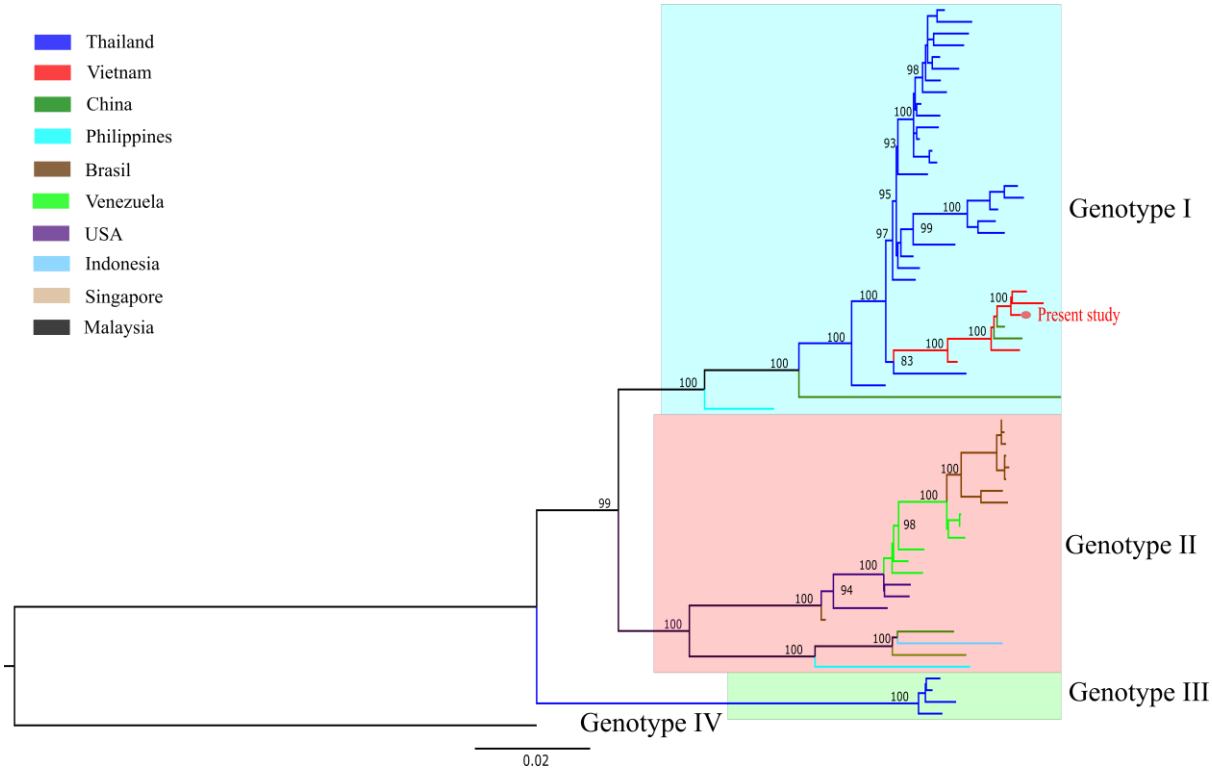


Figure 4.9: Phylogenetic tree illustrating the position of the DENV sequences obtained from the present study.

The consensus including partial NS1, NS2A, NS2B and NS3 was used to construct the phylogenetic tree. The DENV sequences were belonged to DENV genotype I in the context of the four genotypes (I-IV) of DENV serotype 4. Numbers represent bootstrap values.

4.3.2 Study Phase 2 – prospective evaluation of the established pipeline

Cohort characteristics

Phase 2 was prospectively conducted between September 2020 and May 2021. During this period, 151 patients were enrolled in the clinical study. Of these, 14 fulfilled the selection criteria and were subject to real-time metagenomic analysis. Details concerning their demographics, clinical presentation, CSF findings, management, and outcome are shown in Table 4.4.

Results of metagenomic analysis

The 14 CSF samples included were sequenced in 6 MiSeq and 2 MinION runs. The window time from hospital admission to real time metagenomic analysis was 2 – 6 days (median: 3 days), while the mNGS assay turnaround time was from 3 – 12 days (median: 5 days) (Figure 4.9). Results of sequence analysis showed that of the 14 included CSFs, 13 had no evidence of a CNS infection pathogen detected. In the remaining case (Patient P8), a total of 1708 *Klebsiella pneumoniae* sequences accounting for 17% of the 29,869 reads was obtained from a MinION run (Figure 4.10). Otherwise, there was no other pathogen detected in this CSF sample. The turnaround time was 12 days, corresponding to day 15 of hospitalisation (Figure 4.10), attributed to the failure of the first sequencing experiment.

The patient infected with *K. pneumoniae* was a 36-year-old female with diabetes, presenting with clinically suspected bacterial meningitis (Table 4.4). She had a history of illness of one day, and presented with fever, headache, altered consciousness, language dysfunction and neck stiffness on admission, with a GCS of 13. CSF investigations showed an elevated white cell count number (51,728 cells/mm^3) with a predominance of neutrophil (88%), high lactate level (24.3 nmol/l) and a CSF/blood glucose ratio of 0.03. Routine diagnostic tests including India ink, Gram stain, bacterial culture and *S. pneumoniae* PCR were all negative. She received empiric Ceftriaxone prescription for 14 days and was discharged with full recovery after 15 days of hospitalisation.

Table 4.4: Demographics, clinical symptoms, CSF laboratory findings, management, and outcome of 14 patients in prospective mNGS sequencing.

Patient No	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14
Demographics														
Age in years	37	25	29	39	68	19	60	36	55	52	18	73	26	72
Gender	M	M	F	M	M	M	M	F	M	M	M	M	M	M
Clinical symptoms														
Illness day	1	5	10	2	22	5	21	1	2	12	4	20	4	10
Fever	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	U	Y	Y	Y
Headache	Y	Y	U	Y	Y	U	Y	Y	N	Y	N	Y	U	Y
Seizure	N	Y	Y	Y	N	N	N	N	N	N	N	N	Y	N
Psychosis	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
Language dysfunction	N	Y	N	N	N	Y	Y	Y	Y	Y	N	N	N	N
Movement disorder	N	N	Y	Y	N	N	N	N	U	N	N	N	N	N
Convulsion	N	Y	N	N	N	Y	N	N	N	N	Y	N	Y	N
Stiff neck	N	N	N	Y	Y	Y	U	Y	N	Y	N	N	Y	Y
Glasgow coma score	13	9	10	14	12	10	14	13	U	14	7	12	6	8
CSF laboratory results														
WCC/mm^3	3,731	54	2	109	1,693	2,441	349	51,728	160	449	1	7,779	9,230	338
Neutrophil %	91	63	50	34	84	72	48	88	36	20	100	78	86	74
Lymphocyte %	9	37	50	6	16	28	52	12	64	80	U	22	14	25
Protein g/L	1.17	0.47	0.73	0.87	2.04	0.45	0.79	0.27	0.55	3.77	0.41	1.93	1.85	0.91
Lactate nmol/L	3.94	4.15	1.70	3.99	5.23	4.40	3.97	24.30	3.89	3.63	2.03	5.44	11.63	3.35
CSF: blood glucose ratio	0.63	0.81	0.77	0.75	0.57	0.53	0.50	0.03	0.74	0.52	0.59	0.49	0.29	0.45
Conventional diagnostics														
Zn stain	N	N	Y	Y	Y	Y	Y	N	N	Y	N	Y	N	Y
India ink stain	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y
Cryptococcal antigen	N	N	Y	Y	Y	Y	Y	N	N	Y	N	Y	N	Y
Gram stain	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Bacterial culture	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y

Patient No	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14
HSV PCR	N	Y	N	N	N	Y	Y	N	Y	Y	Y	N	N	N
JEV Serology	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
VZV PCR	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
S. pneumoniae PCR	N	N	N	N	N	N	N	Y	N	N	N	N	N	N
Xpert for *M. tuberculosis*	N	N	N	Y	N	N	N	N	N	N	N	N	N	Y
Management														
Ventilation	N	Y	N	N	N	Y	N	N	Y	Y	Y	N	Y	Y
Sedative drug administration	Y	Y	N	N	N	Y	N	N	Y	Y	Y	N	Y	Y
TB treatment	N	N	N	N	N	U	N	N	N	Y	N	N	N	N
Acyclovir	N	Y	N	N	N	U	Y	Y	Y	Y	Y	N	N	N
Antibiotics	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y
Outcome														
Duration of hospitalisation	17	61	57	12	17	37	18	15	7	8	3	19	45	23
Modified Rankin scale	1	5	1	0	0	0	1	0	5	3	6	1	3	3

Note to table 4.4: Y: Yes, N: No, U: Unknown, M: Male, F: Female

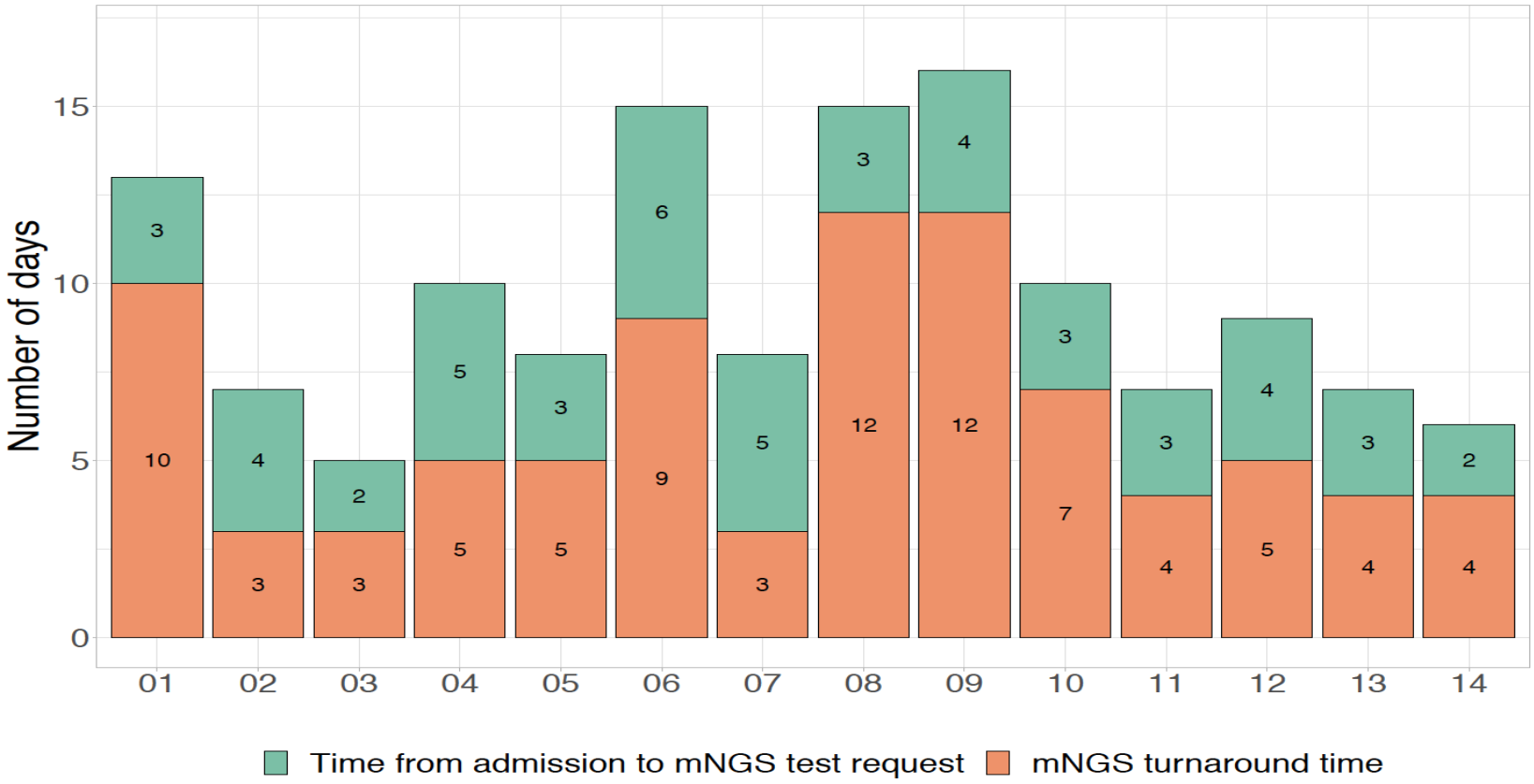


Figure 4.10: Bar chart illustrating the time in days from admission to real-time metagenomic analysis and the assay turnaround time in 14 patients included in phase 2 of the study.

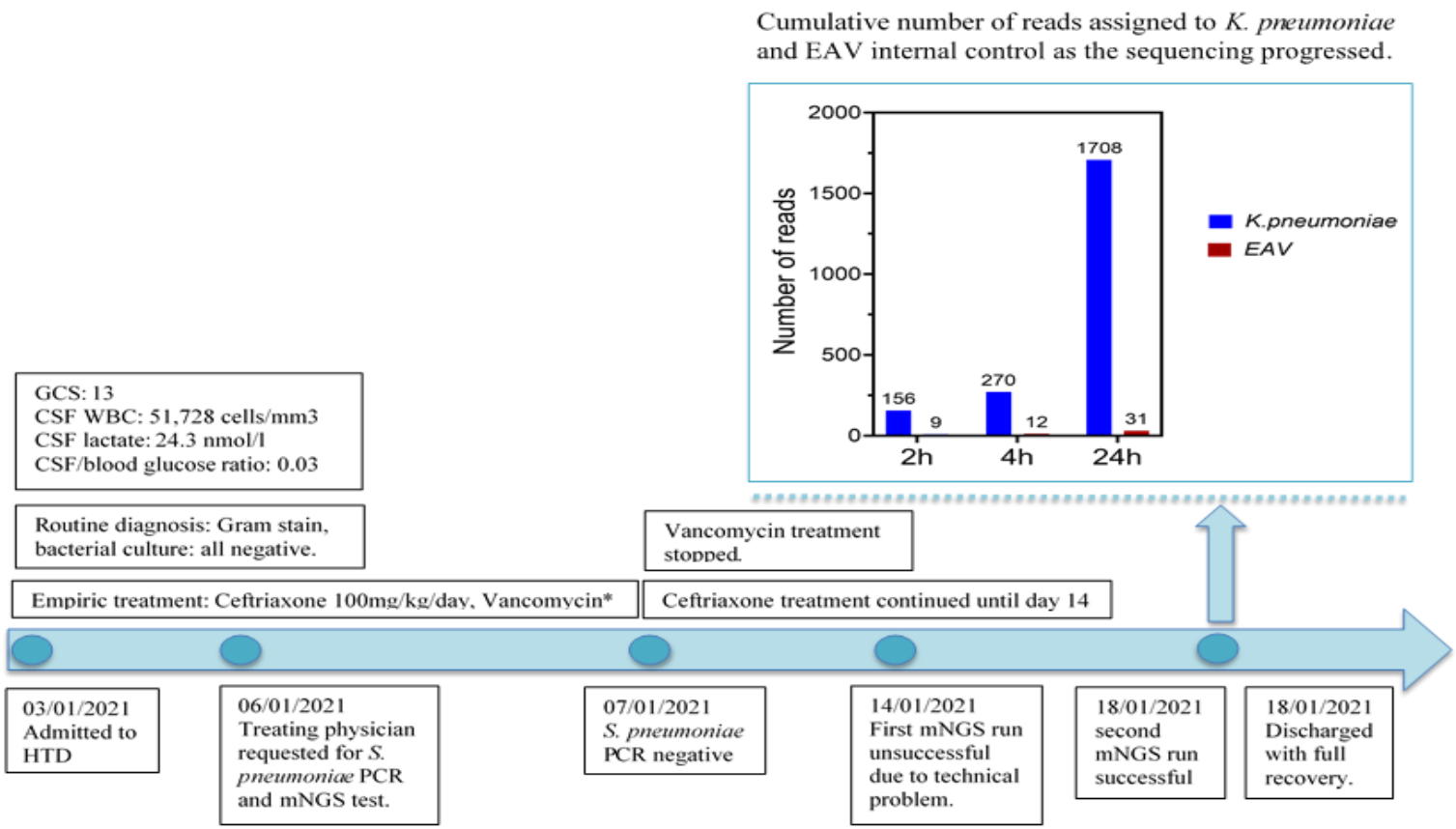


Figure 4.11: Diagram showing the timeline of laboratory investigations and clinical management of the bacterial meningitis patient infected with *K. pneumoniae* diagnosed by MinION based metagenomics.

Note to Figure 4.11: * Vancomycin dose is adjusted by patients' weight and renal function which was not collected as part of the clinical study.

4.4 Discussion and conclusion

In this chapter, I reported the results of the development and prospective evaluation of an internally controlled metagenomic workflow for CNS infections diagnosis in Vietnam. I demonstrated that my established workflow could simultaneously detect both bacteria and viruses in a single experiment, with comparable yields when operated on Illumina MiSeq and ONT MinION sequencers. While the primary focus of the pipeline was to identify the causative agents, the obtained sequences could also be used to comprehensively assess presence of antimicrobial resistance conferring mutations, and/or evolution of the pathogens, supporting previous reports (301,302). Additionally, the performance of the assay was internally monitored with the use of non-human viruses (PhHV and EAV), spiked with CSF samples prior to nucleic acid isolation. Collectively, my established assay offers a new approach to the diagnosis of such diseases that can be caused by diverse pathogens such as CNS infections, especially when routine diagnosis fails to identify a causative agent.

The overall sensitivity and specificity of our metagenomics workflow for pathogen identification relative to the conventional assays was 70% and 100%, respectively. These figures are within the range of previous reports (187,290,295,303). The failure of metagenomics to detect a bacterial/viral pathogen in 30% of the 30 CSF samples of group 1 (patients with an aetiology identified) was likely attributed to several factors (304), but especially pathogen loads. In the absence of quantitative PCR data, we were not able to informatively assess this. However, high Ct values (i.e., low viral loads) were documented in several PCR positive samples (VZV and JEV) that were negative by metagenomics. Additional factors might include the abundance of human host DNA that can be depleted using selective lysis and endonuclease digestion to increase the sensitivity of pathogen detection (305–307). This approach, however, was not applied as part of the present study. Also, the quality of the tested samples might be affected by the long-

term storage before analysis (phase 1), and the genome sizes of the pathogens (e.g., bacteria versus viruses).

In four occasions, metagenomics could detect a CNS infections pathogen, but the conventional diagnostic assays were either negative or failed to identify a specific aetiology. Firstly, metagenomics identified *S. pneumoniae* in CSF of a patient with bacterial meningitis and provided a comprehensive antimicrobial resistance conferring mutations profiles. For this patient, routine diagnostics could only demonstrate evidence of infection by Gram stain analysis. Antibiotic use prior to hospital admission and/or late hospital admission might play a role. Secondly, in two patients presenting with meningoencephalitis of group 3, metagenomics could detect DENV and mumps virus (one each) that were not requested for PCR testing as part of routine diagnosis. Finally, during the prospective evaluation phase, mNGS assay could detect *K. pneumoniae* in a patient with bacterial meningitis of unknown cause. Although except for HSV, antivirals are currently not available for the other CNS infections viruses, rapid and accurate patient diagnosis are important to guide patient management and to avoid unnecessary use of antibiotics (308). Additionally, better understanding of the spectrum of CNS infections pathogens would also help inform policy makers with resource allocations tailored for infections associated with specific local settings, e.g., vaccine preventable diseases (*S. pneumoniae* and mumps virus) (49,262) and vector-borne diseases (DENV) (309). These collective findings emphasise the importance of testing for a wide range of pathogens in patients presenting with CNS infections, in turn underscoring the utility of pan-pathogen assays such as metagenomics.

My study represents one of the first to conduct real-time metagenomic investigations in a clinical setting in LMICs. I have shown that metagenomics can detect a pathogen in patients that were left undiagnosed after routine diagnosis, expanding the spectrum of pathogens causing CNS infections. These findings therefore could aid clinical management and help reduce the the unnecessary prescription of antibiotics. The long turnaround time

achieved during the prospective phase was atributed to the COVID-19 discruption (310) and the availability of the resources. Therefore, I was only able to conduct the analysis on a weekly basis. This lowered the impact of metagenomics in informing the management of patients with CNS infections, especially when a pathogen was detected (311), as for the case of *K. pneumoniae*. Moving forward, metagenomics should be initiated as soon as possible after routine diagnosis fails to identify a pathogen in future studies.

The detection of sequences related to HIV and HBV genomes likely represented incidental findings rather than an association with the ongoing CNS infections. Although HBV genomes were also detected in the CSF of two patients diagnosed with CNS infections in a previous report (312), further investigation is required to understand how HBV might contribute to the observed symptoms and pathology. Thus, this emphasises the importance of a close collaboration between laboratory scientists and treating physicians in interpretation of metagenomic results (287). Additionally, despite the use of dedicated labs for separate steps (nucleic acid isolation, amplification, and sequencing) sequences related to SARS-CoV-2, which were extensively sequenced during the study period in our laboratory at the time, were also detected in metagenomic outputs of one CSF sample (295). These findings suggest that good laboratory practice and well-designed workplace are essential to minimize contamination (187), and warrant further considerations (304).

The strengths of my study include that I used consecutive patients presenting with CNS infections enrolled in the clinical study. Additionally, the study patients were admitted to a specialized department dedicated to CNS infections. And only one laboratory was responsible for patient diagnostics. Collectively, these factors have minimized the potential biases associated with patient selection and diagnostic results. However, my study has some limitations. First, I only focused on bacterial and viral etiologies, whilst CNS infections can also be caused by other pathogens, including *M. tuberculosis*, parasites, and fungi. Second, I did not explore the utility potential of non-CSF

samples (urine and plasma). Notably, JEV has previously been detected in urine of patients presenting with meningoencephalitis (313).

To summarise, I have successfully developed and prospectively evaluated an internally controlled metagenomic pipeline for the diagnosis of bacteria and viruses in CSF of patients with CNS infections. The results highlighted the challenges in establishing the causative agents of CNS infections. Importantly, my findings emphasise that metagenomics cannot replace conventional diagnostic assays but could provide a complementary strategy that might help improve (novel) pathogen identification, especially when routine diagnostics fails to identify a pathogen. In interpretation of the results, care must be taken not to overestimate the contribution of commensal viruses and/or contaminants to the disease under investigation.

Chapter 5

Value of Lipocalin-2 as a Biomarker for Bacterial Meningitis

5.1 Introduction

Using liquid chromatography tandem mass-spectrometry based approach followed by specific ELISA analysis, my research group and I have previously identified Lipocalin 2 (LCN2) as a potential biomarker for bacterial meningitis (BM) in Vietnamese adults (314). LCN2 is an innate immune protein, secreted by neutrophils, hepatocytes and renal tubular cells (315). LCN2 is known to have antibacterial properties. It acts by capturing and depleting siderophores, small iron-binding molecules synthesized by bacteria, thereby interfering the iron uptake of the bacteria (315,316). To inform future research direction about LCN2 in patients with CNS infections, in this chapter, I aimed to further assess the diagnostic performance of LCN2 using an independent cohort of patients with CNS infections, enrolled in the clinical study described in Chapters 2&3.

5.2 Materials and methods

5.2.1 Study design and specimen collection

Admission CSF samples from consecutive patients enrolled in the prospective observational study between March 2019 and May 2021 described in Chapters 2&3 were selected for analysis. The collected CSF samples were immediately stored at -80^{o}C until analysis. The duration of storage collection to analysis was 1-2 years. Additionally, the previously published dataset of LCN2 levels obtained from 364 consecutive patients enrolled in the same clinical study during September 2017 and February 2019 was also used for pooled analyses to assess the overall diagnostic performance of LCN2 (317).

5.2.2 Assignment of CNS syndrome groups

Patients were assigned to different clinical groups based on case definition and the results of standard laboratory investigations as described in Chapters 2&3. In brief, a confirmed diagnosis was established if an infectious

agent was identified in the CSF samples by microbial investigation (routine culture, and/or Gram stain, and/or PCR) or serological tests for JEV and Dengue or detection of specific antibodies against anti-NMDAR. Otherwise, patients were considered as suspected CNS infection syndromes based on treatment responses and/or clinical judgment of the physicians. Meanwhile, CNS infections were excluded in patients who had no meningeal signs, CSF laboratory parameters in normal ranges, and negative results of microbiological and serological investigations. Those patients were grouped into a non-CNS infection group.

5.2.3 Measurement of LCN2 levels

Measurement of LCN2 levels was performed on CSF samples using a commercial monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems, Minneapolis, MN, US). The experiments were performed according to the manufacturer's instructions, and the result was expressed as ng/mL. Detailed procedures are presented in Appendix C (S5.1).

5.2.4 Statistical analysis

SPSS 23.0 (IBM, White Plains, NY, USA) and Prism 9.2.0 (GraphPad Software, La Jolla, CA, USA) were used for data analysis and visualization. The Mann-Whitney U, or the Kruskal-Wallis test was applied to compare between groups of continuous variables. The correlation between continuous variables was evaluated by the Spearman correlation test. The diagnostic performance of LCN2 was evaluated using the area under the receiver operating characteristic curve (AUROC) with the cutoff values corresponding to the highest accumulation of sensitivity and specificity. The diagnostic models of two or more combined parameters were evaluated using a logistic regression model.

5.3 Results

5.3.1 Baseline characteristics of the study population

During the study period (March 2019 – May 2021), a total of 427 patients were enrolled in the clinical study. A CNS-infection cause was identified in 191 (44.7%) patients, with bacterial pathogens other than *Mycobacterium tuberculosis* (MTB) being the leading causes (62/191, 32.5%), followed by MTB (56/191, 29.3%) and viral pathogens (24/191, 12.6%) (Figure 5.1 and Table 5.1). Accordingly, based on the results of laboratory analysis, the included patients were assigned to 8 clinical entities, with major groups including those with viral meningoencephalitis (VME), bacterial meningitis (BM), tuberculous meningitis (TBM) and non-CNS infections (Figure 5.1). Additionally, the frequency of clinical conditions of 70 patients without CNS infection syndromes is demonstrated in Appendix D (Table S5.1). Due to the overlap with data presented in Chapter 3, I presented details about demographic features, clinical symptoms, laboratory findings and outcomes of the study population in Appendix D (Table S5.2).

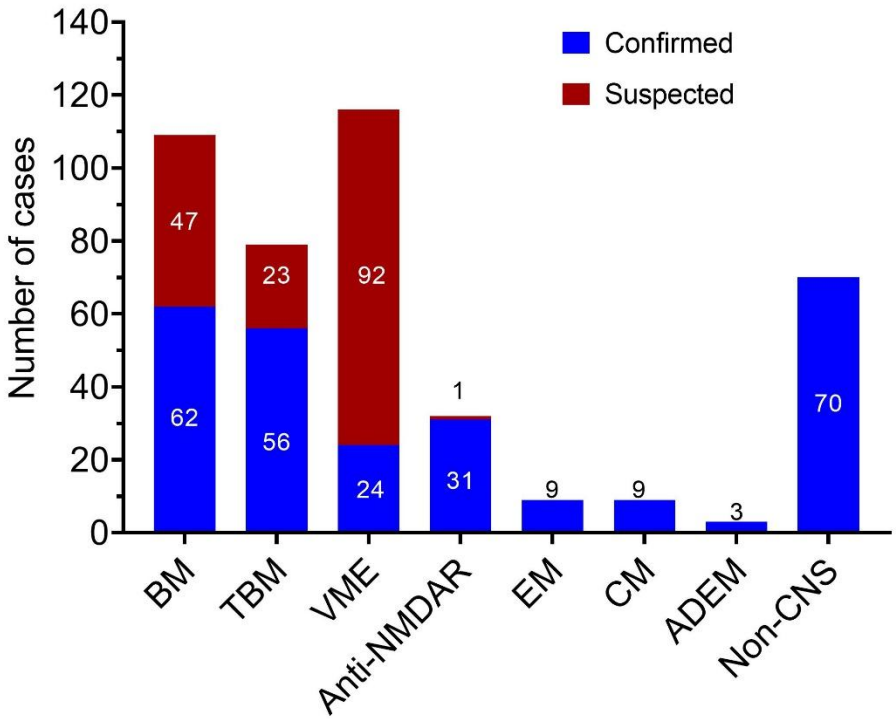


Figure 5.1: Clinical groups of CNS infection syndromes in the validation phase (N=427).

Note to Figure 5.1: VME: Viral meningoencephalitis, BM: Bacterial meningitis, TBM: Tuberculous meningitis, CM: Cryptococcal meningitis, EM: Eosinophilic meningitis, Anti-NMDAR: Anti-N-Methyl-D-Aspartate Receptor encephalitis, ADEM: Acute disseminated encephalomyelitis, Non-CNS: Non-central nervous system infections.

Table 5.1: List of aetiological agents detected in patients with BM and VME.

Pathogens	Number of cases (n)
S. suis	24
S. pneumoniae	9
E. coli	9
K. pneumoniae	7
B. pseudomallei	2
E. faecium	1
E. faecalis	1
Salmonella spp	1
S. constellatus	1
S. gallolyticus	1
S. agalactiae	1
L. monocytogenes	1
Gram staining positive only	4
HSV	13
VZV	6
DENV	2
JEV	3

5.3.2 CSF LCN2 concentrations

Results of ELISA analysis showed that LCN2 concentrations were significantly different among the included patient groups with the highest concentration observed in the BM group (median: 325.84 ng/mL, range: 0.85-3,630.78 ng/mL), followed by TBM (median: 30.20 ng/mL, range: 1.15-478.63 ng/mL) and VME (media: 4.42 ng/mL, range: 0.14-707.95 ng/mL) (Figure 5.2A). In contrast, LCN2 was almost absent or detected at very low levels in CSFs of patients presenting with autoimmune encephalitis, EM or CM and non-CNS infections (median: 1.26 ng/mL, range: 0.03-234.42 ng/mL).

Of the patients with BM, CSF LCN2 levels were higher in those with a confirmed diagnosis than in those without a cause identified: median (range), 447.09 ng/mL (0.85-3,630.78) vs. 147.92 ng/mL (4.07-1,000) (Figure 5.2B). Additionally, CSF LCN2 concentrations were comparable between major pathogen groups (*S. suis, S. pneumoniae, E. coli and K. pneumoniae*) (Figure 5.2C)

5.3.3 Diagnostic value of LCN2

For diagnostic performance analysis, because of the low levels of CSF LCN2 values and the small sample size, I grouped patients with CM, EM, and NMDAR encephalitis into one group, namely others. Patients with TBM and VME were separately analysed. The results showed that LCN2 could accurately discriminate BM patients from those with TBM, VME and other clinical entities, AUROC: 0.93 (95% CI, 0.90-0.96). Accordingly, the obtained sensitivity and specificity were 0.89 (95% CI, 0.85-0.92) and 0.85 (95% CI, 0.77-0.91), respectively, and the DOR was 45.85 (95% CI, 24.27-86.62) (Figure 5.3). As indicated by the ELISA results, subgroup analysis showed the diagnostic values of LCN2 in discriminating BM from VME were higher than that in discriminating BM from TBM (Figure 5.3).

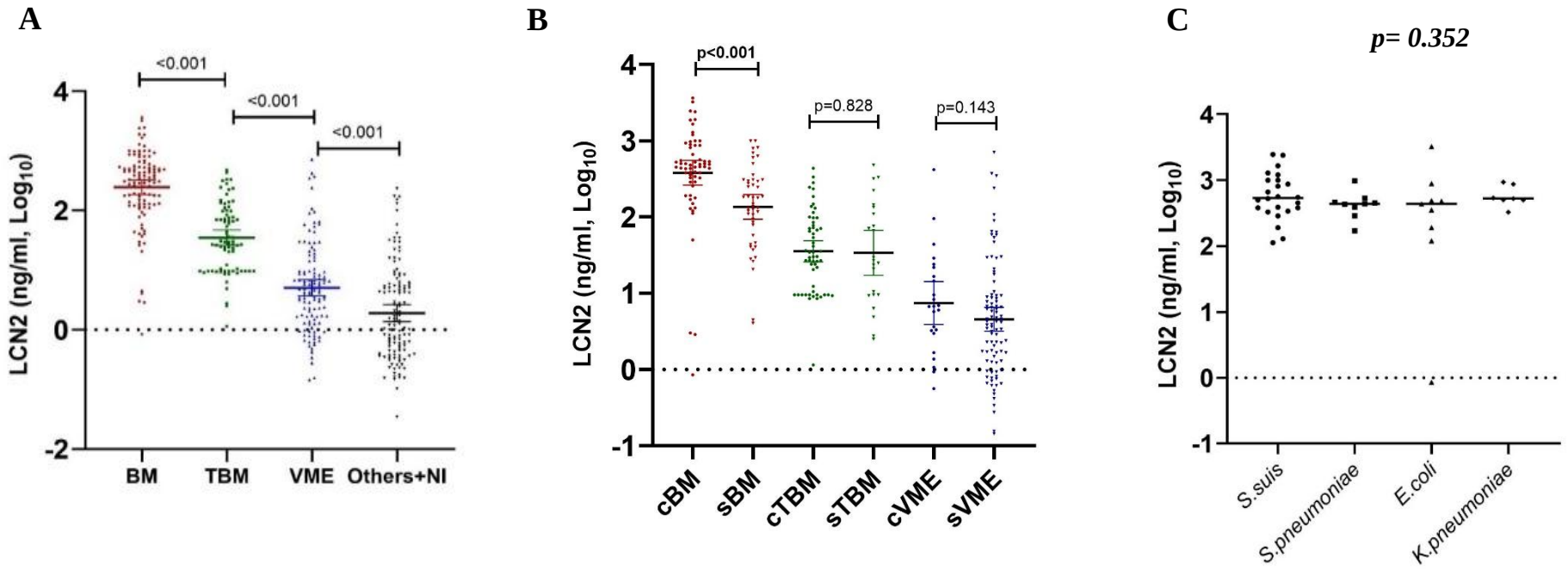


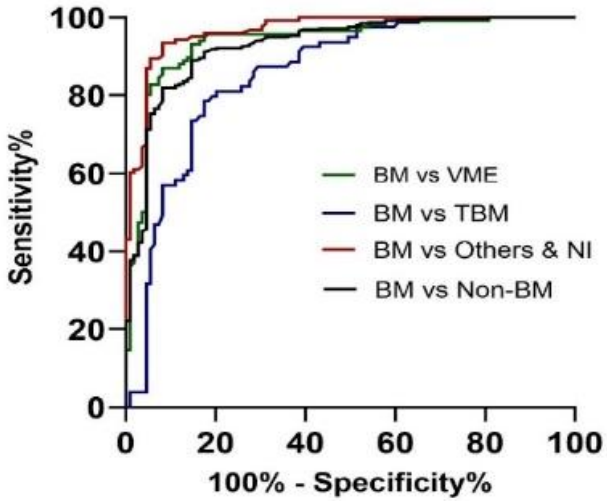
Figure 5.2: Comparisons of CSF LCN2 concentrations in different groups

(A) Comparisons of CSF LCN2 concentrations among the CNS syndromes groups

(B) Comparisons of CSF LCN2 concentrations between confirmed and suspected groups.

(C) Comparisons of CSF LCN2 concentrations between major bacterial agents

Note to Figure 5.2: Others: CM, anti-NMDAR encephalitis, EM, neurotoxoplasmosis, NI: non-CNS infections



BM vs.	LCN2 cut-off (ng/ml)	AUROC (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	DOR (95%CI)
TBM	103.51	0.86 (0.80-0.91)	0.78 (0.68-0.86)	0.83 (0.74-0.89)	17.31 (8.33-35.85)
VME	33.50	0.94 (0.90-0.97)	0.87 (0.80-0.92)	0.92 (0.86-0.96)	76.96 (31.30-178.83)
Others	35.89	0.97 (0.95-0.99)	0.94 (0.88-0.97)	0.92 (0.85-0.96)	180.17 (66.99-484.56)
Non-BM	78.16	0.93 (0.90-0.96)	0.89 (0.85-0.92)	0.85 (0.77-0.91)	45.85 (24.27-86.62)

Figure 5.3: Diagnostic value of LCN2 as a biomarker for BM

Note to Figure 5.3: Others: CM, anti-NMDAR encephalitis, EM, neurotoxoplasmosis, NI: non-CNS infections, non-BM: nonbacterial meningitis.

5.3.4 Diagnostic values of LCN2 in comparison with routine CSF biomarkers

The diagnostic values of LCN2 in predicting BM in comparison with routine CSF biomarkers (leukocytes, protein, lactate, and glucose) are displayed in Figure 5.4. LCN2 outperformed all these four routine CSF biomarkers in discriminating BM patients from patients with non-BM (TBM, VME, anti-NMDAR encephalitis, CM, EM, and non-CNS infections). The obtained AUROC for leukocytes, protein, lactate, and glucose were 0.89 (95% CI 0.86-0.93), 0.81 (95%CI: 0.77-0.86), 0.87 (95% CI: 0.83-0.91) and 0.82 (95% CI 0.77-0.86), respectively. These figures were considerably lower than that of LCN2, 0.93 (95% CI: 0.90-0.96).

5.3.5 Diagnostic models for BM patients

To this end, the key question is whether LCN2 could provide any extra value to the BM diagnostic model that is based on currently used routine CSF markers (leukocytes, protein, lactate, and glucose). Adding LCN2 to the diagnostic model based on a combination of leukocytes, protein, lactate, and glucose increased the AUROC and DOR from 0.90 (95%CI: 0.86–0.94) to 0.94 (95%CI: 0.91-0.97), and 54.08 (95%CI: 18.90-154-76) to 70.64 (95%CI: 24.69-202.14) (Figure 5. 5 and Table 5.2). Likewise, subgroup analyses showed that adding LCN2 to the diagnostic model consisting of leukocytes, protein, lactate, and glucose improved the AUROC in differentiating between BM and TBM (from 0.82 to 0.89), between BM and VME (from 0.92 to 0.95) and between BM and patients belonging to the others group (from 0.94 to 0.97) (Figure 5.5B). Pooled analysis using data from patients of the present study (n=427) and a dataset (n=364) that my research group and I have previously published (314) reproduced findings of individual cohorts (Figure 5.6B and Table 5.2).

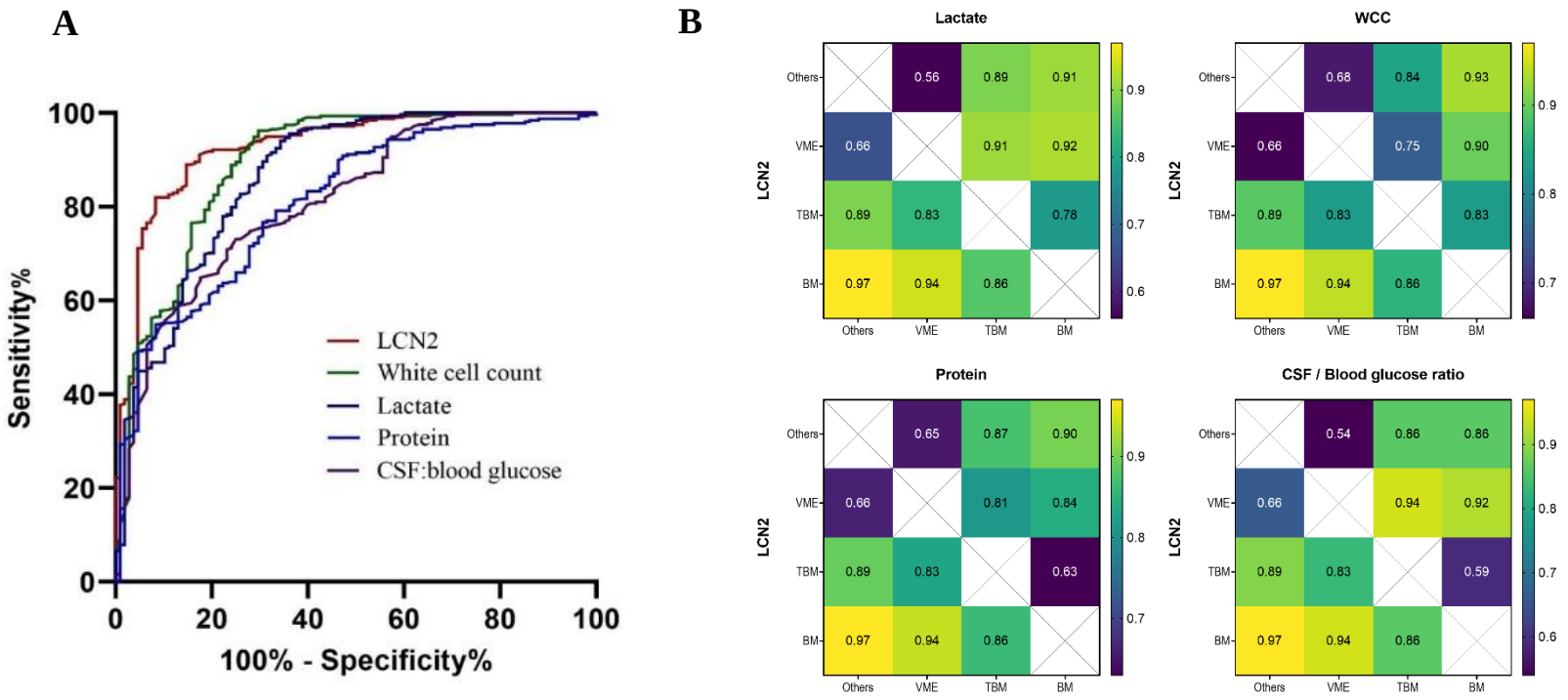


Figure 5.4: Comparisons of diagnostic values of LCN2 and existing CSF biomarkers for predicting BM from other groups with CNS infection syndromes.

(A) AUROC analysis showing the outperformance of LCN2 in comparisons with existing CSF parameters (leukocyte, lactate, protein and CSF: blood glucose ratio. Other groups included anti-NMDAR encephalitis, cryptococcal meningitis, eosinophilic meningitis, ADEM and non-CNS infections.

(B) AUROC values of subgroup analysis in comparisons of LCN2 and individual CSF parameters

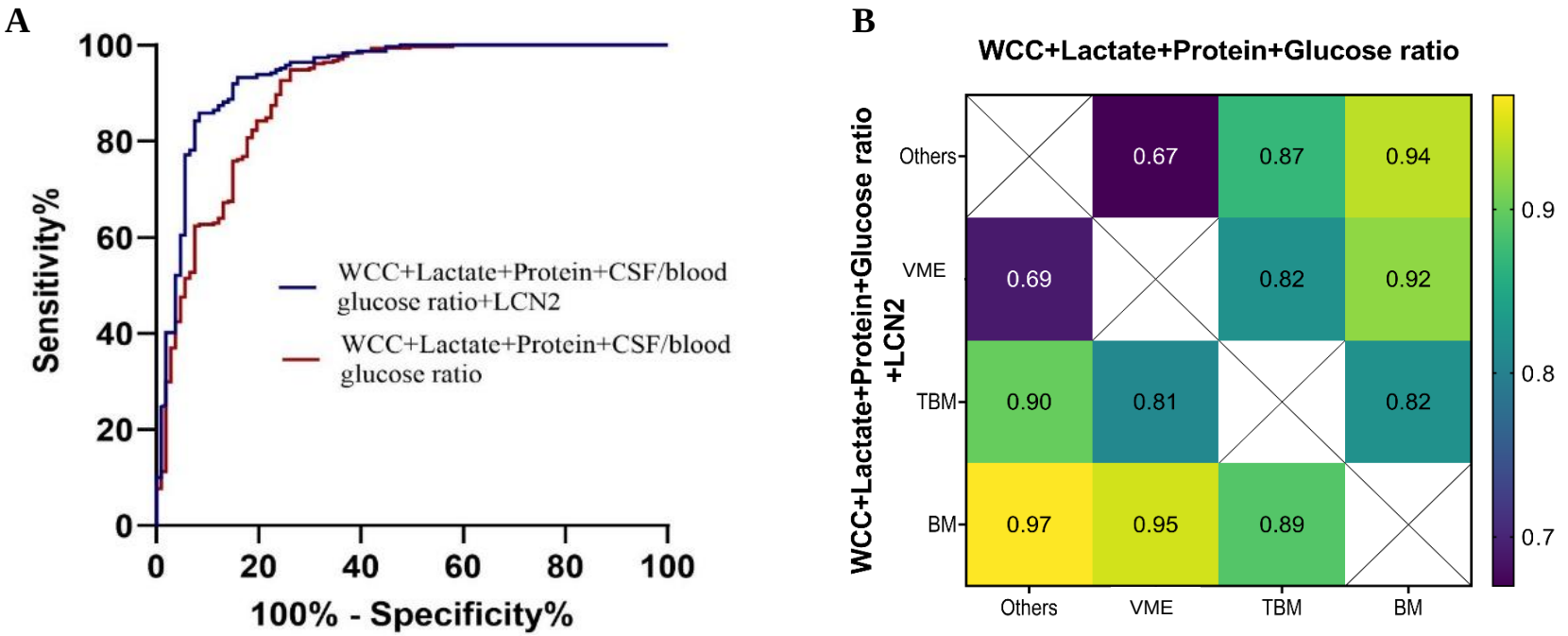


Figure 5.5: Diagnostic models of BM with the combinations of different CSF diagnostic biomarkers.

(A) AUROC analysis showing the diagnostic performance of two diagnostic models including the combination of LCN2 and current diagnostic biomarkers and combination of only these CSF parameters.

(B) Comparisons of AUROC values of subgroups in comparison of two diagnostic models

Table 5.2: Summary of diagnostic performance of individual CSF biomarkers and combination model of existing biomarker and CSF LCN2 in discriminating patients with BM from those with non-BM.

BM vs. non-BM	Cut-off	AUROC (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	DOR (95%CI)
Validation phase 1 (318)					
WCC (cells per mm^3)	709	0.89 (0.84-0.94)	0.75 (0.63-0.84)	0.91(0.87-0.94)	30.33 (15.21-60.49)
Lactate (nmol/L)	5.77	0.88 (0.83-0.93)	0.75 (0.63-0.84)	0.84 (0.79-0.88)	15.75 (8.27-30.01)
Protein (g/L)	1.13	0.75 (0.69-0.82)	0.78 (0.67-0.87)	0.61 (0.56-0.67)	5.67 (2.3-10.71)
CSF/blood glucose ratio	0.33	0.70 (0.62-0.77)	0.76 (0.71-0.81)	0.52 (0.40-0.63)	3.43 (1.82-6.48)
LCN2 (ng/mL)	221	0.95 (0.92-0.98)	0.88 (0.77-0.94)	0.91 (0.88-0.94)	73.77 (31.75-171.38)
WCC+lactate+protein+CSF/blood glucose ratio	NA	0.94 (0.90-0.98)	0.86 (0.75-0.92)	0.92 (0.88-0.94)	66.2 (29.32-149.68)
WCC+lactate+protein+CSF/blood glucose ratio + LCN2	NA	0.96 (0.93-0.99)	0.91 (0.81-0.96)	0.97 (0.94-0.98)	308.26 (105.65-899.44)
Validation phase 2					
WCC (cells per mm^3)	1,216	0.89 (0.86-0.93)	0.96 (0.93-0.98)	0.70 (0.61-0.78)	56.00 (19.57-160.25)
Lactate (nmol/L)	8.18	0.87 (0.83-0.91)	0.94 (0.91-0.96)	0.66 (0.56-0.74)	30.41 (10.63-87.02)
Protein (g/L)	1.67	0.81 (0.77-0.86)	0.77 (0.72-0.81)	0.69 (0.60-0.77)	14.06 (4.91-40.23)
CSF/blood glucose ratio	0.44	0.82 (0.77-0.86)	0.73 (0.68-0.78)	0.75 (0.66-0.82)	8.11 (2.83-23.21)
LCN2 (ng/mL)	78.16	0.93 (0.90-0.96)	0.89 (0.85-0.92)	0.85 (0.77-0.91)	45.85 (24.27-86.62)
WCC+lactate+protein+CSF/blood glucose ratio	NA	0.90 (0.86-0.94)	0.95 (0.92-0.97)	0.74 (0.65-0.81)	54.08 (18.90-154-76)

WCC+lactate+protein+CSF/blood glucose ratio + LCN2	NA	0.94 (0.91-0.97)	0.86 (0.82-0.89)	0.92 (0.85-0.96)	70.64 (24.69-202.14)
Pooled analysis of 2 validation phases					
WCC (cells per mm^3)	716	0.89 (0.86-0.92)	0.91 (0.88-0.93)	0.75 (0.68-0.80)	30.33 (19.59-46.98)
Lactate (nmol/L)	8.43	0.87 (0.84-0.90)	0.94 (0.92-0.96)	0.64 (0.57-0.71)	27.85 (17.50-44.34)
Protein (g/L)	1.67	0.79 (0.75-0.83)	075 (0.72-0.78)	0.65 (0.58-0.72)	5.57 (3.88-8.01)
CSF/blood glucose ratio	0.44	0.76 (0.72-0.80)	0.76 (0.63-0.71)	0.71 (0.63-0.77)	7.75 (4.10-14.65)
LCN2 (ng/mL)	106.66	0.93 (0.90-0.95)	0.86(0.83-0.89)	0.86 (0.80-0.90)	37.74 (19.97-71.29)
WCC+lactate+protein+CSF/blood glucose ratio	NA	0.91 (0.88-0.94)	0.95 (0.93-0.97)	0.74 (0.67-0.80)	54.08 (28.62-102.17)
WCC+lactate+protein+CSF/blood glucose ratio + LCN2	NA	0.94 (0.92-0.96)	0.91 (0.88-0.93)	0.85 (0.79-0.89)	57.30 (30.33-108.25)

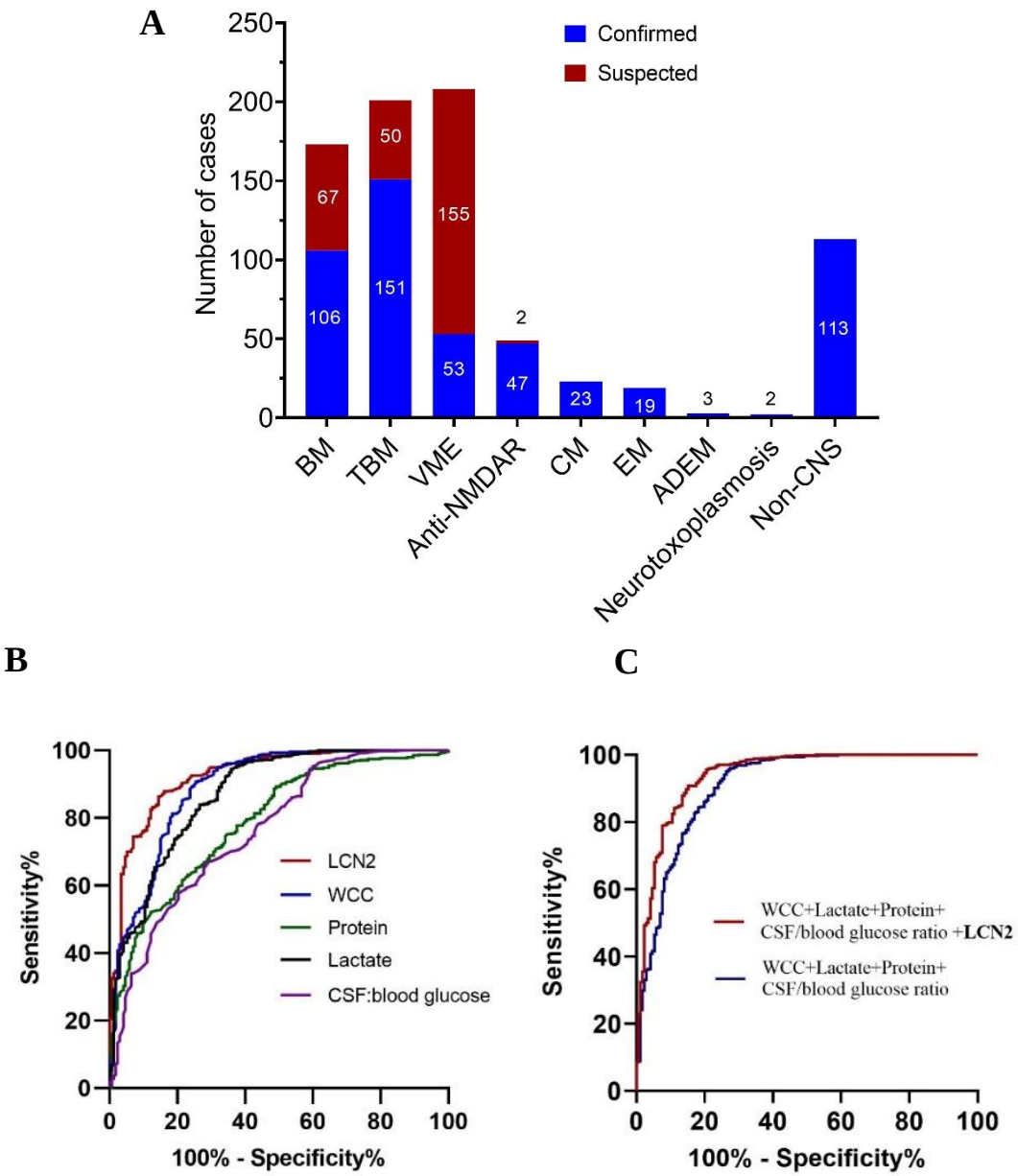


Figure 5.6: Pooled analysis of LCN2 diagnostic performance in two validation phases (N=791).

(A) Clinical groups of CNS infection syndromes

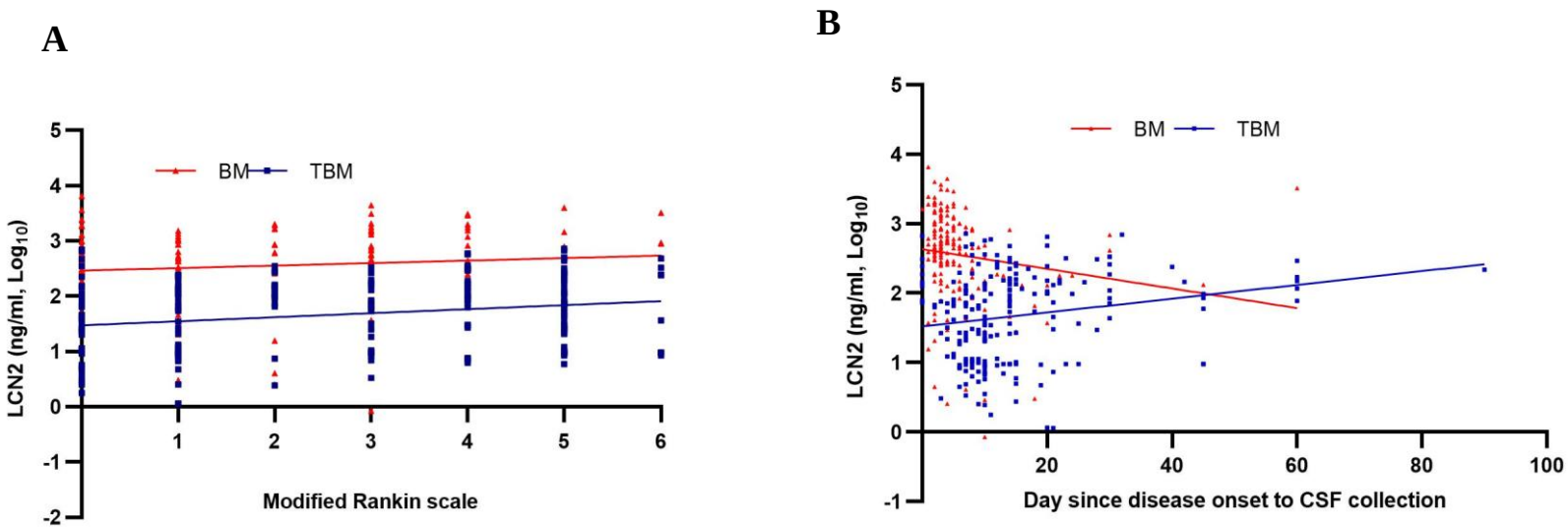
(B) Comparisons of diagnostic values of LCN2 and existing CSF biomarkers for predicting BM

(C) Comparisons of value of combination diagnostic model for BM

5.3.6 Correlation between LCN2 levels and patient outcome, illness day and routine CSF makers

There was no association between LCN2 levels and in-hospital outcomes assessed using mRS in patients with BM (Spearman R, 0.148; p, 0.054). But in patients with TBM, LCN2 levels were associated with poor outcomes at discharge (Spearman R, 0.206; p, 0.004), (Figure 5.7A).

In patients with BM, CSF LCN2 levels were correlated negatively with the duration of illness at admission (Spearman R, -0.297; $p<0.001$). However, in patients with TBM, there was a positive correlation between illness day and LCN2 levels (Spearman R, 0.209; p, 0.003) (Figure 5.7B). For both groups of patients with BM and TBM, CSF LCN2 concentrations were correlated positively with the levels of total CSF protein, white cell counts and lactate but negatively correlate with CSF: blood glucose ratio (Figure 5.8 and Table 5.3).



	Correlation	CSF LCN2	
		BM	TBM
Modified Rankin scale	Spearman r	0.148	0.206
	P value	0.054	0.004

Correlation		CSF LCN2	
		BM	TBM
Illness day	Spearman r	-0.297	0.209
	P value	<0.001	0.003

Figure 5.7: Association between CSF LCN2 levels and other parameters of BM and TBM groups.

(A) Association between CSF LCN2 levels and outcome at discharge

(B) Association between LCN2 levels and illness duration

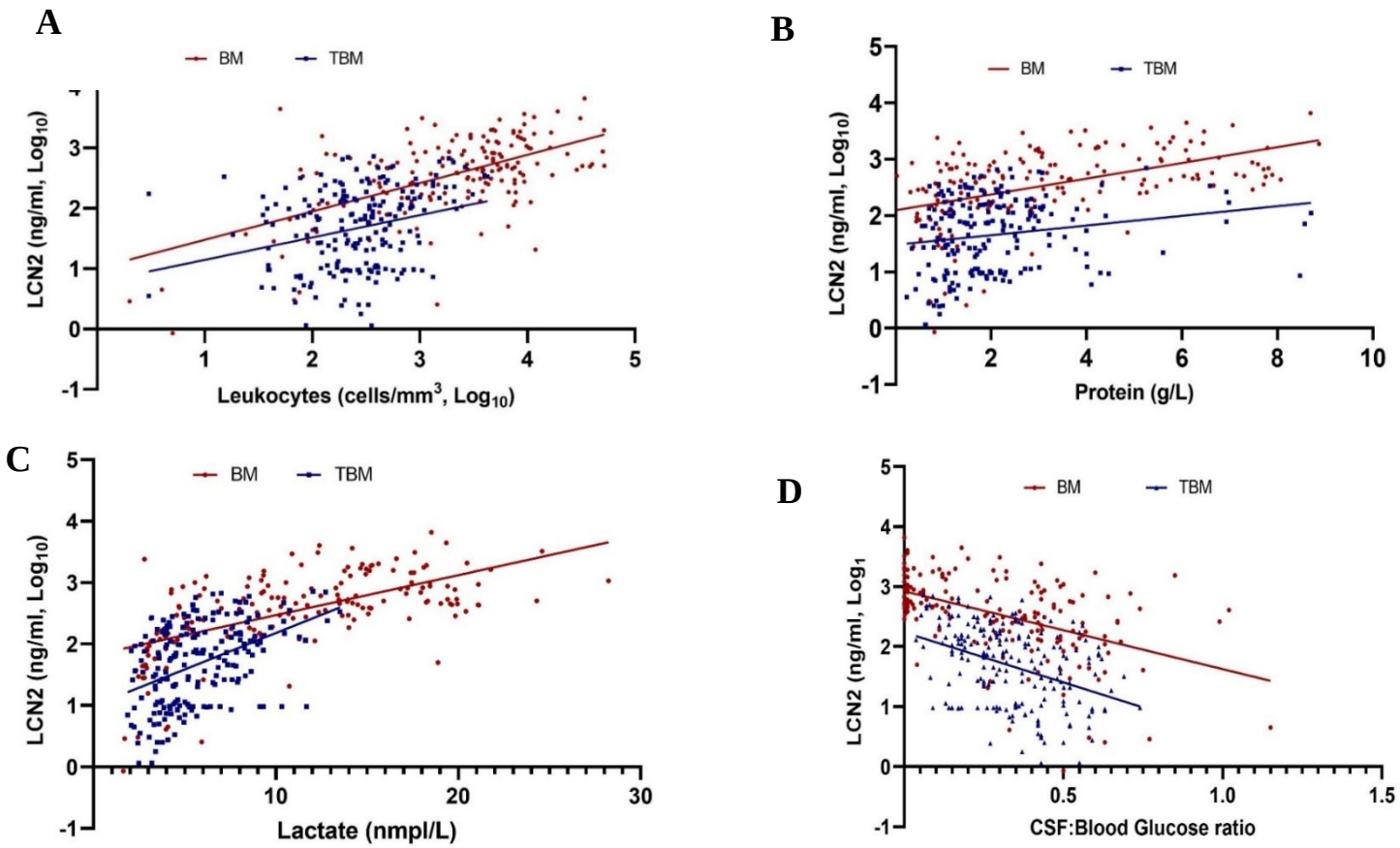


Figure 5.8: Correlation between CSF LCN2 levels and other laboratory parameters in BM and TBM groups.

(A) LCN2 vs Leukocytes, (B) LCN2 vs protein, (C) LCN2 vs Lactate, (D) LCN2 vs CSF: Blood Glucose ratio

Table 5.3: Correlation analysis of LCN2 and four current CSF parameters (leukocyte, protein, lactate, and glucose).

Correlation		CSF LCN2	
		BM	TBM
Leukocyte	**Spearman r**	0.470	0.285
	P value	<0.0001	<0.0001
Total protein	**Spearman r**	0.539	0.245
	P value	<0.0001	0.0005
Lactate	**Spearman r**	0.622	0.437
	P value	<0.0001	<0.0001
CSF: Blood Glucose ratio	**Spearman r**	-0.507	-0.413
	P value	<0.0001	<0.0001

5.4 Discussion and Conclusion

In this chapter, I validated the diagnostic values of LCN2 in patients with CNS infection syndromes. Using a large cohort of 427 consecutive patients, I demonstrated that LCN2 is a highly sensitive and specific biomarker for accurate prediction of BM in Vietnamese adults presenting with clinically suspected CNS infections. Additionally, my findings showed that LCN2 is more sensitive than routine CSF biomarkers (leukocytes, glucose, protein, and lactate) in discriminating between patients with BM and those with non-BM. Accordingly, a diagnostic model combining LCN2, and the four currently used CSF parameters (leukocytes, lactate, glucose, and protein) provides the best diagnostic model for patients with BM. These findings are in agreement with findings from the previous validation phase conducted by my research group and me as well as the obtained results from previous reports (243,319,320).

However, previous studies conducted by others only focused on quantifying LCN2 concentrations in patients with confirmed BM and viral encephalitis and did not compare the performance of LCN2 against routine CSF markers (leukocytes, glucose, protein and lactate) (243,319,320). My study included patients with a wide spectrum of CNS infection syndromes (including bacterial, fungal, tuberculous, viral, and parasitic meningitis). Additionally, I also compared the diagnostic performance of LCN2 against that of CSF markers commonly used as part of routine care worldwide. As such, my findings have expanded the existing body of knowledge about the association between LCN2 and CNS infections, and for the first time provide robust evidence that LCN2 is a highly sensitive and specific biomarker for discriminating BM from a broad-spectrum of CNS infection syndromes.

The differences in CSF LCN2 levels between laboratory confirmed and clinically suspected BM groups parallel the associations between LCN2 levels and routine CSF parameters (glucose, lactate, white cell counts and protein) and the illness day at admission. These data pointed to the association between the host responses and an on-going infection (i.e., the presence of a

bacterial pathogen in clinical samples at the time of collection). Notably, the association between LCN2 and bacterial infections has also previously been documented in patients with pneumonia and febrile (321). Collectively, the data suggested that LCN2 can be a useful marker to inform antibiotics use in patients with bacterial infections more broadly, hence helping improve the antibiotic stewardship.

Consistent with findings from our previous validation study (318), in the present study I also found no significant difference in CSF LCN2 levels among bacterial etiologies of CNS infections (including Gram-positive and Gram-negative bacteria) in our two validation phases. Therefore, the production of LCN2 in response to bacterial infections is likely species independent. The contribution of LCN2 to the pathophysiology of bacterial infections of the central nervous system and more broadly invasive diseases caused by bacterial pathogens warrants further research.

The limitation of my study included that it was conducted at a single major tertiary referral hospital. Although all routine diagnostic approaches and patient assessments were consistent over the course of the study, minimizing potential bias, it would be worth considering collaborating with other research groups to examine the diagnostic accuracy of this marker in other settings. Additionally, my study has so far only focused on Vietnamese adult cohort, leaving the utility potential of LCN2 in pediatric CNS infections and especially more broadly in other unknown settings.

To summarise, the obtained results have consistently shown that LCN2 is a highly sensitive and specific biomarker for accurate prediction of BM in adults, especially when used alongside other standard CSF parameters. Prospective studies are needed to assess the utility potential of LCN2 in the diagnosis and management of CNS infections, including children, and whether it can be used in settings with limited laboratory capacity to improve outcomes from these devastating conditions.

Chapter 6

Validation of a Disintegrin and Metalloprotease like Decysin as a Biomarker for Central Nervous System Infections

6.1 Introduction

In addition to the discovery of LCN2, the original mass-spectrometry analysis conducted by my research group and me has also revealed several other biomarker candidates in patients with CNS infections (318). Of these, disintegrin and metalloprotease like decysin (ADAMDEC1) was predominantly found in patients presenting with tuberculosis meningitis but not in those with other syndromes (bacterial meningitis, viral encephalitis, and non-CNS infections). ADAMDEC1 is selectively expressed in mature dendritic cells and macrophages of the gastrointestinal tract and secondary lymphoid tissue (322,323). Mature ADAMDEC1 has proteolytic activity with capability of cleaving macromolecular substrates (322). The biological function of ADAMDEC1 is unknown. Yet it has been hypothesised to play a role in immunity. In this chapter, I aimed to define the diagnostic value of ADAMDEC1 in patients with CNS infection syndromes. Specifically, I applied ADAMDEC1 specific ELISA to verify the original mass-spectrometry results and then expanded the ELISA testing to a larger cohort of patients with CNS syndromes enrolled in the clinical study described in Chapters 2 and 3.

6.2 Materials and Methods

6.2.1 Study design and specimen collection

The patient cohorts and CSF samples used in this chapter were derived from three clinical studies conducted in Viet-Anh ward of HTD, and have previously been described (314). Briefly, study #1 was conducted during January 2015 and September 2016, and aimed to improve the laboratory diagnosis of TBM and meningoencephalitis in Vietnamese adults (123). As per the study protocol, admission CSF samples were collected from adult patients (≥18 years) with clinically suspected CNS infections alongside meta-

clinical data and hospital outcomes. Study #2 aimed to investigate the immunological responses in bacterial meningitis patients, especially those infected with *Streptococcus suis* during 2015 and 2017. Accordingly, admission CSF samples were collected from patients (≥16 years) with clinically suspected bacterial meningitis alongside meta-clinical data and hospital outcomes. Study #3 was the 3-year prospective observational study described in Chapters 2 and 3. CSF samples in study# 3 were collected for routine diagnosis and were stored at -80^{o}C for the study purpose and the duration of storage before ADAMDEC1 analysis was 2-3 years.

6.2.2 Measurement of ADAMDEC1 levels

Because this Chapter was built on the previous mass-spectrometry work (318) (Appendix E), here I applied ADAMDEC1 specific ELISA to verify the original mass-spectrometry findings, using CSF of the clinical studies #1&2. And I then futher validated the results using samples of the clinical study #3.

Measurement of ADAMDEC1 levels was carried out using a commercial sandwich enzyme-linked immune-sorbent assay (ELISA) (Aviva Systems Biology, San Diego, CA, US). The experiments and result interpretation were performed according to the manufacturer's instructions as described in Appendix E (S6.2). Sample dilution was applied to target protein concentrations in the middle of the assay linear dynamic range.

6.2.3 Statistical analysis

SPSS 23.0 (IBM, White Plains, NY, USA) was used for data analysis and GraphPad Prism 9.2.0 (GraphPad Software, La Jolla, CA, USA) was applied to generate figures. The Mann-Whitney U test or the Kruskal-Wallis test was used to compare groups of continuous variables as appropriate. The correlation between continuous variables was evaluated by the Spearman correlation test. The diagnostic performance of candidate biomarkers was assessed using the area under the receiver operating characteristic curve (AUROC). The cutoff values for result interpretations were selected based

on the highest accumulation of sensitivity and specificity. The diagnostic performance of two or more combined variables was evaluated using a logistic regression model.

6.3 Results

6.3.1 Baseline characteristics of study population

Overview about the contribution of the three clinical cohorts to the analyses described in the present Chapter is shown in Figure 6.1.

Verification cohort

Due to the availability of the materials, only 34/45 CSF samples selected for the original mass-spectrometry-based discovery phase were included for analysis. The 34 included CSF samples consisted of 15 patients with confirmed TBM, 8 patients with confirmed BM, 6 patients with confirmed VME and 5 patients with non-CNS infections for analysis The baseline characteristics of these 34 patients are presented in Appendix E (Table S6.1).

Validation cohort

Of 364 patients of the study #3, 362 had admission CSF available for analysis (Figure 6.1). The baseline characteristics, including demographic features, clinical symptoms, laboratory findings and outcomes are presented Appendix E (Table S6.2) and etiological agents are shown in Table 6.1. The frequency of clinical conditions of the 43 patients without CNS infections is detailed in Appendix E (Table S6.3). Of the 207 patients with a confirmed diagnosis, *M. tuberculosis* was the most common cause (46.9%, 97/207), followed by bacterial agents (20.3%, 42/207) and viral pathogens (12.1%, 25/207). The remaining 43 patients included those with anti-NMDAR encephalitis (8.2%, 17/207), cryptococcal meningitis (6.8%14/207), parasitic eosinophilic meningitis (4.8%10/207) and neurotoxoplasmosis (1.0%, 2/207) (Figure 6.1).

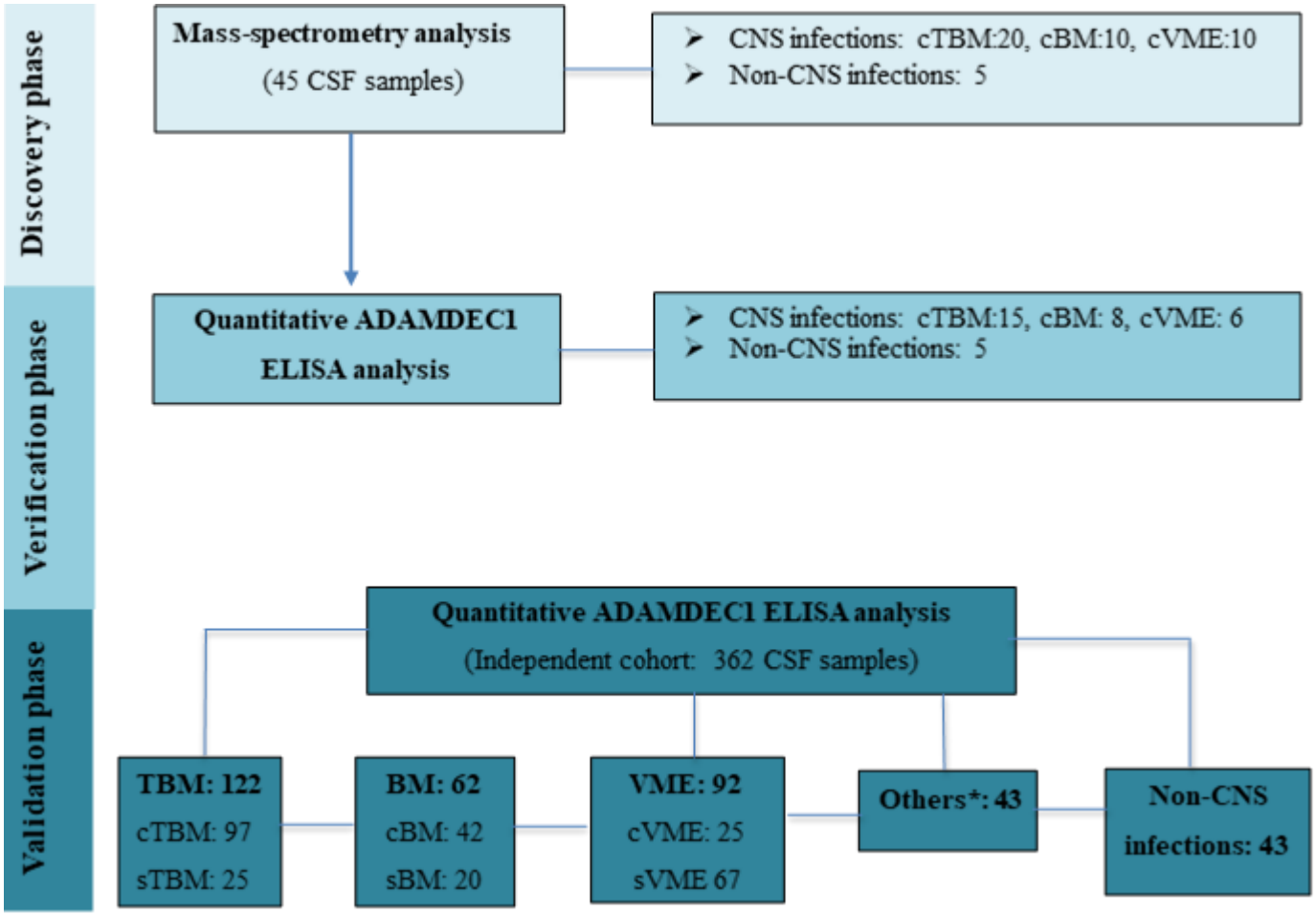


Figure 6.1: An overview of origin of clinical samples used for the analysis.

Note to Figure 6.1. TBM: tuberculous meningitis (TBM), cTBM: confirmed TBM, sTBM: clinically suspected TBM, BM: bacterial meningitis (BM), cBM: confirmed BM, sBM: clinically suspected BM, VME: viral meningoencephalitis (VME), cVME: confirmed VME, sVME: clinically suspected VME.

*Including: cryptococcal meningitis (n=14), anti-NMDAR encephalitis (n=17), eosinophilic meningitis (n=10), neurotoxoplasmosis (n=2)

Table 6.1: List of aetiological agents detected in patients with confirmed BM and VME.

Pathogens	Number of cases
S. suis	20
S. pneumoniae	5
E. coli	5
N. meningitides	2
E. gallinarum	1
S. agalactiae	1
B. pseudomallei	1
S. gallolyticus	1
E. faecalis	1
S.aureus	1
Gram staining positive only	4
HSV	11
VZV	7
DENV	5
JEV	2

6.3.2 Results of ADAMDEC1 ELISA analysis – verification phase

The obtained results showed that ADAMDEC1 levels of TBM group were significantly higher than that of VME and non-CNS infection groups ($p < 0.001$). ADAMDEC1 levels of the TBM group were also higher than that of the BM group but the difference was not statistically significant ($p = 0.264$) (Figure 6.2A). These collective findings supported the original results of mass-spectrometry analysis.

ADAMDEC1 level with a cutoff 20 ng/ml could distinguished TBM patients from those with VME or non-CNS infections with an AUROC of 0.92 (95% CI, 0.82-1), corresponding to the sensitivity of 0.91 (95% CI, 0.62-1) and specificity of 0.87 (95% CI, 0.62-0.98) (Figure 6.2B). However, ADAMDEC1 could not accurately discriminate TBM patients from those with BM; AUROC: 0.68 (95% CI, 0.31-0.95), corresponding to a sensitivity of 0.63 (95% CI, 0.31-0.86) and a specificity of 0.93 (95% CI, 0.70-1).

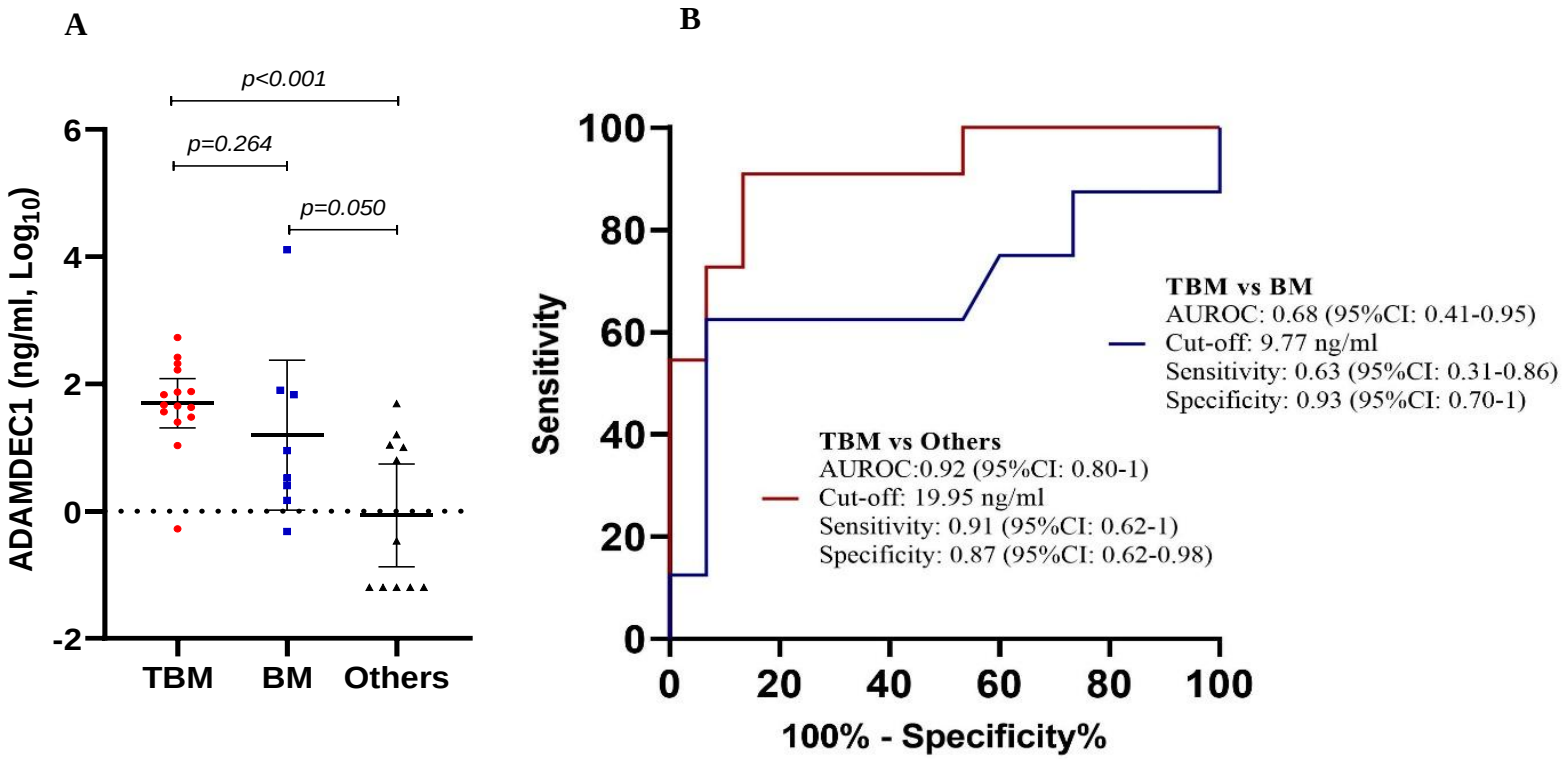


Figure 6.2: Results of mass-spectrometry and ADAMDEC1 ELISA analysis of the discovery cohort.

(**A**) Dot plots demonstrating the differences in CSF ADAMDEC1 levels obtained from quantitative ELISA analysis

(**B**) AUROC curve based on ADEMDEC-1 levels measured by quantitative ELISA analysis

Note to Figure 6.2. TBM: tuberculous meningitis, BM: bacterial meningitis, Others: viral meningoencephalitis and non-CNS infections

6.3.3 Results of ADAMDEC1 ELISA analysis – validation phase

Results of ELISA analysis of the verification phase indicated that ADAMDEC1 can be a diagnostic marker for patients with TBM. Thus, I conducted validation experiments using CSF of the clinical study #3 to further assess the robustness of the ADAMDEC1 diagnostic value. The obtained results showed that ADAMDEC1 levels were significantly different among the CNS infection groups (Figure 6.4). However, in contrast with the results of the verification phase, ADAMDEC1 level of BM patients was higher than that of the TBM patients; median (range): 114 ng/mL (0.06-5560 ng/mL) vs. 14 ng/mL; (0.06-323 ng/mL) (Figure 6.3A). Additionally, ADAMDEC1 was almost absent or detected at very low levels in CSF of patients with VME (median, 0.06 ng/mL; range, 0.06-461 ng/mL) or other forms of CNS infection syndromes (cryptococcal meningitis, eosinophilic meningitis, neurotoxoplasmosis, anti-NMDAR encephalitis) (median, 0.06 ng/mL; range, 0.06-144 ng/mL) and without CNS infection (median, 0.06 ng/mL; range, 0.06-25 ng/mL) (Figure 6.3A).

Subgroup analysis showed that ADAMDEC1 levels were higher in patients with confirmed diagnosis than in those without an aetiological agent identified (Figure 6.3B). Of the patients with BM, ADAMDEC1 levels were comparable between the pathogen groups (Figure 6.3C).

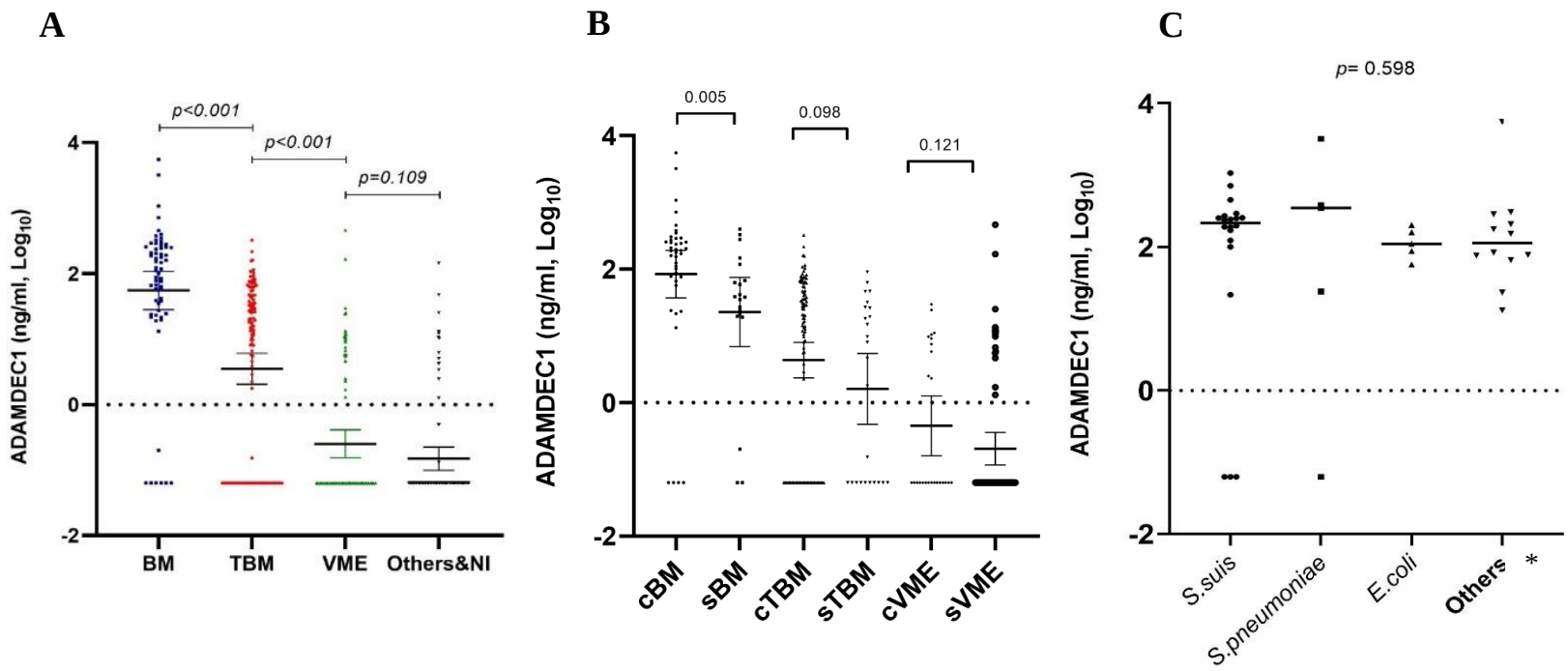
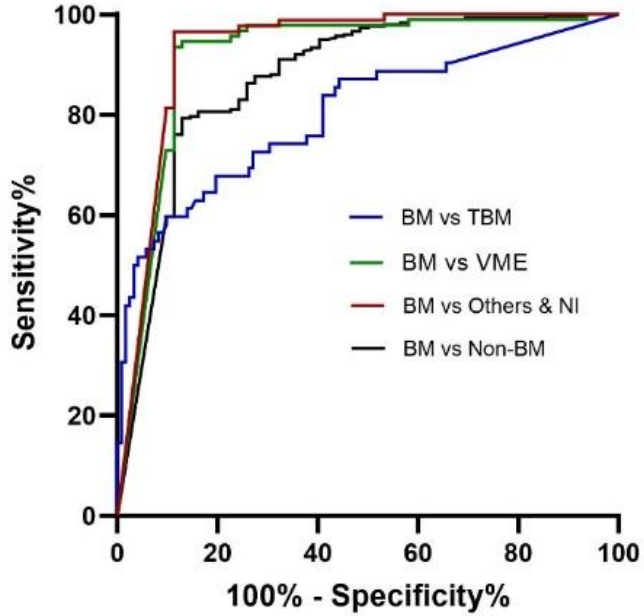


Figure 6.3: Comparison of ADAMDEC1 levels in different patient groups

6.3.4 Diagnostic performance of CSF ADAMDEC1

Analysis of ADAMDEC1 levels of the validation phase showed that ADAMDEC1 could discriminate BM from VME and other CNS infection syndromes (CM, EM, neurotoxoplasmosis, anti-NMDAR encephalitis) and non-CNS infections better than BM from TBM (Figure 6.4). With an ADAMDEC1 concentration cutoff of 13 ng/mL, the obtained AUROC and DOR were 0.92 and 112.6 (for BM vs VME) and 0.93 and 326.1 (for BM vs others plus non-CNS infections), respectively. However, AUROC and DOR for distinguishing BM from TBM were 0.80 and 13.6, respectively. More details are presented in Table 6.2 and Figure 6.4.

Back-to-back comparison showed that although ADAMDEC1 could distinguish BM from other patient groups, its diagnostic value was comparable with those of routine CSF makers, especially WCC (Figure 6.5). Accordingly, a diagnostic model consisting of a combination of ADAMDEC1 with CSF leukocytes, protein, lactate, and glucose levels did not provide extra values to the diagnostic model using a combination of these four parameters (Figure 6.6).



BM vs	ADAMDEC1 cut-off (ng/ml)	AUROC (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	DOR
TBM	75.86	0.80 (0.73-0.88)	0.60 (0.47-0.71)	0.90 (0.84-0.94)	13.57 (6.20-29.67)
VME	13.03	0.92 (0.87-0.97)	0.93 (0.86-0.97)	0.89 (0.78-0.94)	112.62 (35.96-352.74)
Others&NI	13.12	0.93 (0.88-0.98)	0.97 (0.90-0.99)	0.89 (0.78-0.94)	326.07 (65.31-1,628.00)
Non-BM	18.66	0.87 (0.82-0.92)	0.79 (0.74-0.84)	0.87 (0.77-0.93)	25.18 (8.80-72.06)

Figure 6.4: Diagnostic performance of ADAMDEC1 in the validation cohort.

Note to Figure 6.4: Others: patients with other CNS infections (CM, anti-NMDAR encephalitis, neurotoxoplasmosis, or EM), NI: non-CNS infections, Non-BM: non bacterial meningitis

Table 6.2: Diagnostic performance of existing and candidate biomarkers and their combinations.

BM vs. non-BM	AUROC (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	DOR (95%CI)
WCC (cells per mm^3)	0.89 (0.84-0.93)	0.91 (0.87-0.94	0.74 (0.62-0.83)	81.81 (28.59-234.11)
Lactate (nmol/L)	0.88 (0.83-0.93)	0.81 (0.76-0.85)	0.74 (0.62-0.83)	31.26 (10.92-89.45)
Protein (g/L)	0.75 (0.69-0.82)	0.61 (0.56-0.67)	0.77 (0.66-0.86)	4.69 (1.64-13.42)
CSF/blood glucose ratio	0.70 (0.62-0.78)	0.60 (0.55-0.66)	0.66 (0.54-0.77)	3.50 (1.22-10.02)
ADAMDEC1 (ng/mL)	0.87 (0.82-0.93)	0.79 (0.74-0.84)	0.87 (0.77-0.93)	25.18 (8.80-72.06)
WCC+lactate+protein+CSF/blood glucose ratio	0.93 (0.88-0.97)	0.87 (0.83-0.91)	0.89 (0.78-0.94)	53.55 (22.73-126.18)
WCC+lactate+protein+CSF/blood glucose ratio + ADAMDEC1	0.93 (0.89-0.97)	0.90 (0.86-0.93)	0.87 (0.77-0.93)	60.08 (26.12-138,17)

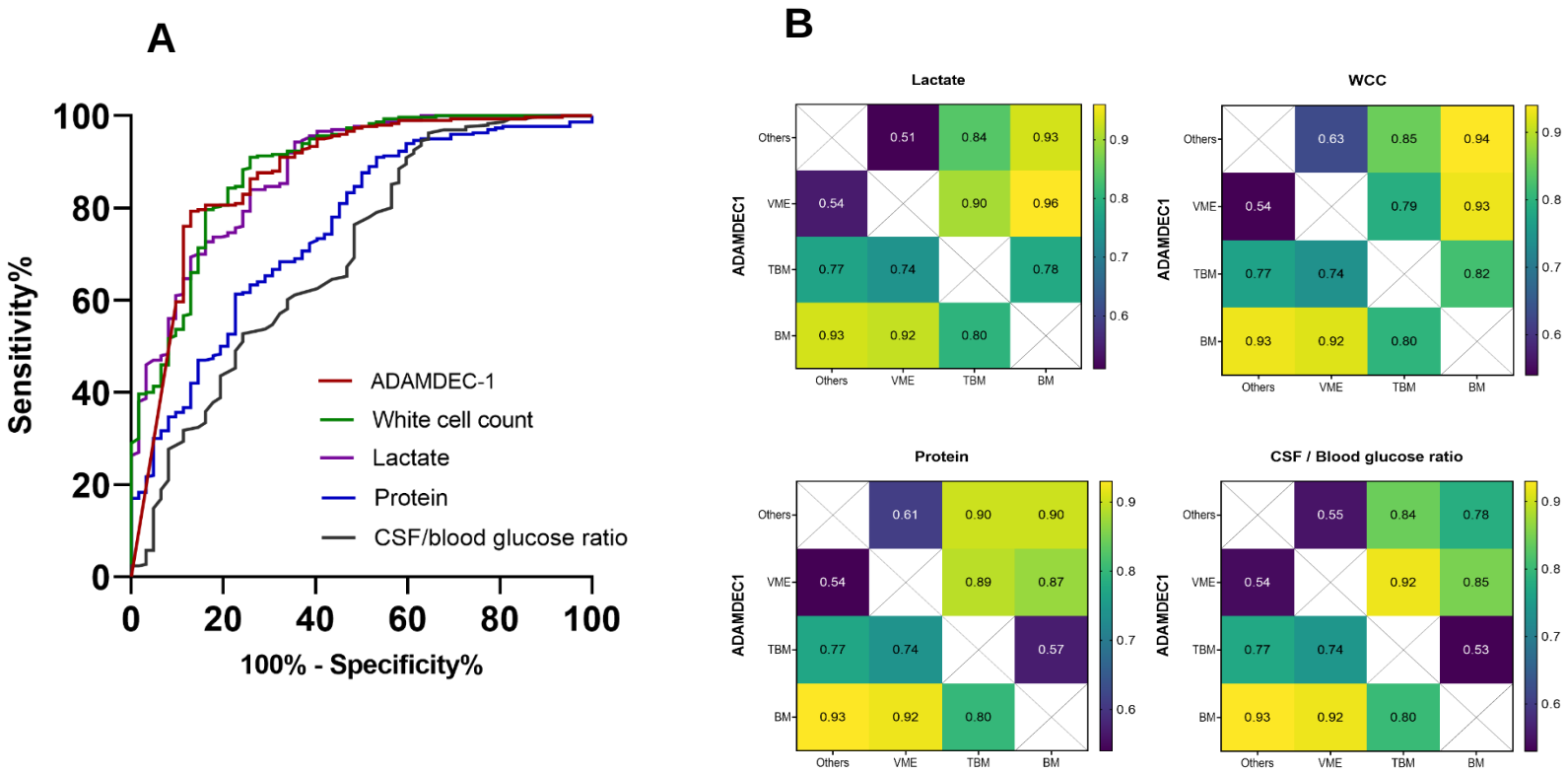


Figure 6.5: Diagnostic values of ADAMDEC1 in predicting bacterial meningitis in comparison with existing CSF parameters and novel candidate biomarkers.

(**A**) AUROC curve of ADAMDEC1 in comparison of existing CSF parameters in distinguishing between bacterial meningitis with other CNS infections (TBM, encephalitis, anti-NMDAR encephalitis, cryptococcal meningitis, neurotoxoplasmosis, eosinophilic meningitis or non-CNS infections), (**B**) AUCROC values of subgroup analyses

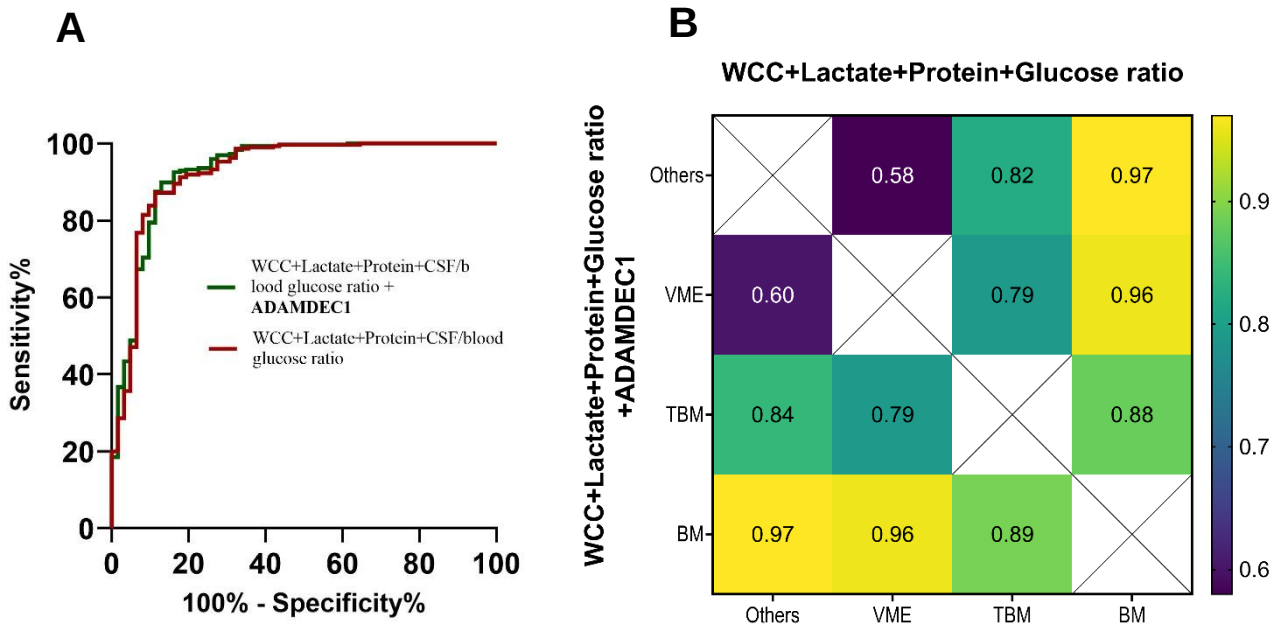


Figure 6.6: Diagnostic values of combination diagnostic models for discriminating bacterial meningitis from other groups of CNS infection and other diagnosis.

(A) AUROC of combination of existing protein markers and their combination with ADAMDEC1

(**B**) AUCROC values of subgroup analyses

6.4 Discussion and conclusion

Informed by the results of the original mass-spectrometry based discovery work (318). In this Chapter, I performed specific ELISA analysis to assess the diagnostic value potential of ADAMDEC1 in patients with CNS infections. The results of the verification phase confirmed the original findings using mass-spectrometry, demonstrating that ADAMDEC1 could be a candidate diagnostic biomarker for TBM patients. However, additional work on a larger cohort of the validation cohort failed to replicate the original findings. ADAMDEC1 level was highest in patients with BM of the validation but not in those with TBM as suggested by the results of the verification phase. Importantly, ADAMDEC1 did not provide any extra diagnostic values as compared to the routine CSF biomarkers (WCC lactate, glucose, and protein) in distinguishing between BM and non-BM patients.

There are several likely reasons for the discrepancy between the original mass spectrometry findings and the results in validation phase of ADAMDEC1. The low level of ADAMDEC1 in CSF samples of BM patients of the discovery phase could be explained by the small sample size of this cohort (248,324). Another contributing factor could be the quality of the CSF samples after a long-term storage and shipment to the UK for mass-spectrometry analysis (325).

In conclusion, in this Chapter I showed that ADAMDEC1 could be a biomarker for prediction of BM in Vietnamese adults but it did not offer any extra diagnostic values as compared to current routine CSF biomarkers. My findings emphasise the importance of conducting a proper validation step following the initial findings of the discovery and verification phases of the biomarker discovery work.

Chapter 7
Summary and Future Directions

7.1 Findings of this thesis

My PhD thesis consists of a series of studies, conducting at the clinical laboratories of OUCRU, and the brain infections department of HTD in Ho Chi Minh City, Vietnam. HTD is a tertiary referral hospital for Southern Vietnam with a population of over 40 million. The project aimed at improving the diagnosis in Vietnamese adults with CNS infection syndromes, using a combination of conventional diagnostic assays coupled with advanced technologies, including next-generation sequencing and mass-spectrometry. The results are presented in Chapters 2-6 and are summarised below.

In **Chapter 2**, I investigated the causes and epidemiology of 581 Vietnamese adults presenting with CNS infection syndromes enrolled in the clinical study over three years, September 2017 and September 2020. Of the enrolled patients, TBM was the most common clinical entity, accounting for 32.4% (188/581) of cases, followed by VME (29.6%, 172/581) and BM (22.9%, 133/581). Extensive diagnostic workup using combination of routine assays and enhanced testing for a wide range of pathogens could identify a cause in 58.7% (341/581) of the study participants. Diverse infectious agents encompassing bacteria, viruses, fungi, and parasites were detected. Of the detected bacterial pathogens, *S. suis* was the major cause of BM, accounting for 29.3% (39/133), whereas HSV and VZV were the main causes of VME, accounting for 9.0% (20/221) and 5.0% (11/ 221) of the encephalitis cases, respectively. Alongside the detection of infectious agents, anti-NMDAR encephalitis was detected in 21.3% (47/221) of patients with clinically suspected VME, exceeding the detection rates of HSV, VZV, Dengue and JEV combined in clinically suspected VME patients. Patients with CNS infection syndromes came from all provinces in southern Vietnam and were admitted to my hospital throughout the year. Only TBM patients exhibited clear annual peaks in February-March and October-November.

In **Chapter 3**, I explored the available meta-clinical data to answer the research question about the clinical and laboratory features, and outcomes associated with specific causes of CNS infection syndromes in Vietnamese adults. The results demonstrated that CNS infection syndromes in my setting were associated with diverse but overlapped clinical features, and substantial morbidity and mortality. Fever, headache, neck stiffness and consciousness reduction were the common features found in all clinical entities, but they can hardly be used for accurate differential diagnosis. Poor hospital outcomes (death and moderate to severe sequelae combined) were recorded in 45.6% (264/579) of the study patients, in part likely attributable to the high detection rates of (multi-) drug resistant bacterial pathogens, for example, *S. suis, S. pneumoniae*, *E. coli* and *M. tuberculosis*. Additionally, I conducted 6- and 12-month follow-up in those with a confirmed anti-NMDAR encephalitis diagnosis after discharge aiming to understand more about this disease and its burden, which may help inform future research directions. Notably, residual disability was recorded in 7/15 (46.7%) NMDAR encephalitis patients successfully followed up at 12 months after discharge. Collectively, the results of Chapters 2 and 3 have provided significant insights into the epidemiology, causes, clinical features and (long-term) outcomes in Vietnamese adults with CNS infection syndromes. Yet, despite extensive diagnostic work-up, the causes were established in only 58.7% of the 581 included patients.

Therefore, in subsequent Chapters (4-6), I aimed to address the diagnostic challenge of CNS infection syndromes. In **Chapter 4,** I developed (phase 1) and prospectively evaluated (phase 2) an internally controlled metagenomic pipeline for simultaneous detection of both bacterial and viral causes of CNS infections. In phase 1, I demonstrated that my established workflow could simultaneously detect both bacteria other than *M. tuberculosis* and viruses in a single experiment, with comparable yields when operated on Illumina MiSeq and ONT MinION sequencers. Sequences related to common contaminants and commensal viruses/bacteria were also detected. The overall sensitivity and specificity of our metagenomics workflow for pathogen

identification relative to the conventional assays was 70% and 100%, respectively. While the primary focus of the pipeline was to identify the causative agents, the obtained sequences could also be used to comprehensively assess the presence of antimicrobial resistance conferring mutations, and/or evolution of the pathogens. Additionally, the performance of the assay was internally monitored with the use of non-human viruses (PhHV and EAV), spiked with CSF samples prior to nucleic acid isolation to control the quality of the whole procedure. In phase 2, my established assay successfully idenditified *Klebsiella pneumoniae* infection in a meningititis patient of unknown cause. Collectively, the findings of Chapter 4 emphasised that metagenomics could not replace conventional diagnostic assays but could provide a complementary approach that can improve (novel) pathogen identification, especially when routine diagnosis fails to identify a causative agent. In interpretation of the results, care must be taken not to overestimate the contribution of commensal viruses and/or contaminants to the disease under investigation, underscoring the importance of a close cooperation between laboratory scientists and treating physicians.

Efforts to tackle the diagnostic challenges of CNS infection syndromes continued in **Chapters 5 and 6.** Specifically, I evaluated the diagnostic performance of two potential novel CSF biomarkers, LCN2 and ADAMDEC1 initially discovered in CSF of patients with CNS infection syndromes using mass-spectrometry. In **Chapter 5**, my collective findings using LCN2 data obtained from a total of 791 patients with CNS infection syndromes (validation cohort 1: n=364 and cohort 2: n=427) showed that LCN2 could accurately discriminate BM from other clinical entities of CNS infections syndromes, including TBM, VME, EM, CM and auto-immune encephalitis, with AUROC of 0.93 (95% CI: 0.90-0.95), corresponding to the sensitivity of 0.86 (95% CI: 0.83-0.89), the specificity of 0.86 (95% CI: 0.80-0.90) and the DOR of 37.74 (95% CI: 19.97-71.29). In comparative analysis, LCN2 outperformed existing CSF parameters (leukocytes, protein, lactate, and glucose) in discriminating BM from other clinical groups of CNS infection syndromes. Accordingly, a diagnostic model combining LCN2 with

leukocytes, protein, lactate, and glucose gave a higher diagnostic value for BM patients as compared to the model using those 4 routine CSF parameters, AUROC: 0.94 (95% CI: 0.92-0.96) vs. AUROC: 0.91 (95% CI: 0.88-0.94). Collectively, LCN2 can act as an independent diagnostic biomarker for discriminating BM from other entities of CNS infection syndromes or in combination with other CSF parameters.

My analysis in **Chapter 6** however showed that ADAMDEC1 was not a useful marker in discriminating between patients with CNS infection syndromes. Indeed, the diagnostic model consisting of a combination of CSF ADAMDEC1 with leukocytes, protein, lactate, and glucose did not provide additional diagnostic values in discriminating between BM and other clinical groups as compared to the diagnostic model using routine CSF parameters (leukocytes, protein, lactate, and glucose), AUROC: 0.93, 95% CI: 0.89-0.97 vs AUROC: 0.93, 95% CI: 0.88-0.97). These findings, albeit unexpected, emphasise the importance of conducting extensive validation experiments after the initial discovery phase in mass spectrometry-based biomarker discovery work.

Collectively, my PhD research has provided significant insights into the causes, epidemiological, clinical and laboratory features, and outcome associated with CNS infection syndromes in Vietnamses adults. The project has also advanced our knowledge about the application of novel approaches, especially metagenomics and mass spectrometry-based proteomics, in clinical practice and pandemic prepareness in Vietnam, a low- and middle income country.

7.2 Limitations of the thesis

Despite the novel findings outlined above, there are still several limitations in my study. Firstly, the patients were recruited only at a referral hospital in Ho Chi Minh, and the study population was restricted to adults, as the result, the data in this study might not well be generalised for a wider patient population with CNS infections in Vietnam. Secondly, in terms of diagnosis, although metagenomics did not reveal any rickettsial pathogens or leptospirosis, these

are potentially important zoonotic causes of CNS infections in Vietnam and should be screened by specific and sensitive PCR testing. Additionally, I only tested *A cantonensis*, leaving the contribution of the other parasites such as *Baylisascaris procyonis* and *Gnathostoma spinigerum* to the burden of eosinophilic meningitis in Vietnam remained unknown. Likewise, in patients with clinically suspected autoimmune encephalitis, testing for antibodies against other receptor types (such as anti-glutamate receptor type AMPA, anti-LGI1, anti-GAPA, anti-DPPX, anti-CASPR2) should be conducted. Thirdly, for assessment of discharge and long-term outcomes of anti-NMDAR encephalitis, a control group was not included for comparison. In addition, the modified Rankin scale was used for assessment but mRS only provides basic information about physical disability but not about emotional problems or ability to return to work. An alternative multidimensional assessment could be used for further evaluation of recovery. Fourthly, my study represents one of the first to conduct real-time metagenomic investigations in a clinical setting in LMICs. However, my study only focused on bacterial and viral etiologies while other causes of CNS infections including *M. tuberculosis*, parasites, and fungi were not investigated. Also, I did not explore the utility potential of non-CSF samples (urine and plasma) in patients presenting with meningoencephalitis, leaving the detection of causative agents in these samples unknown. Lastly, the sample size of the discovery cohort in the proteomics pipeline were small and the samples were stored for a long time that may affect the chance of finding novel markers. Lastly, the validation phase was conducted at a single major tertiary referral hospital and only focused on Vietnamese adults. Collaboration with other research groups would be worth examining the diagnostic accuracy of LCN2 in other settings and the utility potential of LCN2 in pediatric CNS infections.

7.3 Future directions

The findings of this study have also opened up new research opportunities for CNS infection syndromes in Vietnam that I will discuss herein.

Anti-NMDAR encephalitis and autoimmune encephalitis more broadly have been recognised as important differential diagnoses in patients with encephalitis worldwide. Therefore, clinicians should be aware of this new clinical entity, and testing for autoimune encephalitis, in particular anti-NMDAR encephalitis, should be considered in patients with clinical signs and symptoms suggestive of anti-NMDAR encephalitis to avoid extensive diagnostics and delay in treatment. Future studies should also look into other types of autoimmune encephalitis such as anti-glutamate receptor type AMPA, anti-LGI1, anti-GAPA, anti-DPPX, anti-CASPR2, and the prevalence of autoimmune encephalitis in Vietnamese children. Albeit beyond my expertise, clinical trials finding the optimal treatment pathways for autoimmune encephalitis patients are also urgently needed.

As exemplified by SARS-CoV-2 and other emerging pathogens, clinicians have played a key role in early recognition of new infections. Therefore, the application of metagenomics in clinical setting would undoudtedly be critial to pandemic preparedness and response. However, little is known about the extent to which metagenomics might help improve clinical outcome of life-threatening infections, this should be addressed in future studies. Of equal importance is to establish standard criteria for accreditation of metagenomic laboratories, which is currently not available.

LCN2 is a sensitive and specific biomarker for discriminating bacterial meningitis from a broad spectrum of other central nervous system infections. Future studies should assess the utility of LCN2 in other settings, especially in children. My group and I have started to address this through an ongoing collaboration with Professor Shaun Morris at Department of Pediatrics, University of Toronto, Canada, looking into the diagnostic values of LCN2 in BM in children. And we will discuss with colleagues within the Wellcome

funded AAP network, especially in Laos (15,24,326), to further study the performance of LCN2 in the diagnosis of CNS infections more broadly in Southeast Asia. Additionally, a clinically qualified point-of-care test of LCN2 that can be used at the bedside might be helpful for clinicians in making early diagnosis; discussion is ongoing with collaborators at the DxDhub in Singapore to develop a LCN2 rapid test. Of equal importance is to assess the extent to which LCN2 might help inform the admission or withdrawal of antimicrobial therapies, thereby improving the antibiotic stewardship.

Advances in MS instruments and workflows have greatly accelerated the process of biomarker discovery. These findings have revealed significant insights into pathophysiology, and potentially offered new diagnostic approaches to life-threatening infections (247,327–329). Novel biomarker discovery studies should be continued with larger sample sizes to increase the chance of finding novel markers and the robustness of the discovery workflows.

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Appendices

Appendix A: Supplementary materials for Chapter 2

Table S2.1 Laboratory-based evidence for CNS infection confirmation

Diagnosis group	First-line diagnosis	Enhanced diagnostic testing
Bacterial meningitis	Bacterial culture, and/or Gram stain	Real-time PCR
Tuberculous meningitis	Ziehl-Neelsen stain, and/or GeneXpert and/or MGIT*	NA
Viral meningoencephalitis	HSV and VZV PCR JEV IgM and Dengue virus IgM	Dengue, JEV, Enterovirus and Mumps virus PCR
Cryptococcal meningitis	India Ink stain, and/or Cryptococcal FLA and/or fungal culture	NA
Cerebral toxoplasmosis	Brain imaging and serology	NA
Eosinophilic meningitis	Hematology	*Angiostrongylus cantonensis PCR*
Auto-immune encephalitis	ND	IgG against NMDA receptor

*Mycobacteria Growth Indicator Tubes

Table S2.2: The frequency of diagnosis at discharge among 98 patients without CNS infection syndromes in the cohort of 679 patients of the clinical study

Syndrome	Number of cases n (%)
Sepsis	22 (22.4)
Post viral infection syndrome	18 (18.4)
Psychiatric disorder	10 (10.2)
Epileptic	8 (8.2)
Pneumonia	5 (5.1)
Alcoholic	3 (3.1)
Toxic	3 (3.1)
Cerebral tumor	2 (2.0)
Guillain Barre	2 (2.0)
Hypertension	2 (2.0)
Myasthenia gravis	1 (1.0)
Opioid disorder	1 (1.0)
Cerebral infarction	1 (1.0)
Post malaria neurological syndrome	1 (1.0)
Cirrhosis	1 (1.0)
Endocarditis	1 (1.0)
Spleen abscess	1 1.0)
Wilson	1 1.0)
Others	15 (15.3)

Appendix B: Supplementary materials for Chapter 3

Table S3.1: Baseline characteristics, clinical and laboratory findings, treatment, and outcomes of patients with confirmed and suspected BM, TBM and VME.

Characteristics	BM				TBM				VME			
	Whole group (N=-133)	Confirmed (N=78)	Suspected (N=55)	*P* value	Whole group (N=188)	Confirmed (N=142)	Suspected (N=46)	*P* value	Whole group (N=172)	Confirmed (N=40)	Suspected (N=132)	*P* value
Demographics												
Median age in years (IQR)	55 (41-65)	55 (42-63)	56 (37-70)	0.716	40 (31-53)	39 (29-52)	43 (34-61)	0.123	32 (23-45)	35 (25-53)	31 (23-45)	0.249
Male, n (%)	86 (64.7)	51 (65.4)	35 (63.6)	0.856	139 (73.9)	106 (74.6)	33 (71.7)	0.696	110 (64.0)	30 (75.0)	80 (60.6)	0.097
HCMC origin, n (%)	33 (24.8)	13 (16.7)	20 (36.4)	0.014	40 (21.3)	34 (23.9)	6 (13.0)	0.116	44 (25.6)	10 (25.0)	34 (25.8)	0.923
Clinical findings												
Median illness day on admission, n (IQR)	4 (2-6)	3 (2-5)	5 (3-9)	0.013	12 (8-18)	13 (9-20)	9 (8-15)	0.044	6 (4-7)	5 (4-7)	6 (3-8)	0.815
Positive HIV test, n (%)	0	0	0		40 (21.3)	37 (26.1)	3 (6.5)	0.004	1 (0.6)	1 (2.5)	0 (0.0)	0.069
Fever, n (%)	122 (91.7)	72 (92.3)	50 (90.9)	0.101	181 (96.3)	137 (96.5)	44 (95.7)	0.748	159 (92.4)	36 (90.0)	123 (93.2)	0.285

Characteristics	BM				TBM				VME			
	Whole group (N=-133)	Confirmed (N=78)	Suspected (N=55)	*P* value	Whole group (N=188)	Confirmed (N=142)	Suspected (N=46)	*P* value	Whole group (N=172)	Confirmed (N=40)	Suspected (N=132)	*P* value
Weight loss, n (%)	5 (3.8)	2 (2.6)	3 (5.5)	0.106	69 (36.7)	53 (37.3)	16 (34.8)	0.362	7 (4.1)	1 (2.5)	6 (4.5)	0.715
Sweat, n (%)	2 (1.5)	2 (2.6)	0 (0.0)	0.103	23 (12.2)	16 (11.3)	7 (15.2)	0.110	3 (1.7)	0 (0.0)	3 (2.3)	0.583
Cough > 2 weeks, n (%)	2 (1.5)	1 (1.3)	1 (1.8)	0.816	23 (12.2)	16 (11.3)	7 (15.2)	0.650	5 (2.9)	2 (5.0)	3 (2.3)	0.638
Headache, n (%)	109 (82.0)	69 (88.5)	40 (72.7)	0.034	174 (92.6)	132 (93)	42 (91.3)	0.884	123 (71.5)	24 (60.0)	99 (75.0)	0.102
Consciousness reduction, n (%)	124 (93.2)	72 (92.3)	52 (94.5)	0.680	134 (71.3)	108 (76.1)	26 (56.5)	0.011	147 (85.5)	37 (92.5)	110 (83.3)	0.202
Local seizure, n (%)	3 (2.3)	3 (3.8)	0 (0.0)	0.337	2 (1.1)	1 (0.7)	1 (2.2)	0.263	17 (9.9)	4 (10.0)	13 (9.8)	0.885
General seizure, n (%)	7 (5.3)	3 (3.8)	4 (7.3)	0.242	5 (2.7)	4 (2.8)	1 (2.2)	0.349	64 (37.2)	16 (40.0)	48 (36.4)	0.749
Psychosis, n (%)	12 (9.0)	6 (7.7)	6 (10.9)	0.529	5 (2.7)	3 (2.1)	2 (4.3)	0.728	28 (16.3)	7 (17.5)	21 (15.9)	0.737
Language change, n (%)	18 (13.5)	8 (10.3)	10.18.2)	0.347	12 (6.4)	11 (7.7)	1 (2.2)	0.203	17 (17.0)	4 (10.0)	13 (9.8)	0.834
Hemiplegia, n (%)	7 (5.3)	5 (6.4)	2 (3.6)	0.759	17 (9.0)	16 (11.3)	1 (2.2)	0.088	7 (4.1)	5 (12.5)	2 (1.5)	0.008
Limb weakness (Paraplegia/ Tetraplegia), n	4 (3.0)	2 (2.6)	1 (1.8)	0.775	20 (10.6)	14 (9.9)	1 (2.2)	0.131	11 (6.4)	2 (5.0)	9 (6.8)	0.681

Characteristics	BM				TBM				VME			
	Whole group (N=-133)	**Confirmed (N=78)**	**Suspected (N=55)**	***P* value**	**Whole group (N=188)**	**Confirmed (N=142)**	**Suspected (N=46)**	***P* value**	**Whole group (N=172)**	**Confirmed (N=40)**	**Suspected (N=132)**	***P* value**
(%)												
Convulsion, n (%)	3 (2.3)	2 (2.6)	1 (1.8)	0.775	3 (1.6)	2 (1.4)	1 (2.2)	0.571	29 (16.9)	5 (12.5)	24 (18.2)	0.478
Stiff neck, n (%)	107 (80.5)	65 (83.3)	42 (76.4)	0.608	103 (54.8)	73 (54.1)	30 (65.2)	0.323	69 (40.1)	15 (37.5)	54 (40.9)	0.126
Abnormal movement												
Face-mouth-tongue	0 (0.0)	0 (0.0)	0 (0.0)		2 (1.1)	0 (0.0)	2 (4.3)	0.024	17 (9.9)	4 (10.0)	13 (9.8)	0.665
Trunk	0 (0.0)	0 (0.0)	0 (0.0)		1 (0.5)	1 (0.7)	0 (0.0)	0.435	11 (6.4)	2 (5.0)	9 (6.8)	0.177
Extremity	8 (6.1)	2 (2.6)	0 (0.0)	0.476	4 (2.1)	4 (2.8)	0 (0.0)	0.366	10 (5.8)	3 (7.5)	7 (5.3)	0.163
Glasgow Coma Scale ≤ 9, n (%)	25 (19.7)	20 (25.6)	5 (9.1)	0.032	31 (16.8)	28 (19.7)	3 (6.5)	0.091	45 (26.2)	10 (25.0)	35 (26.5)	0.258
CSF findings												
Opening pressure cmCSF, median (IQR)	22 (16-30)	23 (18-36)	18 (14-23)	**<0.001**	20 (14-27)	20 (14-28)	18 (15-22)	0.338	16 (13-20)	18 (14-22)	16 (12-19)	0.047
CSF Hematology												
WCC/mm^3, median (IQR)	2,215 (630-5,628)	3,806 (942-7,666)	1,010 (369-3,993)	**0.003**	286 (142-493)	273 (153-503)	320 (139-475)	0.886	49 (11-187)	139 (28-420)	37 (9-138)	0.003

Characteristics	BM				TBM				VME			
	Whole group (N=-133)	Confirmed (N=78)	Suspected (N=55)	*P* value	Whole group (N=188)	Confirmed (N=142)	Suspected (N=46)	*P* value	Whole group (N=172)	Confirmed (N=40)	Suspected (N=132)	*P* value
Neutrophil %, median (IQR)	82 (67-92)	87 (77-94)	74 (42-87)	**<0.001**	27 (13-60)	34 (17-67)	14 (11-31)	**<0.001**	16 (12-33)	13 (11-18)	17 (12-38)	0.010
Lymphocyte %, median (IQR)	18 (8-33)	13 (6-23)	26 (13-58)	**<0.001**	72 (40-87)	64 (33-83)	86 (69-88)	**<0.001**	83 (63-88)	87 (83-89)	80 (52-88)	0.001
Biochemistry												
Protein g/L, median (IQR)	2.7 (1.2-5.1)	4.0 (1.7-6.1)	1.5 (1.0-3.1)	**<0.001**	1.8 (1.1-2.4)	1.8 (1.2-2.4)	1.7 (1.0-2.4)	0.630	0.6 (0.4-1.0)	0.7 (0.5-1.3)	0.6 (0.3-1.0)	0.027
Lactate nmol/L, median (IQR)	10.2 (5.0-15.1)	14.1 (10.0-17.4)	5.6 (3.8-8.8)	**<0.001**	5.2 (3.8-7.5)	5.5 (3.9-7.8)	4.5 (3.6-5.7)	0.021	2.6 (2.1-3.2)	2.6 (2.2-3.2)	2.7 (2.1-3.3)	0.575
CSF: blood glucose ratio, median (IQR)	0.3 (0.1-0.5)	0.1 (0.0-0.3)	0.5 (0.4-0.6)	**<0.001**	0.3 (0.2-0.4)	0.3 (0.2-0.4)	0.4 (0.3-0.5)	0.009	0.6 (0.6-0.7)	0.6 (0.5-0,7)	0.7 (0.6-0.7)	0.032
Interventions												
Ventilation, n (%)	18 (13.5)	13 (16.7)	5 (9.1)	0.233	24 (12.8)	21 (14.8)	3 (6.5)	0.253	35 (20.3)	5 (12.5)	30 (22.7)	0.078
Sedative drug administration, n (%)	24 (18.0)	11 (14.1)	13 (23.6)	0.169	20 (10.6)	16 (11.3)	4 (8.7)	0.629	40 (23.3)	8 (20.0)	32 (24.2)	0.798
TB treatment, n (%)	4 (3.0)	2 (2.6)	2 (3.6)	0.903	174 (92.6)	134 (94.4)	40 (87.0)	0.096	5 (2.9)	1 (2.5)	4 (3.0)	0.904
Acyclovir, n (%)	7 (5.3)	3 (3.8)	4 (7.3)	0.558	9 (4.8)	3 (2.1)	6 (13.0)	0.009	116 (67.4)	34 (85.0)	82 (62.1)	0.007

Characteristics	BM				TBM				VME			
	Whole group (N=-133)	Confirmed (N=78)	Suspected (N=55)	*P* value	Whole group (N=188)	Confirmed (N=142)	Suspected (N=46)	*P* value	Whole group (N=172)	Confirmed (N=40)	Suspected (N=132)	*P* value
Antibiotics, n (%)	130 (97.7)	77 (98.7)	53 (96.4)	0.569	64 (34.0)	45 (31.7)	19 (41.3)	0.283	70 (40.7)	18 (45.0)	52 (39.4)	0.583
Outcome												
Duration of hospitalisation in days, median (IQR)	16 (12-23	15 (11-24)	17 (12-22)	0.740	27 (3-40)	27 (3-40)	26 (3-42)	0.626	11 (7-19)	19 (13-25)	10 (6-14)	<0.001
Rankin scale, n (%)				0.257				0.338				0.352
0	47 (35.6)	23 (29.9)	24 (43.6)		31 (16.5)	20 (14.1)	11 (23.9)		68 (39.5)	10 (25.0)	58 (43.9)	
1-2	34 (25.8)	19 (24.7)	15 (27.2)		51 (27.2)	40 (28.2)	11 (23.9)		52 (30.2)	14 (35.0)	38 (28.8)	
3-4	36 (37.2)	26 (22.5)	10 (18.2)		43 (22.9)	33 (23.3)	10 (21.8)		29 (16.8)	9 (22.5)	20 (15.1)	
5	9 (6.8)	5 (6.5)	4 (7.3)		47 (25.0)	36 (25.4)	11 (23.9)		21 (12.2)	7 (17.5)	14 (10.6)	
6	6 (4.5)	4 (5.2)	2 (3.6)		16 (8.5)	13 (9.2)	6 (6.5)		2 (1.2)	0 (0.0)	2 (1.5)	

Appendix C: Supplementary materials for Chapter 4

Table S4.1: Diagnostic tests carried out as part of routine care and/or as per the study protocol when appropriate.

Test	Reference
Gram stain	Standard procedure
Bacterial culture	Standard procedure
Ziehl-Neelsen staining	Standard procedure
GenXpert	Standard procedure
MGIT	Standard procedure
S. suis PCR	(330)
S. pneumoniae PCR	(331)
N. meningitidis PCR	(331)
HSV PCR	(332)
VZV PCR	(333)
DENV PCR	(334)
JEV PCR	(335)
Flavivirus PCR	(336)
Enterovirus PCR	(337)
Influenza A virus	(338)
Angiostrongylus cantonensis PCR	(256)
Cryptococcal FLA	Standard procedure
DENV serology	(215)
JEV serology	(215)
Anti-NMDAR	EUROIMMUN, Luebeck, Germany

Table S4.2: The frequency of clinical conditions among 25 patients with non-CNS infections in the development phase of mNGS pipeline

Clinical conditions	Number of cases, n (%)
Sepsis	6 (24)
Epilepsy	3 (12)
Cerebral infarction	3 (12)
Alcoholic	3 (12)
Post viral infection syndrome	2 (8)
Guillain Barre	1 (4)
Myasthenia gravis	1 (4)
Spleen abscess	1 (4)
Wilson	1 (4)
Cirrhosis	1 (4)
Urinary tract infections	1 (4)
Psychiatric disorder	1 (4)
Toxicity	1 (4)

Table S4.3: Agreement between MiSeq- and MinION-based metagenomic workflows

CSF No	Diagnosis at discharge	Routine diagnostics		MiSeq	MinION
		Diagnosed by	Aetiology	Results	Results
1	Confirmed BM	Culture	*N. meningitidis*	Not detected	Not detected
2	Confirmed BM	Culture	*E. coli*	Not detected	Not detected
3	Confirmed BM	Gram stain + culture	*S. angionosus* + *E. coli*	Not detected	Not detected
4	Confirmed BM	Culture	*S. suis*	*S. suis*	*S. suis*
5	Confirmed BM	Culture	*S. suis*	*S. suis*	*S. suis*
6	Confirmed BM	Gram stain + culture	*S. suis*	*S. suis*	*S. suis*
7	Confirmed VM	PCR	HSV	HSV	HSV
8	Confirmed VM	PCR	VZV	Not detected	Not detected
9	Confirmed VM	Serology and PCR	JEV	Not detected	Not detected
10	Suspected VM	NA	Not detected	Dengue	Dengue

Appendix D: Supplementary materials for Chapter 5

S5.1: Human Lipocalin-2/NGAL immunoassay procedure (Quantikine®ELISA)

ASSAY PROCEDURE

The Human Lipocalin-2 Conjugate must remain at 2-8 °C during use. Bring all other reagents and samples to room temperature before use. It is recommended that all standards and samples be assayed in duplicate.

Note: *High concentrations of Lipocalin-2 are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-52 to each well. *Assay Diluent RD1-52 may contain a precipitate. Mix well before and during use.*
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. **Incubate for 2 hours at 2-8 °C.** A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of **cold** Human Lipocalin-2 Conjugate to each well. Cover with a new adhesive strip. **Incubate for 2 hours at 2-8 °C.**
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes **at room temperature. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

Table S5.1: The frequency of clinical conditions among 70 patients with non-CNS infections for LCN2 validation phase 2

Clinical conditions	Number of cases (n)
Psychiatric disorder	14 (20.00)
Sepsis	13 (18.57)
Pneumonia	10 (14.29)
Post viral infection syndrome	10 (14.29)
Epileptic	6 (8.57)
Lupus	2 (2.86)
Pulmonary tuberculosis	2 (2.86)
Cerebral infarction	2 (2.86)
Toxic	2 (2.86)
Alcoholic	1 (1.43)
Endocarditis	1 (1.43)
Guillain Barre	1 (1.43)
Post rickettsial fever	1 (1.43)
Parkinson	1 (1.43)
Diabetes	1 (1.43)
Sinusitis	1 (1.43)
Thrombocytopenia	1 (1.43)
Unclear cause	1 (1.43)

Table S5.2: Baseline characteristics of the cohort of 427 patients in the validation phase 2 of LCN2

Characteristics	Whole group (N=427)	BM (N=109)	TBM (N=79)	VME (N=116)	Anti-NMDAR (N=32)	Eosinophilic encephalitis (N=9)	Cryptococcal meningitis (N=9)	Non-CNS infections (N=70)
Demographics								
Median age in years (IQR)	41 (27-57)	55 (38-66)	41 (29-56)	32 (24-47)	22 (18-31)	47 (37-53)	59 (36-63)	38 (27-61)
Male, n (%)	257 (60.2)	70 (64.2)	49 (62.0)	79 (68.1)	12 (37.5)	6 (66.7)	6 (66.7)	35 (50.0)
HCMC origin, n (%)	91 (21.3)	31 (28.4)	11 (13.9)	23 (19.8)	4 (12.5)	1 (11.1)	2 (22.2)	18 (25.7)
Clinical findings								
Median illness day on admission, n (IQR)	7 (4-11)	4 (2-8)	11 (8-20)	6 (4-7)	13 (7-30)	18 (12-25)	20 (10-60)	6 (3-9)
HIV positive status, n (%)	2 (0.5)	0	0	0	0	0	1 (11.1)	1 (1.4)
Fever, n (%)	393 (92.0)	100 (91.7)	75 (94.9)	111 (95.7)	27 (84.4)	7 (77.8)	8 (88.9)	62 (88.6)
Weight loss, n (%)	24 (5.6)	3 (2.8)	17 (21.5)	3 (2.6)	0	0	0	1 (1.4)
Sweat, n (%)	3 (0.7)	3 (2.8)	0	0	0	0	0	0
Cough > 2 weeks, n (%)	13 (3.0)	4 (3.7)	4 (5.1)	4 (3.4)	1 (3.1)	0	2 (22.2)	5 (5.7)
Headache, n (%)	311 (72.8)	83 (76.1)	75 (94.9)	81 (69.8)	16 (50.0)	8 (88.9)	9 (100)	70 (100)
Consciousness reduction, n (%)	386 (90.4)	102 (93.6)	64 (81.0)	104 (89.7)	32 (100)	8 (88.9)	8 (88.9)	65 (92.9)
Local seizure, n (%)	32 (7.4)	4 (3.7)	1 (1.3)	14 (12.1)	5 (15.6)	0	0	8 (11.4)
General seizure, n (%)	84 (19.7)	10 (9.2)	1 (1.3)	47 (40.5)	8 (25.0)	0	1 (11.1)	17 (24.3)
Psychosis, n (%)	76 (17.8)	12 (11.0)	5 (6.3)	15 (12.9)	26 (81.3)	1 (11.1)	2 (22.2)	15 (21.4)

Characteristics	Whole group (N=427)	BM (N=109)	TBM (N=79)	VME (N=116)	Anti-NMDAR (N=32)	Eosinophilic encephalitis (N=9)	Cryptococcal meningitis (N=9)	Non-CNS infections (N=70)
Language change, n (%)	99 (23.2)	15 (13.8)	17 (21.5)	23 (19.8)	20 (62.5)	3 (33.3)	1 (11.1)	18 (25.7)
Movement disorder, n (%)	58 (13.6)	3 (2.8)	3 (3.8)	19 (16.4)	23 (71.9)	0	0	9 (12.9)
Hemiplegia, n (%)	27 (6.3)	8 (7.3)	6 (7.6)	5 (4.3)	1 (3.1)	0	1 (11.1)	6 (8.6)
Limb weakness (Paraplegia/ Tetraplegia), n (%)	32 (7.5)	4 (3.7)	9 (11.4)	10 (8.6)	1 (3.1)	4 (44.4)	0	4 (5.7)
Convulsion, n (%)	44 (10.3)	6 (5.5)	0	23 (19.8)	5 (15.6)	0	0	10 (14.3)
Stiff neck, n (%)	267 (61.5)	93 (85.3)	61 (77.2)	57 (49.1)	9 (28.1)	8 (88.9)	8 (88.0)	29 (41.4)
Abnormal movement, n (%)								
Face-mouth-tongue	49 (11.5)	1 (0.9)	0	16 (13.8)	19 (59.4)	1 (11.1)	0	11 (15.7)
Trunk	15 (3.5)	0	0	8 (6.9)	5 (15.6)	0	0	2 (2.9)
Extremity	20 (4.7)	1 (0.9)	0	6 (5.2)	10 (31.3)	0	0	3 (4.3)
Glasgow Coma Scale ≤ 9, n (%)	92 (21.5)	21 (19.3)	13 (16.5)	32 (27.6)	12 (37.5)	0	2 (22.2)	12 (17.1)
CSF investigations								
CSF Hematology								
Log (10) WCC/mm^3, median (IQR)	2.2 (1.3-2.9)	3.5 (2.8-3.9)	2.4 (2.2-2.7)	1.9 (1.3-2.3)	1.2 (0.9-1.4)	2.6 (2.5-2.9)	2.5 (2.4-2.7)	0.7 (0.3-1.4)
Neutrophil %, median (IQR)	34 (14-75)	84 (69-92)	31 (15-59)	18 (12-38)	14 (12-28)	16 (14-26)	34 (24-49)	26 (14-60)
Lymphocyte %, median (IQR)	65 (24-85)	15 (8-30)	69 (42-85)	82 (60-88)	86 (70-88)	59 (42-65)	66 (51-76)	73 (40-85)
Eosinophil%, median (IQR)	0	0	0	0	0	26 (12-39)	0	0

Characteristics	Whole group (N=427)	BM (N=109)	TBM (N=79)	VME (N=116)	Anti-NMDAR (N=32)	Eosinophilic encephalitis (N=9)	Cryptococcal meningitis (N=9)	Non-CNS infections (N=70)
Biochemistry								
Protein g/L, median (IQR)	1.0 (0.5-2.3)	2.8 (1.2-5.3)	1.8 (1.2-2.5)	0.6 (0.4-1.3)	0.3 (0.2- 0.4)	1.0 (0.6-1.6)	1.3 (1.0-2.4)	0.4 (0.3-0.8)
Lactate nmol/L, median (IQR)	3.8 (2.4-7.2)	11.4 (5.3-15.6)	5.7 (4.4-7.7)	2.7 (2.1-3.6)	1.9 (1.7-2.3)	3.8 (3.2-4.4)	5.6 (4.9-9.7)	2.5 (2.0-3.3)
CSF: blood glucose ratio, median (IQR)	0.5 (0.3-0.7)	0.3 (0.02-0.4)	0.3 (0.2-0.4)	0.6 (0.6-0.7)	0.7 (0.6-0.9)	0.3 (0.3-0.5)	0.1 (0.1-0.2)	0.6 (0.5-0.7)
Outcome								
Day of hospitalisation (IQR)	17 (9-30)	17 (14-26)	30 (4-42)	13 (9-22)	38 (25-56)	27 (16-28)	25 (20-32)	9 (7-17)
Rankin scale, n (%) (N=418)								
0	175/418 (41.9)	60/108 (55.6)	15/77 (19.5)	67/112 (59.8)	3/31 (9.7)	2 (22.2)	4 (44.4)	24/69 (34.8)
1-2	102/418 (24.4)	28/108 (25.9)	17/77 (22.1)	23/112 (20.6)	14/31 (45.2)	3 (33.3)	1 (11.1)	15/69 (21.7)
3-4	50/418 (11.9)	8/108 (7.4)	15/77 (19.5)	8/112 (5.4)	8/31 (25.8)	1 (11.1)	0	12/69 (17.4)
5	73/418 (17.5)	8/108 (7.4)	23/77 (29.9)	15/112 (13.4)	4/31 (12.9)	3 (33.3)	3 (33.3)	15/69 (21.7)
6	18/418 (4.3)	4/108 (3.7)	7/77 (9.1)	1/112 (0.9)	2/31 (6.5)	0	1 (11.1)	3/69 (4.3)

Appendix E: Supplementary materials for Chapter 6

S6.1: Biomarker discovery for discriminating CNS infections groups.

In the discovery phase, a total of 1012 proteins were identified by LC-MS/MS analysis of 45 CSF samples. Subsequent analysis showed that there were 729 protein biomarkers relevant to clinical diagnosis of CNS infections, especially for patients with BM and TBM. Among that, 60 protein signatures were expressed dominantly in CSFs of patients with BM diagnosis and the other 19 biomarkers were presented significantly in CSFs of patients with TBM (Table S6.3 and Table S6.4). There was no biomarker candidate identified in CSFs of patients with VME. Of the protein markers obtained, a disintegrin and metalloprotease like decysin (ADAMDEC1) was demonstrated having a potential to differentiate TBM from other groups (Figure S6.1 and Table S6.4) because ADAMDEC1 was presented merely in patients with TBM but was absent in patients with non-TBM diagnosis.

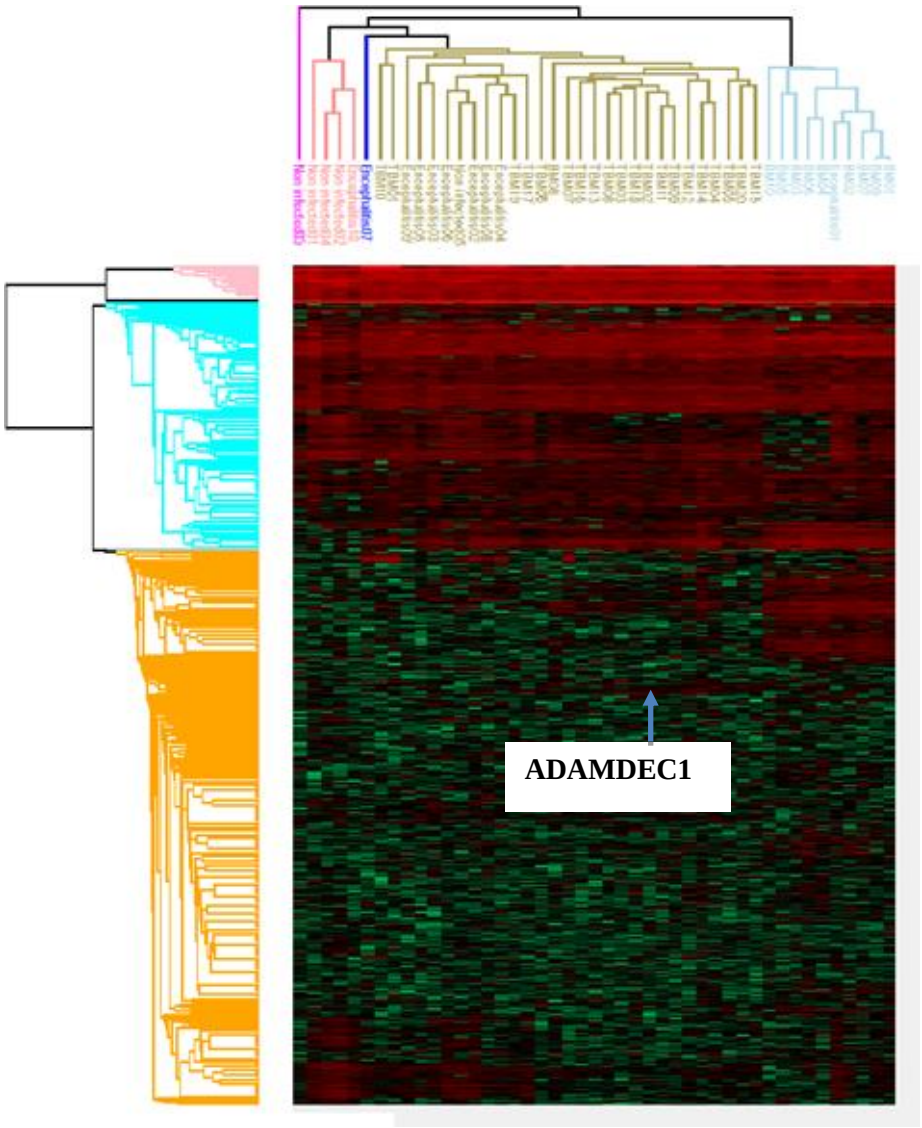

Figure S6.1: An overview of protein marker discovery phase using quantitative mass-spectrometry analysis of 45 CSFs from patients with CNS infections.

The heat map shows clustering profiles of protein/peptide markers (rows) in different clinical entities (columns). LCN2 and ADAMDEC1 are 2 candidate biomarkers for BM and TBM, respectively.

S6.2: ADAMDEC1 ELISA procedure (Avia Systems Biology)

10. Assay Procedure

- Equilibrate all reagents and materials to ambient room temperature prior to use in the procedure.
- Optimal results for intra- and inter-assay reproducibility will be obtained when performing incubation steps at 37°C as indicated below.

10.1 Determine the required number of wells and return any remaining unused wells and desiccant to the pouch.

10.2 Add 100 µL of serially titrated standards, diluted samples or blank into wells of the **Anti-ADAMDEC1 Microplate**. At least two replicates of each standard, sample or blank is recommended.

10.3 Cover the plate with the well plate lid and incubate at 37°C for 2 hours.

10.4 Remove the plate lid and discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

10.5 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.6 Add 100 µL of prepared **1X Biotinylated ADAMDEC1 Detector Antibody** to each well.

10.7 Cover with the well-plate lid and incubate at 37°C for 60 minutes.

10.8 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

10.9 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.10 Wash plate 3 times with **1X Wash Buffer** as follows:

10.10.1 Add 300 µL of **1X Wash Buffer** to each assay well.

10.10.2 Incubate for 2 minute.

10.10.3 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle.

10.10.4 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.10.5 Repeat steps 10.10.1 through 10.10.4 **two** more times.

10.11 Add 100 µL of prepared **1XAvidin-HRP Conjugate** into each well and incubate at 37°C for 60 minutes.

10.12 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

10.13 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.14 Wash plate **5 times** with **1X Wash Buffer** as in Step 10.10.

10.15 Add 90 µL of **TMB Substrate** to each well and incubate at 37°C **in the dark** for 15-30 minutes. Wells should change to gradations of blue. If the color is too deep, reduce the incubation time.

(NOTE: optimal incubation time must be determined by the user. Optimal development can be visualized by blue shading in the top four standard wells, while the remaining standards are still clear.)

10.16 Add 50 µL of **Stop Solution** to each well. Well color should change to yellow immediately. Add the **Stop Solution** in the same well order as done for the **TMB Substrate**.

10.17 Read the O.D. absorbance at 450 nm with a standard microplate reader within 5 minutes of stopping the reaction in step 10.16. If wavelength correction is available, set to 540 nm or 570 nm.

Table S6.1: Baseline characteristics of 34 patients for the verification phase of ADAMDEC1

Characteristics	BM (N=8)	TBM (N=15)	VME (N=6)	Non-CNS infections (N=5)
Demographics				
Age in years (median, IQR)	46 (28-64)	39 (30-53)	51 (19-48)	58 (43-66)
Male, n (%)	7 (87.5)	7 (46.7)	5 (83.3)	2 (40.0)
Ho Chi Minh City origin, n (%)	4 (50.0)	3 (20.0)	2 (33.3)	2 (40.0)
Illness day on admission (median, IQR)	2 (0-12)	14 (5-21)	4 (2-8)	4 (3-12)
Length of hospital stay (median, IQR)	18 (13-26)	27 (1-33)	4 (3-15)	2 (0-13)
Clinical findings				
Fever, n (%)	NA	14 (93.3)	6 (100)	5 (100)
Headache, n (%)	5 (62.5)	14 (93.3)	6 (100)	3 (60.0)
Cranial nerve palsy, n (%)	NA	3 (20.0)	0	0
Hemiplegia n (%)	NA	1 (6.7)	1 (16.7)	0
Paraplegia, n (%)	NA	0	0	0
Tetraplegia, n (%)	NA	0	0	0
Convulsions, n (%)	NA	1 (6.7)	0	0
Neck stiffness, n (%)	NA	14 (93.3)	6 (100)	4 (80.0)
GCS at enrolment (median, IQR)	NA	14 (11-15)	11 (9-13)	14 (12-15)
HIV positive, n (%)	NA	1 (6.7)	0	0
CSF examinations				

Characteristics	BM (N=8)	TBM (N=15)	VME (N=6)	Non-CNS infections (N=5)
WCC/mm^3, median (median, IQR)	20,050 (15,350-31,000)	288 (154-396)	331 (51-1,127)	5 (2-49)
Neutrophil %, median (median, IQR)	91.6 (88.6-95.4)	25.0 (14.0-44.0)	5.0 (1.0-13.0)	66.6 (20-87)
Lymphocyte %, median (median, IQR)	4.2 (3.6-5.2)	75.0 (56.0-86.0)	91.0 (42.5-96.5)	80 (13-99.9)
Biochemistry				
Protein g/L (median, IQR)	NA	2.3 (1.9-2.8)	0.9 (0.7-1.6)	0.5 (0.3-1.8
Lactate nmol/L (median, IQR)	NA	7.4 (5.4-8.1)	3.1 (2.4-3.3)	3.7 (2.2-5.6)
CSF: blood glucose ratio (median, IQR)	NA	0.18 (0.15-0.25)	0.62 (0.58-1.63)	0.65 (0.47-0.78)
Discharge mRS				
0	NA	3 (20.0)	0	1 (20.0)
1	NA	3 (20.0)	2 (33.3)	0
2	NA	3 (20.0)	0	2 (40.0)
3	NA	3 (20.0)	2 (33.3)	1 (20.0)
4	NA	0	1 (16.7)	0
5	NA	2 (13.4)	1 (16.7)	0
6	NA	1 (6.7)	0	1 (20.0)

Table S6.2: Baseline characteristics of 362 patients for the validation phase 1 of ADAMDEC1

Characteristics	Whole group (N=362)	BM (N=62)	TBM (N=122)	VME (N=92)	Anti-NMDAR (N=17)	Eosinophilic encephalitis (N=10)	Cryptococcal meningitis (N=14)	Non-CNS infections (N=43)
Demographics								
Median age in years (IQR)	40 (27-56)	57 (40-67)	41 (30-54)	31 (22-44)	25 (22-28)	30 (20-40)	36 (30-59)	48 (33-64)
Male, n (%)	242 (66.9)	42 (66.7)	97 (79.5)	54 (58.7)	9 (52.9)	4 (40.0)	10 (71.4)	25 (58.1)
HCMC origin, n (%)	88 (24.3)	12 (19.4)	29 (23.8)	27 (29.3)	3 (17.6)	2 (20.0)	4 (28.6)	10 (23.3)
Clinical findings								
Median illness day on admission, n (IQR)	7 (4-14)	4 (2-6)	11 (8-18)	6 (4-8)	22 (10-30)	26 (16-43)	18 (12-30)	5 (3-8)
HIV positive status, n (%)	25 (6.9)	0	22 (18.0)	0	0	1 (10.0)	1 (7.1)	1 (2.3)
Fever, n (%)	327 (90.3)	57 (91.9)	117 (95.9)	83 (90.2)	13 (76.5)	7 (70.0)	12 (85.7)	36 (83.7)
Weight loss, n (%)	72 (19.9)	4 (6.5)	48 (39.3)	6 (6.5)	0	3 (30.0)	6 (42.9)	3 (7.0)
Sweat, n (%)	25 (6.9)	0	18 (14.8)	3 (3.3)	1 (5.9)	1 (10.0)	0	1 (2.3)
Cough > 2 weeks, n (%)	24 (6.6)	1 (1.6)	18 (14.8)	3 (3.3)	0	1 (10.0)	0	1 (2.3)
Headache, n (%)	281 (77.6)	55 (88.7)	113 (92.6)	64 (69.6)	9 (52.9)	10 (100)	13 (92.9)	15 (34.9)
Consciousness reduction, n (%)	282 (77.9)	57 (91.9)	83 (68.0)	76 (82.6)	16 (94.1)	4 (40.0)	10 (71.4)	34 (79.1)
Local seizure, n (%)	14 (3.9)	2 (3.2)	1 (0.8)	6 (6.5)	3 (17.6)	0	0	2 (4.7)
General seizure, n (%)	60 (16.6)	3 (4.8)	4 (3.3)	28 (30.4)	8 (47.1)	0	3 (21.4)	14 (32.6)

Characteristics	Whole group (N=362)	BM (N=62)	TBM (N=122)	VME (N=92)	Anti-NMDAR (N=17)	Eosinophilic encephalitis (N=10)	Cryptococcal meningitis (N=14)	Non-CNS infections (N=43)
Psychosis, n (%)	37 (10.2)	1 (1.6)	1 (0.8)	15 (16.3)	12 (70.6)	0	0	8 (18.6)
Language change, n (%)	36 (9.9)	4 (6.5)	5 (4.1)	10 (10.9)	10 (58.8)	0	0	7 (16.3)
Movement disorder, n (%)	36 (9.9)	0	7 (5.7)	15 (16.3)	10 (58.8)	1 (10.0)	0	3 (7.0)
Hemiplegia, n (%)	25 (6.9)	4 (6.5)	12 (9.8)	4 (4.3)	0	1 (10.0)	1 (7.1)	3 (7.0)
Limb weakness (Paraplegia/ Tetraplegia), n (%)	34 (9.4)	2 (3.2)	15 (12.3)	6 (9.9)	0	1 (10.0)	2 (14.2)	8 (18.6)
Convulsion, n (%)	32 (8.9)	2 (3.2)	3 (2.4)	19 (20.6)	2 (11.8)	0	0	6 (13.9)
Stiff neck, n (%)	159 (43.9)	46 (74.2)	57 (46.7)	30 (32.6)	5 (29.4)	5 (50.0)	7 (50.0)	8 (18.6)
Abnormal movement, n (%)								
Face-mouth-tongue	15 (4.1)	0	2 (1.6)	15 (4.1)	10 (58.8)	0	0	0
Trunk	13 (3.6)	0	1 (0.8)	5 (5.4)	7 (41.2)	0	0	0
Extremity	19 (5.2)	0	4 (3.3)	7 (7.6)	7 (41.2)	0	0	1 (2.3)
Glasgow Coma Scale ≤ 9, n (%)	66 (18.2)	12 (19.4)	16 (13.1)	24 (26.1)	6 (35.3)	1 (10.0)	3 (21.3)	2 (4.6)
Blood and CSF findings								
Opening pressure cmCSF, median (IQR)	17 (13-25)	22 (17-31)	19 (13-26)	16 (12-20)	10 (8-18)	24 (13-35)	25 (20-40)	15 (11-17)
CSF Hematology								
WCC/mm^3, median (IQR)	167 (24-507)	1,924 (610-5,120)	312 (139-503)	43 (8-178)	23 (7-55)	502 (258-977)	37 (14-122)	2 (1-6)

Characteristics	Whole group (N=362)	BM (N=62)	TBM (N=122)	VME (N=92)	Anti-NMDAR (N=17)	Eosinophilic encephalitis (N=10)	Cryptococcal meningitis (N=14)	Non-CNS infections (N=43)
Neutrophil %, median (IQR)	25 (12-66)	83 (72-92)	26 (13-60)	14 (11-24)	14 (11-22)	12 (10-15)	23 (16-36)	30 (12-50)
Lymphocyte %, median (IQR)	67 (26-86)	17 (8-28)	73 (40-87)	84 (63-88)	86 (78-89)	47 (34-51)	68 (64-81)	50 (0-70)
Eosinophil%, median (IQR)	0	0	0	0	0	42 (28-59)	0	0
Biochemistry								
Protein g/L, median (IQR)	1.0 (0.5-2.1)	2.5 (1.1-4.4)	1.9 (1.1-2.8)	0.7 (0.3-1.0)	0.3 (0.2-0.4)	0.8 (0.6-1.3)	0.6 (0.5-1.1)	0.4 (0.3-0.5)
Lactate nmol/L, median (IQR)	3.6 (2.5-6.0)	9.9 (5.4-15.1)	4.9 (4.5-6.4)	2.5 (2.1-3.1)	1.9 (1.7-2.1)	2.7 (2.4-3.3)	4.9 (4.5-6.4)	2.5 (2.0-3.0)
CSF: blood glucose ratio, median (IQR)	0.5 (0.3-0.7)	0.3 (0.1-0.5)	0.3 (0.2-0.4)	0.7 (0.6-0.7)	0.8 (0.7-0.9)	0.4 (0.4-0.5)	0.3 (0.2-0.4)	0.7 (0.6-0.7)
Outcome								
Day of hospitalisation	13 (6-29)	14 (11-23)	26 (2-39)	11 (7-19)	41 (32-52)	16 (8-23)	20 (4-60)	8 (4-14)
Rankin scale, n (%)								
0	52 (14.4)	8 (12.9)	22 (18.0)	17 (18.5)	0	0	0	5 (11.6)
1-2	109 (30.1)	16 (25.8)	31 (25.4)	38 (41.4)	2 (11.8)	7 (70.0)	1 (7.1)	14 (32.6)
3-4	128 (35.4)	31 (50.0)	36 (29.5)	25 (27.1)	9 (52.9)	2 (20.0)	7 (50.0)	17 (39.5)
5	44 (12.2)	3 (4.8)	17 (13.9)	10 (10.9)	5 (29.4)	1 (10.0)	1 (7.1)	6 (14.0)
6	29 (8.0)	4 (6.5)	16 (13.1)	2 (2.2)	1 (5.9)	0	5 (35.7)	1 (2.3)

Table S6.3: The frequency of clinical conditions among 43 patients with non-CNS infections in the cohort of ADAMDEC1 validation phase 2

Clinical conditions	Number of cases (n)
Alcoholic	3
Cerebral infarction	4
Cerebral tumor	1
Cirrhosis	1
Endocrine disorder	1
Epileptic	7
Hypertension	1
Myasthenia gravis	1
Opioid disorder	1
Pneumonia	1
Post malaria neurological syndrome	1
Post viral infection syndrome	5
Psychiatric disorder	2
Sepsis	12
Spleen abscess	1
Wilson	1

Table S6.4: List of biomarker candidates for BM identified by mass spectrometry analysis

No	Protein ID	Protein name	Gene name	Mean intensity of BM (log2)	Mean intensity of Other (log2)	Difference in intensity between BM and Others	Minus Log (p value)
1	P06744	Glucose-6-phosphate isomerase	GPI	-19.99	-24.54	-4.55	7.74
2	P60660-2	Myosin light polypeptide 6	MYL6	-19.77	-26.05	-6.28	7.30
3	P28676	Grancalcin	GCA	-21.18	-26.58	-5.40	7.11
4	P11413-2	Glucose-6-phosphate 1-dehydrogenase	G6PD	-21.17	-26.40	-5.23	6.31
5	P26583	High mobility group protein B2	HMGB2	-20.83	-26.23	-5.41	5.93
6	P05109	Protein S100-A8	S100A8	-14.87	-20.83	-5.96	5.87
7	P05164-2	Myeloperoxidase	MPO	-18.03	-23.71	-5.68	5.84
8	P06702	Protein S100-A9	S100A9	-14.55	-21.29	-6.75	5.84
9	P43490	Nicotinamide phosphoribosyltransferase	NAMPT	-21.54	-26.07	-4.53	5.82
10	**P80188-2**	**Neutrophil gelatinase-associated lipocalin**	**LCN2**	**-17.82**	**-23.60**	**-5.78**	**5.81**
11	P22894	Neutrophil collagenase	MMP8	-19.34	-24.09	-4.75	5.77
12	P50395	Rab GDP dissociation inhibitor beta	GDI2	-20.20	-24.66	-4.46	5.74
13	P20160	Azurocidin	AZU1	-20.45	-26.12	-5.67	5.61
14	P41218	Myeloid cell nuclear differentiation antigen	MNDA	-19.89	-24.52	-4.63	5.60
15	P61160	Actin-related protein 2	ACTR2	-21.19	-25.46	-4.27	5.47
16	O15144	Actin-related protein 2/3 complex subunit 2	ARPC2	-19.85	-25.15	-5.29	5.47
17	P08670	Vimentin	VIM	-18.54	-22.20	-3.66	5.46
18	P08107	Heat shock 70 kDa protein 1A	HSPA1A	-19.45	-24.10	-4.65	5.37
19	P30044-2	Peroxiredoxin-5, mitochondrial	PRDX5	-20.84	-25.66	-4.82	5.23

20	P04040	Catalase	CAT	-19.23	-24.71	-5.48	5.22
21	P09429	High mobility group protein B1	HMGB1	-21.35	-25.89	-4.53	5.12
22	P61158	Actin-related protein 3	ACTR3	-20.73	-25.39	-4.66	5.03
23	P35579	Myosin-9	MYH9	-21.17	-26.72	-5.54	5.02
24	P04083	Annexin A1	ANXA1	-19.86	-25.38	-5.52	4.81
25	P49913	Cathelicidin antimicrobial peptide	CAMP	-20.72	-24.60	-3.88	4.74
26	P12814-3	Alpha-actinin-1	ACTN1	-20.87	-25.06	-4.18	4.73
27	U3KPS2	Myeloblastin	PRTN3	-19.02	-22.98	-3.96	4.71
28	P01040	Cystatin-A	CSTA	-18.79	-24.16	-5.37	4.70
29	Q6UX06	Olfactomedin-4	OLFM4	-22.21	-26.55	-4.33	4.69
30	P52209-2	6-phosphogluconate dehydrogenase, decarboxylating	PGD	-19.40	-24.03	-4.63	4.67
31	P37837	Transaldolase	TALDO1	-19.90	-24.88	-4.98	4.60
32	P51149	Ras-related protein Rab-7a	RAB7A	-21.76	-25.96	-4.21	4.59
33	P08246	Neutrophil elastase	ELANE	-17.89	-23.13	-5.24	4.59
34	O15143	Actin-related protein 2/3 complex subunit 1B	ARPC1B	-21.64	-26.41	-4.77	4.58
35	O43707	Alpha-actinin-4	ACTN4	-21.84	-25.85	-4.01	4.52
36	P08311	Cathepsin G	CTSG	-19.38	-24.24	-4.86	4.48
37	P59998-3	Actin-related protein 2/3 complex subunit 4	ARPC4	-19.92	-24.16	-4.24	4.39
38	P61626	Lysozyme C	LYZ	-16.34	-18.05	-1.71	4.39
39	P30041	Peroxiredoxin-6	PRDX6	-21.56	-25.35	-3.79	4.35
40	P00338-3	L-lactate dehydrogenase A chain	LDHA	-20.19	-23.10	-2.91	4.24
41	Q05315	Galectin-10	CLC	-21.36	-25.34	-3.98	4.18

42	P09960	Leukotriene A-4 hydrolase	LTA4H	-21.47	-24.95	-3.48	4.15
43	O14950	Myosin regulatory light chain 12B	MYL12B	-21.26	-25.66	-4.40	4.12
44	P09211	Glutathione S-transferase P	GSTP1	-18.82	-23.38	-4.56	4.10
45	P00491	Purine nucleoside phosphorylase	PNP	-21.14	-25.57	-4.43	4.07
46	P18428	Lipopolysaccharide-binding protein	LBP	-20.82	-24.91	-4.09	4.05
47	P60709	Actin, cytoplasmic 1	ACTB	-16.18	-17.82	-1.65	4.02
48	P21333-2	Filamin-A	FLNA	-21.85	-26.23	-4.38	4.01
49	Q9ULZ3-2	Apoptosis-associated speck-like protein containing a CARD	PYCARD	-20.63	-24.76	-4.13	3.88
50	P47756-2	F-actin-capping protein subunit beta	CAPZB	-21.89	-26.33	-4.44	3.85
51	P62491-2	Ras-related protein Rab-11A	RAB11A	-21.91	-26.14	-4.23	3.82
52	Q01518	Adenylyl cyclase-associated protein 1	CAP1	-20.93	-25.40	-4.47	3.77
53	O15145	Actin-related protein 2/3 complex subunit 3	ARPC3	-21.08	-24.75	-3.67	3.77
54	O00299	Chloride intracellular channel protein 1	CLIC1	-21.69	-26.07	-4.38	3.75
55	P35754	Glutaredoxin-1	GLRX	-20.49	-24.84	-4.36	3.62
56	E9PR52	Chitinase-3-like protein 2	CHI3L2	-21.29	-25.72	-4.43	3.60
57	P02788-2	Lactotransferrin	LTF	-18.80	-24.04	-5.24	3.38
58	P18206-2	Vinculin	VCL	-22.71	-26.28	-3.58	3.34
59	P52566	Rho GDP-dissociation inhibitor 2	ARHGDIB	-18.87	-22.34	-3.47	3.31
60	P62942	Peptidyl-prolyl cis-trans isomerase FKBP1A	FKBP1A	-19.83	-24.10	-4.27	3.27

Table S6.5: List of biomarker candidates for TBM identified by mass spectrometry analysis

No	Protein ID	Protein name	Gene name	Mean intensity of BM (log2)	Mean intensity of Other (log2)	Difference in intensity between BM and Others	Minus Log (p value)
1	P25311	Zinc-alpha-2-glycoprotein	AZGP1	-16.21	-17.29	-1.08	9.22
2	P23381	Tryptophan-tRNA ligase	WARS	-20.06	-23.81	-3.75	5.58
3	P29622	Kallistatin	SERPINA4	-20.65	-22.77	-2.13	4.39
4	P02746	Complement C1q subcomponent subunit B	C1QB	-18.32	-19.31	-0.99	4.35
5	A0A075B6J0	Immunoglobulin lambda variable 1-40	IGLV1-40	-17.35	-20.25	-2.90	4.25
6	P02749	Beta-2-glycoprotein 1	APOH	-18.32	-19.33	-1.01	4.13
7	P32455	Guanylate-binding protein 1	GBP1	-23.23	-25.44	-2.21	3.41
8	P16070-10	CD44 antigen	CD44	-22.26	-23.46	-1.19	3.23
9	P02747	Complement C1q subcomponent subunit C	C1QC	-17.80	-18.93	-1.13	3.14
10	P01591	Immunoglobulin J chain	JCHAIN	-19.12	-22.10	-2.98	2.93
11	Q8WVN6	Secreted and transmembrane protein 1	SECTM1	-20.96	-23.77	-2.81	2.88
12	Q96IY4	Carboxypeptidase B2	CPB2	-21.79	-24.05	-2.26	2.83
13	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	-22.42	-24.14	-1.72	2.82
14	P01625	Immunoglobulin kappa variable 4-1	IGKV4-1	-23.58	-26.87	-3.29	2.78
15	O15204	ADAM DEC1	ADAMDEC1	-22.75	-25.26	-2.51	2.64
16	P19971-2	Thymidine phosphorylase	TYMP	-22.78	-25.01	-2.24	2.57

17	A0A075B6J9	Immunoglobulin lambda variable 2-18	IGLV2-18	-21.35	-24.22	-2.87	2.37
18	P01596	Immunoglobulin kappa variable 1-5	IGKV1-5	-21.02	-23.70	-2.69	2.36
19	P02743	Serum amyloid P-component	APCS	-23.61	-25.81	-2.19	2.30
14	P01625	Immunoglobulin kappa variable 4-1	IGKV4-1	-23.58	-26.87	-3.29	2.78
15	**O15204**	**ADAMDEC1**	**ADAMDEC1**	**-22.75**	**-25.26**	**-2.51**	**2.64**
16	P19971-2	Thymidine phosphorylase	TYMP	-22.78	-25.01	-2.24	2.57
17	A0A075B6J9	Immunoglobulin lambda variable 2-18	IGLV2-18	-21.35	-24.22	-2.87	2.37
18	P01596	Immunoglobulin kappa variable 1-5	IGKV1-5	-21.02	-23.70	-2.69	2.36
19	P02743	Serum amyloid P-component	APCS	-23.61	-25.81	-2.19	2.30