Diagnostic Cerebrospinal Fluid Biomarker Discovery and Validation in Patients with Central Nervous System Infections

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ABSTRACT

Background: Central nervous system (CNS) infections are common causes of morbidity and mortality worldwide. Rapid, accurate identification of the likely cause is essential for clinical management and the early initiation of antimicrobial therapy, which potentially improves clinical outcome.

Methods: We applied liquid chromatography tandem mass-spectrometry on 45 cerebrospinal fluid (CSF) samples from a cohort of adults with/without CNS infections to discover potential diagnostic protein biomarkers. We then validated the diagnostic performance of a selected biomarker candidate in an independent cohort of 364 consecutively treated adults with CNS infections admitted to a referral hospital in southern Vietnam.

Results: In the discovery cohort, we identified lipocalin 2 (LCN2) as a potential biomarker of bacterial meningitis. The analysis of the validation cohort showed that LCN2 could discriminate bacterial meningitis from other CNS infections, including tuberculous meningitis, cryptococcal meningitis and viral/antibody-mediated encephalitis (sensitivity: 0.88 (95% confident interval (CI): 0.77–0.94), specificity: 0.91 (95%CI: 0.88–0.94) and diagnostic odd ratio: 73.8 (95%CI: 31.8–171.4)). LCN2 outperformed other CSF markers (leukocytes, glucose, protein and lactate) commonly used in routine care worldwide. The combination of LCN2 and these four routine CSF markers resulted in the highest diagnostic performance for bacterial meningitis (area under receiver-operating-characteristic-curve 0.96; 95%CI: 0.93–0.99).

Conclusions: Our results suggest that LCN2 is a sensitive and specific biomarker for discriminating bacterial meningitis from a broad spectrum of CNS infections. A prospective study is needed to further assess the diagnostic utility of LCN2 in the diagnosis and management of CNS infections.

INTRODUCTION

Central nervous system (CNS) infections cause significant mortality and morbidity worldwide, but especially in low- and middle-income countries (1). Common CNS infections include bacterial meningitis (BM), viral encephalitis, tuberculous meningitis (TBM) and cryptococcal meningitis (2), but there are >100 documented infectious causes of CNS infections (3). Additionally, over the last decade, antibody-mediated causes of encephalitis (e.g. anti-N-methyl-D-aspartate receptor (anti-NMDAR) encephalitis) have been recognized (4), which further challenges routine diagnostics.

Clinical features are often insufficient to discriminate the likely cause and standard laboratory investigations identify the causative agent in <60% of cases (5, 6). Critically, the clinical management of CNS infections varies according to its aetiology. Thus, rapid and accurate identification of the likely cause of the infection is essential to initiate appropriate therapy and improve patient outcome.

Over the last decade, mass-spectrometry has emerged as a sensitive, hypothesis-free approach for the discovery of novel diagnostic biomarkers in both communicable (e.g. CNS infections) and non-communicable diseases (7-9). However, previous biomarker-discovery studies of CNS infections have either been limited in sample size or have not included a validation phase (7). Here, using a mass-spectrometry based approach we first searched for novel diagnostic biomarkers in cerebrospinal fluid (CSF) samples from a discovery cohort of 45 patients with brain infections. We then sought to validate our findings in an independent cohort of 364 consecutively treated adults with CNS infections.

MATERIALS AND METHODS

Setting and the clinical studies

CSF samples were derived from three different clinical studies: study #1, #2 and #3 (Figure 1), conducted in the brain infections ward of the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City, Vietnam. HTD is a tertiary referral hospital for severe infectious diseases, including suspected CNS infections, occurring in the southern provinces of Vietnam, with a population of >40 million.

The clinical study #1 entitled "expanding the laboratory diagnosis of tuberculous meningitis and meningoencephalitis in Vietnam" was conducted during January 2015–September 2016 (10). As per the study protocol, any adult (≥18 years) with a suspected CNS infection and requirement for lumbar puncture was eligible for enrolment. Patients were excluded if pyogenic bacterial meningitis (very cloudy or pus-like CSF) was suspected, lumbar puncture was contra-indicated, or no informed consent was obtained.

The clinical study #2 focused on the immunological responses in bacterial meningitis patients, especially those infected with *Streptococcus suis*, and was conducted during 2015 and 2017. Any patient (≥16 years) with suspected pyogenic bacterial meningitis (very cloudy or pus-like CSF) was eligible for enrolment. Patient was excluded if lumbar puncture was contra-indicated, or no informed consent was obtained.

The clinical study #3 started in September 2017 and is on-going. The study aims to explore the potential diagnostic utility of next-generation sequencing and mass-spectrometry in CNS infections. Any patient (≥16 years) with suspected CNS infection and requirement for lumbar puncture was eligible for enrolment. Patients were excluded if no written informed consent was obtained.

For all the aforementioned studies, CSF and plasma samples were collected at presentation alongside demographic and clinical data and the results of routine diagnostic tests. All specimens were stored at -80°C until analysis.

Routine diagnosis

As part of routine care, CSF specimens of patients with suspected CNS infections were cultured and/or examined by microscopy for the detection of bacteria, fungi and *Mycobacterium tuberculosis* with the use of standard methods (Table S1) (11). Herpes simplex virus (HSV) PCR was performed on CSF from those with suspected viral encephalitis. Varicella zoster virus (VZV) PCR, and serological testing for dengue virus (DENV) IgM, Japanese encephalitis virus (JEV) or mumps virus (MuV) was performed if clinically indicated and when testing for other pathogens was negative. Diagnosis of measles was based on compatible clinical features and the presence of measles IgM.

Assignment of CNS infection diagnosis

Assignment of the CNS infection cause (TBM, BM, cryptococcal meningitis, eosinophilic meningitis, or anti-NMDAR encephalitis) was first based on the results of standard laboratory investigations. The diagnosis was confirmed if the relevant infectious agent was identified in the CSF. Otherwise, patients were considered as having clinically suspected CNS infections (TBM/BM/encephalitis) based on treatment responses and/or clinical judgment of treating physicians. Because of the focus of the present study, probable and possible TBM (defined by the Marais criteria (12)) were regarded as clinically suspected TBM. CNS infection was excluded in those with no meningeal signs, CSF laboratory parameters were in normal ranges, and all microbiological and serological investigations were negative.

Sample preparation and mass-spectrometry analysis

CSF was analyzed as individual samples using proteomic platforms available at the Target Discovery Institute, University of Oxford. Briefly, CSF was digested in solution after reduction/alkylation with DTT/Iodoacetamide, followed by protein precipitation with Chloroform/Methanol (13). After tryptic digest, peptides were desalted using SepPak C18 cartridges (Waters) and injected into a LC-MS/MS platform consisting of Dionex Ultimate3000 nHPLC (Thermo) and either Q-Exactive or Q-Exactive HF. Peptides were separated with a linear gradient of 5-35% Acetonitrile in 0.1% Formic acid over 60 minutes using a 50cm x 75µm Easy Spray column (Thermo). MS1 resolution was set to 70,000 (HF 60,000) with an AGC target of 3E6. Fragment spectra were acquired with a resolution of 17,500 (HF 30,000) for up to 128ms (HF 45ms) and an AGC target of 1E5 ions. The output data were searched for human proteome (Uniprot, 05/2016 and 05/2018) using Mascot 2.5 or the Central Proteomics Pipeline (14). Peptide FDR was adjusted to 1% and lable free quantitation was conducted with either SINQ (15) within the CPFP or Progenesis QI version 3.1.4003.30577. Separation of CNS infection diagnostic groups based on the obtained peptide/protein profiles was performed using Perseus software version 1.6.6.0 (16)

Measurement of lipocalin-2 concentration by quantitative ELISA

Measurement of lipocalin 2 (LCN2) concentrations was performed on CSF samples of the discovery and validation cohort as well as a subset of plasma samples of the validation cohort using Quantikine® ELISA kits (R&D Systems, Minneapolis, MN, US). The experiments were performed according to the manufacturer's instruction.

Statistical analysis

Continuous variables were compared using the Mann-Whitney *U* test or the Kruskal-Wallis test or Wilcoxon signed-rank test. The correlation between continuous variables was assessed using Spearman correlation test. All statistical tests were performed two-sided. The area under the receiver operating characteristic curve (AUROC) was used to quantify the diagnostic performance of biomarkers for a given diagnosis. The cutoff values for outcome prediction were selected based on the highest sum of sensitivity and specificity. A logistic regression model was used to evaluate the diagnostic performance of two or more variables combined. All continuous variables were modeled as linear terms. All analyses were performed in SPSS V23.0 (IBM Corp, NY, US), and all figures were generated using GraphPad PRISM® V5.04 (GraphPad Software Inc, CA, US).

Ethics

The study was approved by Institutional Review Board of HTD and the Oxford Tropical Research Ethics Committee (OxTREC). Written informed consents were obtained from each participant or a relative if the patient was incapacitated.

RESULTS

Baseline characteristics of the study population

Discovery cohort: For the initial mass-spectrometry analysis, we selected a total of 45 patients enrolled in the clinical study #1 and #2. This consisted of 40 patients with laboratory confirmed CNS infections: TBM (n=20), BM (n=10), encephalitis (n=10), and five patients with non-CNS infection (Figure 1). Of the 10 patients with BM, seven were infected with *S. suis* and three with *S. pneumoniae*. Of the patients with encephalitis, herpes simplex virus was the cause in 5, DENV in 3, JEV in 1 and mumps virus in 1. The cohort's clinical characteristics and outcomes are presented in Table S2.

Validation cohort: To validate the results of the discovery phase, we selected 364 consecutive adult patients enrolled in the study #3 (Figure 1). The baseline characteristics, clinical outcomes, and results of etiological investigations of the cohort are presented in Table S2 and the footnote of Figure 1, respectively. After the exclusion of 43 patients without CNS infections, the etiology was confirmed in 63% of the 321 patients with CNS infections. TBM was the most frequent diagnosis, followed by viral encephalitis and BM. The remaining patients included those with anti-NMDAR encephalitis, cryptococcal meningitis, parasitic eosinophilic meningitis and neurotoxoplasmosis (Figure 1). Of the patients with TBM, BM and viral encephalitis, a confirmed diagnosis was established in 97/122 (80%), 44/64 (69%) and 29/92 (32%), respectively. Of the 44 laboratory-confirmed bacterial meningitis patients, *S. suis*, was the commonest cause (n=20), followed by *S. pneumoniae* (n=6) and *Escherichia coli* (n=5). Of the 29 patients with laboratory confirmed viral encephalitis, HSV was the commonest cause (n=11), followed by VZV (n=7), and DENV (n=5) (Figure. 1).

Biomarker discovery

Tandem mass-spectrometry analysis of 45 CSF samples of the discovery cohort identified a total of 1,012 proteins. Of these, 891 were included in the analysis based on the number of peptides and sequence coverage. Subsequent analysis identified a total of 729 quantifiable protein signatures that were clinical-entity specific, especially for patients with BM (Figure 2A). Of these, 60 and 19 were significantly expressed in the CSF of patients with BM and TBM, respectively (Table S3). No diagnostic biomarker candidate was found in patients with viral encephalitis.

Of the protein candidates identified in the BM group, lipocalin 2 (LCN2), also known as neutrophil gelatinase-associated lipocalin, had a sensitivity of 1 (95%CI: 0.73-1) and a specificity of 0.89 (95%CI: 0.74-0.95) for prediction of BM (AUROC: 0.97 [95% confidence interval [CI], 0.9–1]). Because of its known biological significance in bacterial infections (17-19), previous reports of high concentrations in bacterial meningitis (20, 21), and the availability of a quantitative ELISA assay, LCN2 was thus selected for further evaluation.

LCN2 ELISA analysis to verify the results of original LC-MS/MS analysis

In order to verify the mass-spectrometry findings, we performed quantitative ELISA analysis of the 45 CSF samples used for the discovery phase. Subsequently, the result suggested that LCN2 concentration of 159 ng/ml or above could accurately distinguish BM from TBM, encephalitis and non-CNS infections groups; AUROC curve: 0.97 (95%CI: 0.92–1), corresponding to the sensitivity of 1 (95%CI: 0.72–1) and the specificity of 0.86 (95%CI: 0.71–0.94) (Figures 2B and 2C). Thus, the diagnostic values of LCN2 based on the results of quantitative ELISA analysis confirmed the original finding of LC-MS/MS analysis.

CSF LCN2 concentrations in the validation cohort

LCN2 was quantified in the CSF of the 364 consecutively treated adults with CNS infections enrolled in the study #3 (Figure 1). The results showed that LCN2 concentrations were significantly different amongst the diagnostic groups with the highest concentration observed in the BM group (median: 778. 8 ng/ml, range: 2.5–6566.3), followed by TBM groups (median: 86.3 ng/ml, range: 1.1–723.4) (Figure 3A). In contrast, LCN2 was almost absent or detected at very low levels in CSF of patients presenting with anti-NMDAR encephalitis (median: 0.9 ng/ml, range: 0.2–27.8) or in those without CNS infection (median: 0.2 ng/ml, range: 0.2–120.3) (Figure S1). Of the patients with BM, CSF LCN2 levels were higher in those with a confirmed diagnosis than in those without a bacteria identified (Figure S1), while the duration of illness at enrolment was similar between the two groups (data not shown).

Diagnostic performance of CSF LCN2

Analysis of LCN2 concentrations obtained from the validation cohort demonstrated that LCN2 could accurately discriminate bacterial meningitis from other CNS infections with AUROC curves ranging from 0.9 (for BM vs. TBM, LCN2 concentration cut-off: 365 ng/ml) to 0.99 (BM vs. other CNS infections (i.e. non-encephalitis or non-TBM), LCN2 concentration cut-off: 134 ng/ml) and a diagnostic odd ratio (DOR) of 44.8 or above (Figure 3B).

Currently, CSF parameters such as leukocytes, protein, lactate and glucose concentrations are routinely used as diagnostic makers in the primary assessment of patients presenting with CNS infections. We thus compared the diagnostic performance of LCN2 alone and in combination with these markers..

LCN2 outperformed the existing biomarkers in discriminating between BM and other CNS infections (including TBM and encephalitis) (Figures 4A and 4B). When LCN2 was combined with leukocytes, protein, lactate and glucose concentrations in CSF, the diagnostic model consisting of LCN2 and these four CSF parameters provided the highest discriminatory ability for BM (Figure 4C). More specifically, in terms of discriminating between BM and all other CNS infections, the predictive values for BM based on AUROC curves and DOR increased from 0.94 (95%CI: 0.80–0.98) to 0.96 (95%CI: 0.93–0.99), and 66.2 to 308.3 when LCN2 was added to the CSF parameters based model (Figure 4C). Similar results were obtained when assessing the utility of LCN2 in discriminating between BM and other specific clinical entities (TBM or encephalitis) (Figure 4D and Figure S2). LCN2 did not, however, help distinguish confirmed from suspected BM (Table S4).

Association between CSF and plasma concentrations of LCN2

LCN2 is a ubiquitous protein which can be found in bodily fluids of healthy individuals (22). We assessed if plasma LCN2 can be a surrogate of CSF LCN2 in a subset of 22 patients with BM (laboratory confirmed: n=14 and clinically suspected: n=8). Plasma LCN2 concentration was, however, significantly lower than that of CSF; median: 147.9 ng/ml, range: 33.7–194.8 vs. CSF LCN2: median: 472.1 ng/ml, range: 15.7–3102.3, P<0.001. There was no correlation between CSF and plasma LCN2 (Spearman R: 0.37, P=0.08), suggesting that LCN2 is intrathecally produced in response to the bacterial invasion of the CNS.

DISCUSSION

Here, using a mass-spectrometry-based approach, we initially identified LCN2 as a potential diagnostic marker for BM. Additional validation work on an independent cohort showed that LCN2 could accurately discriminate BM from other CNS infections. LCN2 also outperformed existing BM diagnostic makers (CSF leukocytes, and protein, glucose and lactate concentrations) that are currently used as part of routine care. A diagnostic model consisting of LCN2 and these four CSF parameters gave the best diagnostic performance for BM. Our data thus suggest that LCN2 can act as an independent diagnostic maker of BM alone or in combination with other CSF parameters.

LCN2 is secreted by neutrophils, hepatocytes and renal tubular cells (23). It is encoded by LCN2 gene and is known to have antibacterial properties because of its ability to inhibit the bacterial growth via the interference of bacterial iron uptake (23). LCN2 has recently been recognized as a sensitive biomarker for the diagnosis of severe blood stream infection (24) and pneumonia caused by *S. pneumoniae* (18). High concentrations of LCN2 in the CSF of patients with BM have been previously reported (20, 21). However, previous studies only focused on quantifying LCN2 concentrations in patients with confirmed BM and viral encephalitis and did not compare the performance of LCN2 against commonly used CSF markers such as leukocytes, glucose, protein and lactate. Our study was conducted in Vietnam and included patients with many different CNS infections, including bacterial, fungal, tuberculous, viral and parasitic meningitis, and anti-NMDAR encephalitis). Additionally, we also compared the diagnostic performance of LCN2 against that of CSF markers commonly used as part of routine care worldwide. As such, our results have expanded our knowledge about the relation between LCN2 and CNS infections,

and for the first time provide strong evidence that LCN2 is a highly sensitive biomarker for discriminating BM from a broad-spectrum of CNS infections.

The differences in CSF LCN2 levels between laboratory confirmed and clinically suspected BM groups 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). This is in agreement with previous studies showing that the decrease of plasma LCN2 level was correlated with the success of antibiotic treatment in patients with bacteremia (17). Collectively, CSF LCN2 might also be a useful marker for treatment response assessment. Therefore, further research should aim at defining the optimal cut-off of LCN2 concentrations that can be used to inform the administration or withdrawal of antibiotics in patients with BM.

Our study has some of limitations. A part from LCN2, we did not explore the utility potential of the other biomarker candidates identified in the discovery cohort (e.g. CSF cathelicidin for BM (25)) detected by original mass-spectrometry analysis). Likewise, we did not assess the diagnostic performance of LCN2 against and/or in combination newly proposed biomarkers for CNS infections such as procalcitonin and heparin-binding protein for BM (7, 9, 26-28), and CSF lipoarabinomannan for TBM (29). Additionally, we only focused our analysis on adults, leaving the utility potential of LCN2 in pediatric CNS infections unknown.

In spite of these limitations, the strengths of our study include that it represents the largest and most comprehensive mass-spectrometry-based biomarker discovery investigation focusing on patients with various clinical entities of CNS infections to date (7). The study was also conducted in Vietnam and therefore includes all the major infectious causes of CNS infections seen globally. Additionally, because our study was conducted at a single major tertiary referral

hospital, all routine diagnostic approaches and patient assessments were consistent over the course of the study, thereby minimizing potential bias.

To summarize, our study showed for the first time that LCN2 is a highly sensitive 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.

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LEGENDS TO FIGURES

Figure 1: An overview of protein marker discovery phases and origin of clinical samples used for the analysis

Note to Figure 1: TBM: tuberculous meningitis, cTBM: confirmed tuberculous meningitis, sTBM: clinically suspected tuberculous meningitis, BM: bacterial meningitis, cBM: confirmed bacterial meningitis, sBM: clinically suspected bacterial meningitis, EN: encephalitis, cEN: confirmed encephalitis, sEN: clinically suspected encephalitis, NI: non-CNS infections

[#]Including: *S. suis* (n=20), *S. pneumonia* (n=6), *E. coli* (n=5), *N. meningitides* (n=2), *B. pseudomallei* (n=1), *E. faecalis* (n=1), *E. gallinarum* (n=1), *S. agalactiae* (n=1), *S. aureus* (n=1), *S. gallolyticus* (n=1) and gram staining positive only (n=5)
[*]including: herpes simplex virus (n=11), varicella zoster virus (n=7), dengue virus (n=5), Japanese encephalitis virus (n=2), dengue virus/Japanese encephalitis virus (n=1), mumps virus (n=1), measles virus (n=1) and influenza A virus (n=1)
[$]Including: cryptococcal meningitis (n=14), anti-NMDAR encephalitis (n=17), eosinophilic meningitis (n=10), neurotoxoplasmosis (n=2)

Figure 2. Results of mass-spectrometry and LCN2 ELISA analysis of the discovery cohort. (**A**) Heatmap showing clinical entities clustering based on the protein/peptide profiles obtained from label-free quantitative mass-spectrometry analysis of 45 patients of the discovery phase. Columns represent clinical entities, while rows represent individual proteins, (**B)** Dot plots demonstrating the difference in CSF LCN2 levels between BM and non-BM groups obtained from quantitative ELISA analysis, (**C**) AUROC curve based on LCN2 levels measured by quantitative ELISA analysis

Note to Figure 2. Non-BM: non bacterial meningitis (encephalitis, tuberculous meningitis or non-CNS infections)

Figure 3. Results of LCN2 ELISA and AUROC analysis of the validation cohort. (**A**) LCN2 concentrations in patients with meningitis, tuberculous meningitis, encephalitis and others (cryptococcal meningitis, anti-NMDAR encephalitis, eosinophilic meningitis, neurotoxoplasmosis and non-CNS infections), (**B**) AUROC curves showing the diagnostic values of LCN2 in discriminating bacterial meningitis from other CNS infections entities

Note to Figure 3: Others: patients with other CNS infections (cryptococcal meningitis, anti-NMDAR encephalitis, neurotoxoplasmosis, or eosinophilic meningitis) or non-CNS infections, Non-BM: non bacterial meningitis

Figure 4. Diagnostic values of LCN2 in predicting bacterial meningitis in comparison and in combination with existing CSF parameters. (**A**) AUROC curves showing that LCN2 is better than the existing CSF parameters in distinguishing between bacterial meningitis with other CNS infections (tuberculous meningitis, encephalitis, anti-NMDAR encephalitis, cryptococcal meningitis, neurotoxoplasmosis, eosinophilic meningitis or non-CNS infections), (**B**) AUCROC values of subgroup analyses, (**C**) AUROC curves showing that LCN2 significantly improves the

discriminatory ability of the diagnostic model for bacterial meningitis using the remaining CNS infections groups as controls, (**D**) AUROC values of subgroup analyses

Note to Figure 4: WCC: white blood cell count (leukocyte count)

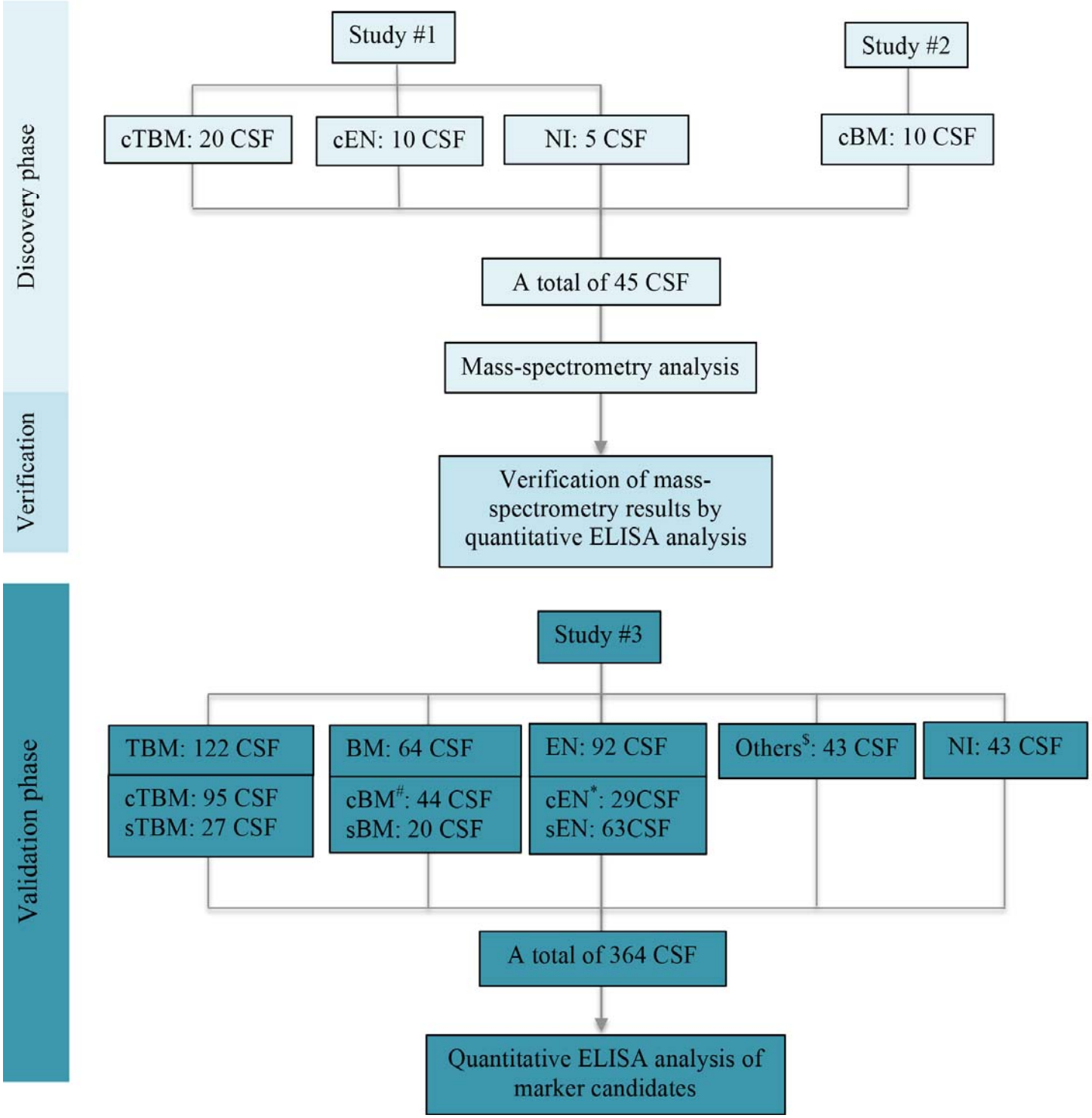


Figure 1: An overview of protein marker discovery phases and origin of clinical samples used for the analysis

Note to Figure 1: TBM: tuberculous meningitis, cTBM: confirmed tuberculous meningitis, sTBM: clinically suspected tuberculous meningitis, BM: bacterial meningitis, cBM: confirmed bacterial meningitis, sBM: clinically suspected bacterial meningitis, EN: encephalitis, cEN: confirmed encephalitis, sEN: clinically suspected encephalitis, NI: non-CNS infections

[#]Including: *S. suis* (n=20), *S. pneumonia* (n=6), *E. coli* (n=5), *N. meningitides* (n=2), *B. pseudomallei* (n=1), *E. faecalis* (n=1), *E. gallinarum* (n=1), *S. agalactiae* (n=1), *S. aureus* (n=1), *S. gallolyticus* (n=1) and gram staining positive only (n=5)
[*]including: herpes simplex virus (n=11), varicella zoster virus (n=7), dengue virus (n=5), Japanese encephalitis virus (n=2), dengue virus/Japanese encephalitis virus (n=1), mumps virus (n=1), measles virus (n=1) and influenza A virus (n=1)
[$]Including: cryptococcal meningitis (n=14), anti-NMDAR encephalitis (n=17), eosinophilic meningitis (n=10), neurotoxoplasmosis (n=2)

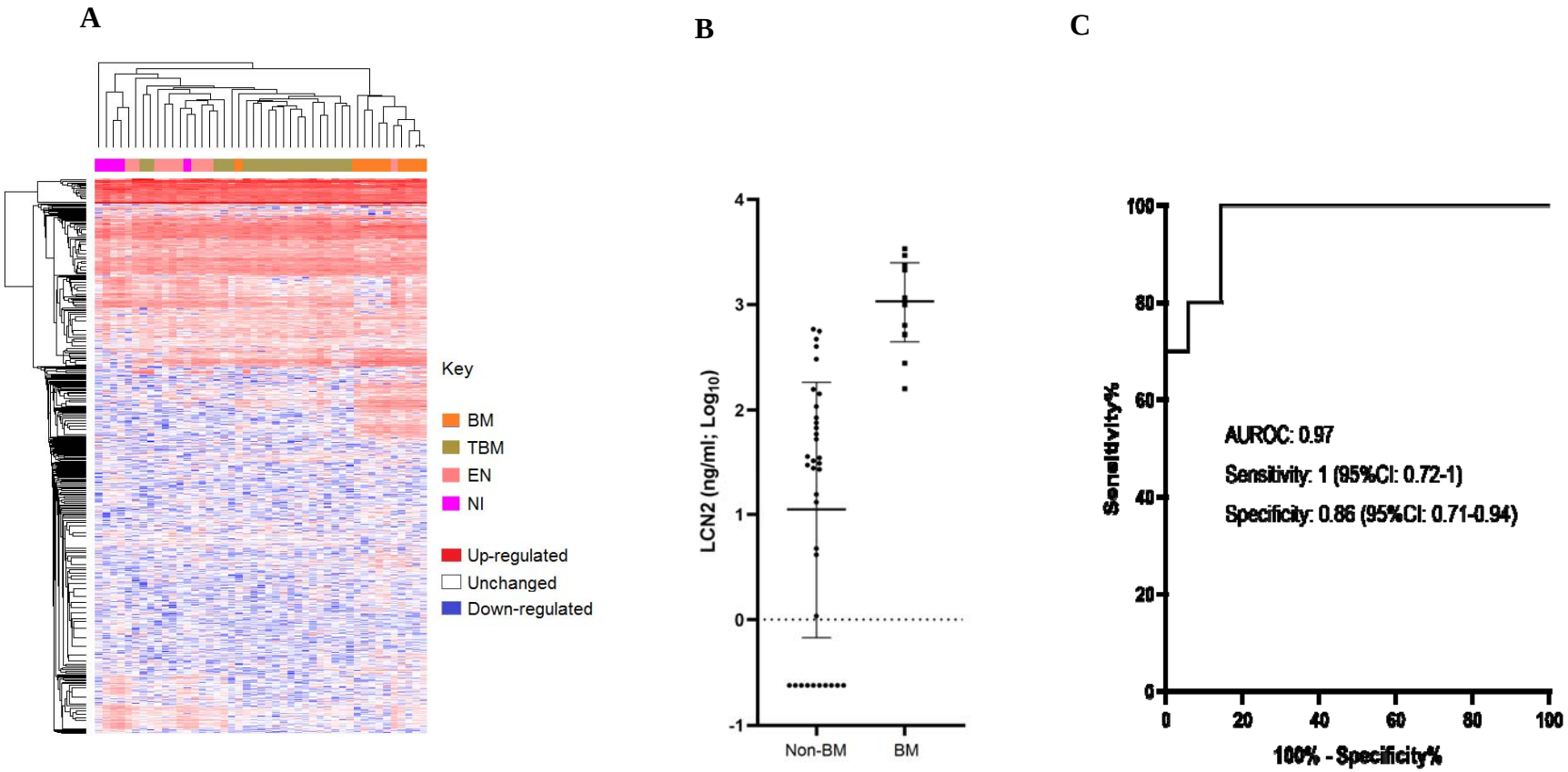
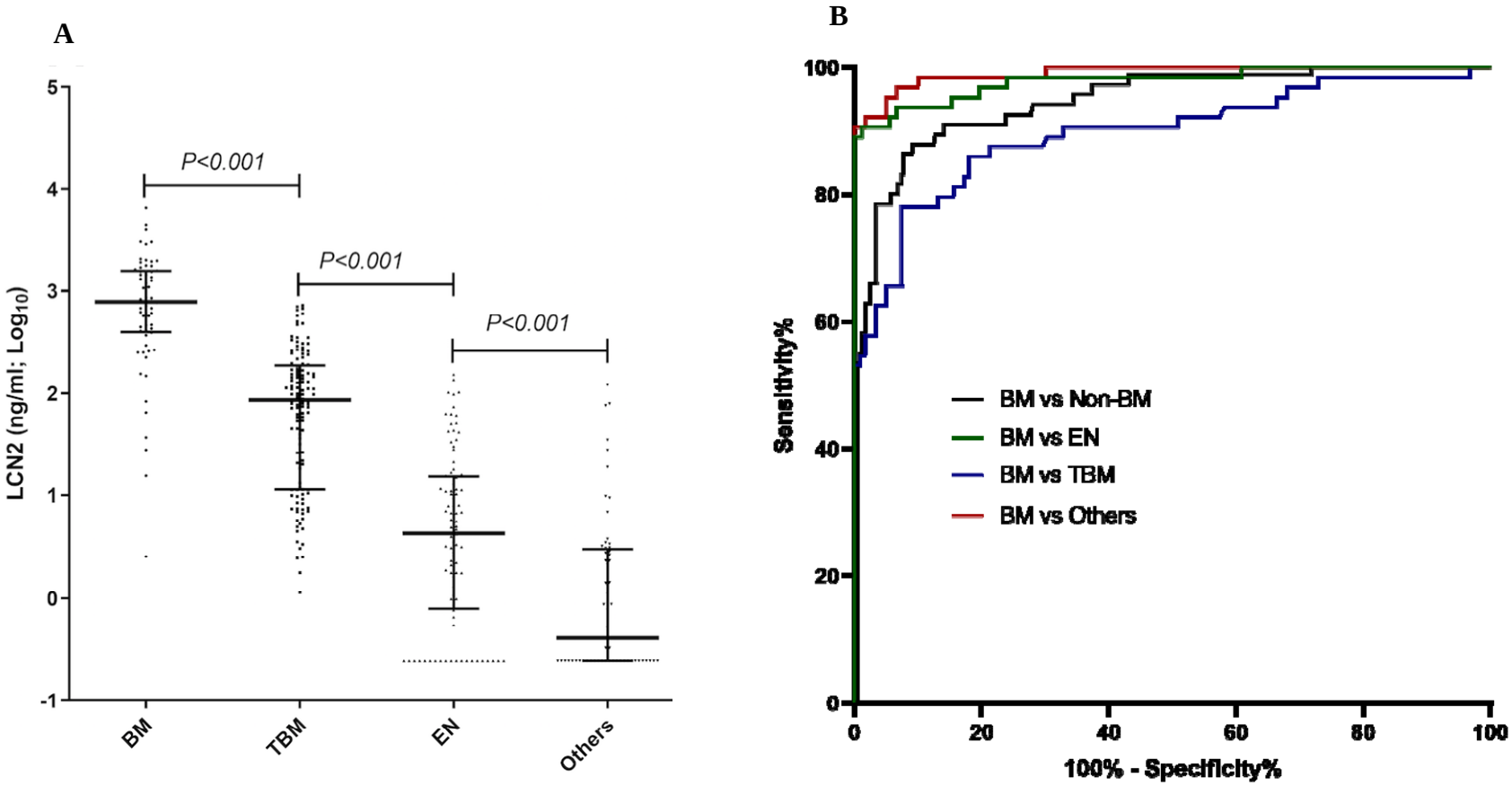


Figure 2. Results of mass-spectrometry and LCN2 ELISA analysis of the discovery cohort. (**A**) Heatmap showing clinical entities clustering based on the protein/peptide profiles obtained from label-free quantitative mass-spectrometry analysis of 45 patients of the discovery phase. Columns represent clinical entities, while rows represent individual proteins, (**B)** Dot plots demonstrating the difference in CSF LCN2 levels between BM and non-BM groups obtained from quantitative ELISA analysis, (**C**) AUROC curve based on LCN2 levels measured by quantitative ELISA analysis

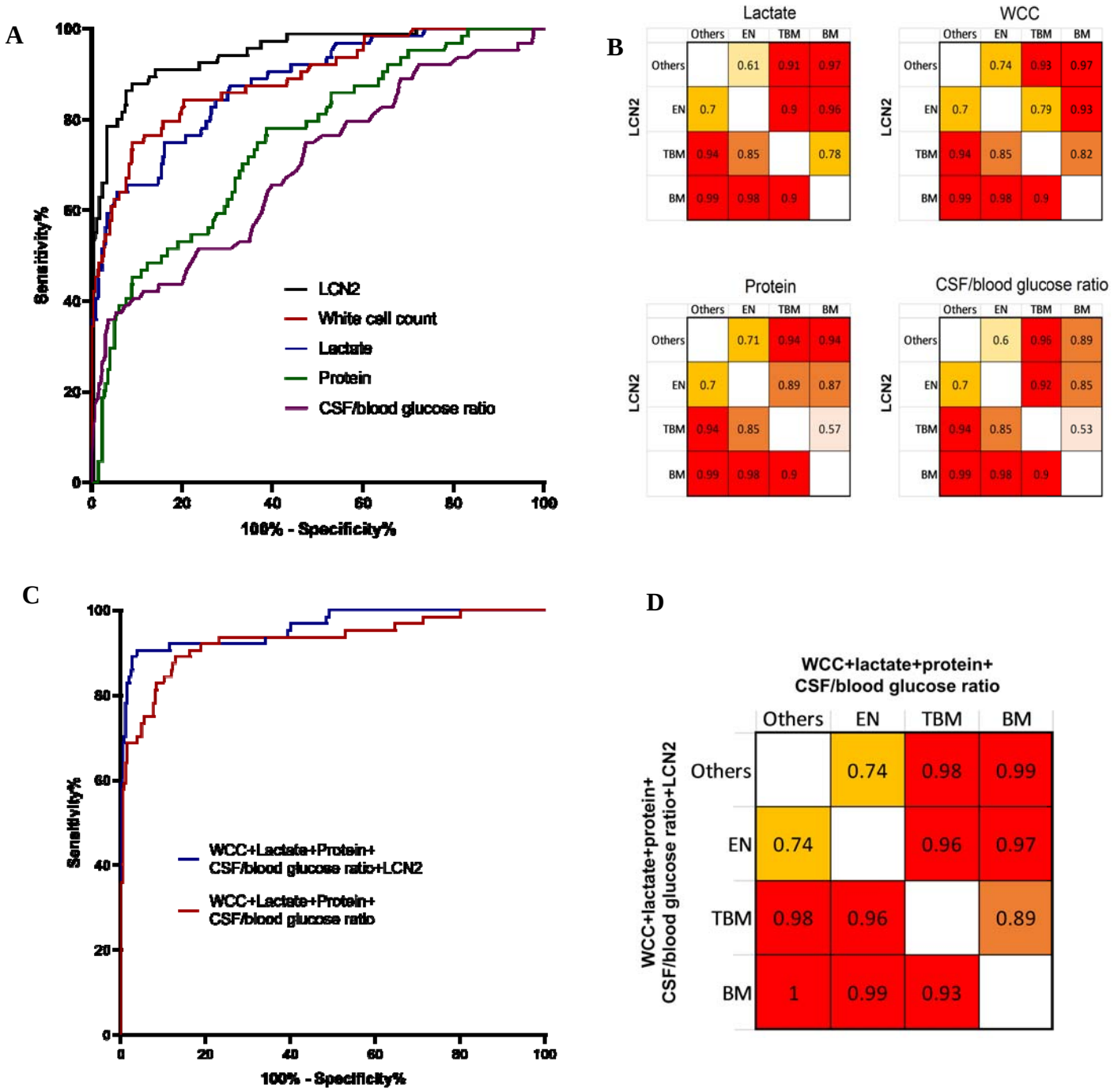
Note to Figure 2. Non-BM: non bacterial meningitis (encephalitis, tuberculous meningitis or non-CNS infections)



BM vs.	LCN2 cut-off (ng/ml)	AUC (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	DOR
Non-BM	221	0.95(0.92–0.98)	0.88(0.77–0.94)	0,91(0.88–0.94)	73.8 (31.8–171.4)
TBM	365	0.90(0.84–0.95)	0.78 (0.67–0.87)	0.93(0.87–0.96)	44.8(18.2–110.4)
EN	142	0.98(0.96–1)	0.91(0.81–0.96)	0.99(0.94–1)	879.7(103.2-7495.1)
Others	134	0.99(0.98–1)	0.91(0.81–0.96)	1(0.94–1)	NA

Figure 3. Results of LCN2 ELISA and AUROC analysis of the validation cohort. (**A**) LCN2 concentrations in patients with meningitis, tuberculous meningitis, encephalitis and others (cryptococcal meningitis, anti-NMDAR encephalitis, eosinophilic meningitis, neurotoxoplasmosis and non-CNS infections), (**B**) AUROC curves showing the diagnostic values of LCN2 in discriminating bacterial meningitis from other CNS infections entities

Note to Figure 3: Others: patients with other CNS infections (cryptococcal meningitis, anti-NMDAR encephalitis, neurotoxoplasmosis, or eosinophilic meningitis) or non-CNS infections, Non-BM: non bacterial meningitis



BM vs. non-BM	WCC+lactate+protein+ CSF/blood glucose ratio	WCC+lactate+protein+ CSF/blood glucose ratio +LCN2
AUC (95%CI)	0.94 (0.90–0.98)	0.96 (0.93–0.99)
Sensitivity (95%CI)	0.86 (0.75–0.92)	0.91 (0.81–0.96)
Specificity (95%CI)	0.92 (0.88–0.94)	0.97 (0.94–0.98)
DOR	66.2 (29.3–149.7)	308.3 (105.6-899.4)

Figure 4. Diagnostic values of LCN2 in predicting bacterial meningitis in comparison or in combination with existing CSF parameters. (**A**) AUROC curves showing that LCN2 is better than the existing CSF parameters in distinguishing between bacterial meningitis with other CNS infections (tuberculous meningitis, encephalitis, anti-NMDAR encephalitis, crytococcal meningitis, neurotoxoplasmosis, eosinophilic meningitis or non-CNS infections), (**B**) AUCROC values of subgroup analyses, (**C**) AUROC curves showing that LCN2 significantly improves the discriminatory ability of the diagnostic model for bacterial meningitis using the remaining CNS infections groups as controls, (**D**) AUROC values of subgroup analyses

Note to Figure 4: WCC: white blood cell count (leukocyte count)

SUPPLEMENTARY MATERIALS

Table S1. Diagnostic tests carried out as part of routine care and/or as per the study protocols

	Study #1	**Study #2**	**Study #3**
Gram stain	Y	Y	Y
Bacterial culture	Y	Y	Y
Ziehl-Neelsen staining	Y	Y	Y
GenXpert	Y	Y	Y
MGIT	Y	Y	Y
S. suis PCR	Y	Y	Y
S. pneumoniae PCR	Y	Y	Y
N. meningitidis PCR	Y	Y	Y
16S rRNA PCR	ND	Y	Y
HSV PCR	Y	Y	Y
VZV PCR	Y	Y	Y
DENV PCR	ND	ND	Y
JEV PCR	ND	ND	Y
Flavivirus PCR	ND	ND	Y
Enterovirus PCR	ND	ND	Y
Influenza A virus	Y	Y	Y
Mumps virus PCR	ND	ND	Y
Zika virus PCR	ND	ND	Y
Angiostrongylus cantonensis PCR	ND	ND	Y
Cryptococcal FLA	Y	Y	Y
DENV serology	Y	Y	Y
JEV serology	Y	Y	Y
Anti-NMDAR	ND	Y	Y

Note to Table S1: Y: yes. ND: not done

Table S2. Baseline characteristics of the discovery and validation cohort

	Discovery cohort				Validation cohort						
	TBM (N=20)	Encephalitis (N=10)	BM♪ (N=10)	Non-CNS infections (N=5)	BM (N=64)#	TBM (N=122)♫	Encephalitis (N=92)$	Anti-NMDAR (N=17)	Eosinophilic meningitis (N=10)	Cryptococcal meningitis (N=14)	Non-CNS infections (N=43)
Demographics											
Age in years	40(23-75)	33(18-53)	49(23-74)	58(0-70)	55 (17-87)	41(17-87)	31 (16-78)	25(17-48)	30(18-60)	35.5(22-68)	48(20-92)
Gender	11/9	8/2	7/3	2/3	44/20	97/25	54/38	9/8	4/6	10/4	25/18
Ho Chi Minh City origin	5(25)	2(20)	4(44.4)	2(40)	12(18.8)	29(23.8)	27(29.3)	3(17.6)	2(20)	4(28.6)	10(23.3)
Illness day at enrollment	14(0-60)	5(0-10)	2(0-13)	4(3-18)	4 (1-30)	12 (2-90)	6(1-90)	22(6-37)	25.5(7-60)	18(4-30)	5(1-60)
Length of hospital stay	24(0-59)	5(0-67)	18(13-26)	2(1-17)	14 (1-119)	26(0-162)	11(0-118)	41(27-102)	11(1-23)	23(0-134)	8(0-75)
Clinical signs/symptoms											
Fever (n,%)	19(95)	10(100)	NA	5(100)	59(96.7)	117(96.7)	83(92.2)	13(76.5)	7(70)	12(85.7)	36(87.8)
Headache (n,%)	18(90)	10(100)	6(66.7)	3(60)	57(91.9)	113(96.6)	64(76.2)	9(64.3)	10(100)	13(92.9)	15(40.5)
Cranial nerve palsy (n,%)	4(20)	2(20)	NA	0	5(7.8)	23(18.9)	9(9.8)	0	3(30)	4(28.6)	3(7)
Hemiplegia (n,%)	1(5)	2(20)	NA	0	1(1.6)	12(9.8)	4(4.3)	0	1(10)	1(7.1)	3(7)
Paraplegia (n,%)	0	0	NA	0	1(1.6)	10(8.2)	3(3.3)	0	1(10)	1(7.1)	4(9.3)
Tetraplegia (n,%)	0	0	NA	0	1(1.6)	5(4.1)	3(3.3)	0	0	1(7.1)	4(9.3)
Convulsions (n,%)	1(5)	1(10)	NA	0	1 (1.6)	2(1.7)	15(16.5)	0	0	0	5(11.9)
Neck stiffness (n,%)	17(85)	9(90)	NA	4(80)	47 (77)	57(47.6)	30(33.7)	5(29.4)	5(50)	7(50)	8(19.5)
GCS** at enrolment (median, range)	14(8-15)	11(7-15)	NA	14(10-15)	12 (3-15)	14(4-15)	11(3-15)	11(6-14)	15(9-15)	13(8-15)	13(7-15)
HIV positive, (n%)	2(20)	1(10)	NA	0	0	22(24.2)	0	0			1(2.3)
CSF examinations											
CSF leukocyte count (per mm3)	317 (58-896)	209 (18-1571)	11000 (500-19200)	5(1-93)	1924 (24-51810)	312(3-3969)	43(1-909)	23(6-187)	501(140-1101)	36.5(2-357)	2(1-2700)
CSF neutrophils (%)	26(3-93)	3(0-18)	91.5(83-98)	20(0-87)	83 (10-98)	26(0-95)	14(0-91)	14(9-65)	12(7-42)	26.5(0-67)	30(0-93)
CSF lymphocytes (%)	74(7-94)	96(0-98)	6(0.9-17)	13(0-99)	17 (2-90)	73(5-92)	84(0-94)	86(35-91)	47(20-70)	67.5(33-86)	50(0-99)
CSF/blood glucose ratio	0.19 (0.04-0.4)	0.61 (0.5-7.66)	NA	0.65 (0.41-0.67)	0.3 (0-1)	0.3(0.1-0.7)	4(1.7-7.7)	0.8(0.5-1.4)	0.4(0.4-0.9)	0.3(0-0.6)	0.7(0.4-1.3)
CSF lactate (mmol/L)	6.77 (3.21-12.43)	2.79 (1.88-3.52)	NA	3.65 (1.78-7.3)	9.9 (2.3-28.2)	5(1.9-12.8)	2.5(1.3-6.6)	1.9(1.4-2.7)	2.7(2.2-4.2)	4.9(2.7-12.7)	2.5(1.4-6.4)
Total protein (g/L)	2(1.1-4.1)	0.8(0.3-2.4)	NA	0.5(0.3-2.6)	2.3 (0.3-8.7)	1.9(0.2-29.8)	0.7(0.1-3.2	0.3(0.2-0.8)	0.8(0.2-3.9)	0.6(0.4-1.8)	0.4(0.2-3.2)
Discharge mRS^				.							
0	3(15)	0	NA	1(20)	8(12.5)	22(18)	17(18.2)	0	0	0	5(11.6)
1	3(15)	2(20)	NA	0	6(9.4)	16(13.1)	19(20.7)	2(11.8)	3(30)	0	6(14)
2	5(25)	0	NA	2(40)	12(18.8)	15(13.2)	19(20.7)	0	4(40)	1(7.1)	8(18.6)
3	4(20)	4(40)	NA	1(20)	20(31.3)	14(11.5)	12(13)	6(35.3)	1(10)	4(28.6)	8(18.6)
4	1(5)	2(20)	NA	0	11(17.2)	22(18)	13(14.1)	3(17.6)	1(10)	3(21.4)	9(20.9)
5	2(10)	2(20)	NA	0	3(4.7)	17(13.7)	10(10.9)	5(29.4)	1(10)	1(7.1)	6(14)
6	2(10)	0	NA	1(20)	4(6.3)	16(13.1)	2(2.2)	1(5.9)	0	5(35.7)	1(2.3)

Note to Table S2: ♪outcomes at discharge were recorded as full recovery (n=4) or neurological deficit (n=4)*****due to the small sample size, data on two cases with neurotoxoplamosis are not shown, #including 44 laboratory confirmed cases, ♫including 95 laboratory confirmed cases, $including 23 laboratory confirmed cases, **Glasgow coma score, ^Modified Rankin Scale (0: full recovery with no symptoms, 1: No significant disability, 2: Slight disability, 3: Moderate disability, 4: Moderately severe disability, 5: Severe disability, and 6: Dead); BM: bacterial meningitis, TBM: tuberculous meningitis; Data are number (%), continuous variables are presented as median (range)

Table S3. List of marker candidates identified by mass spectrometry analysis

BM group							
No	**Protein ID**	**Protein name**	**Gene name**	**Mean intensity of BM (log_2)**	**Mean intensity of Other (log_2)**	**Difference in intensity between BM and Others**	**-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	7.3
3	P28676	Grancalcin	GCA	-21.18	-26.58	-5.4	7.11
4	P11413-2	Glucose-6-phosphate 1-dehydrogenase	G6PD	-21.17	-26.4	-5.23	6.31
5	P26583	High mobility group protein B2	HMGB2	-20.83	-26.23	-5.41	5.3
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	-23.6	-5.78	5.81
11	P22894	Neutrophil collagenase	MMP8	-19.34	-24.07	-4.75	5.77
12	P50395	Rab GDP dissociation inhibitor beta	GDI2	-20.2	-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	-19.86	-25.15	-5.29	5.47
17	P08670	Vimentin	VIM	-18.54	-22.2	-3.66	5.46
18	P08107	Heat shock 70 kDa protein 1A	HSPA1A	-19.45	-24.1	-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	-21.18	-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	-24.6	-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	4.7
29	Q6UX06	Olfactomedin-4	OLFM4	-22.21	-26.55	-4.33	4.69
30	P52209-2	6-phosphogluconate dehydrogenase, decarboxylating	PGD	-19.4	-24.03	-4.63	4.67
31	P37837	Transaldolase	TALDO1	-19.90	-24.88	-4.98	4.6
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	-23.1	-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	-4.4	4.12
44	P09211	Glutathione S-transferase P	GSTP1	-18.82	-23.38	-4.56	4.1
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	-21.9	-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	-25.4	-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	3.6
57	P02788-2	Lactotransferrin	LTF	-18.8	-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	-24.1	-4.27	3.27
TBM group							
No	**Protein ID**	**Protein name**	**Gene name**	**Mean intensity of BM (log_2)**	**Mean intensity of Other (log_2)**	**Difference in intensity between TBM and Others**	**-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	-17.36	-20.25	-2.9	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	-17.8	-18.93	-1.13	3.14
10	P01591	Immunoglobulin J chain	JCHAIN	-19.12	-22.1	-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	-23.7	-2.69	2.36
19	P02743	Serum amyloid P-component	APCS	-23.62	-25.81	-2.19	2.3

Table S4. **Results of analysis comparing the diagnostic value of LCN2 in distinguishing between patients with confirmed and clinically suspected bacterial meningitis**

cBM vs. sBM	**Cut-off**	**AUC (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**DOR (95% CI)**
LCN2 (ng/ml)	452.3	0.74 (0.6-0.88)	0.82 (0.68-0.9)	0.6 (0.39-0.78)	6.8 (2.1-21.9)
CSF leukocytes (cell per mm^3)	1533	0.61 (0.47-0.76)	0.61 (0.47-0.74)	0.65 (0.43-0.82)	3.0 (1-8.9)
CSF lactate (mmol/L)	9.0	0.84 (0.74-0.95)	0.77 (0.63-0.87)	0.8 (0.58-0.92)	13.6 (3.7-50.1)
CSF/blood glucose ratio	0.2	0.76 (0.63-0.88)	0.57 (0.42-0.7)	0.95 (0.76-0.99)	25 (3.1-203.6)
CSF protein (g/L)	2.1	0.74 (0.61-0.86)	0.66 (0.51-0.78)	0.75 (0.53-0.89)	5.8 (1.8-19)
CSF White cell count+lactate+CSF/blood glucose level+CSF protein	NA	0.87 (0.77-0.97)	0.95 (0.85-0.99)	0.65 (0.43-0.82)	39 (7.2-21.4)
CSF White cell count+lactate+CSF/blood glucose level+CSF protein+lipocalin 2	NA	0.87 (0.77-0.96)	0.95 (0.85-0.99)	0.65 (0.43-0.82)	39 (7.2-21.4)

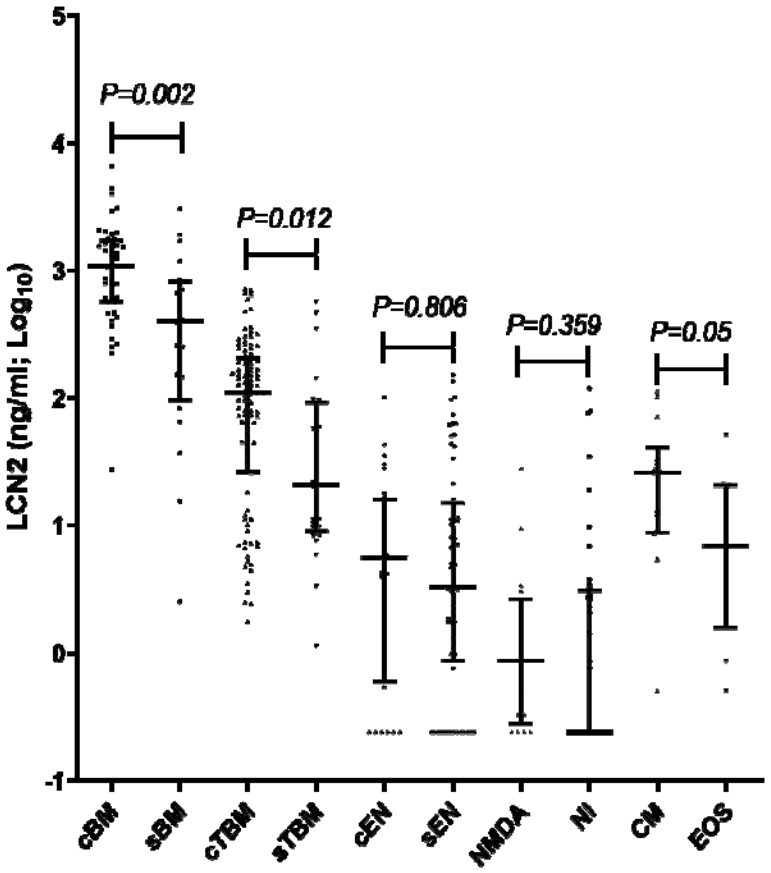
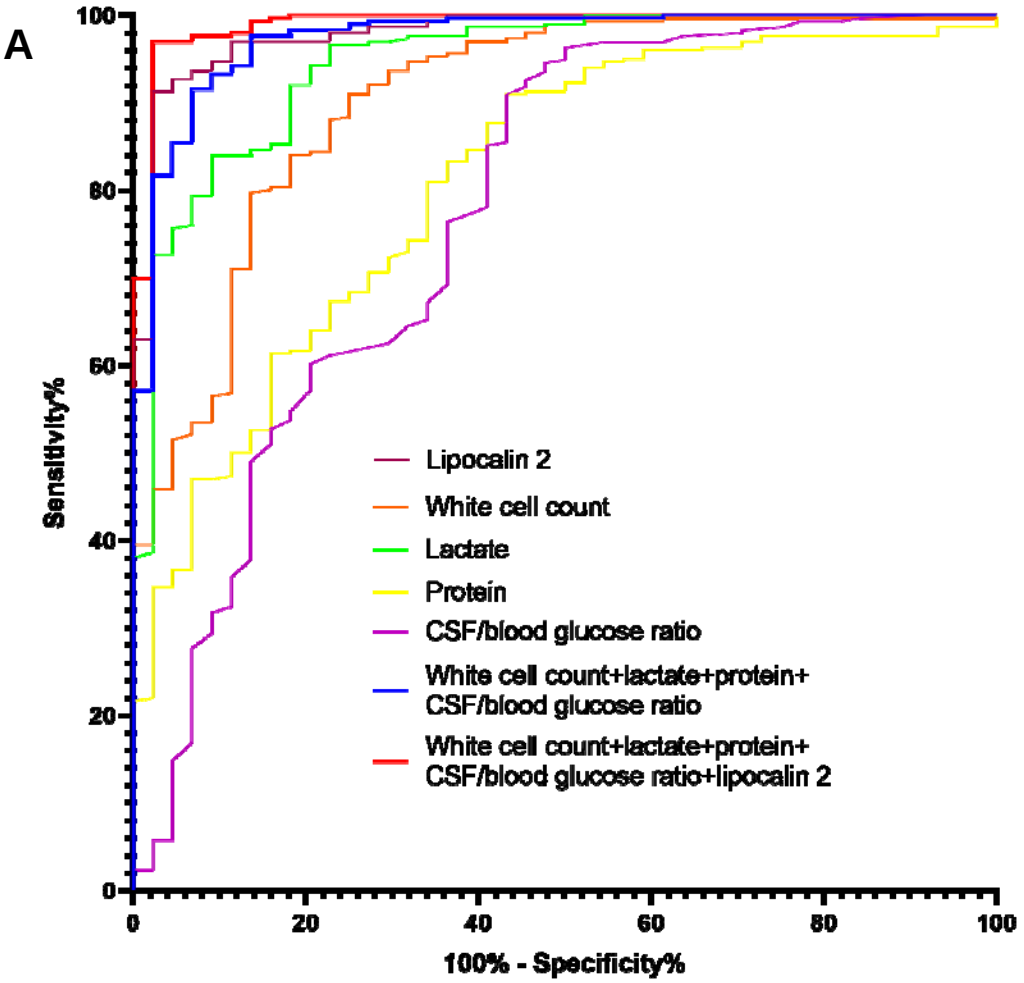
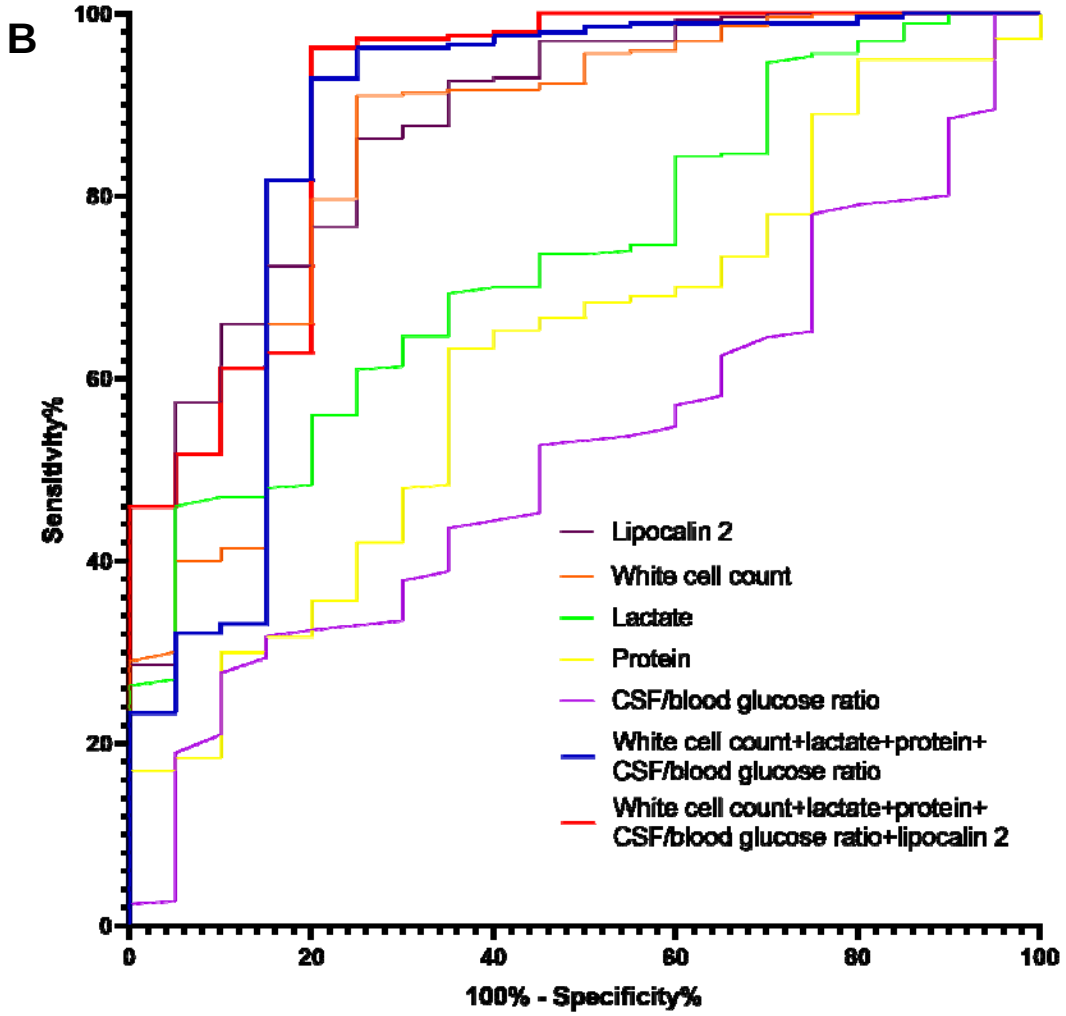


Figure S1. Plots showing the distribution of LCN2 concentrations in patients with laboratory confirmed or clinically suspected CNS infections and non-CNS infections

Note to Figure S1: cBM: confirmed bacterial meningitis, sBM: clinically suspected bacterial meningitis, cTBM: confirmed tuberculous meningitis, sTBM: clinically suspected tuberculous meningitis, cEN: confirmed encephalitis, sEN: clinically suspected encephalitis, NMDA: anti-NDMAR encephalitis, NI: non-CNS infections, CM: crytococcal meningitis, EOS: eosinophilic meningitis



cBM prediction	Cut-off values	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	DOR (95% CI)
Lipocalin 2 (ng/ml)	221.3	0.98 (0.96-1)	0.98 (0.88-1)	0.91 (0.88-0.94)	453.2 (59.9-3426.3)
CSF leukocytes (cell per mm^3)	427	0.91 (0.86-0.96)	0.86 (0.73-0.94)	0.8 (0.75-0.84)	25.3 (10.2-62.7)
CSF lactate (mmol/L)	5.8	0.95 (0.91-0.98)	0.91 (0.79-0.96)	0.84 (0.79-0.88)	52.5 (17.9-153.5)
CSF/blood glucose ratio	<0.2	0.78 (0.69-0.86)	0.57 (0.42-0.7)	0.92 (0.88-0.94)	14.3 (6.9-29.4)
CSF protein (g/L)	2.9	0.81 (0.75-0.88)	0.57 (0.42-0.7)	0.91 (0.87-0.94)	13.3 (6.5-27.2)
CSF white cell count+lactate+CSF/blood glucose level+CSF protein	NA	0.97 (0.95-1)	0.93 (0.82-0.98)	0.92 (0.88-0.94)	148.2 (42.8-512.8)
CSF white cell count+lactate+CSF/blood glucose level+CSF protein+lipocalin 2	NA	0.99 (0.98-1)	0.93 (0.82-0.98)	0.97 (0.94-0.98)	435.8 (113.3-1676.1)



sBM prediction	Cut-off values	AUC (95% CI)	Sensitivity (95% CI)	Specificity 95% CI)	DOR (95% CI)
Lipocalin 2 (ng/ml)	146.5	0.88 (0.79-0.96)	0.75 (0.53-0.89)	0.86 (0.82-0.9)	18.9 (6.5-54.9)
CSF white cell count (per mm^3)	709	0.85 (0.75-0.95)	0.75 (0.53-0.89)	0.91 (0.87-0.94)	30.3 (10.2-89.9)
CSF lactate (mmol/L)	3.0	0.73 (0.64-0.83)	0.95 (0.76-0.99)	0.46 (0.41-0.52)	16.2 (2.1-122.5)
CSF/blood glucose ratio	<0.7	0.52 (0.41-0.64)	0.9 (0.7-0.97)	0.28 (0.24-0.34)	3.6 (0.8-15.7)
CSF protein (g/L)	1.2	0.62 (0.51-0.74)	0.65 (0.43-0.82)	0.63 (0.58-0.69)	3.2 (1.2-8.3)
CSF white cell count+lactate+CSF/blood glucose level+CSF protein	NA	0.87 (0.76-0.98)	0.8 (0.58-0.92)	0.93 (0.89-0.95)	52.4 (16.1-170.8)
CSF white cell count+lactate+CSF/blood glucose level+CSF protein+lipocalin 2	NA	0.9 (0.83-0.98)	0.8 (0.58-0.92)	0.96 (0.94-0.98)	103.6 (29.7-361.8)

Figure S2. Diagnostic performance of LCN2 in discriminating between laboratory confirmed (A) or clinically suspected bacterial meningitis patients (B) and other clinical entities and in comparison with existing biomarkers.

Note to Figure S2: NA: not applicable