Clinical predictors, biomarkers and pathogen discovery sequencing in suspected central nervous system infections

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CLINICAL PREDICTORS, BIOMARKERS AND PATHOGEN DISCOVERY SEQUENCING IN SUSPECTED CENTRAL NERVOUS SYSTEM INFECTIONS

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Clinical predictors, biomarkers and pathogen discovery sequencing in suspected central nervous system infections

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CHAPTER 1

INTRODUCTION

INTRODUCTION

Infections of the central nervous system (CNS) are associated with high morbidity and mortality, depending on the causative pathogen. Invasion of the CNS by a pathogen can cause either an infection of the membranes surrounding the brain and spinal cord (meningitis), the brain itself (encephalitis), the spinal cord (myelitis) or a combination of these. Encephalitis is mostly referred to as a clinical syndrome, characterized by an altered mental status, combined fever, seizures, focal neurological deficits, elevated cerebrospinal fluid (CSF) leukocyte count, abnormalities on neuroimaging and/or an abnormal electroencephalogram.[1] Clinical symptoms of CNS infections can be diverse and vary by the site of infection of the CNS and causative pathogen.[2] Virtually all pathogens can infect the brain, including viruses, bacteria, fungi and parasites. Treatment and prognosis of CNS infections differ per cause.

The incidence and etiology of CNS infections vary greatly by age and geographical location. Overall incidence rate, including viral and bacterial meningitis and encephalitis, has been reported to be 8.1 per 100.000 population per year in Denmark.[3] For viral meningitis, numbers ranging from 2.7-3.6/100.000 have been described in Europe[3,4], whereas for bacterial meningitis rates of 0.7-3.1/100.000 were reported in Europe and the United States.[3,5,6] In poor-income countries however, especially sub-Saharan Africa, the incidence of bacterial meningitis ranges from 10-40 cases per 100.000 population per year.[7] Common viruses causing meningitis are enteroviruses and herpes viruses, in which case prognosis is usually relatively good. However, for example in patients with enterovirus meningitis, 6% does have an unfavorable outcome after 6 months.[8] In community-acquired bacterial meningitis, most cases are caused by *Streptococcus pneumoniae* and in lesser extent by *Neisseria meningitidis* and various other bacteria.[7,9] In contrast to viral meningitis, bacterial meningitis has a more severe clinical course and immediate treatment with antibiotics and dexamethasone is required to improve prognosis.[9-11] The same accounts for viral encephalitis, in which outcome is generally poor. For encephalitis in general, incidence rates of 7/100.000 have been described in the United States, of which 10-25% of the cases are attributable to viral infection, with herpes viruses being the most common cause in infectious encephalitis.[2,12] Of all viral encephalitides, outcome has been studied most extensively in herpes simplex virus (HSV) encephalitis, in which early start of treatment with acyclovir has also been associated with improved outcome.[13,14]

Because of the potentially poor prognosis and risk of mortality in suspected cases of bacterial meningitis or HSV encephalitis, treatment with antimicrobial agents should be initiated as soon as possible. Empirical treatment without confirmation of the pathogen,

has its drawbacks, as complications like antimicrobial resistance to antibiotics or kidney damage caused by acyclovir may occur. Therefore a timely and accurate diagnosis is of the essence. Besides different types of CNS infections the differential diagnosis in patients suspected of a CNS infection also includes inflammatory CNS diseases, systemic infections without CNS involvement and neurological diseases such as epilepsy or stroke.[12,15,16] Differentiating between these diseases can be difficult based on clinical and laboratory parameters.[15,17] The classical triad of fever, neck stiffness and an altered mental status is present in only 41% of patients with bacterial meningitis, and 95% has two out of four symptoms of headache, fever, altered mental status and neck stiffness.[9,17,18] An elevated number of leukocytes in the CSF so far best predicts having a CNS infection or not, with a sensitivity of 94%, however, with a specificity of only 68%.[15] Several diagnostic prediction models have been proposed, combining clinical and laboratory characteristics to predict the risk of acute bacterial meningitis in a patient.[17] Although some of them have been validated externally, many of them have not and often only use cohorts including patients with bacterial and viral meningitis. They should therefore be used with caution, and validation in a broader population consisting of all consecutive patients suspected of any CNS infection should be performed.

Various studies have examined other diagnostic markers for the diagnosis of bacterial meningitis in both blood and CSF.[17] The concentration of lactate in CSF, for example, is one of those markers that has been studied extensively, and is widely available as diagnostic test. However, in most studies the comparison was between patients with bacterial or viral meningitis, whereas the concentration also seems elevated in patients with other CNS diseases.[19,20] Moreover, in one meta-analysis, sensitivity of CSF lactate concentration to differentiate between bacterial and viral meningitis decreased from 93% to 49% in patients who were treated with antibiotics prior to the lumbar puncture.[20] Usefulness in clinical practice therefore seems limited. For both the diagnosis of bacterial meningitis or viral encephalitis, several other markers have been evaluated, but so far no additional value in clinical practice has been established.[12,17]

In a substantial proportion of patients in whom we do find the diagnosis of a CNS infection to be extremely likely, it is not possible to detect the causative pathogen with currently available microbiological methods. Bacterial CSF cultures remain negative in 4-50% of patients with bacterial meningitis[21], and polymerase chain reaction (PCR) of the CSF remains negative in 35-42% of viral CNS infections.[3,4,15] In patients with bacterial meningitis in Burkina Faso, PCR was able to identify a pathogen in 34% of patients in whom conventional methods did not lead to the definitive diagnosis.[22] Since PCR only targets specific pathogens, multiplex PCR's have been developed, enabling testing for multiple pathogens with only one test. One of them is the FilmArray meningitis

or encephalitis panel (BioFire Diagnostics LLC, Salt Lake City, UT, USA), which can simultaneously test for 16 bacterial, viral and fungal pathogens. A meta-analysis of the FilmArray meningitis or encephalitis panel, found a sensitivity of 90% and a specificity of 97% for detection of all pathogens.[23] However, diagnostic analysis using this test is complicated by the number of false-positives, with the highest percentage of false-positives per individual pathogen being for *S. pneumoniae* (17.5%).[23]

Metagenomic sequencing has emerged in recent years as a promising microbiological method to detect pathogens hypothesis free. With metagenomic sequencing, all nucleic acid is being extracted and sequenced, which in theory would enable detection of all pathogens present in a sample, including known, unexpected and novel species. It is being researched extensively in patients with suspected CNS infections, with promising results.[24,25] However, thus far these kind of methods are not ready to replace conventional microbiological techniques and are being used in addition to other available diagnostic methods.

In 2012, the PACEM (Pediatric and Adult Causes of Encephalitis and Meningitis) study was set up in the Amsterdam University Medical Centers (AUMC) by the Neuro-infections research group.[15] Up until 2015, the PACEM study prospectively included all consecutive patients in the AUMC, location AMC, in whom a lumbar puncture was performed because of the suspicion of a CNS infection, and determined diagnostic accuracy of clinical and laboratory characteristics. The study confirmed that CSF leukocyte count thus far is the best individual predictor for the diagnosis of CNS infections, but lacks specificity.[15]

These results were the reason to set up the I-PACE (Improving Prognosis by using innovative methods to diagnose Causes of Encephalitis) study in 2017, which is by now an ongoing, prospective cohort study in multiple hospitals in the Netherlands (Figure 1). The aim of the I-PACE study is to improve the timely cause-specific diagnosis for encephalitis and thereby improve outcome of these patients.

Inclusion criteria are identical to the PACEM study, and of all included patients, clinical data, blood, pharyngeal and rectal swabs and CSF are being stored in a biobank. With all these patient materials, various clinical predictors, biomarkers and pathogen sequencing techniques will be developed, applied and validated in this cohort. Besides patients with CNS infections, final diagnoses of included patients consist of CNS inflammatory disease, systemic infections and other neurological disorders like epilepsy or stroke.[15] This population reflects clinical practice and is, therefore, an ideal cohort for diagnostic studies.

Figure 1. Participating hospitals in the I-PACE study

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is to assess and improve diagnostic accuracy of clinical predictors, biomarkers and pathogen discovery sequencing for diagnosing CNS infections. To address this aim, several methods were studied or validated in patients included in the PACEM and/or I-PACE studies.

First, in **Chapter 2** an external validation of existing diagnostic prediction models for bacterial meningitis is presented. We performed a search of the literature and systematic review of the identified prediction models, and validated them in adults from the PACEM cohort. Since a substantial part of the identified prediction models in **Chapter 2** was originally developed in cohorts of children, **Chapter 3** presents the results of a validation of diagnostic prediction models for bacterial meningitis in children from the PACEM study.

In addition to currently available clinical signs and laboratory tests that can assist in diagnosing a CNS infection, the search for new biomarkers is ongoing. **Chapter 4** presents a study on the diagnostic accuracy of neurofilament light chain (NfL) as diagnostic marker for CNS infections. NfL is a component of the axonal skeleton and in several CNS diseases identified as marker for axonal damage. NfL was measured in the CSF of adult patients from the PACEM cohort, initially suspected of a CNS infection.

Furthermore, microbiological techniques that can consistently identify the specific causative pathogen in suspected CNS infections are needed. **Chapter 5, 6 and 7** describe the use of viral metagenomic sequencing technique called virus discovery cDNA amplified fragment length polymorphism next generation sequencing (VIDISCA-NGS) in patients with suspected CNS infections. In **Chapter 5** the performance of VIDISCA-NGS is evaluated in CSF samples, in which previously a virus was detected by qPCR to test the reproducibility of the method. In **Chapter 6** we present the results of a study on the diagnostic accuracy of VIDSCA-NGS in CSF of patients with and without proven viral CNS infections from the PACEM cohort. For this study we selected patients with a clinical diagnosis of a viral CNS infections and patients in whom finally a different diagnosis was established. CSF of these patients was analyzed with VIDISCA-NGS. Subsequently, in **Chapter 7** we explored the possibility of VIDISCA-NGS being able to detect bacterial RNA in CSF in addition to viruses.

Finally, **Chapter 8 and 9** describe clinical and laboratory characteristics in specific subgroups of patients with the initial suspicion of a CNS infection, whereas **Chapter 10** focused on outcome. One specific subgroup were patients with the suspicion of a nosocomial CNS infection, not related to trauma or neurosurgery. **Chapter 8** gives an overview of their clinical characteristics, the diagnostic accuracy of these characteristics and what final diagnoses were established in this group of non-surgical nosocomial CNS infections. A different subgroup was studied in **Chapter 9**, which presents the results of a study in patients who present with a seizure and receive a lumbar puncture for the suspicion of a CNS infection. The study in **Chapter 10** focused on outcome of the entire cohort of patients in the PACEM and I-PACE cohort and determined predictors for poor outcome.

In **Chapter 11** the results of the presented studies and its implications for the future are being discussed. In the **Appendices** we summarize de results of this thesis.

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CHAPTER 2

SYSTEMATIC REVIEW AND VALIDATION OF DIAGNOSTIC PREDICTION MODELS IN PATIENTS SUSPECTED OF MENINGITIS

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ABSTRACT

Objectives

Diagnostic prediction models have been developed to assess the likelihood of bacterial meningitis (BM) in patients presented with suspected central nervous system (CNS) infection. External validation in patients suspected of meningitis is essential to determine the diagnostic accuracy of these models.

Methods

We prospectively included patients who underwent a lumbar puncture for suspected CNS infection. After a systematic review of the literature, we applied identified models for BM to our cohort. We calculated sensitivity, specificity, predictive values, area under the curve (AUC) and, if possible, we evaluated the calibration of the models.

Results

From 2012-2015 we included 363 episodes. In 89 (24%) episodes, the patient received a final diagnosis of a CNS infection, of whom 27 had BM. Seventeen prediction models for BM were identified. Sensitivity of these models ranged from 37% to 100%. Specificity of these models ranged from 44% to 99%. The cerebrospinal fluid model of Oostenbrink reached the highest AUC of 0.95 (95% CI 0.91-0.997). Calibration showed over- or underestimation in all models.

Conclusion

None of the existing models performed well enough to recommend as routine use in individual patient management. Future research should focus on differences between diagnostic accuracy of the prediction models and physician's therapeutic decisions.

INTRODUCTION

Acute community-acquired bacterial meningitis is a severe disease that requires immediate medical attention. Mortality is high and up to half of the survivors suffer from neurological and cognitive sequelae.[1,2] Early start of treatment improves the prognosis and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines recommended to treat all patients with suspected bacterial meningitis as soon as possible and not later than within one hour after emergency presentation.[3-5] A cohort study of patients with suspected central nervous system (CNS) infections showed that bacterial meningitis was diagnosed in only 7% of cases, and that 75% did not have a CNS infection.[6] To avoid unnecessary antibiotic treatment but still treat all patients with bacterial meningitis early, it is important to confirm or exclude the diagnosis as quick as possible.

Several diagnostic prediction models have been developed to guide clinicians in these situations. Most of these models combine clinical and laboratory findings to predict the probability of acute bacterial meningitis, compared to more benign viral meningitis or no bacterial meningitis.[7-10] Although some of these models have been validated externally, they should be used with caution.[11] The population used for derivation varies per model, often comparing patients with viral and bacterial meningitis only, and different gold standards for the diagnosis were used. Therefore, it is necessary to look at the value of these models in a broader population consisting of all patients suspected of CNS infection.

We performed a single center study in adult patients with suspected CNS infections in whom a lumbar puncture was performed.[6] The aim of this current study was to validate existing diagnostic prediction models for bacterial meningitis in this cohort.

METHODS

Systematic review

We systematically reviewed the literature in MEDLINE and EMBASE to identify models that predict the probability of acute bacterial meningitis. Previously a search filter for prediction models was validated.[12] We combined this filter with terms for meningitis including both Medical Subject Heading (MeSH) terms and terms in title and abstract (Supplementary Material 1). We searched for full text articles in scientific peer-reviewed journals between January 1946, the earliest date of included publications in MEDLINE, and August 2018, the moment of our search. We only included papers published in

English, German, French, Spanish or Dutch, which were the languages we were able to translate.

For the purpose of this review we defined a prediction model as a decision-making tool that provides risk categories or probabilities for the diagnosis of bacterial meningitis or suggests a diagnostic or therapeutic course of action, containing at least three variables obtained from history, physical examination or simple diagnostic tests.[13] Publications were included if they contained the development, an update or a validation of a prediction model. Publications focusing on tuberculous meningitis or neuroborreliosis were excluded because of the different, sub-acute, clinical presentation of these diseases. Quality of the included studies was assessed according to the TRIPOD criteria.[14,15]

Patients

We validated the identified prediction models using data from 363 episodes in adults (≥16 years) with suspected neurological infections.[6] Between 2012-2015 patients presenting at the emergency department or inpatients were included if examination of cerebrospinal fluid (CSF) was performed because CNS infection was suspected. Exclusion criteria were neurosurgery or traumatic brain injury within the last three months prior to the suspected infection, or a neurosurgical device in situ. Methods have been described in detail previously.[6] In this cohort 89 patients (24%) were diagnosed with a CNS infection, of which 27 (7%) had bacterial meningitis. Other diagnostic categories included inflammatory CNS diseases, systemic infections without CNS infection and other neurological diseases such as stroke or epilepsy. We validated all identified prediction models in these 363 episodes. If specific predictors or a valid proxy were not available, the prediction model was validated without that particular variable and the suggested cut-off for defining high probability was adjusted accordingly.

Statistics

The performance of the prediction models was assessed by the evaluation of discrimination and calibration.[16-18] Discrimination was evaluated by constructing receiver operating characteristic (ROC) curves and calculating the area under the curve (AUC) with 95% confidence intervals (CI). Higher AUC values were considered to indicate better discriminatory ability, as follows: "excellent discrimination" with an AUC of ≥0.90; "good discrimination" for 0.80 ≤ AUC < 0.90; "fair discrimination" with 0.70 ≤ AUC < 0.80; and "poor discrimination" with an AUC < 0.70.[19,20] Calibration was evaluated by building a calibration curve and assessing the calibration slope. Additionally we calculated the calibration-in-the-large, which is the difference between the mean observed proportion and mean predicted proportion. For some of the prediction models based on a

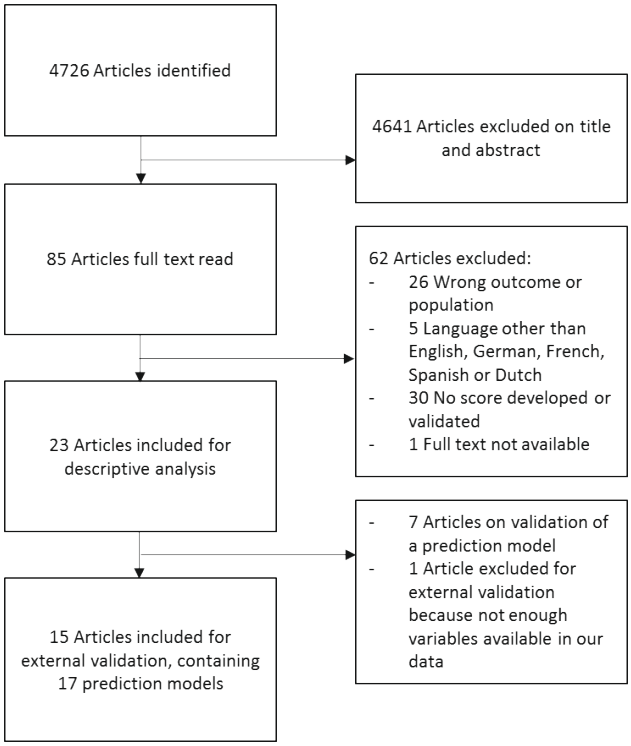
multivariable logistic regression model, we were not able to retrieve all beta coefficients from the original publication. Whenever this was the case, or if a model was not based on multivariable logistic regression model at all, we used the observed proportions in the respective risk categories, as reported in the original paper, as expected proportions in those risk groups in the validation data. For these models we used the Hosmer-Lemeshow (HL)-test to test the goodness-of-fit, instead of calculating the calibration slope. To evaluate the impact of the method we used, we performed a simulation study for two of the models for which we had both the entire multivariable logistic regression model and the observed proportions in the respective risk categories. This showed comparable results with regard to calibration (Supplementary Table 1). When cut-offs for defining high or low risk of bacterial meningitis were reported in the original publication, sensitivity, specificity, positive and negative predictive values were calculated with 95% confidence intervals. For the models for which we had the complete multivariable logistic regression model, we calculated proportions of patients assigned to different risk groups, to clearly display the spread of predicted probabilities and the clinical significance of this spread. We defined a probability of <0.1 as "low risk" and >0.8 as "high risk" , based on a consensus in a discussion with two clinicians, prior to the analysis. Probabilities in between were considered insignificant for clinical decision making. The median number of missing values was 2% (interquartile range 0-6.75%). Missing data were handled by multiple imputation using the R package MICE. We used 40 variables from medical history, physical examination and laboratory results as predictors to impute missing values.[21] For discrimination and calibration we used R packages pROC[22] and predictABEL.[23] We used Rubin's rule and bootstrapping to estimate proportions and c-statistics based on eight imputation sets. All statistical tests were two-tailed and p-values of <0.05 were considered statistically significant.

RESULTS

Systematic review

Our literature search yielded 4726 articles of which 4641 (98%) were excluded based on title or abstract and 85 (2%) articles were read in full (Figure 1). We included 23 publications on 17 different diagnostic prediction models for acute bacterial meningitis.[7-10,24-43] Seventeen publications described the development or update of a prediction model, of which two also validated another existing model in their dataset. The publications of Spanos[10] and Oostenbrink[9,40,41] both reported two separate models, of which the latter combined two logistic regression models into one score.

Figure 1. Inclusion process



An overview of the characteristics of the included prediction models is given in Table 1. All models were based on clinical characteristics and/or laboratory test results from blood and CSF. Different statistical methods have been used to develop the models (Table 1). Characteristics of the derivation cohorts on which the models were based are presented in Table 2, as well as the previously reported performance measures. Eleven models were developed in children[8,9,25,27,30,31,34,35,40,41,43], two in adults[28,42], four in both[7,10,29]. The evaluation of the quality of the included publications is shown in supplementary table 2. The most frequent limitations were retrospective derivation cohorts, small numbers of patients and little information about all model-building procedures such as selection of predictors.

Table 1. Characteristics of included prediction models

	Population	Modelling method	Items	Cut-off
Bonsu (2004)	Children >1 month-18 years, with pleiocytosis	Logistic regression model	$1/(1+e^{-L})$, in which L= 11.448 +0.003× CSF total neutrophil count(cells/mm3)-34.802×$(10^{-2}$×CSF protein (mg/dL))$^{0.5}$+21.991×$(10^{-2}$×CSF protein (mg/dL)-0.345×age (years)	Bact. men. is unlikely if score <0,1
Bonsu 2 (2008)	Children >4 weeks – 18 years with acute meningitis	Recursive partitioning analysis	Blood leucocytes >17.000 cells/ml, bands-to-neutrophils ratio in blood >10%[a], CSF leucocyte count >600 cells/ul, CSF neutrophils >75%, CSF total protein >100mg/dL, CSF glucose <40mg/dL. One point per item, add points.	Bact. men. is unlikely if score 0
Boyer (1980)	Children 1 month-15 years with meningitis	Univariate analysis	Temp≥39.5=1, purpura=2, neurological signs (obnubilation/coma/focal/seizures) = 1, CSF protein level 0.9-1.4g/L= 1, ≥1,4=2, CSF glucose 0.35-0.2 g/L or 2-1 mmol/L=1, ≤0.2 g/L or ≤1 mmol/L=2, CSF leukocytes/mm3 1000-4000=1, ≥4000= 2, CSF ≥60% polynuclears= 1, blood leucocytes ≥15000/mm3=1	If score ≥5 treat for bact. men., if score ≤2 no treatment required, if score 3-4 repeat LP after 12-24h hours if clinically stable
Brivet (2005)	Adults (>16 years) with meningitis	Predictors based on logistic regression	Severity (altered consciousness, seizures, focal neurological findings, shock) or CSF absolute neutrophil count above 1,000/mm3 mm	Bact. men. is not ruled out if one characteristic is present
Chavanet (2007)	Children >3 months and adults with meningitis	Categorical analysis regression tree method	Children: CSF leukocytes ≥1800 cells/ul= 2 points, CSF neutrophils >80%=3 points, CSF protein >1.2 g/L=3 points, CSF/blood glucose ratio ≤0.3=3 points. Adults: blood leukocyte count >15/ul=3 points, CSF leukocytes ≥1700=4 points; <1700=2 points; <250=1 point, CSF neutrophils >90%=4 points; >80%=2points; >25%=1 point, CSF protein >2.3 g/L=5 points; >0.8=2 points, CSF/blood glucose ratio ≤0.35=4 points	Children: ≥2 high risk on bact. men.; Adults: >6 high risk on bact. men.
De Cauwer (2007)	Children 0-15 years with meningitis	Not specified (searched for values with satisfactory margins)	CSF neutrophils percentage >80%, CSF glucose <52 mg/dl, CRP count ≥2.0 mg/dl, CSF protein ≥100 mg/dl	Bact. men. likely if ≥1 point or more on 4-point scale
Deivanayagam (1993)	Children 2 months – 11 years with suspected meningitis	Not specified	CSF leukocytes >300/mm3 and CSF neutrophils >60% and CSF/blood glucose ratio <0.5 or absolute CSF glucose <30 mg/dL	With all three CSF findings (low CSF/blood glucose ratio or low absolute glucose in CSF) empirical diagnosis of bact. men. can be reasonably made

Table 1. (Continued)

	Population	Modelling method	Items	Cut-off
Dubos (2007)	Children 29 days-16 years with meningitis	Chosen variables based on literature and Bacterial Meningitis Score	Convulsions, purpera, toxic appearance (lethargy, irritability, altered peripheral perfusion, hypo- or hyperventilation), blood procalcitonin ≥0.5 ng/ml, CSF protein ≥0.5 g/L, positive CSF Gram stain	Patient should be treated with antibiotics if one characteristic is present
Freedman (2001)	Children 2 months – 17 years in whom LP performed for ruling out BM	Predictors based on logistic regression	CSF leucocytes >30/ul, younger than 6 months, abnormal CSF glucose/ ratio/protein/gram stain/ peripheral band count[a])	Patient should be treated as bact. men. if one characteristic is present
Hoen (1995)	Children >1 month and adults with meningitis	Logistic regression model	$1/(1+e^{-L})$, in which L=32.13×10^{-4}×CSF PMN count (10^6/L)+2.365×CSF protein (g/L)+0·6143×blood glucose (mmol/L)+0·2086×white blood cell count (10^9/L)[11]	Bact. men. is unlikely if score <0,1
Nigrovic (Bacterial Meningitis Score, 2002)	Children 29 days- 19 years with meningitis	Score based on multivariate logistic regression and recursive partitioning analysis	Positive CSF Gram stain=2, CSF protein ≥0·8 g/L =1, peripheral absolute neutrophil count ≥10 000 cells per µL=1, seizures before or on admission=1, CSF absolute neutrophil count >1000 cells per µL=1	Bact. men. unlikely if score 0 on 6-point scale
Oostenbrink (2001-2004)	Children >1 month – 15 years with meningeal irritation	Logistic regression model clinical	-6.83582+ 0.82676×vomiting+ 3.08052× disturbed consciousness (reaction to pain or no reaction) +1.59488×petechiae or ecchymosis +3.04784×meningeal irritation+ 0.39908× duration complaints(days, max 10)+ 0.07384× CRP count (mg/L)+ 2.56222×cyanosis	No bacterial meningitis cases if score <8.5 on 40-point scale
		Logistic regression model CSF	-0.07305158 +1.084134×log of CSF PMN counts- 4.489344× CSF-blood glucose ratio	No cut-off
		Combined score	Duration of complaints=1 point per day (maximum 10[a]), vomiting=2, meningeal irritation=7.5, cyanosis=6.5[a], petechiae or ecchymosis=4, disturbed consciousness=8, CRP=0.5 points per 50 mg/L increase (max 2pts), CSF PMN count: 10-99=1, 100-999=2, 1000-9999= 3, 10.000+ = 4, CSF to blood glucose ratio= -0.5 points per 0.1 decrease (max -5 pts)	Different cut-offs per combination of clinical and CSF scores

Table 1. (Continued)

	Population	Modelling method	Items	Cut-off
Spanos (1989)	Children >1 month and adults with meningitis	CSF predictors for ABM based on probability	CSF glucose concentration <1·9 mmol/L, CSF to blood glucose ratio <0·23, CSF protein concentration >2·2 g/L, CSF leucocyte count >2000/µL, CSF neutrophil count >1180/µL	Bact. men. is likely if one CSF characteristic is present
		Logistic regression model	$1/(1+e^{-L})$, in which L=+ 2.29 x age (years) + 2.79 if age is ≤1 year; -2.71 x age + 7.79 if >1 year but ≤2 years;-0.159 x age+ 2.69 if >2 years but ≤22 years; + 0.100 x age-3.01 if > 22 years; + 0.52 x number of months from August 1; -12.76 x CSF-blood glucose ratio (use 0.6 if ratio exceeds 0.6); + 0.341 x (CSF PMN count x 106/L)0.333. If the Gram stain was positive, a probability of .99 would be assumed.	No cut-off
Tokuda (2009)	Adults (≥16 years) with meningitis	Recursive partitioning analysis	*High risk*: positive Gram stain or CSF neutrophil count >150/mm3 and >15% (cells/mm3), or neutrophils >15% and <150/mm3 and mental status change (GCS ≤14). *Low risk:* Gram stain negative and CSF neutrophils <15% or Gram negative, neutrophils >15% and <150/mm3 and no mental status change	High vs. low risk

CSF= cerebrospinal fluid, PMN= polymorphonuclear leucocytes, (A)BM= (acute) bacterial meningitis, CRP= C-reactive protein, GCS= Glasgow Coma Scale score, LP= lumbar puncture

[a] Not enough data available for this variable in validation cohort

Table 2. Derivation and previous validation of identified prediction models

	Population	Source of data	Participants	Sample size	Original model performance	Internal/external validation
Bonsu (2004)	Children >1 month-18 years, with pleiocytosis	Retrospective database cohort, n= 142	1998-2002+1992-1999, USA, 2 centers, children with pleocytosis	60 ABM, 82 enterovirus	Sens 98%, spec 62%, AUC 0.97, HL-test p=0.53	Dubos 2006 (r, n=166): sens 100%, spec 57%, Chavanet 2006 (r, n=175 children/n=100 adults): sens 100%, spec 85%/0%
Bonsu 2 (2008)	Children >4 weeks - 18 years with pleocytosis	Retrospective database cohort, n= 78	1998-2002, USA, children with pleocytosis	19 BM, 59 enterovirus	Sens 100%, spec 34%, AUC 0.98	Bonsu 2008 (r, n=158): sens 100%, spec 42%, Bonsu 2008 (r, n=871): sens 100%, spec 44%, AUC 0.97
Boyer (1980)	Children 1 month-15 years with meningitis	Retrospective database cohort, n= 149	1970-1978, France, children with meningitis	18 certain BM, 79 certain VM, 36 uncertain	x	Chavanet 2006 (r, n=175 children/n=100 adults): sens 89%/88%, spec 100%
Brivet (2005)	Adults (>16 years) with meningitis	Retrospective database cohort, n= 144	1982-2005, France, hospitalized patients with meningitis	90 BM	Sens 99%, spec 98%	x
Chavanet (2007)	Children >3 months and adults with meningitis	Retrospective database cohort, n=275	1995-2002 France, hospitalized for acute meningitis	Children: 19 BM, 105 VM, 51 undetermined meningitis. Adults: 17 BM, 53 VM, 30 undetermined	Sens 100%, spec children 85%, spec adults 71%	x
De Cauwer (2007)	Children 0-15 years with meningitis	Retrospective database cohort, n=92	1997-2005, Belgium	21 BM, 71 VM	x	x
Deivanayagam (1993)	Children 2 months - 11 years, suspected of meningitis	Prospective cohort, n=114	1989-1990, India, children with suspected meningitis	55 definitive BM	Sens 80%, spec 56%	x
Dubos (2007)	Children 29 days-16 years with meningitis	Retrospective database cohort, n=167	1995-2004, France, hospitalized for meningitis	146 VM, 21 BM	Sens 100%, spec 51%	Dubos 2010 (r, n=198), sens 100%, spec 36%

Table 2. (Continued)

	Population	Source of data	Participants	Sample size	Original model performance	Internal/external validation
Freedman (2001)	Children 2 months – 17 years in whom LP performed for ruling out BM	Retrospective database cohort, n= 1617	1992-1996, Canada, lumbar puncture for ruling out BM	29 BM	Sens 100%, spec 47.7%, LR 1.91	Dubos 2006 (r, n=166), sens 100%, spec 13%
Hoen (1995)	Children >1 month and adults with meningitis	Retrospective database cohort, n=398	1983-1991, France, final diagnosis of acute community acquired meningitis, >1 month	115 ABM, 283 AVM, 102 uncertain	Sens 97%, spec 93%, AUC 0.991	Jaeger 2000 (r, n=103 in children <3.5 months): sens 97.7%, spec 94.4%, Leblebicioglu 1996 (r, n=30): sens 100%, spec 82%, AUC 0.993, Baty 2000 (p, n=109): sens 80%, spec 95%, Dubos 2006 (r, n=166): sens 94%, spec 92%, Chavanet 2006 (r, n=175 children/ n=100 adults): sens 89%/100%, spec 94%/70%
Nigrovic (Bacterial Meningitis Score, 2002)	Children 29 days-19 years with meningitis	Retrospective database cohort, n= 456	1992-2000, USA, children with meningitis	86 BM, 370 aseptic meningitis	x	Dubos 2006 (r, n=166), sens 100%, spec 66%, Chavanet 2006 (r, n=175 children/n=100 adults): sens 95%/100%, spec 99%/ 85%, Nigrovic (r, n=240): sens 100%, spec 73%, Nigrovic 2012 (m, n=4896): sens 99.3%, spec 62.1%, Dubos 2010 (r, n=198), sens 100%, spec 52%
Oostenbrink (2001-2004)	Children >1 month – 15 years with meningeal irritation	Retrospective database cohort, n= 286	1988-1999, The Netherlands, meningeal signs	83 BM, 34 VM, 169 other infectious or self-limiting diseases	AUC 0.94	Dubos 2006 (r, n=166): sens 83%, spec 72%, Oostenbrink (r, n=74): AUC 0.92, Oostenbrink 2004 (p, n=226): clinical model AUC 0.94, CSF model AUC 0.97

Table 2. (Continued)

	Population	Source of data	Participants	Sample size	Original model performance	Internal/external validation
Spanos (CSF predictors, 1989)	Children >1 month and adults with meningitis	Retrospective database cohort, n= 422	1969-1980, UK, acute meningitis hospitalized	217 ABM, 205 AVM	x	x
Spanos (logistic model, 1989)	Children >1 month and adults with meningitis	Retrospective database cohort, n= 120	1969-1980, UK, acute meningitis hospitalized	48 ABM, 72 AVM	AUC 0.968	Hoen 1995 (r, n=398): sens 97%, spec 82%, AUC 0.981, Leblebicioglu 1996 (r, n=30): sens 89%, spec 82%, AUC 0.952, McKinney(r, n=150): AUC 0.977, Chavanet 2006 (r, n=175 children/ n=100 adults): sens100%, spec 1%/8%
Tokuda (2009)	Adults (≥16 years) with meningitis	Prospective cohort, n=176	1990-2000, Japan, acute meningitis	101 aseptic meningitis, 66 BM	Sens 99%, spec 89%	Tokuda 2009 (p, n=28): sens 100%, spec 91%

(A)BM= (acute) bacterial meningitis, (A)VM= (acute) viral meningitis, spec= specificity, sens= sensitivity, AUC= area under the curve, r= retrospective study, p= prospective study, HL-test= Hosmer Lemeshow-test, CSF= cerebrospinal fluid, LP=lumbar puncture, LR= likelihood ratio

Validation of prediction models

Sixteen of the seventeen identified prediction models were validated in our cohort. The model of Dubos was excluded from validation because four of the eight predictors in the model were not available in our dataset. [34]

All beta coefficients of the multivariable logistic regression model could be retrieved from the article or were provided by the author for the models of Hoen, the first model of Bonsu, Spanos and Oostenbrink. Sensitivity, specificity and predictive values of the high-risk category could be calculated for thirteen models, since the logistic regression model of Spanos did not provide a cut-off for high or low risk and Oostenbrink was counted as one score.

The model of Freedman and the second model of Bonsu used neutrophil bands in peripheral blood as a predictor, which was not available in our cohort. We did not adjust the original cut-off value for these two scores because the neutrophil bands in peripheral blood variable had a negligible contribution to the determination of the original cut-off value; none of the patients in the original study by Bonsu and only one in the study by Freedman would have been classified differently if this variable would have been ignored. For the model of Oostenbrink we were unable to assign points for cyanosis and we could assign no more than 2 points for duration of symptoms in days. We adjusted the original cut-off of Oostenbrink by reducing the cut-off with the percentage of points that could have been assigned based on the missing values.

Discrimination was good to excellent in all models but one (Table 3). The AUC's in these models ranged from 0.82 (Oostenbrink clinical model) to 0.95 (Oostenbrink CSF model). Only the first model of Bonsu showed an AUC of 0.76 (CI 0.65-0.86), indicating fair discrimination.

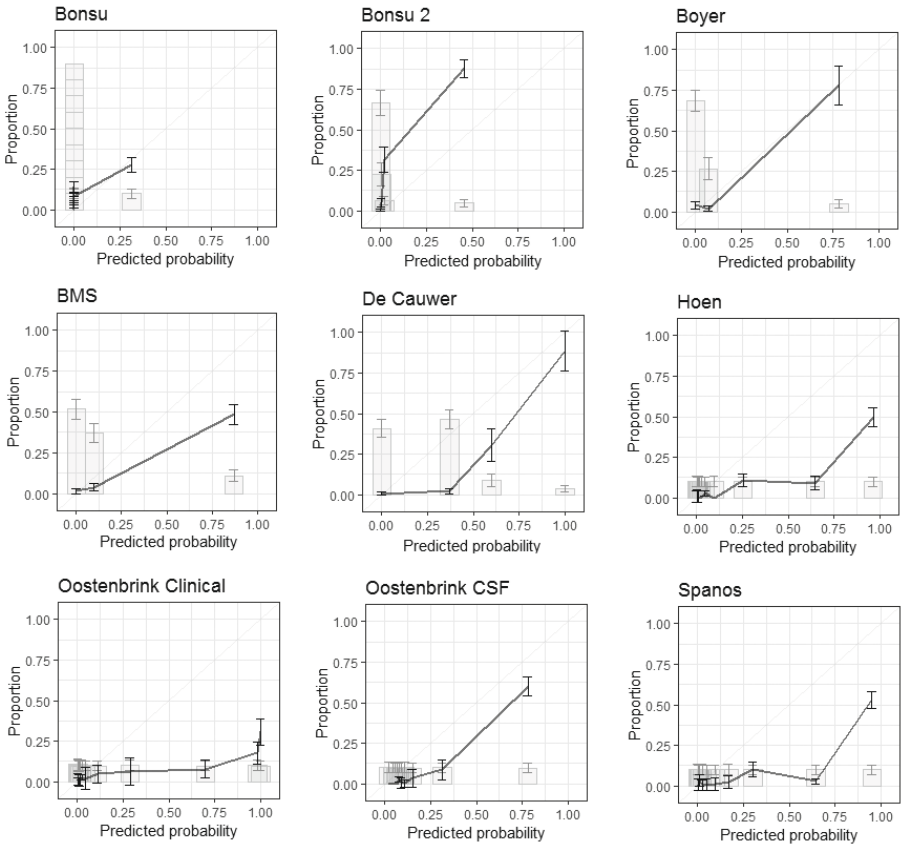
The HL-test and calibration slopes indicated poor fit of all the models. However, the calibration curve of Boyer and the CSF model of Oostenbrink did show reasonable agreement between the predicted and observed probability (Figure 2). All other calibration curves and calibration-in-the-large showed over- or underestimation of the models (Table 3, Figure 2).

Table 3. Discrimination and calibration

	AUC (95% CI)	Calibration in the large (95% CI)	Calibration slope (95% CI) or HL-test
Bonsu	0.76 (0.65-0.86)	-4% (-8 to -10%)	Slope: 0.07 (0.04-0.11), p <0.001
Bonsu 2	0.94 (0.89-0.99)	-5% (-8 to -2%)	HL-test: p <0.001
Boyer	0.90 (0.81-0.98)	-2% (-5 to 2%)	HL-test: p <0.001
Chavanet	0.93 (0.87-0.98)	NA	NA
De Cauwer	0.90 (0.84-0.98)	19% (13 to 24%)	HL-test: p <0.001
Hoen	0.90 (0.83-0.98)	13% (8 to 18%)	Slope: 0.28 (0.18-0.38), p <0.001
Nigrovic	0.88 (0.79-0.96)	6% (1 to 10%)	HL-test: p <0.001
Oostenbrink clinical model	0.82 (0.73-0.91)	24% (19 to 30%)	Slope: 0.09 (0.06-0.13), p <0.001
Oostenbrink CSF model	0.95 (0.91-0.997)	10% (5 to 15%)	Slope: 1.41 (1.00-1.83), p <0.05
Spanos	0.90 (0.82-0.97)	16% (10 to 21%)	Slope: 0.62 (0.43-0.80), p <0.001

AUC= area under the curve, CI= confidence interval, HL= Hosmer-Lemeshow, CSF= cerebrospinal fluid, NA= not applicable

Figure 2. Calibration curves of performance of prediction models for the diagnosis of bacterial meningitis



In our cohort, the model of Freedman was the only one with a sensitivity of 100% (95% CI 84-100%) and a negative predictive value (NPV) of 100% (95% CI 97-100%) (Table 3). However, specificity in Freedman was 52% (95% CI 47-57%) and the positive predictive value (PPV) 14% (95% CI 10-18%).

Highest specificity was reached by the first model of Bonsu and Deivanayagam with 99% (95% CI 99-100%) and 99% (95% CI 98-100%). Chavanet used different cut-off points for children and adults. In adults, a specificity of 95% (95% CI 93-97%) was reached, with a sensitivity of 74% (95% CI 70-79%). When using the cut-off for children this was 82% (95% CI 77-86%) and 85% (95% CI 82-89%) respectively (Table 4).

Table 4. Sensitivity, specificity and predictive values

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Bonsu	37% (32-42%)	99% (99-100%)	83% (80-87%)	95% (93-97%)
Bonsu 2	96% (94-98%)	71% (67-76%)	22% (17-26%)	99.5% (99-100%)
Boyer	87% (83-90%)	80% (76-84%)	26% (21-31%)	99% (97-99.8%)
Brivet	74% (70-79%)	50% (45-55%)	11% (8-14%)	96% (94-98%)
Chavanet - adults	74% (70-79%)	95% (93-97%)	54% (48-59%)	98% (96-99%)
Chavanet - children	85% (82-89%)	82% (77-86%)	27% (22-32%)	99% (97-99,7%)
De Cauwer	96% (94-98%)	44% (39-49%)	12% (9-15%)	99% (98-100%)
Deivanayagam	56% (50-61%)	99% (98-100%)	82% (78-86%)	97% (95-98%)
Freedman	100% (84-100%)	52% (47-57%)	14% (11-18%)	100% (97-100%)
Hoen	93% (90-95%)	71% (66-75%)	20% (16-24%)	99% (98-100%)
Nigrovic	90% (87-93%)	55% (50-60%)	14% (10-18%)	99% (97-99.8%)
Oostenbrink	75% (70-79%)	79% (75-84%)	23% (18-27%)	97% (96-99%)
Oostenbrink[a]	86% (82-90%)	53% (47-58%)	13% (9-16%)	98% (96-99%)
Spanos	56% (50-61%)	96% (94-98%)	53% (48-58%)	96% (95-98%)
Tokuda	85% (82-89%)	70% (65-75%)	19% (15-23%)	98% (97-99.6%)

CI= confidence interval, PPV= positive predictive value, NPV= negative predictive value
[a] With adjusted cut-off

Most patients fell into the low risk categories, although this varied per model (Table 5; Figure 2). The proportion of patients that did not fall into high or low risk, according to our definition, ranged from 0.2% (Bonsu) to 38% (Oostenbrink CSF) (Table 5). For Bonsu, 97% of the patients were assigned to the low risk category, meaning only 3% were assigned to the high risk category.

In our cohort, the CSF model of Oostenbrink scored best in terms of discrimination with an AUC of 0.95 (0.91-0.997). Calibration was reasonable for this model. The second

best AUC was reached by the second model of Bonsu (0.94; 95% CI 0.89-0.99), which also showed the best combination of sensitivity (96%; 95% CI 94-98%) and specificity (71%; 95% CI 67-76%).

One patient with bacterial meningitis from our cohort was missed by all prediction models, except for Freedman. This was a 28-year-old woman diagnosed with acute myeloid leukemia, presenting with neutropenic fever. CSF results showed 1044 cells/^{3} µl, a protein of 0.7 g/l and a glucose of 2.8 (blood glucose 9.7). CSF and blood cultures grew *Rothia mucilaginosa.*

Table 5. Proportions of patients in different risk groups

Probability	<0.1 (95% CI)	≥0.1 and≤0.8 (95% CI)	>0.8 (95% CI)
Bonsu	97% (95-99%)	0.2% (0-0.8%)	3% (1-5%)
Hoen	66% (61-71%)	22% (18-27%)	12% (8-15%)
Oostenbrink clinical	54% (48-59%)	23% (19-27%)	23% (19-28%)
Oostenbrink CSF	58% (53-63%)	38% (33-43%)	4% (2-7%)
Spanos	55% (50-60%)	34% (29-39%)	10% (7-13%)

CI= confidence interval, CSF= cerebrospinal fluid

DISCUSSION

We validated sixteen diagnostic prediction models for bacterial meningitis in a cohort of 363 patients in whom a lumbar puncture was performed for the suspicion of CNS infection. Systematic evaluation showed that the quality of the studies varied widely with regard to design, analyses and reporting of the models. Discrimination was excellent in all models but one. Calibration however, showed relevant over- or underestimation of bacterial meningitis by all models. As we found no model with both few false negatives and few false positives, the models should be used with caution in clinical practice, if they should be used at all.

We identified many models but no more than half were validated previously. [27-31,42] If models were validated, this was done using selective patient groups.[7,28-31,42,43] Many external validation studies did not evaluate discrimination[24,29,32,33,36,39], and none of them evaluated calibration.[7,24,29,32,33,36-41] Most models in our validation study performed worse than previously described.[29,33,36-39] Apart from the fact that prediction models perform better in their derivation cohorts than in external validation cohorts, there are several other likely explanations for this. First, most previous validation studies were performed

in cohorts of patients with proven meningitis, whereas our cohort consisted of all patients suspected of a CNS infection. This is however the at-risk population in which these diagnostic prediction rules will be used and it is therefore essential to validate them in this population. Also, some of the previous validations were performed by the same research group in the same hospital as the original study, which makes it difficult to generalize these results.[24,36] Furthermore, the lack of children in our cohort can explain differences in performance of models developed in children. For example, for the BMS of Nigrovic *et al.* we found a sensitivity of 90% (95% CI 87-93%) and specificity of 55% (95% CI 50-60%), whereas a meta-analysis conducted by the authors in 2012 showed a sensitivity of 99.3% and a specificity of 62.1% in children.[39]

Most prediction models were developed to accurately identify patients with (or without) bacterial meningitis. Because missing bacterial meningitis will have devastating consequences, only 100% sensitivity seems good enough. However, a more reasonable consideration could be whether or not a prediction model adds value in a clinical setting. Baty *et al*. performed a prospective validation study of the model of Hoen, where they compared clinical and computed diagnoses to the definitive diagnosis of the patient.[24] Their conclusion was that it could be a helpful decision-making aid, but still a model like this should not replace the physicians opinion.[24] It is by no means certain that a well validated prediction model will outperform clinical judgement. This is exemplified by a study in New Zealand and Australia that compared three well validated clinical decision rules with excellent test characteristics for traumatic head injury in children with the clinical decisions of treating physicians.[44] They found that in their center accuracy of physicians in terms of sensitivity and specificity was high.[44] Therefore, the decision rules had limited potential to increase the diagnostic accuracy and could even increase the rate of unnecessary CT-scans.[44] The results of both studies show the importance of prospective comparison of physicians' performance with and without prediction models, for instance in a cluster randomized design. The effects of implementing one of the identified prediction models in clinical practice, other than the model of Hoen, have not been studied.

Our study has several limitations. First, 10 of the validated models were developed in cohorts of children only and 4 in cohorts of both children and adults. We did not have any children in our cohort, and our results are therefore limited to adults with suspected CNS infections. Second, some variables used in three models were not recorded in our database. As explained before we do not think it would have changed our results for Freedman and Bonsu. However, for the model of Oostenbrink we had to adjust the different cut-offs, which could have resulted in an overestimation of the number of patients in the high-risk group.

In conclusion, none of the models performed well enough to recommend routine use in individual patient management. In cases where clinical evaluation has been performed, results of the scores could be used as an additional source of information for excluding or confirming the diagnosis. Also, beginning physicians could use them as an aid by comparing their clinical opinion to the outcome of the prediction model. However, caution is especially recommended in complex patients, for example like in our patient with neutropenic fever. Future research should focus on prospective comparison of diagnostic accuracy between prediction models and clinician accuracy.

Declaration of Competing Interest

No potential conflicts of interest relevant to this article exist.

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SUPPLEMENTARY DATA

Supplementary Material 1. Search strategy

MEDLINE

("Meningitis, Bacterial"/ or "Meningitis, Escherichia coli"/ or "Meningitis, Haemophilus"/ or "Meningitis, Listeria"/ or "Meningitis, Meningococcal"/ or "Waterhouse-Friderichsen Syndrome"/ or "Meningitis, Pneumococcal"/ or "Tuberculosis, Meningeal"/ or ((mening* and (bacterial or Escherichia or e-coli or h?emophilus or listeria or meningococcal or pneumococcal or tubercul* or streptococcus)) or "Waterhouse-Friderichsen Syndrome").ti,ab,kf.

AND

Validat$.mp. or Predict$.ti. or Rule$.mp. or (Predict$ and (Outcome$ or Risk$ or Model$)).tw. or ((History or Variable$ or Criteria or Scor$ or Characteristic$ or Finding$ or Factor$) and (Predict$ or Model$ or Decision$ or Identif$ or Prognos$)).tw. or (Decision$.tw. and ((Model$ or Clinical$).tw. or logistic models/)) or (Prognostic and (History or Variable$ or Criteria or Scor$ or Characteristic$ or Finding$ or Factor$ or Model$)).tw. or ("Stratification" or "Discrimination" or "Discriminate" or "c-statistic" or "c statistic" or "Area under the curve" or "AUC" or "Calibration" or "Indices" or "Algorithm" or "Multivariable").tw. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]

AND

(different* or test or tests or diagnos*).ti,ab. or exp DIAGNOSIS/ or exp DIAGNOSIS, DIFFERENTIAL/ or di.fs.)

NOT

exp animals/ not humans/

Embase

(bacterial meningitis/ or group b streptococcal meningitis/ or haemophilus meningitis/ or listeria meningitis/ or exp pneumococcal meningitis/ or tuberculous meningitis/ or Neisseria meningitidis/ or (meningitis/ and exp Escherichia coli/) or ((mening*

and (bacterial or Escherichia or e-coli or h?emophilus or listeria or meningococcal or pneumococcal or tubercul* or streptococcus)) or "Waterhouse-Friderichsen Syndrome").ti,ab,kw.

AND

Validat*.tw. or Predict*.ti. or Rule*.tw. or (Predict* and (Outcome* or Risk* or Model*)).tw. or ((History or Variable* or Criteria or Scor* or Characteristic* or Finding* or Factor*) and (Predict* or Model* or Decision* or Identif* or Prognos*)).tw. or (Decision*.tw. and ((Model* or Clinical*).tw. or statistical model/)) or (Prognostic and (History or Variable* or Criteria or Scor* or Characteristic* or Finding* or Factor* or Model*)).tw. or ("Stratification" or "Discrimination" or "Discriminate" or "c-statistic" or "c statistic" or "Area under the curve" or "AUC" or "Calibration" or "Indices" or "Algorithm" or "Multivariable").tw.

AND

exp diagnosis/ or (different* or test or tests or diagnos*).mp.)

NOT

(exp animal/ or animal.hw. or nonhuman/) not (exp human/ or human cell/ or (human or humans).ti.)

Supplementary Table 1. Comparison in calibration results between using multivariable logistic regression model and observed probability from derivation cohort used as predicted probability

	Multivariable logistic regression model	**Observed probability as predicted probability**
Bonsu		
Calibration-in-the-large	-4% (-8 to -10%)	-3% (-6 to 1%)
Calibration slope/ HL-test	Slope: 0.07 (0.04-0.11), p <0.001	HL-test: p <0.001
Spanos		
Calibration-in-the-large	16% (10 to 21%)	14% (9 to 19%)
Calibration slope/ HL-test	Slope: 0.62, (0.43-0.80), p<0.001	HL-test: p<0.001

HL= Hosmer Lemeshow

Supplementary Table 2. Quality of identified articles on development and validation of prediction models for bacterial meningitis (TRIPOD criteria)

Section		Item	D/V	Checklist item	Baty	Bonsu	Bonsu 2	Boyer	Brivet	Chavanet	De Cauwer	Delvanayagam	Dubos 2006	Dubos 2007	Dubos 2010	Freedman	Hoen	Jaeger	Lebleblicioglu	McKinney	Nigrovic 2002	Nigrovic 2012	Oostenbrink 2001	Oostenbrink 2002	Oostenbrink 2004	Spanos	Tokuda
Title and abstract	Title	1	D;V	Identify the study as developing and/or validating a multivariable prediction model, the target population, and the outcome to be predicted	p. 422	p. 511	p. 437	p. 225	p. 1654	p. 328	p. 343	p. 284	p. 647	p. 434	p. 963	p. 1301	x	p. 418	p. 252	p. 8	p. 711	p. 799	p. 611	p. 1189	p. 109	p. 2700	p. 537
	Abstract	2	D;V	Provide a summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions.	p. 422	p. 511	p. 437	p. 225	p. 1654	p. 328	p. 343	p. 284	p. 647	p. 434, 435	p. 963	p. 1301	p. 267	p. 418	x	p. 8	p. 712	p. 799	p. 611	p. 1189	p. 109	p. 2700	p. 537
Introduction	Background and objectives	3a	D;V	Explain the medical context (including whether diagnostic or prognostic) and rationale for developing or validating the multivariable prediction model, including references to existing models	p. 422	p. 512	p. 438	p. 225	p. 1654, 1655	p. 329	p. 343	p. 284	p. 647	p. 435	p. 963	p. 1301	p. 268	p. 418, 419	x	p. 8	p. 712	p. 799	p. 611	p. 1189	p. 109	p. 2700	p. 538
		3b	D;V	Specify the objectives, including whether the study describes the development or validation of the model or both	p. 423	p. 512	p. 438	p. 225	p. 1655	p. 329	p. 343	p. 284	p. 647	p. 435	p. 964	p. 1302	p. 268	p. 419	x	p. 8	p. 713	p. 799	p. 611	p. 1189	p. 109	p. 2700	p. 538
Methods	Source of data	4a	D;V	Describe the study design or source data (e.g., randomized trial, cohort, or registry data), separately for the development and validation sets, if applicable	p. 423	p. 512	p. 438	x	p. 1655	p. 330, 331	p. 343	p. 284, 285	p. 647	p. 435	p. 964	p. 1302	p. 268	p. 419	p. 252	p. 8	p. 713	p. 799	p. 612	p. 1190	p. 110	p. 2700	p. 538
		4b	D;V	Specify the key study dates, including start of accrual, end of accrual and, if applicable, end of follow up	p. 423	p. 512	p. 438	p. 225	p. 1655	p. 329	p. 343	p. 284	p. 647	p. 435	p. 965	p. 1302	p. 268	p. 419	p. 252	p. 8	p. 713	p. 799	p. 612	p. 1190	p. 110	p. 2700	p. 538

Supplementary Table 2. (Continued)

Section		Item	D/V	Checklist item	Batty	Bonsu	Bonsu 2	Boyer	Brivet	Chavanet	De Cauwer	Delvanayagam	Dubos 2006	Dubos 2007	Dubos 2010	Freedman	Hoen	Jaeger	Lebleblicioglu	McKinney	Nigrovic 2002	Nigrovic 2012	Oostenbrink 2001	Oostenbrink 2002	Oostenbrink 2004	Spanos	Tokuda
	Participants	5a	D;V	Specify key elements of the study setting (e.g., primary care, secondary care, general population) including number and location of centers	p. 423	p. 512	p. 438	p. 225	p. 1655	p. 329	p. 343	p. 285	p. 647	p. 435	p. 963	p. 1302	p. 268	p. 419	p. 252	p. 8	p. 713	p. 800	p. 612	p. 1190	p. 110	p. 2700	p. 538
		5b	D;V	Describe eligibility criteria for participants	p. 423	p. 512	p. 438	p. 227	p. 1655	p. 329	p. 344	p. 284	p. 647	p. 435	p. 964	p. 1302	p. 268	p. 419	p. 252	p. 8,9	p. 713	p. 800	p. 612	p. 1190	p. 110	p. 2700	p. 538
		5c	D;V	Give details of treatments received, if relevant	NA	NA	NA	p. 235	p. 1655	NA	p. 344	NA	NA	NA	NA	p. 1302	p. 268	NA	NA	NA	p. 714	NA	NA	p. 1190	NA	p. 2700	NA
	Outcome	6a	D;V	Clearly define the outcome that is predicted by the prediction model, including how and when assessed	p. 423	p. 512	p. 440	p. 227	p. 1655	p. 329	p. 345	p. 285	p. 647	p. 436	p. 964	p. 1301, 1302	p. 268	p. 419	p. 252	p. 8	p. 713	NA	p. 612	p. 1190	p. 110	p. 2700	p. 538
		6b	D;V	Report any actions to blind assessment of predictors of the outcome to be predicted	x	x	x	x	x	x	x	p. 284	x	p. 436	p. 965	x	x	x	x	x	x	x	x	x	x	x	x
	Predictors	7a	D;V	Clearly define all predictors used in developing or validating the multivariable prediction model, including how and when they were measured	p. 424	p. 512, 513	p. 438	p. 227, 228	p. 1657	p. 329	p. 345	p. 285	p. 648	p. 436	p. 964, 965	p. 1302	p. 268	p. 419	p. 252	p. 9	p. 714	NA	p. 612	p. 1190	p. 110	p. 2701-2705	p. 539
		7b	D;V	Report any actions to blind assessment of predictors for the outcome and other predictors	x	x	x	x	x	x	x	x	x	p. 436	p. 965	x	x	x	x	x	x	x	x	x	x	x	x
	Sample size	8	D;V	Explain how the study size was arrived at	x	p. 512, 513	x	x	x	x	x	x	p. 648	x	x	x	p. 268	x	x	x	p. 714	NA	x	x	x	x	x
		9	D;V	Describe how missing data were handled (e.g. complete-case analysis, single imputation, multiple imputation, with details of any imputation method)	x	p. 512	p. 438, 440	x	x	x	x	x	p. 648	p. 436	p. 965	x	p. 268	x	x	x	p. 714	p. 800	p. 612	p. 1190	p. 113	p. 2700, 2705	x

Supplementary Table 2. (Continued)

Section	Item	D/V	CheckList item	Baty	Bonsu	Bonsu 2	Boyer	Brivet	Chavanet	De Cauwer	Deivanayagam	Dubos 2006	Dubos 2007	Dubos 2010	Freedman	Hoen	Jaeger	Lebleblicioglu	McKinney	Nigrovic 2002	Nigrovic 2012	Oostenbrink 2001	Oostenbrink 2002	Oostenbrink 2004	Spanos	Tokuda
Statistical analysis methods	10a	D	Describe how predictors were handled in the analysis	NA	p. 512, 513	p. 439	p. 228-232	p. 1655	p. 329	p. 345	p. 285	NA	p. 436	NA	p. 1303	p. 268	NA	NA	NA	p. 714-716	NA	p. 612	p. 1190	NA	p. 2701	p. 539
	10b	D	Specify type of model, all model-building procedures (including any predictor selection), and method for internal validation	NA	p. 512, 513	p. 439	p. 228-233	p. 1655	p. 329, 330	p. 345	p. 285	NA	p. 436	NA	p. 1302, 1303	p. 268, 269	NA	NA	NA	p. 713, 714	NA	p. 612	p. 1190	NA	p. 2701-2705	p. 539
	10c	V	For validation, describe how the predictions were calculated	p. 423	p. 515, 516	p. 439	NA	NA	p. 330	NA	NA	p. 648	p. 436	p. 964	NA	p. 268	p. 419	p. 252	p. 8,9	p. 714	p. 800	NA	p. 1191	p. 110	NA	p. 539-540
	10d	D;V	Specify all measures used to assess model performance and, if relevant, to compare with multiple models	p. 423	p. 515, 516	p. 439	x	p. 1655	x	p. 345	p. 285	p. 648	p. 436	p. 965	p. 1303	p. 268	p. 419	p. 252	p. 9	p. 713	p. 800	p. 613	p. 1191	p. 110	x	p. 539
	10e	V	Describe any model updating (e.g., recalibration) arising from the validation, if done	NA	NA	p. 442	NA	NA	NA	NA	NA	NA	p. 436	NA	NA	p. 268, 269	p. 419	NA	NA	NA	NA	NA	p. 1191	p. 112	NA	NA
	11	D;V	Provide details on how risk groups were created, if done	p. 423	p. 515, 516	p. 439	p. 234	p. 1657	p. 331	p. 345	p. 285	p. 649	p. 436	p. 964	p. 1303	p. 269, 270	p. 419	p. 252	p. 8,9	x	NA	p. 615	x	x	p. 2701-2705	p. 539
Development vs. Validation	12	V	For validation, identify any differences from the development data in setting, eligibility criteria, outcome and predictors	p. 423	NA	p. 440, 441	NA	NA	p. 332	NA	NA	x	x	p. 964	NA	p. 272	p. 419	x	x	p. 713, 714	p. 801	NA	p. 1189	p. 111	p. 2701	p. 541

Supplementary Table 2. (Continued)

Section		Item	D/V	Checklist item	Baty	Bonsu	Bonsu 2	Boyer	Brivet	Chavanet	De Cauwer	Delvanayagam	Dubos 2006	Dubos 2007	Dubos 2010	Freedman	Hoen	Jaeger	Lebleblicioglu	McKinney	Nigrovic 2002	Nigrovic 2012	Oostenbrink 2001	Oostenbrink 2002	Oostenbrink 2004	Spanos	Tokuda
Results	Participants	13a	D;V	Describe the flow of participants through the study, including number of participants with and without the outcome and, if applicable, a summary of the follow-up time	p. 424	p. 513	p. 441	p. 233	p. 1656	p. 330, 332	p. 344	p. 285	p. 648, 649	p. 436	p. 964	p. 1304	p. 268	p. 419	p. 253	p. 9	p. 714, 715	p. 803	p. 614	p. 1191	p. 111	p. 2700, 2701, 2705	p. 541
		13b	D;V	Describe the characteristics of the participants (basic demographics, clinical features, available predictors), including the number of participants with missing data for predictors and outcome	p. 424	p. 514	p. 440, 441	x	p. 1656	p. 330	p. 344	p. 286	x	x	p. 964	p. 1305	p. 269	p. 419	p. 252	x	p. 714, 715	NA	p. 614	p. 1191	p. 111	p. 2701	p. 540
		13c	V	For validation, show a comparison with the development data of the distribution of important variables (demographics, predictors and outcome)	x	NA	x	NA	NA	x	NA	NA	x	x	p. 964	NA	x	x	x	x	p. 714	NA	NA	x	p. 111	x	p. 540
	Model development	14a	D	Specify the number of participants and outcome events in each analysis	NA	p. 514	p. 440, 441	p. 233	x	p. 330	p. 344	x	NA	x	NA	p. 1304	p. 269	NA	NA	NA	p. 716	NA	p. 614	p. 1191, 1192	NA	p. 2700, 2701, 2705	p. 540
		14b	D	If done, report the unadjusted association between each candidate predictor and outcome	NA	p. 514	x	x	p. 1657	x	x	x	NA	NA	NA	x	p. 269	NA	NA	NA	p. 715	NA	p. 615	p. 1192	NA	p. 2705	x
	Model specification	15a	D	Present the full prediction model to allow predictions for individuals (i.e., all regression coefficients, and model intercept or baseline survival at given time point)	NA	p. 514	p. 441	p. 233	x	p. 331	p. 345	p. 287	NA	NA	NA	p. 1305	p. 269	NA	NA	NA	p. 715, 716	NA	p. 615	p. 1191	p. 115	p. 2705, 2706	p. 540

Supplementary Table 2. (Continued)

Section		Item	D/V	Checklist item	Batty	Bonsu	Bonsu 2	Boyer	Briyet	Chavanet	De Cauwer	Deivanayagam	Dubos 2006	Dubos 2007	Dubos 2010	Freedman	Hoen	Jaeger	Lebleblcioglu	Mckinney	Nigrovic 2002	Nigrovic 2012	Oostenbrink 2001	Oostenbrink 2002	Oostenbrink 2004	Spanos	Tokuda
		15b	D	Explain how to use the prediction model	NA	p. 514	p. 441	p. 233, 234	p. 1657	p. 329	p. 345	p. 287	NA	p. 437	NA	p. 1305	p. 273	NA	NA	NA	p. 716	NA	p. 613, 615	p. 1192	NA	p. 2705, 2706	p. 540
Model performance		16	D;V	Report performance measures (with CIs) for the prediction model	p. 424	p. 516	p. 441	x	p. 1657	p. 331	p. 345	x	p. 649	p. 436	p. 965	p. 1305	p. 271	p. 420	p. 253	p. 11	p. 716	p. 802	p. 613	p. 1192	p. 112	p. 2705	p. 541
Model-updating		17	V	If done, report the results from any model updating (i.e., model specification, model performance)	NA	NA	p. 441	NA	NA	NA	NA	NA	NA	p. 436	NA	NA	p. 271	NA	NA	NA	NA	NA	NA	p. 1193	p. 112	NA	NA
Limitations	**Discussion**	18	D;V	Discuss any limitations of the study (such as nonrepresentative sample, few events per predictor, missing data)	p. 425, 426	p. 515, 516	p. 442	x	p. 1658	p. 333	p. 346, 347	x	p. 649	p. 437, 438	p. 966	p. 1305, 1306	p. 272, 273	p. 420	x	x	p. 717	p. 803	p. 616	p. 1193	p. 114	p. 2707	x	p. 542
Interpretation		19a	V	For validation, discuss the results with reference to performance in the development data, and any other validation data	p. 425	NA	x	NA	NA	p. 334	NA	NA	x	p. 437	p. 966	NA	p. 272, 273	p. 420	x	x	p. 717	NA	NA	p. 1192	p. 114	x	p. 542
		19b	D;V	Give an overall interpretation of the results, considering objectives, limitations, results from similar studies and other relevant evidence	p. 425, 426	p. 516	p. 443	p. 234	p. 1658, 1659	p. 334	p. 346, 347	p. 287	p. 649, 650	p. 437, 438	p. 966	p. 1305, 1306	p. 272, 273	p. 421	p. 253	p. 11	p. 717	p. 803, 804	p. 616	p. 1193	p. 114	p. 2706, 2707	p. 542
Implications		20	D;V	Discuss the potential clinical use of the model and implications for future research	p. 425, 426	p. 516	p. 442, 443	p. 234	p. 1658, 1659	p. 334	p. 347	p. 287	p. 649, 650	p. 437, 438	p. 966	p. 1305, 1306	p. 273	p. 421	p. 253	p. 11	p. 717	p. 803, 804	p. 616	p. 1193	p. 114	p. 2706, 2707	p. 542

Supplementary Table 2. (Continued)

Section		Item	D/V	Checklist item	Batty	Bonsu	Bonsu 2	Boyer	Briyet	Chavanet	De Cauwer	Deivanayagam	Dubos 2006	Dubos 2007	Dubos 2010	Freedman	Hoen	Jaeger	Lebleblcioglu	McKinney	Nigrovic 2002	Nigrovic 2012	Oostenbrink 2001	Oostenbrink 2002	Oostenbrink 2004	Spanos	Tokuda
Other information	Supplementary information	21	D;V	Provide information about the availability of supplementary resources, such as study protocol, Web calculator and data sets	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	p. 114	x	x
	Funding	22	D;V	Give the source of funding and the role of the funders for the present study	x	x	x	x	x	x	x	p. 284	p. 650	p. 434	p. 966	x	x	x	x	x	p. 717	x	p. 616	p. 1194	x	p. 2707	x

D= development, V= validation, CI= confidence interval.
Green= reported, Orange= reported incomplete, Yellow/NA= not applicable, Red/x= not reported

CHAPTER 3

DIAGNOSTIC PREDICTION MODELS FOR BACTERIAL MENINGITIS IN CHILDREN WITH SUSPECTED MENINGITIS: A SYSTEMATIC REVIEW AND PROSPECTIVE VALIDATION STUDY

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Submitted

ABSTRACT

Objectives

Diagnostic prediction models exist to assess the probability of bacterial meningitis (BM) in paediatric patients with suspected meningitis. To evaluate the diagnostic accuracy of these models in a broad population of children suspected of CNS infection, we performed external validation.

Methods

We performed a systematic literature review in MEDLINE to identify prediction models for BM and validated these models in a prospective cohort of children aged 0-18 years old suspected of a CNS infection.

Primary and secondary outcome measures

We calculated sensitivity, specificity, predictive values, the area under the ROC curve (AUC) and evaluated calibration of the models for diagnosis of bacterial meningitis.

Results

In total 26 prediction models were validated in a cohort of 450 patients suspected of CNS infection included between 2012 and 2015. In 75 patients (17%) the final diagnosis was a CNS infection including 30 with BM (7%). AUC's ranged from 0.69 to 0.94 (median 0.83, IQR 0.77-0.87) overall, from 0.74-0.96 (median 0.87, IQR 0.82-0.91) in children aged ≥28 days of age and from 0.67 to 0.91 (median 0.80, IQR 0.78-0.82) in neonates.

Conclusions

Prediction models show good to excellent test characteristics for excluding BM in children, however, it remains to be shown whether the use of these models in clinical practice improves patient outcome.

INTRODUCTION

Bacterial meningitis (BM) in children is lethal and debilitating, with mortality rates between 4% and 21% and neurological sequelae occurring in up to one third of survivors.[1-3] Early start of treatment is crucial for the prognosis as delay in antibiotic treatment is associated with adverse outcomes.[4] However, limiting unnecessary use of antibiotics is important to minimize antibiotic resistance, adverse reactions, hospital admission and healthcare costs.[5]

Recognition of bacterial meningitis can be difficult. The typical triad of fever, neck stiffness and altered mental status is present in only 41% of adult patients and is even less common in children and infants.[6,7] Diagnostic prediction models have been developed to help identify which child should be treated for bacterial meningitis and in which a watchful waiting approach can be applied.[8] The majority of these models combine clinical and laboratory findings and predict the probability of acute bacterial meningitis, compared to viral meningitis or no meningitis. However, substantial differences between these models exist, especially with respect to patient populations and diagnostic criteria. Validation of prediction models in a broader population of patients suspected of a central nervous system (CNS) infection is necessary but is often lacking. External validation of sixteen diagnostic prediction models for BM in a cohort of 363 adult patients with suspected CNS infection showed that none of the existing models performed well enough to recommend routine use in individual patient management.[8] However, these models were mostly developed for children and might therefore perform better in a paediatric population.

Our aim was to perform a systematic review of prediction models for bacterial meningitis and validate these model using a multicentre cohort of paediatric patients with suspected CNS infections in whom a lumbar puncture was performed.

METHODS

Systematic review

We systematically reviewed the literature in MEDLINE to identify models that predict the probability of acute bacterial meningitis. The Standards for Reporting Diagnostic accuracy studies (STARD) 2015 guidelines and Preferred Reporting items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines were applied.[9,10] We used a previously validated search filter for prediction models.[11] We combined this filter with terms for meningitis and prediction models and searched for full text articles in

scientific peer-reviewed journals from January 1980 to 1 September 2022 in languages English, German, French, Spanish or Dutch. Prediction models were included if they contained at least three variables obtained from history, physical examination or simple laboratory tests and included children or adults. Publications describing the development, refinement or validation of a prediction model were included. Article screening and data extraction were performed by one researcher (N.S.G.) and discrepancies were discussed and resolved by a second and third researcher (M.C.B and M.W.B). Quality of the included studies was assessed according to the Transparent Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis (TRIPOD) criteria, containing 6 domains with in total 22 items.[12] Each item was scored as reported, reported incompletely, not reported.

Validation cohort

Data from the Paediatric and Adult Causes of Encephalitis and Meningitis (PACEM) study were used for validation of the included prediction models. This was a multicentre prospective study in three hospitals in which patients were included if 1) aged 0-18 years old 2) presented to the emergency department or admitted to the paediatric ward between 2012-2015 with suspected CNS infection and 3) cerebrospinal fluid (CSF) examination was performed.[13] A detailed description of the cohort was described previously.[13] Patients with insufficient amount of data were excluded for this study. We performed a sensitivity analysis in neonates (age <28 days) and in children ≥ 28 days of age because of the different presentation of bacterial meningitis at the neonatal age.[14]

Change in mental status was defined as a Paediatric Glasgow Coma Scale (GCS) <14, coma was defined as a GCS <8.[15] Episodes were categorized into six categories regarding final diagnosis; bacterial meningitis, other CNS infection, inflammatory CNS diseases, systemic infections, other neurological diseases and other systemic disease.[16] All episodes were independently assessed by two clinicians (N.S.G. and S.L.S) and discrepancies were discussed and resolved by a third and fourth clinician (M.C.B and M.W.B). Bacterial meningitis was defined as 1) a positive CSF culture, or 2) a negative CSF culture but positive blood culture and elevated CSF leukocyte count, or 3) a negative CSF and blood culture, elevated CSF leukocyte count, elevated infection parameters in blood and clinical parameters suggesting a bacterial infection. Age-specific cut-off values for abnormal CSF leukocyte count, protein and glucose were used. In children below 3 months >9 leukocytes/mm3 was considered elevated, in children of 3 months or older >6 leukocytes/mm^3 was used as cut-off.[17-19] CSF protein >1000 mg/L and CSF glucose levels <60% of blood glucose levels were considered abnormal. CSF leukocyte count was corrected for CSF erythrocytes by subtracting one leukocyte for every 700 erythrocytes/mm^3.

Statistics

The differences in baseline characteristics between bacterial meningitis and non-bacterial meningitis patients were identified with parametric and nonparametric tests. Chi-square tests and Fisher's exact tests were used to compare categorical outcomes.

The performance of the prediction models was assessed by evaluating discrimination and calibration.[20-22] The different prediction models were considered as index test, diagnosis of bacterial meningitis based on positive CSF culture was considered reference standard. Discrimination was assessed by calculating the area under the receiver operating characteristic (ROC) curves (AUC) with 95% confidence intervals (CI). Calibration was evaluated with the calibration curve, assessing the calibration slope and calculating the calibration-in-the-large. Discriminative ability was categorized as follows: excellent discrimination in case of an AUC of ≥ 0.90; good discrimination for 0.80 ≤ AUC < 0.90; fair discrimination for 0.70 ≤AUC < 0.80; and "poor discrimination" in case of an AUC < 0.70.[23]

In prediction models based on a multivariable logistic regression model in which beta coefficients could not be retrieved from the original publication, we used the observed proportions for the different risk categories from the derivation study as expected proportions in the validation data.

In models that reported the complete multivariable logistic regression model, proportions of patients assigned to different probability intervals were calculated, to display the spread of predicted probabilities and the clinical significance of this spread. A probability of <0.1 was defined as low risk and >0.8 as high risk, based on agreement between two clinicians, in advance of the analysis. Probabilities of 0.1-0.8 were considered not significant for clinical decision making.

The median number of missing values per variable was 12% (interquartile range (IQR) 4-42%). Missing data were handled by multiple imputation using the R package MICE. We used 60 variables from medical history, physical examination and laboratory results as predictors to impute missing values.[24] If a specific predictor from the model or a valid proxy, was not available in the PACEM dataset, the prediction model was validated without that particular variable. For discrimination and calibration we used R packages pROC[22,25] and predictABEL.[26]

We used Rubin's rule and bootstrapping to estimate proportions and c-statistics based on 30 imputation sets.[27]All statistical tests were two-tailed and p-values of <0.05 were considered statistically significant.

RESULTS

Systematic review

Our literature search yielded 7,724 articles of which 39 publications on diagnostic prediction models for acute bacterial meningitis were included. In total 28 publications described the derivation of a total of 31 prediction models.[28-62] Of these 31 models a total of 26 prediction models, described in 24 publications, were included for validation in our study (Figure S1). Thirteen publications validated one or more existing models in their dataset (Table 1,2).[28,31,34,43,45,47,49,54,56-60,63] All models were based on clinical characteristics and/or laboratory test results from blood and CSF. Characteristics of the derivation cohorts and performance measures of original models are presented in Table 1 and 2, models published before 2018 were described in detail previously as well.[8] A total of 23 models were developed in children[29-31,33-36,38-43,45,46,48-51,53,61,62,64,65], of which seven in neonates [34,42,53,64,65], with a median cohort size in the derivation studies of 398 (IQR 158-908) patients. Four models were developed in both adults and children[33,40,50] and three in adults.[32,44,52] The most frequent quality limitations were retrospective derivation cohorts, lack of reporting on handling of missing data, and little information about differences in distribution of important variables between the derivation and validation cohort (Table S1).

Description of cohort

Between 2012 and 2015 a total of 468 episodes were included, of which 450 episodes could be used in the analysis (Table 3). Reasons for exclusion were lack of information in online and paper files (n=14), multiple admissions of one patient in a short timeframe (n=2) and age at admission of 18 years or older (n=2). Included patients were female in 194 out of 450 (43%) cases, median age at admission was 1.5 months (IQR 0.4-12). A total of 75% of children was <1 year old, 40% of children was <28 days old and 92 of 179 (51%) of neonates were born prematurely. For the analyses three cohorts were used: the entire cohort of all children (n=450), neonates only (<28 days of age, n=179) and children aged ≥ 28 days (n=271).

Symptoms were present <24 hours in 227 of 402 (56%) patients. Most common symptoms were fever in 268 of 420 (64%), irritability in 193 of 429 (45%), meningeal irritation in 48 of 249 (19%) and a decreased level of consciousness 80 in 450 (18%). Median CSF leukocyte count in all children was 4 (IQR 1-9). CSF examination showed elevated leukocytes (corrected for CSF erythrocyte count) in 70 of 258 (27%) patients below 3 months old and in 32 of 166 (19%) patients of 3 months old or older. CSF protein was elevated in 73 of 419 (17%) in all patients and CSF to blood glucose ratio was decreased in 104 of 263 (39%) in all patients.

Table 1. Characteristics of included prediction models

	Population	Modelling method	Items	Cut-off
Bonsu (2004)	Children >1 month-18 years, with pleiocytosis	Logistic regression model	$1/(1+e^{-L})$, in which L= 11.448 +0.003× CSF total neutrophil count(cells/mm3)-34.802×$(10^{-2}$×CSF protein (mg/dL))$^{0.5}$+21.991×$(10^{-2}$×CSF protein (mg/dL)-0.345×age (years)	BM unlikely if score <0,1
Bonsu 2 (2008)	Children >4 weeks - 18 years with acute meningitis	Recursive partitioning analysis	Blood leucocytes >17.000 cells/ml, bands-to-neutrophils ratio in blood >10%[a], CSF leucocyte count >600 cells/ul, CSF neutrophils >75%, CSF total protein >100mg/dL, CSF glucose <40mg/dL. One point per item, add points.	BM unlikely if score 0
Boum (2019)	Children 2 months-12 years, history of fever and ≥1 sign of CNS involvement	LRM	Neck stiffness, positive Brudzinski or Kernig sign, bulging fontanel, CSF leucocyte count > 100 cells/μL or peripheral neutrophils > 10,000 cells/mm3.	BM likely if ≥1 items present
Boyer (1980)	Children 1 month-15 years with meningitis	Univariate analysis	Temp≥39.5=1, purpura=2, neurological signs (obnubilation/coma/focal/seizures) = 1, CSF protein level 0.9-1.4g/L= 1, ≥1,4=2, CSF glucose 0.35-0.2 g/L or 2-1 mmol/L=1, ≤0.2 g/L or ≤1 mmol/L=2, CSF leukocytes/mm3 1000-4000=1, ≥4000= 2, CSF ≥60% polynuclears= 1, blood leucocytes ≥15000/mm3=1	If score ≥5 treat for BM, if score ≤2 no treatment required, if score 3-4 repeat LP after 12-24h hours if clinically stable
Brivet (2005)	Adults (>16 years) with meningitis	Predictors based on logistic regression	Severity (altered consciousness, seizures, focal neurological findings, shock) or CSF absolute neutrophil count above 1,000/mm3 mm	BM is not ruled out if one characteristic is present
Chavanet (2007)	Children >3 months and adults with meningitis	Categorical analysis regression tree method	Children: CSF leukocytes ≥1800 cells/μl= 2 points, CSF neutrophils >80%=3 points, CSF protein >1.2 g/L=3 points, CSF/blood glucose ratio ≤0.3=3 points. Adults: blood leukocyte count >15/ul=3 points, CSF leukocytes ≥1700=4 points; <1700=2 points; <250=1 point, CSF neutrophils >90%=4 points; >80%=2points; >25%=1 point, CSF protein >2.3 g/L=5 points; >0.8=2 points, CSF/blood glucose ratio ≤0.35=4 points	Children: ≥2 high risk on BM.; Adults: >6 high risk on BM
Chen (2021)	Full-term neonates aged ≤28 days with sepsis and a LP	SRA	Fever, absence source of infection, neurological manifestation (seizure, abnormal muscle tone, irritability or bulging anterior fontanelle) CRP >25 mg/L and PCT >2.5 ng/mL, CRP >50 mg/L and PCT >30 ng/mL	High risk if presence of ≥1 items
Cheng (2022)	Preterm infants with a LP	LR	0.1258 x PCT level on first day after birth + 1.2542 x prenatal glucocorticoid use (yes=1, no=0) -0.1089 x albumin level −0.2557 x 1-min Apgar −0.1838 x BiPAP days −0.0172 x haemoglobin level on first day after birth −0.8131 x sex (female=2, boy=1)	NR

Table 1. (Continued)

	Population	Modelling method	Items	Cut-off
Dalai (2022)	Neonates with a LP	LRM	-6.42 + 0.59 if no apnoea + 2.05 if no irritability + 0.18 if no high-pitched cry + 1.01 if no seizures +1.26 if no neutrophilia + 0.66 if normal CRP – 0.002 x SNAPPE-II score + 1.75 if no leucomalacia	BM unlikely if score 0
De Cauwer (2007)	Children 0-15 years with meningitis	Not specified	CSF neutrophils percentage >80%, CSF glucose <52 mg/dl, CRP count ≥2.0 mg/dl, CSF protein ≥100 mg/dl	BM likely if ≥1 point or more on 4-point scale
Deivanayagam (1993)	Children 2 months - 11 years with suspected meningitis	Not specified	CSF leukocytes >300/mm3 and CSF neutrophils >60% and CSF/blood glucose ratio <0.5 or absolute CSF glucose <30 mg/dL	With all three CSF findings (low CSF/blood glucose ratio or low absolute glucose in CSF) empirical diagnosis of BM can be reasonably made
Dubos (2007)	Children 29 days-16 years with meningitis	Variables based on literature and BMS	Convulsions, purpera, toxic appearance (lethargy, irritability, altered peripheral perfusion, hypo- or hyperventilation), blood procalcitonin ≥0.5 ng/ml, CSF protein ≥0.5 g/L, positive CSF Gram stain	Patient should be treated with antibiotics if one characteristic is present
Freedman (2001)	Children 2 months - 17 years in whom LP performed for ruling out BM	LRM	CSF leucocytes >30/ul, younger than 6 months, abnormal CSF glucose/ ratio/protein/gram stain/ peripheral band count)	Patient should be treated as BM if one characteristic is present
Hoen (1995)	Children >1 month and adults with meningitis	LRM	$1/(1+e^{-L})$, in which $L=32.13\times10^{-4}\times$CSF PMN count ($10^6$/L)+2.365×CSF protein (g/L)+0.6143×blood glucose (mmol/L)+0.2086×white blood cell count (10^9/L)[11]	BM unlikely if score <0,1
Huang (2019)	Term neonates with a LP	LRM	Model 1: −8.47839 + 0.19469 × CSF WBC + 0.00190 × CSF protein + 0.01667 × CSF glucose – 0.00503 × LDH Model 2 = −6.34939 + 0.14552 × CSF WBC + 0.00069 × CSF protein – 0.05915 ×CSF glucose.	1: BM likely if score > −1.7032 2: BM likely if score > −2.8547
Li (2020)	Full-term infants aged ≤28 days or premature infants <40 weeks PMA with a LP	SRA	Gender, birth weight[a], level of glucose in CSF ≤ 2.12, WBCs in CSF>29, hsCRP level in blood >13.3, and LDH level in blood >73	NR. Assumed: BM likely if ≥1 items present

Table 1. (Continued)

	Population	Modelling method	Items	Cut-off
Mentis (2021)	Patients of all ages with meningitis	ML	Group 1 (G1)= CSF neutrophils and CSF Lymphocytes; Group 2 (G2) = G1 + CSF NLR; Group 3 (G3) = G2 + Blood Albumin; Group 4 (G4) = G3 + Gender + Age; Group 5 (G5) = G4 + Blood glucose ; Group 6 (G6) = G5 + Blood CRP; Group 7 (G7) = G6 + Blood soluble urokinase-type plasminogen activator receptor; Group 8 (G8) = G7 + Lymphocytes-to-blood CRP ratio. Cut-off CSF Neutrophils = 287 cells/µl, cut-off CSF NLR = 2. Other cut-offs not reported.	NR
Mintegi (2020)	Children 29 days-14 years with meningitis	LRM	Serum PCT >1.20 ng/mL = 3 points, CSF protein >80 mg/dL = 2 points, CSF ANC >1000/microliter = 1 point, serum CRP >40 mg/L = 1 point	BM likely if score ≥1
Mirkhani (2018)	Patients suspected of meningitis	DTA	Rule 1: CSF WBC count > 32, CSF protein level >51, CSF glucose level ≤50 Rule 2: CSF WBC > 32, CSF protein level >73, CSF glucose level>50	BM likely if all items of rule 1 or 2 are present
Mwaniki (2011)	Infants <90 days of age with meningitis	LRM	History of fever, history of convulsions, bulging fontanel, irritability, axillary temperature ≥39	BM likely if ≥ 1 item present
Nigrovic (Bacterial Meningitis Score, 2002)	Children 29 days-19 years with meningitis	LRM and recursive partitioning analysis	Positive CSF Gram stain=2, CSF protein ≥0.8 g/L =1, peripheral absolute neutrophil count ≥10 000 cells per µL=1, seizures before or on admission=1, CSF absolute neutrophil count >1000 cells per µL=1	BM unlikely if score 0 on 6-point scale
Oostenbrink (2001-2004)	Children >1 month - 15 years with meningeal irritation	LRM clinical	-6.83582+ 0.82676×vomiting+ 3.08052× disturbed consciousness (reaction to pain or no reaction) +1.59488×petechiae or ecchymosis +3.04784×meningeal irritation+ 0.39908× duration complaints(days, max 10)+ 0.07384× CRP count (mg/L)+ 2.56222×cyanosis	No BM cases if score <8.5 on 40-point scale
		LRM CSF	-0.07305158 +1.084134×log of CSF PMN counts- 4.489344× CSF-blood glucose ratio	No cut-off

Table 1. (Continued)

	Population	Modelling method	Items	Cut-off
		Combined score	Duration of complaints=1 point per day (maximum 10[a]), vomiting=2, meningeal irritation=7.5, cyanosis=6.5[a], petechiae or ecchymosis=4, disturbed consciousness=8, CRP=0.5 points per 50 mg/L increase (max 2pts), CSF PMN count: 10-99=1, 100-999=2, 1000-9999= 3, 10.000+ = 4, CSF to blood glucose ratio= -0.5 points per 0.1 decrease (max -5 pts)	Different cut-offs per combination of clinical and CSF scores
Pelkonen (2021)	Infants <90 days of age with a LP	LRM	Age >7 days, weight <2500 g, ill >7 days, seizures on admission, signs of shock or prolonged capillary refill and unclear CSF	BM likely if ≥1 items present
Spanos (1989)	Children >1 month and adults with meningitis	CSF predictors for ABM based on probability	CSF glucose concentration <1.9 mmol/L, CSF to blood glucose ratio <0.23, CSF protein concentration >2.2 g/L, CSF leucocyte count >2000/µL, CSF neutrophil count >1180/µL	BM likely if one CSF characteristic is present
		LRM	$1/(1+e^{-L})$, in which L=+ 2.29 x age (years) + 2.79 if age is ≤1 year; -2.71 x age + 7.79 if >1 year but ≤2 years;-0.159 x age+ 2.69 if >2 years but ≤22 years; + 0.100 x age-3.01 if > 22 years; + 0.52 x number of months from August 1; -12.76 x CSF-blood glucose ratio (use 0.6 if ratio exceeds 0.6); + 0.341 x (CSF PMN count x 106/L)0.333. If the Gram stain was positive, a probability of .99 would be assumed.	No cut-off
Tokuda (2009)	Adults (≥16 years) with meningitis	Recursive partitioning analysis	*High risk*: positive Gram stain or CSF neutrophil count >150/mm3 and >15% (cells/mm3), or neutrophils >15% and <150/mm3 and mental status change (GCS ≤14). *Low risk:* Gram stain negative and CSF neutrophils <15% or Gram negative, neutrophils >15% and <150/mm3 and no mental status change	High vs. low risk
Wang (2021)	Full-term neonates 0–28 days with CSF pleiocytosis	LRM	Peripheral blood ANC ≥10×109 cells/L, CSF protein level ≥1,650 mg/L, CSF ANC ≥84×106 cells/L, a positive CSF Gram stain, and a history of seizure before or at the time of presentation.	Low risk if no item present

CNS = central nervous system, LRM = logistic regression model, CSF= cerebrospinal fluid, BM= bacterial meningitis, LP= lumbar puncture, SRA = stepwise logistic regression analysis, PCT = procalcitonin, CRP= C-reactive protein, LR = Lasso regression, BiPAP = biphasic positive airway pressure, NR = not reported, SNAPPE-II = score for acute neonatal physiology and perinatal extension II, WBC = white blood cell count, LDH = lactate dehydrogenase, PMA = post menstrual age, hsCRP= high-sensitive C-reactive protein, ML= machine learning, NLR = neutrophil-to-lymphocyte ratio, DTA = decision tree algorithm, BMS = Bacterial Meningitis Score, ANC = absolute neutrophil count.

[a] Variables gender and birth weight were not specified for this model

Table 2. Derivation and previous validation of identified prediction models

	Population	Source of data	Sample size	Original model performance	Internal/external validation
Bonsu (2004)	Children >1 month- 18 years, with pleiocytosis, USA, 1998-2002+1992-1999	Retrospective database cohort, n= 142	60 BM, 82 VM	Sens 98%, spec 62%, AUC 0.97, HL-test p=0.53	Dubos 2006 (r, n=166): sens 100%, spec 57%, Chavanet 2006 (r, n=175 children/ n=100 adults): sens 100%, spec 85%/0%
Bonsu 2 (2008)	Children >4 weeks - 18 years with pleiocytosis, USA, 1998-2002	Retrospective database cohort, n= 78	19 BM, 59 VM	Sens 100%, spec 34%, AUC 0.98	Bonsu 2008 (r, n=158): sens 100%, spec 42%, ; Bonsu 2008 (r, n=871): sens 100%, spec 44%, AUC 0.97
Boum (2019)	Children 2 months-12 years with suspected CNS infection, Uganda, 2009-2012	Prospective database cohort, *n*=459	60 BM	Sens 93.3%, spec 64.4%	NA
Boyer (1980)	Children 1 month-15 years with meningitis, France, 1970-1978	Retrospective database cohort, n= 149	18 certain BM, 79 certain VM, 36 uncertain	NR	Chavanet 2006 (r, n=175 children/ n=100 adults): sens 89%/88%, spec 100%
Brivet (2005)	Adults (>16 years) with meningitis, France, 1982-2005	Retrospective database cohort, n= 144	90 BM	Sens 99%, spec 98%	x
Chavanet (2007)	Children >3 months and adults with meningitis, France, 1995-2002	Retrospective database cohort, n=275	Children: 19 BM, 105 VM, 51 undetermined meningitis. Adults: 17 BM, 53 VM, 30 undetermined	Sens 100%, spec children 85%, spec adults 71%	x
Chen (2021)	Full-term neonates aged ≤28 days with sepsis and a LP, China, 2010-2019	Prospective and retrospective database cohort, *n*= 689	102 BM	Sens 96.2%, accuracy 98.9%	Chen 2021 (p, *n*=383): sens 95.9%, accuracy 98.9%
Cheng (2022)	Preterm infants with a LP, China, 2017-2020	Prospective cohort, n=168	77 BM, 91 AM	Brier score: 0.17, c- slope:0.966, C-index: 0.82 (95% CI: 0.75–0.89)	NA

Table 2. (Continued)

	Population	Source of data	Sample size	Original model performance	Internal/external validation
Dalai (2022)	Neonates with suspected sepsis and a LP, India, 2014-2016	Prospective cohort, n=300	121 definite/ possible BM, 191 no BM	C-statistic: 0.67 (95% CI: 0.60-0.73), p-value < 0.001. Accuracy: 66.2%.	NA
De Cauwer (2007)	Children 0-15 years with meningitis, Belgium, 1997-2005	Retrospective database cohort, n=92	21 BM, 71 VM	NR	x
Deivanayagam (1993)	Children 2 months - 11 years, suspected of meningitis, India, 1989-1990	Prospective cohort, n=114	55 definitive BM	Sens 80%, spec 56%	x
Dubos (2007)	Children 29 days-16 years with meningitis, France, 1995–2004	Retrospective database cohort, *n* = 167	21 BM, 146 VM	Sens 100%, spec 51%	Boum 2018 (p, *n*=459), sens 100%, spec 7.3%; Dubos 2010 (r, n=198), sens 100%, spec 36%
Freedman (2001)	Children 2 months-17 years with a LP, Canada, 1992-1996	Retrospective database cohort, n= 1617	29 BM	Sens 100%, spec 47.7%, LR 1.91	Dubos 2006 (r, n=166), sens 100%, spec 13%
Hoen (1995)	Children >1 month and adults with meningitis, France, 1983-1991	Retrospective database cohort, n=398	115 BM, 283 VM, 102 uncertain	Sens 97%, spec 93%, AUC 0.991	Jaeger 2000 (r, n=103 in children <3.5 months): sens 97.7%, spec 94.4%; Leblebicioglu 1996 (r, n=30): sens 100%, spec 82%, AUC 0.993; Baty 2000 (p, n=109): sens 80%, spec 95%; Dubos 2006 (r, n=166): sens 94%, spec 92%; Chavanet 2006 (r, n=175 children/ n=100 adults): sens 89%/100%, spec 94%/70%
Huang (2019)	Term neonates with a LP, China, 2000-2017	Retrospective database cohort, *n*=1830	105 BM, 1725 AM	1: Sens 95.1%, spec 99.7%, AUC 0.98 2: Sens 95.1%, spec 99.7%, AUC 0.98	NA
Li (2020)	Full-term infants ≤28 days or premature <40 weeks PMA, with a LP, China, 2012-2018	Retrospective database cohort, *n*=997	236 BM	Sens 52.97%, spec 96.98%, AUC 0.91	NA

Table 2. (Continued)

	Population	Source of data	Sample size	Original model performance	Internal/external validation
Mentis[a] (2021)	Patients of all ages with CSF samples at the National Meningitis Reference Laboratory, Greece	Retrospective database cohort, n= 4339 cases	1758 BM, 2581 VM	Median sens MLR : 63% (IQR 61-65%) Median sens RF : 67% (IQR 67-70%) Median sens NB : 60% (58-63%)	NA
Mintegi (2020)	Children 29 days-14 years with meningitis, Spain, 2011-2018	Retrospective and prospective database cohort, *n*= 819	61 BM, 758 AM	Sens 100%, spec 83.2%	Mintegi 2020 (p, *n*=190**):** Sens 100%, spec 77.4%, AUC = 0.986, p<0.0001
Mirkhani (2018)	Individuals suspected of meningitis, Iran, 2009-2011	Retrospective database cohort, *n*= 7945	2219 BM, 2291 AM	Sens 87%, spec 70%, AUC 0.84	NA
Mwanaki (2011)	All admissions aged < 60 days with a LP, Kenya, 2001-2007	Prospective database cohort, n=3923	Age <7 days: 31 BM Age 7-59 days: 76 BM	Age <7 days: sens 84%, spec 67% Age 7-59 days: sens 94%, spec 30%	Obiero (Arch) (r, n= 4809), sens: 79%, spec 51%; Mwanaki 2011 (p, *n*=1512): sens 77.8%, spec 55.2%; Pelkonen 2021 (p, *n*=568): sens 45%, spec 68%
Nigrovic (BMS, 2002)	Children 29 days-19 years with meningitis, USA, 1992-2000	Retrospective database cohort, *n*= 456	86 BM, 370 AM	NA	Abdelrahim 2019 (p, *n*=404), sens and spec NR Boum 2019 (p, *n*=459), sens 100%, spec 13.8% Delannoy 2020 (r, *n*=626), sens 100%, spec, 64.1% Mintegi 2020 (r, *n*=190), AUC =0.926, p<0.0001 Mintegi 2020 (r, *n*=1009): sens 97.7%, spec 51.3% Wang 2021 (r+p, *n*=475), sens 100%, spec 19.4%

Table 2. (Continued)

	Population	Source of data	Sample size	Original model performance	Internal/external validation
Oostenbrink (2001-2004)	Children 1 month -15 years with meningeal signs, The Netherlands, 1988-1999	Retrospective database cohort, n= 286	83 BM, 34 VM, 169 other infectious or self-limiting diseases	AUC 0.94	Dubos 2006 (r, n=166): sens 83%, spec 72% Oostenbrink (r, n=74): AUC 0.92, Oostenbrink 2004 (p, n=226): clinical model AUC 0.94, CSF model AUC 0.97
Pelkonen (2021)	Infants <90 days of age with BM or sepsis signs/symptoms with a LP, Angola, 2016-2017	Prospective database cohort, *n*=1088	212 confirmed BM, 88 probable BM	Sens 97%, spec 16%	NA
Spanos (CSF predictors, 1989)	Children >1 month and adults with meningitis, UK, 1969-1980	Retrospective database cohort, n= 422	217 BM, 205 VM	NR	x
Spanos (logistic model, 1989)	Children >1 month and adults with meningitis, UK, 196901980	Retrospective database cohort, n= 120	48 BM, 72 VM	AUC 0.968	Hoen 1995 (r, n=398): sens 97%, spec 82%, AUC 0.981, Leblebicioglu 1996 (r, n=30): sens 89%, spec 82%, AUC 0.952; McKinney(r, n=150): AUC 0.977; Chavanet 2006 (r, n=175 children/ n=100 adults): sens100%, spec 1%/8%
Tokuda (2009)	Adults (≥16 years) with meningitis, Japan, 1990-2000	Prospective cohort, n=176	66 BM, 101 AM	Sens 99%, spec 89%	Tokuda 2009 (p, n=28): sens 100%, spec 91%
Wang (2021)	Full-term neonates 0–28 days with CSF pleiocytosis, China, 2001-2019	Prospective and retrospective database cohort, *n*=475	94 BM (20%), 381 AM (80%)	Sens 100%, spec 70.9%	NA

CNS = central nervous system, BM= bacterial meningitis, spec= specificity, sens= sensitivity, NA= not applicable, LP= lumbar puncture, p= prospective study, r= retrospective study, AM = aseptic meningitis, c-slope: calibration-slope, C-index = concordance index, CI = confidence interval, AUC= area under the curve, PMA = post-menstrual age, CSF= cerebrospinal fluid, MLR = multiple logistic regression, RF = random forest, NB = naïve-Bayes

[a]Only results from patients aged 0-14 years are shown here; medians of the different groups (G1-G8, Table 1) were calculated per statistical model

Table 3. Baseline characteristics validation cohort [a]

		BM (n=30)	No BM (n=420)	*P* value
Female sex		11/30 (37%)	183/420 (44%)	0.57
Age, months		0.5 (0.1-13.5)	1.6 (0.5-12.1)	0.10
Prematurity		8/26 (31%)	84/334 (25%)	0.53
Symptoms <24 hours		15/29 (52%)	212/373 (57%)	0.59
AB started before LP		6/27 (22%)	28/393 (7%)	0.005
Otitis media		4/30 (13%)	24/371 (6%)	0.15
Sinusitis		4/30 (13%)	49/380 (13%)	0.78
Pneumonia		1/30 (3%)	4/386 (1%)	0.31
Endocarditis		0/30 (0%)	0/399 (0%)	>0.99
General symptoms	Fever	17/27 (63%)	251/393 (64%)	>0.99
	Irritability	16/16 (100%)	177/401 (44%)	<0.001
	Vomiting	6/25 (24%)	81/371 (22%)	0.80
	Diarrhoea	3/27 (11%)	48/377 (13%)	>0.99
	Headache	3/21 (14%)	36/318 (11%)	0.72
	Purpura/petechial rash	4/30 (13%)	10/420 (2%)	<0.001
Vital signs	Heart rate (beats/min)[d]	161 (±43)	156 (±33)	0.448
	Systolic BP (mmHg)[e]	93 (±21)	100 (±23)	0.307
	Diastolic BP (mmHg)[e]	53 (±12)	58 (±19)	0.312
Neurological symptoms	Seizures[b]	3/30 (10%)	48/420 (11%)	0.82
	Focal	0/3 (0%)	6/48 (13%)	>0.99
	Generalized	2/3 (67%)	19/48 (40%)	0.56
	GCS <14	5/30 (17%)	43/420 (10%)	0.27
	GCS < 8	1/30 (3%)	20/420 (5%)	>0.99
	Bulging fontanel	0/6 (0%)	6/43 (14%)	>0.99
	Meningeal irritation	5/15 (33%)	43/234 (18%)	0.15
	Focal deficits	1/24 (4%)	23/394 (6%)	0.73
	Aphasia	0/4 (0%)	4/60 (7%)	>0.99
	Ataxia	0/3 (0%)	6/56 (11%)	>0.99
	Cranial nerve palsy	1/7 (14%)	11/127 (9%)	0.61
	Paresis leg	0/22 (0%)	6/376 (2%)	>0.99
	Paresis arm	0/22 (0%)	4/377 (1%)	>0.99
Laboratory findings blood	CRP (mg/L)[f]	47 (5-155)	8 (2-28)	<0.001
	Thrombocyte count (x10^9/L)[g]	207 (142-289)	279 (211-373)	<0.05
	Leukocyte count (x10^9/L)[h]	16.6 (9.1-21.1)	9.8 (7.1-14.1)	<0.001
Laboratory findings CSF	Leukocyte count (cells/mm^3)[c]	191 (54-2244	3 (1-6)	0.001
	Protein level (g/L)[i]	1.23 (0.6-2.1)	0.40 (0.22-0.74)	<0.001
	CSF:blood glucose ratio[k]	0.51 (±0.30)	0.67 (±0.26)	<0.006

BM = bacterial meningitis, BP = blood pressure, GCS = Glasgow Coma Scale, CRP = C-reactive protein, CSF = cerebrospinal fluid, AB = antibiotics, LP = lumbar puncture

Data are presented as n/N (%), median (IQR) or mean (±SD)

[a] Data are presented as no. of patients/no. of patients in which these data are available.

[b] Type of seizure unknown in 20/48 (42%) patients

[c] Corrected for CSF erythrocyte count if possible, data on CSF leukocyte count available in 429 patients, data on CSF erythrocyte count available in 271 patients.

[d] Data available in 331 patients, [e] data available in 123 patients, [f] data available in 425 patients, [g] data available in 427 patients, [h] data available in 431 patients, [i] data available in 421 patients, [k] data available in 265 patients.

CNS infection was diagnosed in 74 of 450 (16%) of patients, of which 30 (41%) had bacterial meningitis, 39 (53%) viral meningitis and 5 (7%) infectious encephalitis. Other diagnose categories included CNS inflammatory disease (3%), systemic infection (61%), other neurological disease (14%) and other systemic disease (6%). CSF culture was positive in 10 of 30 patients (30%) clinically diagnosed with bacterial meningitis and showed *Streptococcus pneumoniae* in 3, *Streptococcus agalactiae* in 3, *Neisseria meningitidis* in 2, *Haemophilus influenzae* in 1 and *Escherichia coli* in 1. Blood culture was positive in 11 of 30 bacterial meningitis patients (37%) and showed *Streptococcus agalactiae* in 4, *Haemophilus influenza* in 1, *Streptococcus pneumoniae* in 2, *Klebsiella pneumoniae* in 1, *Neisseria meningitidis* in 2 and *Escherichia coli* in 1. One patient had a positive CSF culture for *Streptococcus pneumonia* and a negative blood culture, and one patients had a positive blood culture for *Klebsiella pneumoniae* and a negative CSF culture.

Validation of prediction models

We validated 26 of 31 prediction models in our cohort. The models of Cheng, Dalai, Dubos and Mintegi were excluded for validation because these scores could not be adjusted due to the substantial amount of variables that were not available in our PACEM dataset.[38,43,64,65] The model of Mentis was excluded because the model was not reported in sufficient detail to perform validation.[66] In total 36 (80%) of the total number of 45 predictors from the 26 models were available in our dataset (Table S2). As neutrophil bands in peripheral blood, procalcitonin, and lactate dehydrogenase were not available in our cohort, these were left out of the validation scores. As high-sensitive C-reactive protein was not available, normal CRP concentration was used instead for validation of the model of Li. High risk of meningitis was not defined in the model of Li and therefore assumed to be presence of one or more items. Also, the amount of points for the variables gender and birth weight were not specified. These variables were left out of the model. Finally, we could assign no more than two points for duration of symptoms in days for the model of Oostenbrink because this data was not available in our dataset.[48] We adjusted the original cut-off for the model of Oostenbrink by reducing the maximum amount of points with the percentage of points that were not available due to the missing values. Moreover, predictive values were calculated for the combined Oostenbrink model only.

All children

Discrimination was excellent in 2 models in all children and good in 6 of 13 models (Table 4). The AUC's in these models ranged from 0.69 to 0.94 (median 0.83, IQR 0.77-0.87). The models of Bonsu, Nigrovic, Oostenbrink and Spanos showed an AUC below 0.80 indicating fair discrimination. In all children, the second model of Huang scored best in terms of discrimination with an AUC of 0.94 (CI 0.91 -0.97). Moreover, sensitivity of

Huang was 80% (95% CI 76-88%), with 94% specificity (95% CI 91-96%), 47% PPV (95% CI 42-52%) and 98% NPV (95% CI 97-100%).

Table 4. Discrimination and calibration for all children

	AUC (95% CI)	Calibration in the large (95% CI)	Calibration slope (95% CI) or HL-test
Bonsu	0.75 (0.65-0.86)	34% (29 to 39%)	Slope: 0.06 (0.03-0.09), p < 0.001
Bonsu 2	0.87 (0.79-0.95)	-5% (-8 to -2%)	Slope: 1.6 (1.1-2.0), p < 0.001
Boyer	0.83 (0.72-0.93)	3% (-1 to 6%)	Slope: 0.9 (0.6-1.2), p < 0.001
Chavanet - adults	0.88 (0.80-0.95)	NA	NA
Chavanet - children	0.82 (0.73-0.90)	NA	NA
De Cauwer	0.85 (0.78-0.91)	16% (12 to 21%)	Slope: 1.9 (1.3 to 2.6), p < 0.001
Hoen	0.84 (0.76-0.91)	11% (7 to 15%)	Slope: 0.07 (0.04 to 0.11), p < 0.001
Huang - model 1	0.94 (0.90-0.97)	NA	NA
Huang - model 2	0.94 (0.91-0.97)	NA	NA
Nigrovic	0.79 (0.71-0.86)	18% (13 to 22%)	Slope: 1.4 (0.9 to 1.9), p < 0.001
Oostenbrink clinical	0.69 (0.57-0.81)	24% (19 to 29%)	Slope: 0.14 (0.09 to 0.2), p < 0.001
Oostenbrink CSF	0.77 (0.66-0.87)	12% (8 to 17%)	Slope: 0.68 (0.43 to 0.93), p < 0.001
Spanos	0.74 (0.64-0.84)	20% (15-25%)	Slope: 0.4 (0.3 to 0.5), p < 0.001

AUC = area under the curve, CI = confidence interval, HL = Hosmer-Lemeshow, NA = not applicable, CSF = cerebrospinal fluid

The HL-test and calibration slopes indicated poor fit of all the models and none of the calibration curves showed reasonable agreement between the predicted and observed probability. Moreover, calibration-in-the-large showed over- or underestimation in all of the models (Table 4, Figure S2). Median sensitivity of the 24 models was 80% (IQR 73-94%) overall (Table 5). Negative predictive value (NPV) was ≥ 99% in 5/24 models overall.[30,35,39,42,46] None of the models showed a sensitivity and NPV of 100% in all children.

Median specificity was 57% (IQR 33-92%) overall. Highest specificity was reached by the model of Deivanayagam (100%, 95% CI 99-100%) and the second model of Mirkhani (99%, 95% CI 98-100%) overall. Sensitivity of this model was only 30% (95% CI 26-34%). Performance of models that were originally developed in children (n=17) differed from models developed in adults (or both children and adults, n=7), with median sensitivity in child models of 87% (IQR 80-95%) compared to 58% (IQR 44-74%) in adult models, and median specificity of 51% (IQR 28-60%) in child models compared to 91% (IQR 75-95%) in models developed in adults or both. The combination of sensitivity and specificity

was best in the models of de Cauwer (resp. 97%, 50%), Nigrovic (resp. 94%, 51%) and Li (resp. 97%, 52%) and all three showed a NPV of ≥99%.

Median proportion of patients that fell into the low risk categories, according to our definition, was 50% (IQR 42-81%) overall (Table S3). Median proportion of patients in the high risk categories was 11% (IQR 11-16%). The proportion of patients that did not fall into high or low risk, ranged from 1% (model Huang) to 59% (Bonsu).

Table 5. Sensitivity, specificity and predictive values for all children

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Bonsu	92% (89-94%)	20% (16-24%)	8% (5-11%)	97% (95-99%)
Bonsu 2	90% (89-93%)	60% (55-64%)	14% (11-17%)	99% (97-100%)
Boyer	87% (84-90%)	59% (54-64%)	13% (10-16%)	98% (97-100%)
Boum	83% (79-87%)	35% (30-39%)	8% (6-11%)	97% (95-98%)
Brivet	94% (92-97%)	11% (8-14%)	7% (5-9%)	96% (95-98%)
Chavanet - adults	71% (66-75%)	91% (89-94%)	37% (32-41%)	98% (96-99%)
Chavanet - children	77% (73-81%)	80% (76-84%)	22% (18-26%)	98% (96-99%)
Chen	96% (95-98%)	11% (8-14%)	7% (5-10%)	98% (96-99%)
De Cauwer	97% (95-98%)	50% (45-54%)	12% (9-15%)	100% (99-100%)
Deivanayagam	24% (20-28%)	100% (99-100%)	85% (81-88%)	95% (93-97%)
Freedman	97% (95-98%)	28% (23-32%)	9% (6-11%)	99% (98-100%)
Hoen	76% (71-80%)	77% (74-81%)	19% (16-23%)	98% (96-99%)
Huang – model 1	80% (76-84%)	92% (90-95%)	43% (39-48%)	98% (97-100%)
Huang – model 2	80% (76-88%)	94% (91-96%)	47% (42-52%)	98% (97-100%)
Li	97% (95-98%)	54% (49-59%)	13% (9-18%)	100% (97-100%)
Mirkhani – model 1	33% (29-38%)	97% (95-99%)	44% (40-49%)	95% (93-97%)
Mirkhani – model 2	30% (26-34%)	99% (98-100%)	61% (56-66%)	95% (93-97%)
Mwanaki	95% (93-97%)	14% (11-17%)	7% (5-10%)	97% (96-99%)
Nigrovic	94% (91-96%)	51% (46- 56%)	12% (9-15%)	99% (98-100%)
Oostenbrink combined[a]	74% (69-80%)	44% (40-50%)	9% (6-12%)	96% (94-98%)
Pelkonen	76% (72-80%)	7% (5-9%)	6% (3-8%)	80% (75-84%)
Spanos	54% (49-59%)	92% (89-94%)	32% (27-36%)	97% (95-98%)
Tokuda	58% (52-64%)	73% (69-78%)	14% (10-18%)	96% (94-98%)
Wang	80% (76-83%)	60% (56-65%)	12% (9-16%)	98% (96-99%)

CI = confidence interval, PPV = positive predictive value, NPV = negative predictive value, CSF = cerebrospinal fluid

[a] Adjusted cut-off

Neonates

Discrimination was excellent in 2 models in neonates and good in 4 out of 13 models. The AUC's ranged from 0.67 to 0.91 (median 0.80, IQR 0.78-0.82) (Table S4). Calibration-in-the-large showed over- or underestimation in all of the models validated in the neonate cohort. Median sensitivity of the 24 models was 81% (IQR 63-92%) (Table S5). The model of Freedman showed 100% (95% CI 77-100%) sensitivity and NPV (95% CI 51-100%), with specificity of only 17% (95% CI 11-23%). Median specificity was 51% (IQR 21-89%) in neonates. Only the model of Deivanayagam showed a specificity of 100% (95% CI 99-100%), with a sensitivity of 17% (95% CI 11–23%). Median proportion of patients that was categorized into the low risk categories was 49% (IQR 48-79%) and in the high risk categories 11% (IQR 6-15%) (Table S6). The proportion of patients that did not fall into high or low risk, ranged from 0.1% (model Huang) to 61% (model Bonsu).

Children ≥ 28 days of age

Discrimination was excellent in five models and good in seven models in children ≥ 28 days of age (Table S7). The AUC's ranged from 0.74-0.96 (median 0.87, IQR 0.82-0.91). Calibration-in-the-large showed over- or underestimation in all of the models, however the CSF model of Oostenbrink and the model of Boyer showed reasonable calibration with a slope of 1.1 (95% CI 0.6-1.5) and 0.9 (95% CI 0.5-1.3) respectively. Median sensitivity of the models was 86% (IQR 71-94%), with three models that showed a 100% sensitivity (Table S8). Moreover, twelve models in this cohort showed a NPV of 99% or higher. Median specificity was 60% (IQR 39-93%) in this cohort. The models of Chen, De Cauwer and Li all showed a 100% sensitivity and 100% NPV, with specificities ranging from 7-50%.

Median proportion of patients that was categorized into the low risk categories was 43% (IQR 32-81%) and in the high risk categories 12% (IQR 10-13%) (Table S9). The proportion of patients that did not fall into high or low risk, ranged from 1% (model Huang) to 56% (model of Bonsu, clinical model of Oostenbrink and model of Spanos).

Models developed for neonates performed slightly better in the neonate cohort compared to the cohort with all children: median sensitivity was 88% (IQR 88-93%) in neonates compared to 80% (IQR 80-96%) in all children, median specificity was 67% (IQR 63-92%) in neonates compared to 60% (IQR 54-92%) in all children.

DISCUSSION

We validated twenty-six clinical and laboratory-based diagnostic prediction models for bacterial meningitis, identified in a systematic review, using a cohort of 450 children with suspected CNS infection. Quality of the included studies varied widely regarding study design, statistical analyses and reporting on model-building procedures. Discrimination was excellent in two models and good in 6 out of 13 models (4 out of 13 in neonates). Calibration showed relevant over- or underestimation of bacterial meningitis by all models. A sensitivity of 100% and few false positives are requirements for implementation in clinical care due to the devastating consequences of missing this disease. However, none of the models showed these characteristics, suggesting these models cannot be used on their own.

Children models performed worse in our children cohort compared to the adult cohort in which they were validated previously, contrary to our expectations.[8] Moreover, all models validated in this study performed worse than in their original publication. This is expected because prediction models are tailored to their development dataset, resulting in better performance in the derivation cohort compared to an external cohort. However, other explanations could also play a role. We validated prediction models in a broad cohort of patients with suspected CNS infection whereas previous validation studies were mostly performed in children with microbiologically confirmed meningitis. The population in which these models will be used in daily practice are children suspected of CNS infection, thus evaluating performance in meningitis cohorts only could lead to too optimistic conclusions. Moreover, patient's age differed significantly between the derivation cohorts, ranging from models derived in preterm neonates only to patients of all ages or adults, whereas our cohort consisted of children aged 0-18 years old. Bacterial meningitis symptoms in children vary greatly between different age groups, making it difficult to generalize. Symptoms in adults on the other hand are relatively more homogeneous, which could also explain worse performance in our cohort compared to previous validation in an adult population.[8]

Machine-learning based prediction models have shown promising results in different populations, with improved prediction of clinically important traumatic brain injury in children compared to standard clinical prediction rules.[67] Also, machine-learning models were shown to be able to reduce 68.5% of lumbar punctures in young febrile infants with suspected serious bacterial infection.[68] Our review included one machine-learning model but unfortunately the model was not reported in sufficient detail to perform validation.[66] However, the question remains if machine-learning based algorithms outperform clinical judgement. A systematic review on comparing diagnostic prediction

models with clinical judgement for various medical conditions found that prediction models reduced the proportion of missed diagnoses in only 2 out of 46 publications.[69] This was offset by a larger amount of false positives as well. Comparing the combination of clinical judgement assisted by prediction rules to clinical judgement alone would provide the most valuable information on the added value of prediction models on patient outcome, but studies on this topic are lacking thus far.

To date, a large amount of prediction models for bacterial meningitis have been developed but none showed excellent discrimination when validated in a broader population of all patients suspected of a CNS infection. This might suggest that future research should focus on different ways of improving diagnosis in paediatric bacterial meningitis. Better biomarker-based point-of-care tests that can accurately exclude and include bacterial meningitis in children are needed, especially in complex cases in which definite diagnosis is still unclear after conventional CSF examination.

However, comparing the discriminative performance of prediction models for bacterial meningitis to an ideal diagnostic test with excellent discrimination might not be fair, since a diagnostic test for paediatric bacterial meningitis with 100% sensitivity and 100% specificity does not exist in clinical practice. All current tests show limitations that should be taken into account when assessing the results of an individual patient. Diagnostic prediction models could aid in addition to other diagnostic investigations, however should not be used on their own.

Our research has some limitations. First, some models included variables that were not available in our dataset. These models were validated without those variables, which could lead to difference in performance. Second, a substantial proportion of data were missing and had to be imputed. Although 30 different imputation sets were used, this could have led to some distortion of the performance measurements. Third, the number of patients with bacterial meningitis in our validation cohort was limited. Our validation cohort included 450 patients, including 30 patients with bacterial meningitis (7%). Because confidence intervals were broad, performance in larger cohorts could find better performance in larger validation cohorts. Nevertheless, our data show that no model performed good enough for routine use to stand alone in clinical practice.

In conclusion, this review analysed 39 articles on diagnostic prediction models for bacterial meningitis in children and validated 26 prediction models in a multicentre prospective cohort of 450 children suspected of CNS infection. The models showed good to excellent diagnostic accuracy with poor calibration in all models. Therefore, diagnostic prediction models could be of help in the diagnostic work-up of paediatric

bacterial meningitis but are not recommended to use on their own in routine individual patient care. Future research should focus on the added value of prediction models in clinical practice.

Author contributions

NSG, IEZ, MCB, MWB and DvdB contributed to the study design. NSG and IEZ contributed to the literature search and data-collection. NSG, SS and MCB accessed and verified the original data from the manuscript. NSG, MCB and MWB analysed an interpreted the data. NSG prepared the first draft of the manuscript and created the figures and tables. All authors contributed to data interpretation and writing. NSG, MWB and MCB had final responsibility for the decision to submit for publication.

All authors confirm that they had full access to all the data in the study and accept responsibility to submit for publication.

Conflict of Interest

The authors declare no conflicts of interest.

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SUPPLEMENTARY MATERIAL

PRISMA checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	3
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	3
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	4
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	4
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Supplementary methods
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	4
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	4
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	4
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	NA

PRISMA checklist (Continued)

Section and Topic	Item #	Checklist item	Location where item is reported
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	4
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	5,6
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	5,6
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	6
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	NA
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	NA
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	NA
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	NA
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	6
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	NA
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	7, Figure 1
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	7, Figure 1
Study characteristics	17	Cite each included study and present its characteristics.	7, Table 1
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Table S1
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Table 2

PRISMA checklist (Continued)

Section and Topic	Item #	Checklist item	Location where item is reported
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	NA
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	7-11
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	NA
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	NA
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Table S1
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	NA
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	12-13
	23b	Discuss any limitations of the evidence included in the review.	14
	23c	Discuss any limitations of the review processes used.	14
	23d	Discuss implications of the results for practice, policy, and future research.	12-14
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	NR
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	16
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	NA
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	16
Competing interests	26	Declare any competing interests of review authors.	16
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	16

NA = Not applicable, NR = not reported

STROBE checklist

Our study is reported according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement. The STROBE checklist, downloaded from https://www.strobe-statement.org, is shown below.

	Item No	Recommendation	Manuscript page
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	4,5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	NA
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5
Bias	9	Describe any efforts to address potential sources of bias	NA
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6,7
		(b) Describe any methods used to examine subgroups and interactions	6,7
		(c) Explain how missing data were addressed	6,7
		(d) If applicable, explain how loss to follow-up was addressed	NA
		(e) Describe any sensitivity analyses	6,7

STROBE checklist (Continued)

	Item No	Recommendation	Manuscript page
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	7
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	NA
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	7
		(b) Indicate number of participants with missing data for each variable of interest	6
		(c) Summarise follow-up time (eg, average and total amount)	NA
Outcome data	15*	Report numbers of outcome events or summary measures over time	8
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	NA
		(b) Report category boundaries when continuous variables were categorized	8
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Supplementary Tables S1-S9
Discussi**on**			
Key results	18	Summarise key results with reference to study objectives	13
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	13-15
Generalisability	21	Discuss the generalisability (external validity) of the study results	13-15
Other informati**on**			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

NA = not applicable

Literature search

MEDLINE

(((“Meningitis”[MeSH]) AND ((stratification OR ‘‘ROC Curve’’[Mesh] OR discrimination OR discriminate OR c statistic OR c statistic OR area under the curve OR AUC OR calibration OR indices OR algorithm OR multivariable) OR (validate OR predict*[tiab] OR rule*) OR (predict* AND (outcome* OR risk* OR model*)) OR ((history OR variable* OR criteria OR scor* OR characteristic* OR finding* OR factor*) AND (predict* OR model* OR decision* OR identify OR prognosis)) OR (decision* AND (model* OR clinical* OR logistic models/)) OR (prognostic AND (history OR variable* OR criteria OR scor* OR characteristic* OR finding* OR factor* OR model*)))))

Table S1. Quality of included studies according to TRIPOD criteria

Section		Item	D/V	Checklist item	Abdelrahim	Boum	Chen	Cheng	Dalai	Delannoy	Huang	Li	Mintegi	Mirkhani	Mwanaki	Obiero	Pelkonen	Wang
Title and abstract	Title	1	D;V	Identify the study as developing and/or validating a multivariable prediction model, the target population, and the outcome to be predicted	p. 1	p.131	p. 1132	p.1018	p. 99	p.447	p1	p.1	p. 1	p. 141	p. 1	p. 130	p.462	p. 64
	Abstract	2	D;V	Provide a summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions.	p. 1	p.131	p. 132	p.1018	p. 99	p.447	p. 1	p. 1	p. 1	p. 141	p. 1	p. 130	p.462	p. 64
Introduction	Background and objectives	3a	D;V	Explain the medical context (including whether diagnostic or prognostic) and rationale for developing or validating the multivariable prediction model, including references to existing models	p. 2	p. 131	p. 1132, 1133	p.1019	p. 100	p.447	p. 2	p. 1,2	p. 2	p. 141, 142	p. 1,2	p. 130,131	p.462	p. 64,65
		3b	D;V	Specify the objectives, including whether the study describes the development or validation of the model or both	p. 2	p. 131	p. 133	p.1019	p. 100	p.448	p. 2	p. 1,2	p. 2	p. 142	p. 2	p. 131	p.462	p. 65
Methods	Source of data	4a	D;V	Describe the study design or source data (e.g., randomized trial, cohort, or registry data), separately for the development and validation sets, if applicable	p. 2	p. 131	p. 133	p.1019	p. 100	p.448	p. 2	p. 2	p. 2	p. 142	p. 2	p. 131	p.462	p. 65
		4b	D;V	Specify the key study dates, including start of accrual, end of accrual and, if applicable, end of follow up	p. 2	p. 131	p. 133	p.1019	p. 100	p.448	p. 2	p. 2	p. 2	p. 142	p. 2	p. 131	p.462	p. 65
	Participants	5a	D;V	Specify key elements of the study setting (e.g., primary care, secondary care, general population) including number and location of centers	p. 2	p. 131	p. 133	p.1019	p. 100	p.448	p. 2	p. 2	p. 2	x	p. 2	p. 131	p.462	p. 65
		5b	D;V	Describe eligibility criteria for participants	p. 2	p. 131	p. 133	p.1019	p. 100	p.448	p. 2	p. 2	p. 2	p. 142	p. 2	p. 131	p.462	p. 65
		5c	D;V	Give details of treatments received, if relevant	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Outcome	6a	D;V	Clearly define the outcome that is predicted by the prediction model, including how and when assessed	p. 3	p. 131	1134	p.1019	p. 100, 101	p.448	p. 2	p. 2	p. 2	p. 142	p. 2	p. 131	p.462	p. 66
		6b	D;V	Report any actions to blind assessment of predictors of the outcome to be predicted	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Table S1. (Continued)

Section	Item	D/V	Checklist item	Abdelrahim	Boum	Chen	Cheng	Dalai	Delannoy	Huang	Li	Mintegi	Mirkhani	Mwanaki	Obiero	Pelkonen	Wang
Predictors	7a	D;V	Clearly define all predictors used in developing or validating the multivariable prediction model, including how and when they were measured	p. 3	p. 131, 132	p. 1131	p. 1019, 1020	p. 100	p. 448	p. 2.3	p. 2	p. 3	p. 142	p. 2	p. 131	p. 462, 463	p. 64,65
	7b	D;V	Report any actions to blind assessment of predictors for the outcome and other predictors	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Sample size	8	D;V	Explain how the study size was arrived at	NR	NR	p. 1134	NR	p. 101	p. 449	NR	p. 3,4,5	p. 4	p. 142	NR	NR	NR	NR
	9	D;V	Describe how missing data were handled (e.g. complete-case analysis, single imputation, multiple imputation, with details of any imputation method)	NR	NR	p. 1134	NR	NR	NR	NR	p.3	NR	p. 142	NR	NR	NR	p. 67
Statistical methods	10a	D	Describe how predictors were handled in the analysis	NA	p. 131	p. 1134	p.1020	p. 101	p. 449	p. 3	p. 3	p. 3	p. 142	p. 3	NA	p. 463	NA
	10b	D	Specify type of model, all model-building procedures (including any predictor selection), and method for internal validation	NA	p. 131	p. 134	p.1020	p. 101	p. 448, 449	p. 2.3	p. 3	p. 3.4	p. 142	p. 3	NA	p. 463	NA
	10c	V	For validation, describe how the predictions were calculated	p. 3	p. 131	p. 1134	NA	NA	p. 448, 449	NA	NA	p. 3	p. 143	p. 3	p. 131, 132	p. 462, 463	p.66
	10d	D;V	Specify all measures used to assess model performance and, if relevant, to compare with multiple models	NR	p. 132	p. 136	p.1020	p. 101	p. 448	p. 3	p.3	p. 3.4	p. 143	p. 3	p. 131	p. 463	p. 66
	10e	V	Describe any model updating (e.g., recalibration) arising from the validation, if done	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	p. 66
Risk groups	11	D;V	Provide details on how risk groups were created, if done	p. 3	p. 132	p. 1134	p.1020	p. 101	p. 448, 449	p. 3	NA	p. 3,4, 5	p. 142, 143	p. 3	p. 131,132	NA	p. 66

Table S1. (Continued)

Section		Item	D/V	Checklist item	Abdelrahim	Boum	Chen	Cheng	Dalai	Delannoy	Huang	Li	Mintegi	Mirkhani	Mwanaki	Oblero	Pelkonen	Wang
	Development vs. Validation	12	V	For validation, identify any differences from the development data in setting, eligibility criteria, outcome and predictors	p. 1,2	p.131	p. 1140 (online)	NA	NA	p. 448	NA	NA	p. 2, 4	NR	p. 4,5	NR	NR	p. 66
Results	Participants	13a	D;V	Describe the flow of participants through the study, including number of participants with and without the outcome and, if applicable, a summary of the follow-up time	p. 3,4,5	p. 132	p. 134, 1135	p. 1020	p. 101	p. 449, 450	p. 3	p. 3	p. 4	p. 142	p. 5	p. 132	Suppl. 2 (online)	p. 66
		13b	D;V	Describe the characteristics of the participants (basic demographics, clinical features, available predictors), including the number of participants with missing data for predictors and outcome	p. 3,4,5,6	p. 132	p. 135, 140 (online)	p. 1020	p. 101, 102	p. 449	p. 2.3	p. 3	p. 4	p. 142	p. 3,4	p. 131, 132	Suppl. 2,3,4 (online)	p. 66, 67
		13c	V	For validation, show a comparison with the development data of the distribution of important variables (demographics, predictors and outcome)	NR	p. 131, 132	P. 1135, 1140 (online)	NA	NA	NR	NA	NA	p. 4	NR	p. 3,4,5,6	NR	NR	NR
	Model development	14a	D	Specify the number of participants and outcome events in each analysis	NA	p. 132	p. 136	NA	NA	NA	p. 3	p. 8	NR	NR	p. 6,7	NA	NR	NA
		14b	D	If done, report the unadjusted association between each candidate predictor and outcome	NA	NA	p. 1136	NA	NA	NA	p. 4	p. 4	p. 5	NA	p. 6,7	NA	p. 463	NA
	Model specification	15a	D	Present the full prediction model to allow predictions for individuals (i.e., all regression coefficients, and model intercept or baseline survival at given time point)	NA	p. 132	p. 1134, 1135, 1136	p. 1022	p. 104	NA	p.5	p. 4	p. 5	p. 143	p. 8	NA	p. 473	NA
		15b	D	Explain how to use the prediction model	NA	p. 132	NR	p. 1020, 1021	NR	NA	p. 5	NR	p. 5	p. 143	p. 8	NA	NR	NA
	Model performance	16	D;V	Report performance measures (with CIs) for the prediction model	NR	p. 132	p. 132, 1136	p. 1022, 1023	NR	p. 449,450	p. 5	p. 4	p. 5	p. 143	p. 8	p. 134	p. 463	p. 67,68

Table S1. (Continued)

Section	Item	D/V	Checklist item	Abdelrahim	Boum	Chen	Cheng	Dalal	Delannoy	Huang	Li	Mintegi	Mirkhani	Mwanaki	Obiero	Pelkonen	Wang
	17	V	If done, report the results from any model updating (i.e., model specification, model performance)	NA	p. 132	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	p. 68
Discussion	18	D;V	Discuss any limitations of the study (such as nonrepresentative sample, few events per predictor, missing data)	NR	p. 132	p. 1138, 1139	p. 1025	p. 104	p. 452	p. 6	p. 6	p. 6.7	p. 143	p. 9	p. 135	p. 465	p. 69
	19a	V	For validation, discuss the results with reference to performance in the development data, and any other validation data	p. 6,7,8	p. 132	NR	NA	NA	p. 451	NA	NA	p. 6.7	NA	p. 6	p. 134	p. 464	p. 68,69
	19b	D;V	Give an overall interpretation of the results, considering objectives, limitations, results from similar studies and other relevant evidence	p.6,7,8,9	p. 132	p. 137, 1138, 1139	p. 1023, 1024, 1025	p. 103, 104	p. 451, 452	p. 4,5,6	p. 6	p. 6.7	p. 143	p. 6,7,8,9	p. 134, 135	p. 464, 465	p. 68,69
	20	D;V	Discuss the potential clinical use of the model and implications for future research	p.9	p. 132	p. 1138	p. 1023, 1024, 1025	p. 104	p. 451, 452	p. 5,6	p. 6	p. 5	p. 143	p.7,8,9	p. 134, 135	p. 465	p. 69,70
Other information	21	D;V	Provide information about the availability of supplementary resources, such as study protocol, Web calculator and data sets	p.9	NA	p. 1140	NA	NA	NA	p. 6	p. 8,9	p. 4,5	NA	NA	p. 132-135	p. 462	NA
Funding	22	D;V	Give the source of funding and the role of the funders for the present study	p.9	p. 133	p. 1132	p. 1025	NR	p. 452	NR	p.6	p. 8	NR	p. 9	p. 135	p. 462	p. 70
			No. of reported (green) / no. of items applicable	18/26 (70%)	25/31 (81%)	31/32 (97%)	23/25 (92%)	19/25 (76%)	25/27 (93%)	24/28 (96%)	25/27 (93%)	29/32 (91%)	19/29 (66%)	29/31 (94%)	22/26 (85%)	24/31 (77%)	25/27 (93%)

D= development, V= validation, CI= confidence interval. Green= reported, Orange= reported incomplete, Yellow/NA= not applicable, Red/NR= not reported

Table S2. Percentage missing data per variable

	% missing	Model using this variable
Gender	0	Li, Mentis, Spanos
Months from August	0	Spanos
Age	0.2	Bonsu, Freedman, Mentis, Pelkonen
Birth weight	87.8	Li, Pelkonen
Duration of symptoms	11.1	Oostenbrink, Pelkonen
Fever	7.1	Chen, Mwaniki
Seizures	12.4	Boyer, Brivet, Dubos, Mwaniki, Nigrovic, Pelkonen, Wang
Vomiting	12.4	Oostenbrink
Irritability	25.4	Chen, Dubos, Mwaniki
Temperature	20.4	Boyer, Mwaniki
Purpura	2.9	Boyer, Dubos, Oostenbrink
Bulging fontanel	89.2	Boum, Chen, Mwaniki
Neck stiffness	44.9	Boum, Oostenbrink
Glasgow coma scale	63.7	Brivet, Oostenbrink, Tokuda
Cranial nerve palsy	70.4	Boyer, Brivet
Aphasia	85.8	Boyer, Brivet
Ataxia	86.9	Boyer, Brivet
Paresis	11.8	Boyer, Brivet
Blood CRP	6.0	Chen, De Cauwer, Li, Mentis, Mintegi, Oostenbrink
Blood glucose	37.4	Hoen
Blood leukocyte count	4.6	Bonsu 2, Boyer, Chavanet, Hoen
Blood granulocyte count	16.8	Boum, Nigrovic, Wang
CSF leukocyte count	5.1	Bonsu 2, Boum, Boyer, Chavanet, Deivanayagam, Freedman, Huang, Li, Mentis, Mirkhani, Spanos
CSF granulocyte count	66.4	Bonsu, Bonsu 2, Boyer, Brivet, Chavanet, De Cauwer, Deivanayagam, Freedman, Hoen, Mentis, Mintegi, Nigrovic, Oostenbrink, Spanos, Tokuda, Wang
CSF lymphocyte count	95.6	Mentis
CSF glucose	5.8	Bonsu 2, Boyer, De Cauwer, Deivanayagam, Freedman, Huang, Li, Mirkhani, Spanos
CSF: blood glucose ratio	41.4	Chavanet, Deivanayagam, Freedman, Oostenbrink, Spanos
CSF protein count	6.9	Bonsu, Bonsu 2, Boyer, Chavanet, De Cauwer, Dubos, Freedman, Hoen, Huang, Mintegi, Mirkhani, Nigrovic, Spanos, Wang

CRP = C-reactive protein, CSF = cerebrospinal fluid

Table S3. Proportion of patients in different risk groups for all children

	<0.1 (95% CI)	≥0.1 and ≤0.8 (95% CI)	>0.8 (95% CI)
Bonsu	20% (16-23%)	59% (54-63%)	22% (18-27%)
Hoen	73% (69-78%)	15% (11-18%)	11% (9-15%)
Huang – model 1	88% (85-91%)	1% (0-1%)	12% (9-15%)
Huang – model 2	89% (86-92%)	1% (0-1%)	11% (8-14%)
Oostenbrink clinical	50% (45-54%)	30% (26-35%)	20% (16-24%)
Oostenbrink CSF	45% (40-49%)	52% (48-57%)	3% (1-4%)
Spanos	38% (34-42%)	52% (48-57%)	10% (7-12%)

CI = confidence interval, CSF = cerebrospinal fluid

Table S4. Discrimination and calibration for neonates

	AUC (95% CI)	Calibration in the large (95% CI)	Calibration slope (95% CI) or HL-test
Bonsu	0.79 (0.66-0.92)	28% (19 to 36%)	Slope: 0.09 (0.03 to 0.14), p<0.001
Bonsu 2	0.82 (0.70-0.94)	-8% (-13 to -3%)	Slope: 1.46 (0.80 to 2.13), p<0.001
Boyer	0.80 (0.64-0.95)	2% (-5 to 8%)	Slope: 0.79 (0.32 to 1.25), p<0.001
Chavanet - adults	0.85 (0.75-0.96)	NA	NA
Chavanet - children	0.79 (0.68-0.91)	NA	NA
De Cauwer	0.78 (0.68-0.89)	14% (6 to 22%)	Slope: 1.54 (0.74 to 2.35), p<0.001
Hoen	0.80 (0.68-0.92)	7% (-0.2 to 14%)	Slope: 0.08 (0.01 to 0.16), p<0.001
Huang - model 1	0.91 (0.82-0.99)	NA	NA
Huang - model 2	0.91 (0.83-0.99)	NA	NA
Nigrovic	0.75 (0.64-0.85)	15% (7 to 23%)	Slope: 1.53 (0.67-2.39), p<0.001
Oostenbrink clinical	0.70 (0.56-0.82)	24% (20 to 29%)	Slope: 0.15 (0.09 to 0.20), p<0.001
Oostenbrink CSF	0.74 (0.57-0.90)	8% (1 to 16%)	Slope: 0.49 (0.12 to 0.85), p<0.05
Spanos	0.69 (0.56-0.82)	11% (4 to 19%)	Slope: 0.30 (0.12 to 0.49), p<0.001

AUC = area under the curve, CI = confidence interval, HL = Hosmer-Lemeshow, NA = not applicable, CSF = cerebrospinal fluid

Table S5. Sensitivity, specificity and predictive values for neonates

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Bonsu	97% (95-99%)	19% (13-25%)	12% (7-17%)	98% (97-100%)
Bonsu 2	89% (84-93%)	51% (44-59%)	16% (11-22%)	98% (96-100%)
Boyer	88% (82-93%)	51% (43-59%)	16% (10-21%)	98% (95-100%)
Boum	73% (65-81%)	41% (33-49%)	12% (7-17%)	93% (90-97%)
Brivet	91% (86-95%)	7% (3-10%)	9% (5-13%)	86% (79-92%)
Chavanet - adults	65% (58-72%)	88% (84-93%)	37% (30-45%)	96% (93-99%)
Chavanet - children	83% (77-89%)	72% (66-79%)	24% (18-31%)	98% (95-100%)
Chen	93% (89-97%)	19% (13-25%)	11% (6-15%)	96% (94-99%)
De Cauwer	94% (90-97%)	49% (41-56%)	16% (10-21%)	99% (97-100%)
Deivanayagam	17% (11-23%)	100% (99-100%)	94% (91-98%)	92% (88-96%)
Freedman*	100% (77-100%)	17% (11-23%)	11% (6-16%)	100% (51-100%)
Hoen	73% (66-79%)	79% (73-85%)	27% (20-34%)	97% (94-99%)
Huang - model 1	88% (82-92%)	92% (88-96%)	55% (48-63%)	99% (97-100%)
Huang - model 2	88% (83-92%)	93% (89-97%)	58% (51-66%)	99% (97-100%)
Li	94% (90-97%)	63% (55-70%)	21% (15-27%)	99% (97-100%)
Mirkhani - model 1	46% (38-53%)	95% (92-98%)	51% (43-58%)	94% (91-98%)
Mirkhani - model 2	36% (29-44%)	98% (96-100%)	64% (57-72%)	94% (90-97%)
Mwanaki	93% (89-96%)	22% (16-29%)	11% (6-16%)	96% (94-99%)
Nigrovic	94% (91-98%)	43% (36-51%)	15% (9-20%)	99% (97-100%)
Oostenbrink combined**	68% (58-77%)	51% (43-58%)	13% (8-18%)	94% (90-97%)
Pelkonen	57% (49-65%)	19% (13-26%)	7% (3-11%)	80% (73-86%)
Spanos	47% (39-54%)	90% (86-95%)	34% (27-42%)	94% (91-98%)
Tokuda	53% (43-62%)	72% (64-79%)	17% (10-24%)	93% (90-97%)
Wang	73% (66-80%)	67% (60-74%)	19% (13-25%)	96% (93-99%)

CI = confidence interval, PPV = positive predictive value, NPV = negative predictive value, CSF = cerebrospinal fluid. * Model adjusted by leaving out variable age because all patients are <5 months of age in this cohort.
** Adjusted cut-off

Table S6. Proportion of patients in different risk groups for neonates

	<0.1 (95% CI)	≥0.1 and ≤0.8 (95% CI)	>0.8 (95% CI)
Bonsu	18% (12-23%)	61% (53-69%)	21% (15-27%)
Hoen	73% (66-80%)	16% (10-22%)	11% (6-16%)
Huang - model 1	85% (79-90%)	0.1% (-1-2%)	15% (10-20%)
Huang - model 2	85% (80-90%)	NA	15% (9-20%)
Oostenbrink clinical	48% (41-56%)	46% (38-53%)	6% (3-10%)
Oostenbrink CSF	49% (41-56%)	49% (41-57%)	2% (0 -5%)
Spanos	48% (40-55%)	46% (39-54%)	6% (2-9%)

CI = confidence interval, CSF = cerebrospinal fluid, NA = not applicable

Table S7. Discrimination and calibration for children ≥ 28 days of age

	AUC (95% CI)	Calibration in the large (95% CI)	Calibration slope (95% CI) or HL-test
Bonsu	0.74 (0.58-0.90)	38% (31 to 44%)	Slope: 0.05 (0.02 to 0.08), p < 0.001
Bonsu 2	0.91 (0.81-1.0)	-3% (-6 to -0.3%)	Slope: 1.6 (1.0 to 2.2), p < 0.001
Boyer	0.85 (0.71-0.98)	3% (-1 to 7%)	Slope: 0.9 (0.5 to 1.3), p < 0.001
Chavanet - adults	0.90 (0.79-1.0)	NA	NA
Chavanet - children	0.82 (0.68-0.96)	NA	NA
De Cauwer	0.92 (0.86-0.98)	18% (12 to 23%)	Slope: 2.6 (1.6 to 3.7), p < 0.001
Hoen	0.89 (0.81-0.97)	13% (8 to 19%)	Slope: 0.08 (0.04 to 0.12), p < 0.001
Huang - model 1	0.96 (0.93-0.99)	NA	NA
Huang - model 2	0.96 (0.94-0.99)	NA	NA
Nigrovic	0.82 (0.71-0.92)	20% (14 to 26%)	Slope: 1.4 (0.8 to 2.1), p < 0.001
Oostenbrink clinical	0.80 (0.67-0.93)	32% (26 to 38%)	Slope: 0.2 (0.2 to 0.3), p < 0.001
Oostenbrink CSF	0.87 (0.75-0.99)	15% (10 to 20%)	Slope: 1.1 (0.6 to 1.5), p < 0.001
Spanos	0.80 (0.67-0.94)	25% (19 to 31%)	Slope: 0.5 (0.3 to 0.8), p < 0.001

AUC = area under the curve, CI = confidence interval, HL = Hosmer-Lemeshow, NA = not applicable, CSF = cerebrospinal fluid

Table S8. Sensitivity, specificity and predictive values for children ≥ 28 days of age

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Bonsu	86% (81-90%)	22% (17-27%)	6% (3-9%)	96% (94-99%)
Bonsu 2	93% (89-96%)	65% (59-71%)	13% (9-16%)	99% (99-100%)
Boyer	87% (82-91%)	64% (58-70%)	11 (8-15%)	99% (98-100%)
Boum	96% (94-98%)	31% (25-36%)	7% (4-10%)	99% (99-100%)
Brivet	99% (98-99%)	13% (9-18%)	6% (3-9%)	99% (99-100%)
Chavanet - adults	79% (74-83%)	93% (90-96%)	38% (32-44%)	99% (97-100%)
Chavanet - children	71% (66-77%)	85% (80-89%)	20% (15-25%)	98% (97-100%)
Chen	100% (99-100%)	7% (4-10%)	5% (3-8%)	100% (99-100%)
De Cauwer	100% (99-100%)	50% (44-56%)	10% (6-13%)	100% (99-100%)
Deivanayagam	36% (30-41%)	100% (99-100%)	82% (77-87%)	97% (95-99%)
Freedman	93% (90-96%)	43% (38-50%)	8% (5-12%)	99% (98-100%)
Hoen	79% (74-84%)	77% (72-82%)	15% (11-19%)	99% (97-100%)
Huang - model 1	71% (66-77%)	93% (89-96%)	34% (28-40%)	98% (97-100%)
Huang - model 2	71% (66-77%)	94% (91-97%)	38% (32-44%)	98% (97-100%)
Li	100% (90-100%)	48% (42-54%)	9% (6-13%)	100% (97-100%)
Mirkhani - model 1	21% (17-26%)	98% (96-100%)	37% (30-42%)	96% (94-98%)
Mirkhani - model 2	21% (17-26%)	99% (98-100%)	58% (51-64%)	96% (94-98%)
Mwanaki	98% (96-99%)	9% (5-12%)	5% (3-8%)	98% (98-99%)
Nigrovic	93% (90-96%)	55% (49-61%)	10% (6-13%)	99% (98-100%)
Oostenbrink combined*	85% (80-90%)	40% (34-46%)	7% (4-11%)	98% (96-100%)
Pelkonen	77% (67-87%)	35% (29-41%)	6% (3-9%)	97% (95-99%)
Spanos	64% (59-70%)	92% (89-95%)	31% (26-37%)	98% (96-100%)
Tokuda	64% (57-71%)	74% (68-79%)	13% (8-18%)	97% (95-99%)
Wang	89% (85-93%)	56% (50-61%)	10% (6-13%)	99% (98-100%)

CI = confidence interval, PPV = positive predictive value, NPV = negative predictive value
* Adjusted cut-off

Table S9. Proportion of patients in different risk groups for children ≥ 28 days of age

	<0.1 (95% CI)	≥0.1 and ≤0.8 (95% CI)	>0.8 (95% CI)
Bonsu	21% (17-28%)	56% (50-62%)	22% (17-27%)
Hoen	73% (67-79%)	14% (10-18%)	13% (9-17%)
Huang - model 1	89% (86-93%)	1% (-0.3-2%)	10% (6-14%)
Huang - model 2	90% (87-94%)	1% (-0.3-1%)	10% (6-13%)
Oostenbrink clinical	32% (28-38%)	56% (50-61%)	12% (8-16%)
Oostenbrink CSF	43% (37-49%)	54% (48-60%)	3% (1-5%)
Spanos	32% (27-38%)	56% (50-61%)	12% (8-16%)

CI = confidence interval, CSF = cerebrospinal fluid

Table S10. Discrimination and calibration (CSF leukocytes not corrected for erythrocyte count)

	AUC (95% CI)	Calibration in the large (95% CI)	Calibration slope (95% CI) or HL-test
Bonsu	0.75 (0.65-0.86)	34% (29 to 39%)	Slope: 0.06 (0.03 to 0.09) , p<0.001
Bonsu 2	0.87 (0.79-0.94)	-5% (-8 to -2%)	Slope: 1.55 (1.11 to 1.99), p<0.001
Boyer	0.83 (0.72-0.93)	3% (-1.0 to 6%)	Slope: 0.86 (0.57 to 1.14), p<0.001
Chavanet - adults	0.88 (0.81-0.96)	NA	NA
Chavanet - children	0.82 (0.73-0.90)	NA	NA
De Cauwer	0.85 (0.78-0.91)	16% (12 to 21%)	Slope: 2.0 (1.4 to 2.6), p<0.001
Hoen	0.84 (0.77-0.91)	11% (7 to 15%)	Slope: 0.07 (0.04 to 0.11), p < 0.001
Huang - model 1	0.93 (0.89-0.97)	NA	NA
Huang - model 2	0.93 (0.89-0.97)	NA	NA
Nigrovic	0.79 (0.72-0.86)	18% (13 to 23%)	Slope: 1.4 (0.9 to 1.9), p<0.001
Oostenbrink clinical	0.70 (0.58-0.82)	25% (20 to 29%)	Slope: 0.15 (0.09 to 0.20), p<0.001
Oostenbrink CSF	0.81 (0.71-0.91)	12% (8 to 17%)	Slope: 0.76 (0.50 to 1.00), p<0.001
Spanos	0.74 (0.64-0.83)	19% (15 to 24%)	Slope: 0.40 (0.27 to 0.52), p<0.001

AUC = area under the curve, CI = confidence interval, HL = Hosmer-Lemeshow, NA = not applicable, CSF = cerebrospinal fluid

Table S11. Sensitivity, specificity and predictive values (CSF leukocytes not corrected for erythrocyte count)

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Bonsu	92% (89-94%)	20% (16-24%)	8% (5-11%)	97% (95-99%)
Bonsu 2	90% (89-93%)	60% (55-64%)	14% (11-17%)	99% (98-100%)
Boyer	87% (84-90%)	59% (54-64%)	13% (10-16%)	98% (97-100%)
Boum	85% (81-89%)	34% (30-39%)	8% (6-11%)	97% (95-99%)
Brivet	94% (92-97%)	11% (8-14%)	7% (5-9%)	96% (95-98%)
Chavanet - adults	71% (66-75%)	91% (89-94%)	37% (32-41%)	98% (96-99%)
Chavanet - children	77% (73-81%)	80% (76-84%)	22% (18-25%)	98% (97-99%)
Chen	96% (95-98%)	11% (8-14%)	7% (5-10%)	98% (96-99%)
De Cauwer	97% (95-98%)	50% (45-54%)	12% (9-15%)	100% (99-100%)
Deivanayagam	24% (20-28%)	99% (98-100%)	76% (72-80%)	95% (93-97%)
Freedman	97% (95-98%)	27% (23-31%)	9% (6-11%)	99% (98-100%)
Hoen	76% (71-80%)	78% (74-81%)	19% (16-23%)	98% (96-99%)
Huang - model 1	80% (76-84%)	90% (88-93%)	37% (33-42%)	98% (97-100%)
Huang - model 2	80% (76-84%)	92% (90-95%)	43% (38-47%)	98% (97-100%)
Li	97% (95-98%)	53% (48-57%)	13% (10-16%)	100% (99-100%)
Mirkhani - model 1	33% (29-38%)	96% (95-98%)	40% (36-45%)	95% (93-97%)
Mirkhani - model 2	30% (26-34%)	98% (96-99%)	50% (44-54%)	95% (93-97%)
Mwanaki	95% (93-97%)	14% (11-17%)	7% (5-10%)	97% (96-99%)
Nigrovic	94% (91-96%)	51% (46-55%)	12% (9-15%)	99% (98-100%)
Oostenbrink combined*	77% (71-82%)	44% (39-48%)	9% (6-11%)	96% (95-98%)
Pelkonen	76% (72-80%)	7% (5-9%)	6% (3-7%)	80% (76-84%)
Spanos	54% (49-59%)	92% (89-94%)	32% (27-36%)	97% (95-98%)
Tokuda	60% (54-65%)	73% (68-77%)	15% (10-19%)	96% (94-98%)
Wang	80% (76-84%)	60% (55-65%)	13% (9-16%)	98% (96-99%)

CI = confidence interval, PPV = positive predictive value, NPV = negative predictive value, CSF = cerebrospinal fluid. * Adjusted cut-off

Table S12. Proportion of patients in different risk groups (CSF leukocytes not corrected for erythrocyte count)

	<0.1 (95% CI)	≥0.1 and ≤0.8 (95% CI)	>0.8 (95% CI)
Bonsu	19% (16-23%)	59% (54-63%)	22% (18-26%)
Hoen	73% (69-78%)	15% (11-18%)	12% (9-15%)
Huang - model 1	86% (83-89%)	1% (0.2-2%)	13% (10-16%)
Huang - model 2	87% (84-91%)	NA	13% (10-16%)
Oostenbrink clinical	50% (45-54%)	30% (26-35%)	20% (16-24%)
Oostenbrink CSF	47% (42-51%)	51% (46-55%)	3% (1-4%)
Spanos	42% (37-46%)	49% (44-53%)	10% (7-12%)

CI = confidence interval, CSF = cerebrospinal fluid

Figure S1. Inclusion process

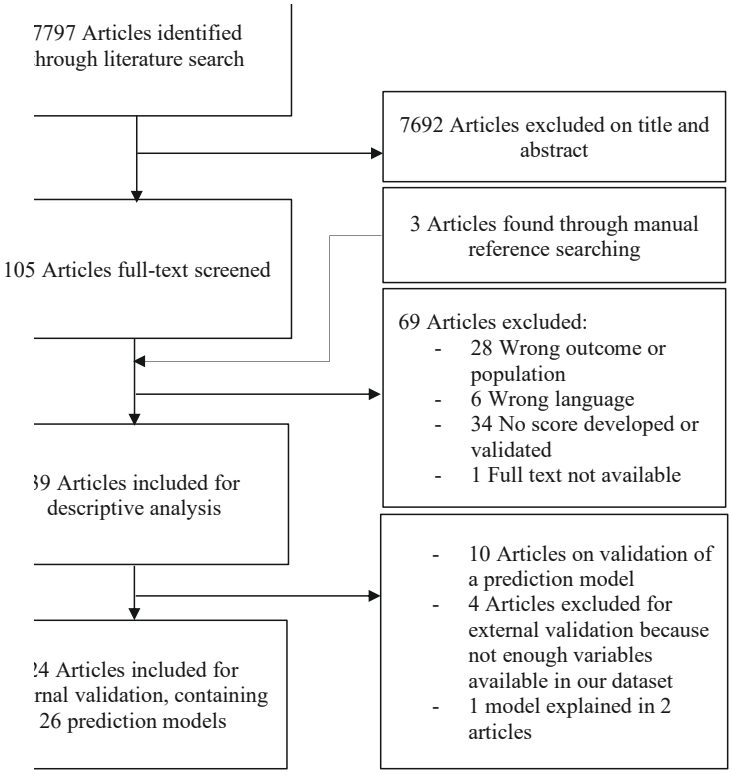
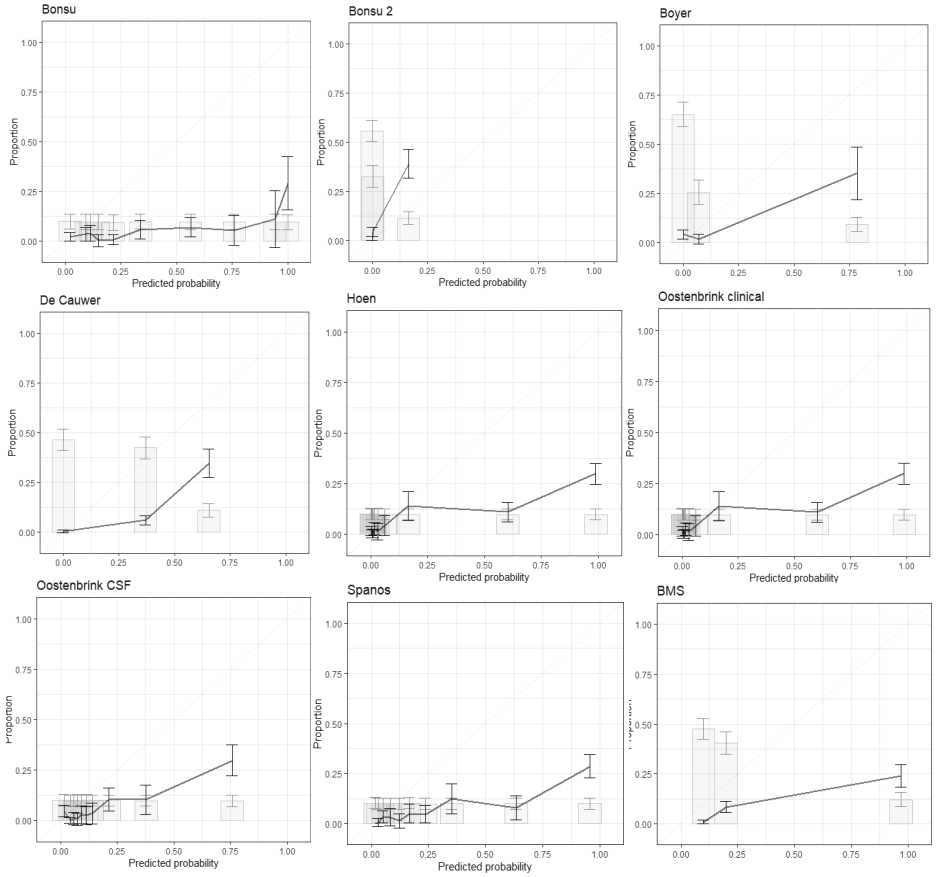


Figure S2. Calibration curves of prediction model performance in all children



CHAPTER 4

NEUROFILAMENT LIGHT CHAIN IN CENTRAL NERVOUS SYSTEM INFECTIONS: A PROSPECTIVE STUDY OF DIAGNOSTIC ACCURACY

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ABSTRACT

Background

Diagnosing central nervous system (CNS) infections quickly is often difficult. Neurofilament light chain (NfL) is a component of the axonal cytoskeleton and identified as marker of neuronal damage in several CNS diseases. We evaluated the diagnostic accuracy of NfL for diagnosing CNS infections.

Methods

We included patients from a prospective cohort of consecutive patients in whom a lumbar puncture was performed for suspected CNS infection in an academic hospital in The Netherlands. The index test was NfL in cerebrospinal fluid (CSF) and reference standard the final clinical diagnosis. Diagnostic accuracy was determined using the area-under-the-curve (AUC) with 95% confidence intervals (CI). The association of CSF NfL with clinical characteristics, diagnosis and outcome was evaluated.

Results

Between 2012 and 2015, 273 episodes in adults of which sufficient CSF was available were included. CNS infection was diagnosed in 26%(n=70), CNS inflammatory disease in 7%(n=20), systemic infection in 32%(n=87), and other neurological disorders in 33%(n=90). Median CSF NfL level was 593 pg/ml (IQR 249-1569) and did not discriminate between diagnostic categories or CNS infection subcategories. AUC for diagnosing any CNS infection compared to patients without CNS infections was 0.50 (95%CI 0.42-0.59). Patients presenting with an altered mental status had higher NfL levels compared to other patients.

Conclusions

NfL cannot discriminate between causes in patients suspected of CNS infections. High concentrations of NfL are associated with severe neurological disease and the prognostic value of NfL in patients with CNS infections should be investigated in future research.

INTRODUCTION

Patients suspected of a central nervous system (CNS) infection often pose a diagnostic dilemma. In a substantial part (76%) of the patients initially suspected of a CNS infection another diagnosis is made, including systemic infections without CNS involvement, metabolic encephalopathies, epilepsy or inflammatory diseases of the CNS. [1] Clinical characteristics fail to differentiate between these causes and the best predictor of a CNS infection is the cerebrospinal fluid (CSF) leukocyte count, but its sensitivity and specificity are still insufficient.[1,2] CNS infection can be caused by a wide variety of pathogens including bacteria, viruses, tuberculosis and fungi. In these patients the outcome depends on early initiation of targeted treatment.[3,4] Cultures or polymerase chain reaction (PCR), however, remain negative in a substantial proportion of the clinically suspected patients, ranging from 35-42% in viral CNS infections and 4-50% in bacterial meningitis.[1,5-7]

Neurofilament light chain (NfL) is a component of the axonal cytoskeleton, of which low levels are being constitutively released from axons into CSF and blood.[8] Increased NfL concentration has been identified as marker of neuronal damage due to a variety of central nervous system diseases.[9] These include multiple sclerosis (MS), Alzheimer's disease, frontotemporal dementia, amyotrophic lateral sclerosis, traumatic brain injury and atypical Parkinsonian disorders.[9-11] NfL has been suggested to be of value in diagnosing some of these CNS diseases, but has also shown value as serum marker for response to treatment and prognosis.[9-11] Few studies have described neurofilaments in patients with CNS infections, of which one showed higher CSF levels of neurofilament heavy in children with bacterial meningitis compared to controls.[12] A recent study from our group showed CSF NfL level in bacterial meningitis patients was associated with poor prognosis, and showed levels differed significantly between causative pathogens.[13] Two other studies showed higher serum and CSF levels in patients with varicella zoster virus (VZV) encephalitis compared to VZV meningitis.[14,15] CSF levels of NfL HIV patients showed elevated levels of NfL were mostly found in those with HIV-associated dementia, but the diagnostic accuracy has not been studied in the at risk population.[16] Finally, in the past two years the value of CSF NfL levels has been evaluated in patients with neurological complications of COVID19, showing elevated levels in some, but no diagnostic value in differentiation between healthy controls or between those neurological manifestations in f COVID19.[17,18]

Our objective was to determine the diagnostic accuracy of NfL for the diagnosis of CNS infections. We hypothesized that NfL might be increased in patients with CNS infections in general and could function as a diagnostic biomarker in patients suspected of CNS

infections. We measured levels of NfL in CSF of consecutive patients with suspected CNS infections from a previously collected, prospective cohort and evaluated the diagnostic accuracy of NfL. Furthermore, we analyzed whether NfL was associated with clinical characteristics and outcome in these patients.

METHODS

Patients and samples

To assess the diagnostic accuracy of CSF NfL, the index test, for CNS infections, we analyzed patients who had been prospectively included in a cohort study of diagnostic parameters in suspected CNS infections, of which methods have been described in detail previously.[1] In brief, all consecutive episodes of inpatients or patients presenting to the emergency department of the Academic Medical Center in Amsterdam, The Netherlands, were prospectively included if they were ≥16 years and underwent a lumbar puncture for the suspicion of a CNS infection. When patients had multiple episodes of suspected CNS infections during the study period, each episode was included as separate entry in the study. Exclusion criteria were a neurosurgical procedure or severe neurotrauma less than three months prior to the lumbar puncture, or a neurosurgical device in situ. Data on clinical presentation, ancillary investigations and outcome were collected. Patients were then divided into groups based on their final clinical diagnosis: CNS infections, CNS inflammation without infection, systemic infection without CNS involvement, non-infectious non-inflammatory neurological disorders, and other systemic disorders. CNS infections were then subdivided into three categories: bacterial meningitis, viral meningitis and other CNS infections. Episodes were considered to be a CNS infection if there was microbiological evidence of infection, or when two neurologists independently classified the episodes as being due to a bacterial, viral or other CNS infection based on all available clinical data. Disagreements on the final diagnosis between the two neurologists were resolved by discussion with a third neurologist (kappa 0.76). This final diagnosis was considered the reference standard, to reflect clinical practice.

For this study, only episodes of patients with a sufficient amount of CSF available for NfL measurement (5 µl), the index test, were included. The CSF obtained during the first lumbar puncture at presentation or during admission was used. This was centrifuged after withdrawal and, after performance of regular diagnostics, frozen and stored in -80º Celsius.

NfL measuremens: Simoa

The index test, NfL, was measured in 5 µl of CSF using Simoa NF-light Advantage Kit (ref. 103186) on a HD-X instrument (Quanterix, Massachusetts, USA) at the Neurochemistry

Laboratory at Amsterdam UMC, location VUmc, according to the manufacturer's instructions. The investigators performing the NfL measurements were blinded to the reference standard.

Statistical analysis

Statistical analysis was done using IBM SPSS Statistics for Windows, version 26 (Armonk, NY: IBM Corp.). Values are displayed as median with interquartile range (IQR) or absolute number with percentage. Continuous variables were compared by using the two-sample t-test or Mann-Whitney U test, depending on the distribution. Categorical data were compared using a Chi-square or Fisher's exact test, depending on sample size. A p-value <0.05 was considered statistically significant. We performed logistic regression analysis to determine the predictive value of NfL concentration in CSF with correction for age because of previous studies reporting an association of age and NfL levels in CSF. The area under the curve (AUC) of the receiver operating characteristic (ROC) curve was calculated to evaluate the diagnostic accuracy of NfL, with 95% confidence intervals (CI). There were no missing data on index and reference standard. As no prior data of the test characteristic of the index tests was available, no power calculation could be performed and the study is considered an exploratory diagnostic accuracy study. This study was reported according to the STARD criteria (Supplementary Table 1).[19]

RESULTS

Between 2012 and 2015, 363 episodes in 349 patients were included in the cohort with all consecutive episodes suspected of CNS infections (Figure 1). In 273 episodes (75%) occurring in 264 patients, a sufficient amount of CSF was available for current analysis. Nine patients were included with two separate episodes of suspected CNS infections. The median age in these 273 episodes was 50 years (IQR 35-65) and 52% (n=142) of the episodes occurred in women (Table 1). Overall median CSF leukocyte count was $4x10^6$/L (IQR 2-22) and median CSF total protein level was 0.4 g/L (IQR 0.3-0.7). A CNS infection was diagnosed in 26% (n=70), CNS inflammatory disease in 7% (n=20), systemic infection without CNS involvement in 32% (n=87), non-infectious or -inflammatory neurological disorders in 33% (n=90) and other systemic diseases in 2% (n=6). Within the group of CNS infections, there were 15 episodes of bacterial CNS infection, 37 of viral CNS infection and 18 of other CNS infection (e.g. cryptococcal meningitis, tuberculous meningitis and cerebral toxoplasmosis). Mortality in all episodes was 11% (n=30) and 26% (n=72) had an unfavorable outcome, defined as a Glasgow Outcome Scale score of less than 5.[20]

Table 1. Baseline characteristics

	All (n=273)	CNS infection (n=70)	CNS inflammatory disease (n=20)	Systemic infection (n=87)	Neurological disorder (n=90)	Other systemic disease (n=6)
Age (years)	50 (35-65)	45 (34-59)	50 (38-62)	52 (34-68)	53 (39-63)	53 (39-63)
Female sex	142 (52)	33 (47)	11 (55)	46 (53)	49 (54)	3 (50)
Clinical presentation						
Headache	159/266 (60)	46/67 (69)	16/19 (84)	55/84 (65)	40/90 (44)	2/6 (33)
Fever (≥38°)	106/271 (39)	31/70 (44)	1/19 (5)	52/87 (60)	21/89 (24)	1/6 (17)
Seizures	30/270 (11)	4/70 (6)	0/20 (0)	7/87 (8)	13/90 (14)	0/6 (0)
GCS <14	77/273 (28)	19/70 (27)	3/20 (15)	25/87 (29)	30/90 (33)	0/6 (0)
GCS ≤8	25/273 (9)	5/70 (7)	0/20 (0)	7/87 (8)	13/90 (14)	0/6 (0)
Focal neurol. deficits	60/273 (22)	16/70 (23)	5/20 (25)	10/87 (11)	29/90 (32)	0/6 (0)
CSF parameters						
CSF leukocytes (10^6/L)	4 (2-22)	147 (25-387)	16 (9-73)	4 (2-5)	4 (2-18)	3 (2-4)
CSF total protein (g/L)	0.4 (0.3-0.7)	0.8 (0.5-1.4)	0.7 (0.5-2.2)	0.3 (0.2-0.5)	0.4 (0.4-0.5)	0.4 (0.3-0.6)
Outcome						
Dead	30 (11)	9 (13)	2 (10)	8 (9)	10 (11)	1 (11)
Unfavorable*	72 (26)	19 (27)	5 (25)	17 (20)	30 (33)	1 (11)

Values are medians (interquartile range) or n/N (%). CNS = central nervous system, GCS= Glasgow Coma Scale score, CSF= cerebrospinal fluid

* Unfavorable outcome is defined as a Glasgow Outcome Scale score of <5

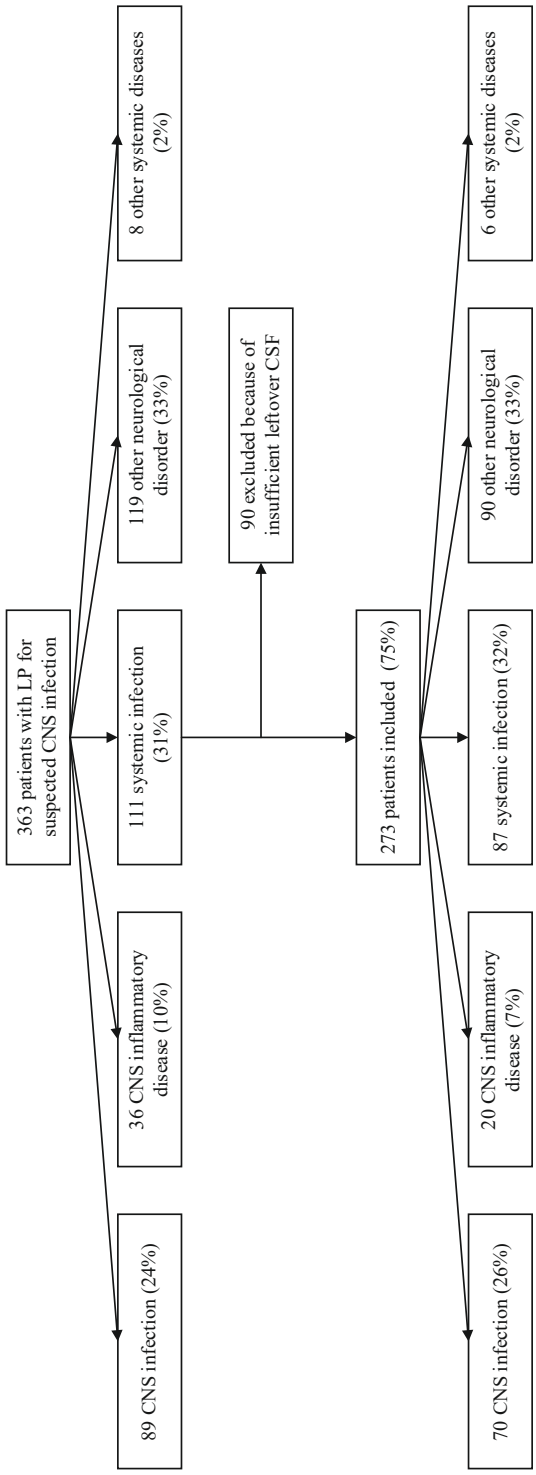
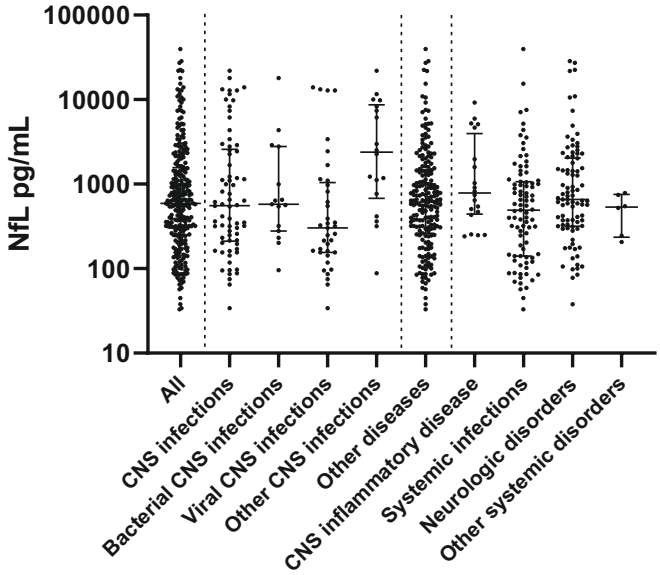


Figure 1. Inclusion flow chart

The median level of NfL was 593 pg/ml (IQR 249-1569) and did not differ between the different diagnostic categories (Kruskal-Wallis test, P=0.44; Figure 2; Supplementary Table 2). In episodes with CNS infections median NfL level was 558 pg/ml (IQR 212-2588) versus 615 pg/ml (IQR 263-1455) in other episodes (P=0.70). Episodes of patients with bacterial meningitis had a median NfL level of 576 pg/ml (IQR 278-2777) compared to 603 pg/ml (IQR 248-1557; P=0.99) in all other episodes, and compared to 303 pg/ml (IQR 156-1041; P=0.22) in viral CNS infections. There were also no differences between groups after correction for age.

Figure 2. Concentration of NfL per diagnosis

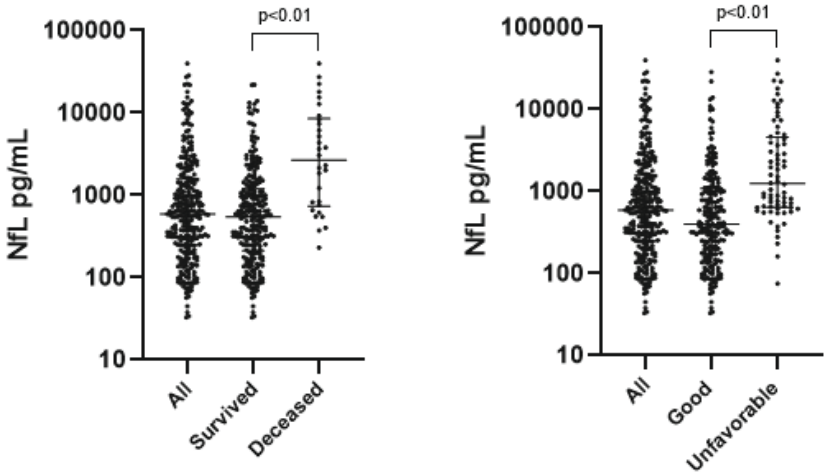


The AUC for diagnosing a CNS infection was 0.50 (95% CI 0.42-0.59). The AUC for diagnosing bacterial meningitis within all patients initially suspected of a CNS infection was 0.52 (95% CI 0.38-0.66). The AUC for differentiating bacterial meningitis from a viral CNS infection was 0.65 (95% CI 0.50-0.81).

Episodes presenting with seizures had higher NfL levels (median 938 pg/ml [IQR 609-2422] vs. 547 pg/ml [IQR 218-1439]; P=0.004), as did episodes presenting with focal neurological deficits (912 pg/ml [IQR 325-2525] vs. 556 pg/ml [IQR 206-1373]; P=0.01). However, after correction for age, there was no association with seizure and focal deficits. In episodes in which the patient had an altered mental state, defined as a score on the Glasgow Coma Scale [GCS] <14, NfL levels were significantly higher compared to

patients with GCS scores of 14 or 15 (1051 pg/ml [IQR 590-2868] vs. 405 pg/ml [IQR196-1104]; P<0.001). In comatose patients (GCS ≤8) this was 1996 pg/ml (IQR 641-4980) and 547 pg/ml (IQR 217-1384) in non-comatose patients (P=0.001). This association remained significant after correction for age. A weak correlation between NfL and age was found (r=0.5, P<0.01) as well as between NfL and CSF protein (r=0.4, P<0.01). No correlation between NfL and sex or CSF leukocytes was found. Corrected for age, NfL was associated with mortality and unfavorable outcome (Figure 3; odds ratio 1.16 [95% CI 1.08-1.24] and 1.14 [95% CI 1.06-1.22] per 1000 pg/ml).

Figure 3. Concentration of NfL per outcome



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DISCUSSION

Our study shows that the diagnostic accuracy for the diagnosis of CNS infections in all patients initially suspected of CNS infections is poor. No difference was found in levels of NfL in CSF between different diagnostic categories. NfL level in CSF was related to neurological symptoms, being associated with an altered mental status as well as seizures and focal neurological abnormalities, although the latter two were not significant after correction for age. Furthermore, higher levels of NfL were associated with mortality and unfavorable outcome.

In non-infectious neurological diseases, varying results have been reported on the diagnostic value of NfL.[9,10] The main limitation of these studies is the selection of patients to determine the diagnostic accuracy of NfL. In general these studies compared patients

with the neurological disease to healthy controls, while this is not the population in which the differentiation needs to be made in clinical practice. For instance, in a study on diagnostic accuracy in Alzheimer's disease, NfL in CSF was able to differentiate between one of the Alzheimer's classification subgroups – patients with either tau pathology or neurodegeneration - and healthy controls, although only with an AUC of 0.69.[21] Differentiation between Alzheimer's disease and frontotemporal dementia was poor, with an AUC of 0.54.[21] A meta-analysis of 15 retrospective studies showed that NfL was higher in patients with multiple sclerosis compared to healthy controls and could differentiate between these categories.[22] A good diagnostic accuracy was found for sporadic Creutzfeldt-Jakob's disease (sCJD), although this was compared to a group with both psychiatric and a variety of non-neurodegenerative neurological diseases.[23] Differentiation between sCJD and several other neurodegenerative diseases was less accurate.[23] These findings are probably due to the fact that NfL is a very sensitive marker for axonal damage, but not specific. Therefore, it appears to discriminate best between neurological diseases with different degrees of axonal damage, rather than between disease categories.[9,10] Our study, including all patients suspected of CNS infections, does reflect clinical practice. However, the group of patients presenting with a suspected acute CNS infection probably is too heterogeneous to use CSF NfL as a diagnostic biomarker, since patients with other neurological diagnoses are also included.

We found NfL levels to be associated with an altered mental status in patients suspected of a CNS infection. This is consistent with NfL being a marker of axonal loss, which can be expected by generalized damage to the brain associated with this clinical characteristic. In several other neurological diseases such as multiple sclerosis and peripheral neuropathies NfL has been suggested to be helpful in monitoring disease activity and response to treatment.[24,25] This could especially be of help in diseases in which clinical evaluation is difficult and additional biomarkers are desirable that reflect the disease activity, like chronic auto-immune meningitis. A recent study in bacterial meningitis showed that NfL in bacterial meningitis was an independent predictor for unfavourable outcome, after correction for age, cranial nerve palsy, and high serum CRP levels.[13] This study also showed that CSF levels of Nfl correlated to the presence of an altered mental status and focal cerebral deficits, confirming CSF NfL levels reflect neuronal damage in CNS infections. In patients with suspected CNS infections, the prediction of outcome is however less informative, because a wide spectrum of diagnoses is still under consideration and NfL levels do not guide the differential diagnosis enabling targeted treatment.[1]

Our study has several limitations. First, the current analysis was a retrospective study on biobanked CSF samples. Clinical data, however, were collected prospectively and bias

should therefore be limited. Second, we performed just one measurement of NfL level per patient, so the course of NfL in CSF of these patients was not evaluated. We did not have data on the exact date of onset of symptoms in our database and it might therefore be possible that patients reached higher levels of NfL later during the disease. For the diagnostic evaluation of NfL this would not matter, since the diagnosis is needed as soon as possible and preferably in the initial CSF sample. However, for prognostic purposes measuring several time-points could provide additional information.[12] Preferably, this would be done in blood samples instead of CSF because of the easier accessibility. The correlation between serum and CSF concentrations should, however, be evaluated in patients with CNS infections more thoroughly, since this correlation can vary in different disease types.[24,26,27] Finally, in only 75% of the patients from the prospectively collected cohort a sufficient amount of CSF was available. Since the distribution of diagnoses corresponds well to the original cohort, selection bias because of this is limited.

CONCLUSION

In conclusion, NfL in CSF has a poor diagnostic accuracy in patients suspected of CNS infections. NfL is associated with clinical signs associated with damage to the nervous system disease and unfavourable outcome. The use of NfL for prognosis or therapy monitoring for CNS infections in patients with elevated levels should be investigated in future research.

Ethics approval

This study was approved by the Biobank Ethical Review Committee of the Amsterdam UMC (number METC 2014_290). Written informed consent was obtained from all participants or their representatives. All experiments were performed in accordance with relevant guidelines and regulations.

Consent to participate

Written informed consent was obtained from all participants or their representatives.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

No potential conflicts of interest relevant to this article exist.

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Authors' contributions

IZ contributed to data gathering, data analyses, data interpretation and writing the first draft of the manuscript. LH contributed to critique of the report. HH contributed to data gathering and data interpretation. CT contributed to data gathering, interpretation, review and critique of the report. DB contributed to review and critique of the report. MB contributed to study design, data gathering, data analysis, data interpretation, review, and critique of the report.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. STARD guideline checklist

Section and topic	No	Item	Page no. manuscript
Title or abstract			
	1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)	1
Abstract			
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)	1
Introduction			
	3	Scientific and clinical background, including the intended use and clinical role of the index test	3
	4	Study objectives and hypotheses	3
Methods			
Study design	5	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)	4
Participants	6	Eligibility criteria	4
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)	4
	8	Where and when potentially eligible participants were identified (setting, location, and dates)	4
	9	Whether participants formed a consecutive, random, or convenience series	4
Test methods	10a	Index test, in sufficient detail to allow replication	4
	10b	Reference standard, in sufficient detail to allow replication	4
	11	Rationale for choosing the reference standard (if alternatives exist)	4
	12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory	NA
	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory	4
	13a	Whether clinical information and reference standard results were available to the performers or readers of the index test	4
	13b	Whether clinical information and index test results were available to the assessors of the reference standard	4
Analysis	14	Methods for estimating or comparing measures of diagnostic accuracy	5
	15	How indeterminate index test or reference standard results were handled	5
	16	How missing data on the index test and reference standard were handled	5

Supplementary Table 1. (Continued)

Section and topic	No	Item	Page no. manuscript
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	5
	18	Intended sample size and how it was determined	5
Results			
Participants	19	Flow of participants, using a diagram	Page 5; Figure 1
	20	Baseline demographic and clinical characteristics of participants	Page 5; Table 1
	21a	Distribution of severity of disease in those with the target condition	Page 6; Table 1
	21b	Distribution of alternative diagnoses in those without the target condition	Page 5; Figure 1
	22	Time interval and any clinical interventions between index test and reference standard	NA
Test results	23	Cross tabulation of the index test results (or their distribution) by the results of the reference standard	Page 6; Figure 2; Supplementary Table 2
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)	6
	25	Any adverse events from performing the index test or the reference standard	NA
Discussion			
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability	8
	27	Implications for practice, including the intended use and clinical role of the index test	9
Other information			
	28	Registration number and name of registry	5
	29	Where the full study protocol can be accessed	NA
	30	Sources of funding and other support; role of funders	9

NA= not applicable

Supplementary Table 2. NfL concentrations per subcategory

Diagnosis	Median NfL concentration (pg/ml)
CNS infections (n=70)	558 (212-2588)
Bacterial meningitis (n=15)	576 (278-2777)
Viral meningitis (n=25)	196 (131-321)
Viral encephalitis (12)	959 (400-10,474)
Progressive multifocal leucencephalopathy (n=3)	7393 (NA)
Cryptococcal meningitis (n=3)	2258 (NA)
Tuberculous meningitis (n=3)	1114 (NA)
Parasitic encephalitis (n=2)	5408 (NA)
Cerebral toxoplasmosis (n=2)	11601 (NA)
Leptospirosis meningitis (n=2)	251 (NA)
CNS inflammatory disease (n=20)	783 (441-3980)
Auto-immune encephalitis (n=4)	3310 (973-4994)
Chronic meningitis (n=5)	912 (498-5130)
Recurrent aseptic meningitis (n=4)	451 (295-526)
Neurosarcoidosis	3113 (NA)
Systemic infection (n=87)	491 (141-1070)
Respiratory tract infection (n=20)	999 (423-3610)
Bacteremia/ systemic bacterial infection (n=15)	402 (149-745)
Systemic viral infection (n=10)	165 (69-450)
Ear, nose or throat infection (n=12)	313 (96-791)
Gastro-intestinal infection (n=11)	204 (86-651)
Genito-urinary tract infection (n=7)	1628 (321-7562)
Skin/soft tissue infection (n=5)	1020 (718-1942)
Fever or sepsis eci (n=4)	345 (78-2803)
Other neurological disease (n=90)	659 (319-2036)
Headache syndrome (n=29)	373 (122-864)
Metabolic or toxic encephalopathy (n=21)	761 (416-2575)
Epilepsy (n=14)	934 (549-1596)
Stroke (n=11)	1418 (658-4931)
Intracerebral tumor (n=6)	1652 (443-9777)
Functional neurological symptoms (n=3)	317 (NA)
Other systemic disease (n=6)	536 (235-754)
Psychiatric condition (n=3)	742 (NA)
Systemic auto-immune disease (n=2)	536 (NA)

NfL= neurofilament light chain, CNS= central nervous system, NA= not applicable

CHAPTER 5

VIRAL METAGENOMICS ON CEREBROSPINAL FLUID

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Genes. 2019 Apr 30;19(5):332

ABSTRACT

Identifying the causative pathogen in central nervous system (CNS) infections is crucial for patient management and prognosis. Many viruses can cause CNS infections, yet screening for each individually is costly and time-consuming. Most metagenomic assays can theoretically detect all pathogens, but often fail to detect viruses because of their small genome and low viral load. Viral metagenomics overcomes this by enrichment of the viral genomic content in a sample. VIDISCA-NGS is one of the available workflows for viral metagenomics, which requires only a small input volume and allows multiplexing of multiple samples per run. The performance of VIDISCA-NGS was tested on 45 cerebrospinal fluid (CSF) samples from patients with suspected CNS infections in which a virus was identified and quantified by polymerase chain reaction. Eighteen were positive for an RNA virus, and 34 for a herpesvirus. VIDISCA-NGS detected all RNA viruses with a viral load >2 × 10^4 RNA copies/mL (n = 6) and 8 of 12 of the remaining low load samples. Only one herpesvirus was identified by VIDISCA-NGS, however, when withholding a DNase treatment, 11 of 18 samples with a herpesvirus load >10^4 DNA copies/mL were detected. Our results indicate that VIDISCA-NGS has the capacity to detect low load RNA viruses in CSF. Herpesvirus DNA in clinical samples is probably non-encapsidated and therefore difficult to detect by VIDISCA-NGS.

INTRODUCTION

For patients with a suspected central nervous system (CNS) infection, rapid and accurate diagnosis is vital to determine treatment and improve prognosis.[1] The differential diagnosis of such patients includes infectious aetiologies, of which viruses are the most common[2], but also non-infectious aetiologies, such as auto immune diseases.[3] Nonetheless, in more than half of cases, the cause remains unknown.[4] Identification of a virus can aid in patient management as it may initiate specific antiviral treatment, or cease or prevent ineffective antiviral, antibiotic, and/or immunosuppressive treatments, which all have potential harmful side effects. For example, when differentiating between an auto-immune and viral origin, immune suppression could lead to deleterious outcomes when caused by an unidentified virus.[5]

During the last two decades, conventional diagnostics for viral CNS infections have shifted from non-specific culturing techniques towards highly specific viral nucleic acid amplification tests, like quantitative polymerase chain reaction (qPCR), or the detection of host-mediated antibody production to the virus (e.g., ELISA). Although these latter assays have greatly increased diagnostic sensitivity, a limitation is that they only target an individual virus or a subset of related viruses. The number of viruses that have been associated with CNS infections currently comprises more than 100[6], with several more discovered in the last decade.[7,8,9,10] Consequently, a comprehensive diagnostic panel would include many specific tests. Since this is unachievable for routine diagnostics, only a small selection of viruses commonly associated with CNS infections are included in most diagnostic panels (e.g., herpes simplex virus 1/2, enteroviruses, and parechoviruses). Other pathogens are usually not examined, or are tested for at a later stage of the disease, by which time irreversible pathology could have occurred.

Metagenomics is a recent and promising development in microbiology, which is theoretically able to detect all viruses, including known, unexpected, and novel species.[5] The sensitivity of such assays is generally determined by three factors: (1) The concentration of viruses in a clinical sample, (2) the amount of background (competing) RNA and DNA, and (3) the sequencing depth. Generally, metagenomics assays are poor or unable to detect viruses in a clinical specimen because of the low viral load relative to the high concentration of background RNA and DNA. To overcome this, viral metagenomic assays enrich the viral content of a sample. Virus discovery cDNA-AFLP (amplified fragment length polymorphism) next-generation sequencing (VIDISCA-NGS) is one of the available assays for viral metagenomics. Characteristic for VIDISCA-NGS is the fragmentation of ds(c)DNA, which is done using a frequent-cutting restriction enzyme, and thus different from the random shearing, random PCR amplification, or

transposon-based shearing techniques used in most viral metagenomic assays.[11,12] The method was first described with the discovery of human coronavirus NL63[13], and since has discovered and detected a wide range of viruses in various sample types.[14,15,16,17,18] VIDISCA-NGS could be an ideal tool for the broad range detection of viruses in cerebrospinal fluid (CSF).

CSF is a distinct bodily fluid containing a relatively low number of host cells. Even with mild pleocytosis, as seen during most viral CNS infections, CSF has a far lower cellular content than a similar volume of blood, respiratory, or faecal material. This low amount of background could influence NGS results in two ways: (1) It may decrease the nucleic acid extraction yield if the total nucleic acid content is too low, or (2) it may be beneficial, as proportionally less sequence space is taken by competing background RNA or DNA. Considering the potential benefit viral metagenomics may have for future viral diagnostics in encephalitis, we determined the capability of VIDISCA-NGS to detect viruses in CSF samples from patients with suspected CNS infections.

METHODS

CSF samples which previously tested positive by viral qPCR were selected from two biobanks of the departments of medical microbiology and neurology of the Amsterdam UMC (location AMC). An HIV-1 qPCR was performed using the RealTime HIV-1 Viral Load Assay (Abbott Molecular, Abbott Park, IL, USA), the other viruses were tested by in-house qPCRs using previously published methods.[19] The first sample set consisted of anonymized leftover CSF samples (n = 27), sent in from patients with suspected CNS infection. The second set of CSF samples (n = 18) were selected from a clinical study on the etiology of encephalitis and meningitis in adult patients.[2] The study was approved by the medical ethics committee of the Academic Medical Centre, Amsterdam, The Netherlands (reference number 2014_290). All samples had a quantifiable viral load and were stored at −80 °C until library preparation for VIDISCA-NGS.

VIDISCA library preparation was performed as previously described.[9,17] Briefly, CSF samples were centrifuged and the supernatant was treated with TURBO™ DNase (Thermo Fisher Scientific, Waltham, MA, USA) to remove naked chromosomal or bacterial DNA. Nucleic acids were extracted using the Boom method[20], followed by reverse transcription with non-ribosomal random hexamers[21] and second strand synthesis. DNA was digested with MseI (T^TAA; New England Biolabs, Ipswich, MA, USA) and ligated to adapters containing a sample identifier sequence. During the fragmentation in VIDISCA, the sample cannot be “over-digested” as fragmentation

relies entirely on the presence of restriction enzyme recognition sites and not on the duration of fragmentation. Ligation to adaptors leads to loss of the restriction enzyme recognition site (after ligation to an adaptor the sequence is TTAT) whereas ligation to another DNA fragment will restore it, allowing re-digestion. Next, size selection with AMPure XP beads (Beckman Coulter, Brea, CA, USA) was performed to remove small DNA fragments prior to a 28-cycle PCR using adaptor-annealing primers. Small and large size selection was performed with AMPure XP beads to select DNA-strands with a length ranging between 100 and 400 nucleotides. Libraries were analyzed using the Bioanalyzer (High Sensitivity Kit, Agilent Genomics, Santa Clara, CA, USA) and Qubit (dsDNA HS Assay Kit, Thermo Fisher Scientific) instruments to quantify DNA length and concentration, respectively. Seventy sample libraries were pooled at the equimolar concentration. The current number of 70 samples was chosen because this has worked for other sample types (non-CSF).[16,17] In total, 50 pmol DNA of the pool was clonally amplified on beads using the Ion Chef System (Thermo Fisher Scientific) and sequencing was performed on the Ion PGM™ System (Thermo Fisher Scientific) with the ION 316 Chip (400 bp read length and 2 million sequences per run). The method for the DNase-free VIDISCA library preparation omitted the TURBO™ DNase step.

All VIDISCA-NGS reads with a minimum length of 45 nucleotides were translated into protein sequences and aligned to a local database of the NCBI eukaryotic viral Identical Protein Groups (downloaded March 2018) using UBLAST[22], the VIDISCA bioinformatics workflow[23], and an online metagenomic profiler (Taxonomer)[24] for identification of probable viral reads and background sequence classification. Probable viral reads were subsequently confirmed when the original VIDISCA-NGS read could be aligned to a reference sequence of the virus with a nucleotide identity of at least 80% using CodonCode Aligner (version 6.0.2). Each alignment was manually inspected for confirmation. Samples were considered VIDISCA-NGS positive when at least one VIDISCA-NGS read could be identified. The number of reads aligned to a reference sequence in CodonCode Aligner was taken as the number of viral reads per sample. Analysis by VIDISCA-NGS was performed blind to qPCR results to avoid biased analysis. All statistical analyses were performed in R (version 3.5.1), and graphs were plotted using R package ggplot2 (version 3.1.0).

RESULTS

Sample Description and qPCR Results

Forty-five CSF samples from patients with a suspected CNS infection were examined. Samples had been tested by routine diagnostic for enterovirus, human immunodeficiency virus 1 (HIV-1, in case the patients were HIV-1 seropositive), parechovirus, and herpesviruses (herpes simplex virus 1 and 2 (HSV-1/2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), and human herpesvirus 7 (HHV-7). The CSF samples contained either a single virus (n = 36) or multiple viruses (n = 9), and tested positive for HIV-1 (n = 10), enterovirus (n = 8), HSV-1/2 (n = 14), VZV (n = 8), EBV (n = 12), CMV (n = 2), and HHV-7 (n = 2). All details concerning the qPCR-results, viral loads, total sequence reads obtained via VIDISCA-NGS, and number of viral sequences are available in the Supplementary Table S1.

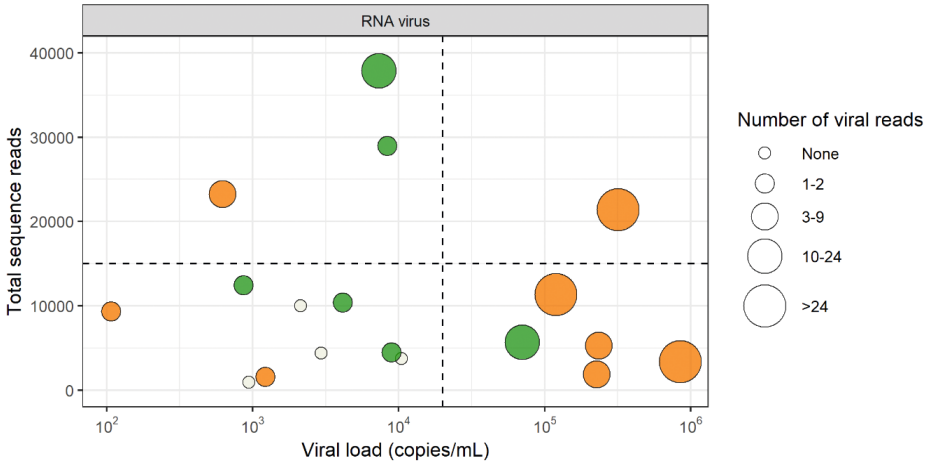
RNA Virus Detection by VIDISCA-NGS

Six samples were positive for enterovirus and eight for HIV-1 by VIDISCA-NGS, all of which were also qPCR positive (Figure 1). The RNA virus concentration in the VIDISCA-NGS positive samples ranged between 1.07×10^2 RNA copies/mL and 8.64×10^5 RNA copies/mL (median: 8.63×10^3 RNA copies/mL). Two samples positive for enterovirus and two for HIV-1 by qPCR were missed by VIDISCA-NGS, with viral loads ranging from 9.40 × 102 to 1.05×10^4 RNA copies/mL (median 2.54×10^3 RNA copies/mL).

To exclude that competition by background nucleic acids or other viruses might have hampered virus detection, we assessed whether co-infection by other pathogens or large quantities of the host genomic background had competed with viral sequences in the four samples that were negative in VIDISCA-NGS. The profile of the background sequences of the negative samples was similar to those of the positive samples, indicating that no major sequence competition was present (Figure 2). Next, we determined whether the sequencing depth of the four negative samples, in combination with the low viral load, may have been insufficient. All four missed samples had fewer than 10,000 sequence reads and had a viral load below 2×10^4 copies/mL, as depicted in the lower left quadrant of Figure 1. Overall, this quadrant contained nine samples of which five were positive and four were negative by VIDISCA-NGS. The five positive samples had only one (n = 4) or two (n = 1) reads mapped to the detected RNA virus. These small numbers of viral reads suggest that such samples (with low viral load, combined with a low sequencing depth) were on the detection limit of VIDISCA-NGS. Samples with a similarly low viral load, but with a higher sequence depth (upper left quadrant of Figure 1), had, on average, more than 5 viral reads per sample. Moreover, a correlation between sequence depth and viral

read number was seen for all samples below 10^4 RNA copies/mL (rho = 0.64 p = 0.02, Spearman's rank correlation test).

Figure 1. Detection of RNA viruses by virus discovery cDNA-AFLP (amplified fragment length polymorphism) next-generation sequencing (VIDISCA-NGS) in cerebrospinal fluid (CSF). Green dots: samples that were positive by VIDISCA-NGS for enterovirus, orange dots: samples that were positive by VIDISCA-NGS for HIV-1, white dots: samples that were negative by VIDISCA-NGS. The size of the dots corresponds to the number of viral reads. On the x-axis, the viral load in CSF is displayed; on the y-axis, the total number of sequence reads. Samples are divided into segments by a horizontal line at 15,000 reads and a vertical line at 2×10^4 RNA copies/mL



DNA Virus Detection by VIDISCA-NGS

Only one sample was VIDISCA-NGS positive for a herpesvirus (VZV), which was also qPCR positive at a concentration of 9.29×10^7 DNA copies/mL. Among the samples that remained herpesvirus negative by VIDISCA-NGS, 33 were positive for at least one herpesvirus by qPCR (median: 9.01×10^3, range: 5.28×10^3–1.62×10^7 DNA copies/mL). Because of the poor performance of VIDISCA-NGS, we hypothesized that our library preparation method, which uses a specific restriction enzyme, may have hampered herpesvirus detection. We examined the number of putative VIDISCA-NGS fragments (the number of unique genomic fragments that can theoretically be detected by VIDISCA-NGS based on the location of the Mse1 restriction enzyme recognition sites and resulting fragments lengths) in the human herpesvirus genomes. All human herpesviruses genomes have at least 16 putative VIDISCA fragments (Table 1). By comparison, the enterovirus and HIV-1 genomes produced a nearly equal number of fragments and were detected at a high success rate as described above.

Figure 2. Background sequences in VIDISCA-NGS. Green dots: samples that were positive by VIDISCA-NGS, white dots: samples that were negative by VIDISCA-NGS, orange dots: the four samples containing an RNA virus not found by VIDISCA-NGS. On top the p-values are shown for the Mann-Whitney U test between the positive and negative VIDISCA-NGS samples. "Human" indicates human mitochondrial or genomic background, "Bacterial" indicates prokaryotic background, "Ambiguous" represents sequences with simultaneous hits to eukaryotes and prokaryotes, and "Unknown" are the sequences that do not match with any reference sequence

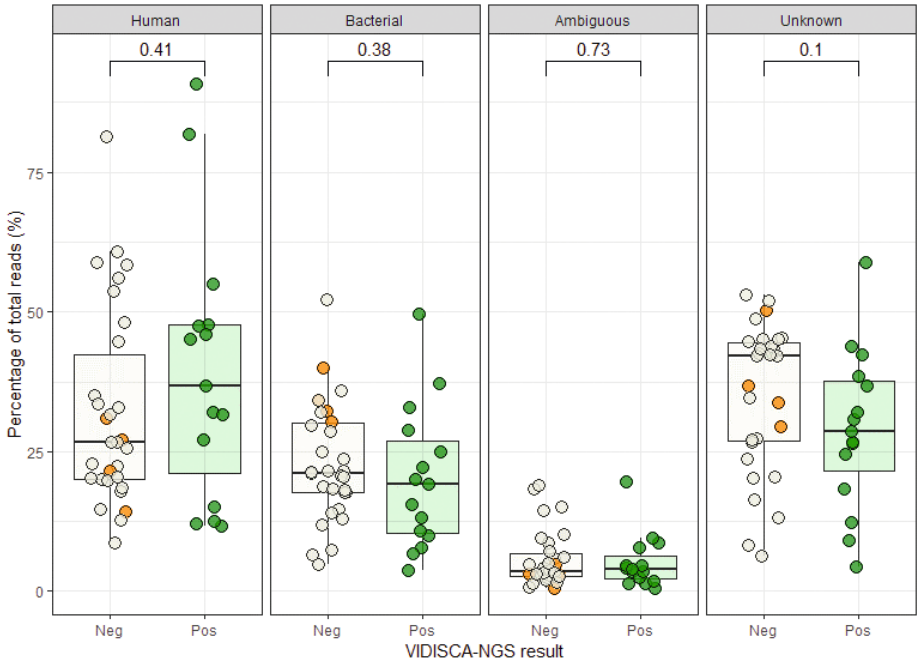


Table 1. Putative number of VIDISCA fragments per virus

Virus	Fragments (n)
HSV-1	40 [1]
HSV-2	16
VZV	352
EBV	129
CMV	137
HHV-7	473
Enterovirus	22
HIV-1	19

[1]Number of putative VIDISCA fragments as determined by the number of genomic regions demarcated by two MseI restriction enzyme recognition sites with a length of 100 to 400 nucleotides. HSV-1: herpes simplex virus 1, HSV-2: herpes simplex virus 2, VZV: varicella-zoster virus, EBV: Epstein-Barr virus, CMV: cytomegalovirus, HHV-7: human herpes virus 7, HIV-1: human immunodeficiency virus 1.

Next, we hypothesized that the nuclease treatment may have hampered the detection of herpesvirus DNA. DNase treatment is done prior to nucleic acid extraction to remove naked chromosomal and bacterial DNA. It is assumed that viral genomic DNA is protected from DNase by the virus particle, however, if viral DNA is non-encapsidated, it will also be degraded. We therefore repeated the library preparation for all 45 CSF samples, now without a DNase treatment.

Virus Detection by DNase-Free VIDISCA-NGS

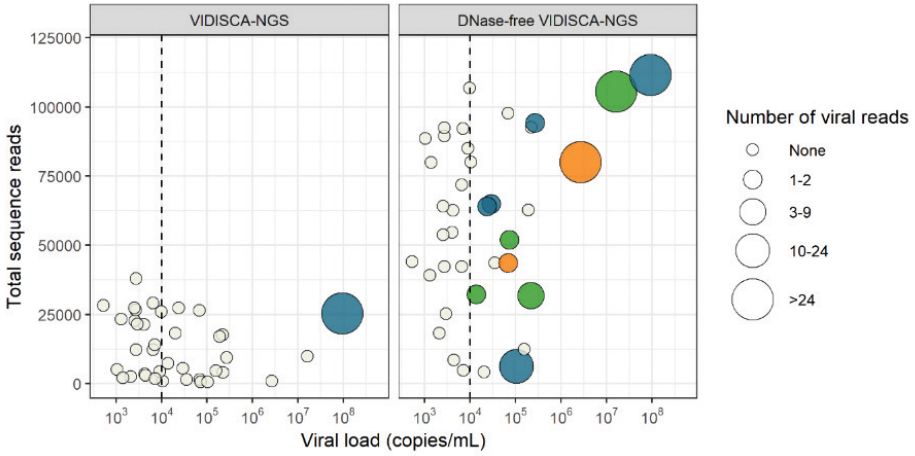
With the DNase-free VIDISCA-NGS, only eight samples contained sequences of an RNA virus (six HIV-1 and two enterovirus) (Table 2), indicating that background DNA seriously hampered detection of RNA viruses. On the other hand, detection of herpesviruses greatly increased. Without a DNase treatment, 11 samples became VIDISCA-NGS positive: four for HSV-1/2, five for VZV, and two for CMV (Figure 3). The viral load of the nuclease-free VIDISCA-NGS herpesvirus positive samples was higher (median: 1.04×10^5) than the negative samples (median: 4.42×10^3, $p < 0.001$, Mann Whitney U test). This association between the virus load and VIDISCA-detection became more visible when 10^4 DNA copies/mL was taken as a threshold; 11 of 18 samples positive by qPCR with $>10^4$ DNA copies/mL were also positive by VIDISCA-NGS, but none below.

Table 2. Performance of VIDISCA-NGS to detect viruses compared to quantitative polymerase chain reaction (qPCR) in CSF

Virus	VIDISCA-NGS	DNase-free VIDISCA-NGS
RNA virus		
Enterovirus	6/8 [1]	2/8
HIV-1	8/10	6/10
Total	14/18	8/18
Herpesvirus		
HSV-1/2	0/14	4/14
VZV	1/8	5/8
EBV	0/12	0/12
CMV	0/2	2/2
HHV-7	0/2	0/2
Total	1/38	11/38

[1] Results shown as: VIDISCA-NGS positives samples / qPCR positive samples.

Figure 3. Detection of herpesviruses by VIDISCA-NGS in CSF. The results of regular VIDISCA-NGS are in the left panel, results of DNase-free VIDICSA-NGS are in the right panel. If a sample contained multiple viruses, multiple data points are displayed for each of the co-infecting viruses. A vertical line is drawn to separate samples above and below 10^4 DNA copies/mL. Green dots: samples that were positive by VIDISCA-NGS for HSV-1/2, blue dots: samples that were positive by VIDISCA-NGS for VZV, orange dots: samples that were positive by VIDISCA-NGS for CMV, white dots: samples that were negative by VIDISCA-NGS. The size of the dots corresponds to the number of viral reads. On the x-axis, the viral load in CSF is displayed; on the y-axis, the total number of sequence reads



Effect of a DNase Treatment on Virus Detection by VIDISCA-NGS

We identified several co-infecting DNA viruses (torque teno virus (TTV), n = 5; human papillomavirus (HPVs), n = 5; and hepatitis B virus (HBV), n = 1), which were not included in the routine diagnostics of the CSF samples, but were identified by VIDISCA-NGS (n = 11). Similar to the effects we observed for herpesvirus detection, we hypothesized that more non-herpes DNA viruses would be detected under the DNase-free condition. Surprisingly, no additional non-herpes DNA viruses were identified using the DNase-free method. On the contrary, of the 11 samples containing non-herpes DNA viruses detected by regular VIDISCA-NGS, only four samples were positive when excluding a DNase treatment (Figure 4).

To assess the overall effect of a DNase treatment, we determined the ratio of viral reads, adjusted for sequencing depth, between the two treatment arms for all viruses identified by VIDISCA-NGS in this study (Figure 5). All herpesviruses had substantially more, or a roughly equal number of viral reads in the DNase-free condition. In contrast, the opposite was true for non-herpes DNA and RNA viruses.

Figure 4. Effect of DNase on the detection of non-herpes DNA viruses by VIDISCA-NGS. On the x-axis, the viral species is displayed; on the y-axis, the total number of sequence reads. Left panel: Normal VIDISCA-NGS, right panel: DNase-free VIDSCA-NGS. Green dots: samples positive for the indicated virus, white dots: samples negative for the indicated virus. The size of the dots corresponds to the number of viral reads

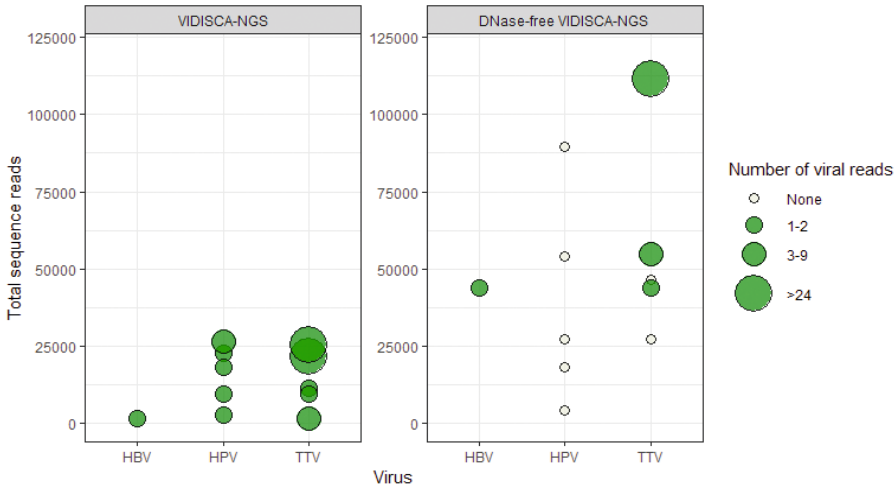
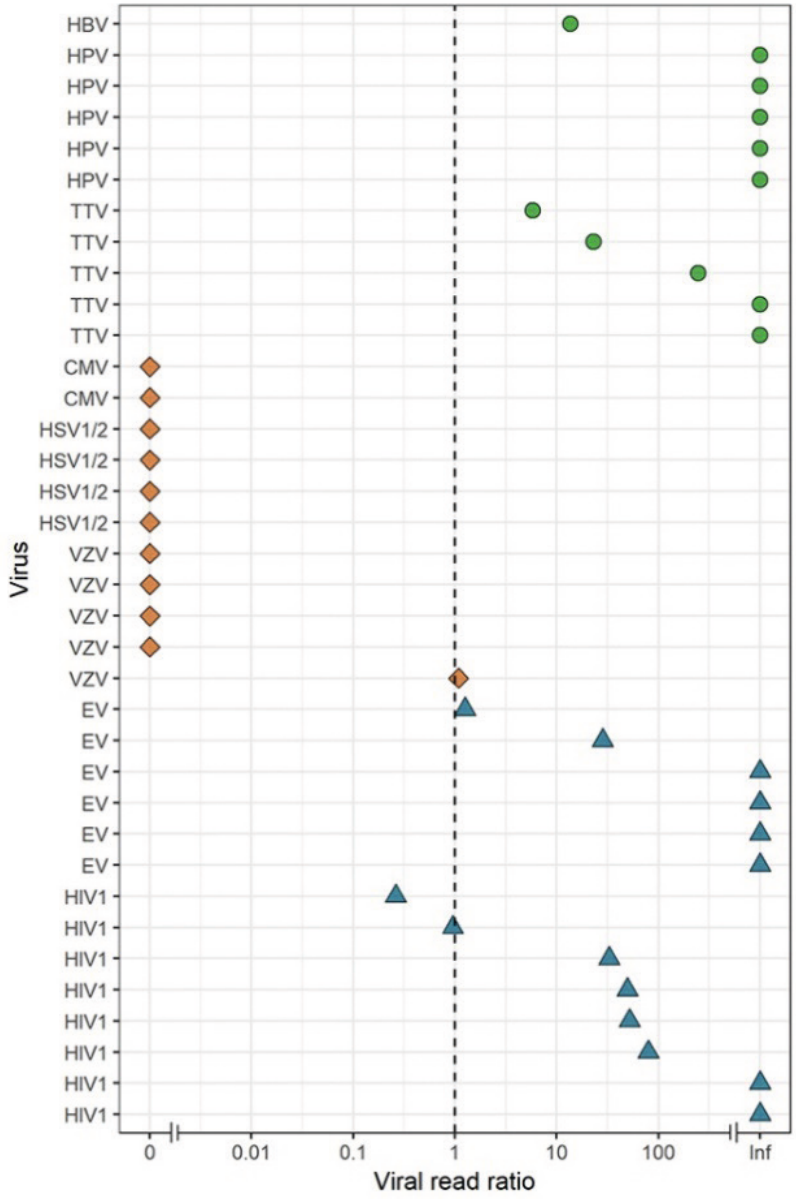


Figure 5. Effect of DNase on the detection of RNA and DNA viruses by VIDISCA-NGS in CSF. Viral read ratio (x-axis) is calculated as the ratio between the number of viral reads for samples with and without a DNase treatment, adjusted for the sequencing depth. Samples with a ratio >1 favour regular library preparation whereas samples with a ratio <1 favour a DNase-free treatment. Green dots: non-herpes DNA viruses, orange diamonds: herpesviruses, blue triangles: RNA viruses. On the y-axis, the viral species are displayed



DISCUSSION

Metagenomic assays have the potential to benefit the diagnosis of CNS-infections. To this end, they need to meet certain prerequisites: Besides being broad—preferably detecting all viruses—an assay should be fast, sensitive, and affordable. VIDISCA-NGS is a unique method for viral metagenomics, which requires a relatively limited sequence depth and allows multiplexing, which reduces costs and runtime per sample.[23] As limited sequence depth, multiplexing, and speed may come at the expense of sensitivity, we evaluated the performance of VIDISCA-NGS on 45 clinical CSF samples containing viruses, quantified via conventional diagnostics (qPCR). VIDISCA-NGS detected an RNA virus in all medium to high viral load samples (>2 × 10^4 RNA copies/mL) and most (67%) of the low viral load samples. One VIDICSA-NGS positive HIV-1 sample had only 1.07 × 10^2 RNA copies/mL, demonstrating the capability to detect even very low load viruses.

Metagenomics has been used to detect novel or unexpected viruses in CSF in several studies[7,8,9,10], but only a limited number of studies have evaluated the performance. Two studies investigated the limit of detection using dilutions of spiked HIV-1 in CSF. One study used the Ribo-SPIA pipeline[25], the second used a tailor-made protocol, including Nextera, to fragment and amplify.[26,27] Both studies used >5 million reads per sample and found a limit of detection of ≈10^2 RNA copies/mL for HIV-1, comparable to that of VIDISCA-NGS when 10,000 reads are used.

Besides the pathogens detected in the current study, VIDISCA-NGS has been able to detect a large number of other viruses, including members of the *Adenoviridae*, *Anelloviridae*, *Caliciviridae*, *Coronaviridae*, *Flaviviridae*, *Hepadnaviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Parvoviridae*, *Peribunyaviridae*, *Picornaviridae*, *Pneumoviridae*, *Polyomaviridae*, and *Retroviridae*, in several types of clinical material (stool, serum, plasma, respiratory swabs).[14,15,16,17,18,28,29,30,31,32] Thus, it is likely that VIDISCA-NGS is able to detect viruses from these families in CSF with similar sensitivities. However, our current findings now indicate there is one viral family difficult to detect with VIDISCA-NGS, namely the Herpesviridae. VIDISCA-NGS was only able to detect one high load herpesvirus (VZV, 9.29 × 10^7 DNA copies/mL) out of 34 qPCR positive samples. We hypothesized that our nuclease treatment hindered herpesvirus detection, and omitting a DNase treatment indeed yielded an additional 10 samples that were positive for herpesvirus. Of the medium to high load herpesviruses (>10^4 RNA copies/mL), DNase-free VIDISCA-NGS detected 61%.

The vulnerability of herpesviruses to DNase is not unexpected. Boom et al. found that CMV DNA in serum and plasma is highly fragmented and susceptible to DNases.[33]

Similarly, Perlejewski *et al*. described a four-fold decrease in HSV-1 reads when using a DNase treatment for metagenomics on CSF.[34] Our study expands on this knowledge by showing that the vulnerability to DNase also applies to the other herpesviruses. This vulnerability signifies that the performance of metagenomic assays should not be evaluated on spiked samples. Herpesvirus culture harvests contain infectious virions with non-fragmented DNA[33,35], whereas herpesvirus in cell-free clinical material is non-infectious and, as mentioned above, contains highly fragmented DNA.[33,36] The only two studies that examined the performance of a metagenomics assay to detect herpesviruses used virus culture harvests, and found low limits of detection (≈10^1 and 10^3 DNA copies/mL for CMV and HSV-1, respectively).[25,26] Caution should be taken to translate these findings to a clinical setting, as virus culture harvests are, especially for herpesviruses, not a correct representative of reality.

Herpesviruses have large DNA genomes and use rolling-circle amplification to produce head-to-tail concatemers of progeny virus.[37] During the lytic replication phase, large amounts of non-infective naked progeny virus are released from the cell and may enter the CSF if replication occurs in the CNS compartment. Because of the high genome copy number and the generally low DNase activity in CSF[38], degradation may take a significant amount of time. Naked herpesvirus DNA could thus persist for an extensive amount of time in CSF, even after the local infection has ceased. In theory, the persistence of naked DNA could also occur for other DNA viruses, such as HPV and TTV. These viruses use similar replication strategies to herpesviruses. The detection of these DNA viruses by VIDISCA-NGS was, however, not hampered by a DNase treatment (Figure 4), indicating that the viral DNA of these viruses was part of an intact virion.

Without amplification, the nucleic acid yield from CSF is generally too low for effective NGS library preparation for metagenomics.[39] For that reason, VIDISCA-NGS implements an amplification step to increase the number of viral genomic fragments from CSF. We previously found that viruses with a concentration of >10^4 copies/mL were detected when 5,000 sequence reads or more were generated per sample from nasopharyngeal swabs.[17] Since then, we have used this number as a threshold to ensure that a sufficient sequence depth was achieved for virus detection. Our current results suggest this threshold may have to be increased for CSF. All RNA virus samples missed by VIDISCA-NGS had fewer than 10,000 reads and a strong correlation between the sequencing depth and number of viral reads was observed. Increasing the sequence depth could therefore increase the detection of low load RNA viruses. As such, we recommend generating 10,000 or more reads per sample.

In the current study, we multiplexed 70 samples per VIDISCA-run. While it is uncommon for a large number of patients with encephalitis to present at the same time, this method could be of substantial benefit in outbreaks[40] and research settings where large cohorts of patients have to be screened at the same time. Because the performance of VIDISCA-NGS remains lower than qPCR, especially for the detection of herpesviruses, VIDISCA-NGS cannot replace conventional diagnostics. Nonetheless, we suggest the use of standard VIDISCA-NGS (including a DNase) in parallel with conventional diagnostics, as this provides a cheap, low-input, and sensitive method to detect known, rare, and novel viruses in CSF.

Author Contributions

Conceptualization, A.W.D.E., M.D., I.E.v.Z., D.v.d.B., M.C.B., L.v.d.H.; methodology, A.W.D.E., M.D., C.M.K. and L.v.d.H.; investigation, A.W.D.E., M.D., M.F.J., C.M.K. and L.v.d.H.; resources, I.E.v.Z., M.B., D.v.d.B. and M.C.B.; data curation, A.W.D.E., M.D., C.M.K. and I.E.v.Z.; writing—original draft preparation, A.W.D.E. and L.v.d.H.; writing—review and editing, M.D., C.M.K., I.E.v.Z., D.v.d.B. and M.C.B.

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Conflicts of Interest

The authors declare no conflict of interest.

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SUPPLEMENTARY TABLE

Table S1 – VIDISCA-NGS and qPCR data for all samples

Sample	Virus	qPCR result	Viral load (copies/mL)	Total seq reads[1]	DNase-free tot seq reads[2]	Viral reads[3]	DNase-free vir reads[4]	Human reads	Bacterial reads	Ambiguous reads	Unknown reads
1	HSV1/2	Pos	6520	12361	42257	0	0	1569	3526	1085	5371
1	HHV7	Pos	2740	12361	42257	0	0	1569	3526	1085	5371
2	HSV2	Pos	13800	7289	32308	0	1	1300	1375	1340	3199
3	VZV	Pos	268000	9442	94294	0	2	2513	1713	571	4607
4	HSV1	Pos	218000	17609	31925	0	7	5927	3785	542	6080
5	EV	Pos	4120	10408	84357	1	0	8512	704	192	955
6	VZV	Pos	528	28311	44136	0	0	17200	1837	1699	3741
7	HIV1	Pos	317895	21434	54646	77	6	10199	2158	1665	9075
7	EBV	Pos	4070	21434	54646	0	0	10199	2158	1665	9075
7	TTV	NT[5]		21434	54646	27	3	10199	2158	1665	9075
8	EBV	Pos	2570	22750	53860	0	0	1994	11879	568	6079
8	HPV	NT		22750	53860	1	0	1994	11879	568	6079
9	EBV	Pos	1050	5162	88644	0	0	3012	1086	142	844
10	HSV1/2	Pos	20300	18198	4285	0	0	3623	5393	604	8270
10	HPV	NT		18198	4285	1	0	3623	5393	604	8270
11	EBV	Pos	68300	26482	97739	0	0	15581	1253	5032	2181
12	HIV1	Pos	119445	11371	46613	39	2	3653	2535	532	4372
12	TTV	NT		11371	46613	2	0	3653	2535	532	4372
13	EV	Pos	862	12469	37485	1	0	6870	1927	165	3290
14	EBV	Pos	2710	26556	89554	0	0	5331	4695	1300	11296
14	HPV	NT		26556	89554	3	0	5331	4695	1300	11296
15	HIV1	Pos	1220	1593	43667	1	0	759	124	22	489
15	HPB	NT		1593	43667	1	2	759	124	22	489
15	CMV	Pos	69000	1593	43667	0	2	759	124	22	489
15	TTV	NT		1593	43667	9	1	759	124	22	489
15	EBV	Pos	35200	1593	43667	0	0	759	124	22	489
16	HSV1/2	Pos	9870	26014	106809	0	0	6925	3073	3742	10960
17	EBV	Pos	2110	2577	18265	0	0	579	643	110	1155
17	HBV	NT		2577	18265	1	0	579	643	110	1155
18	HSV1/2	Pos	224000	4051	92601	0	0	925	740	200	2149
19	HSV1	Pos	73000	666	52047	0	1	136	138	22	301
20	VZV	Pos	29300	5549	65009	0	1	1750	978	395	2335
21	HIV1	Pos	846080	3409	62677	38	14	395	852	85	2010
21	EBV	Pos	4280	3409	62677	0	0	395	852	85	2010
22	HIV1	Pos	107	9343	27134	1	0	2532	1797	806	4101
22	HPV	NT		9343	27134	2	0	2532	1797	806	4101
22	TTV	NT		9343	27134	2	0	2532	1797	806	4101
23	VZV	Pos	104000	680	6343	0	23	126	95	103	354
24	HSV1/2	Pos	190000	17045	62707	0	0	5605	3648	446	7233
25	HIV1	Pos	234220	5280	34480	8	1	2382	1061	205	1513
26	HSV1	Pos	16200000	9925	105692	0	44	2552	2030	937	4312
27	VZV	Pos	7000	14016	92184	0	0	7871	1821	1414	2842
28	HSV1/2	Pos	1410	2067	79948	0	0	924	489	58	563
29	HSV1/2	Pos	2940	21485	25351	0	0	3149	7710	599	9150
30	HIV1	Pos	620	23269	39097	7	45	21117	887	127	1047
30	EBV	Pos	1300	23269	39097	0	0	21117	887	127	1047
31	VZV	Pos	23700	27314	64126	0	1	22213	2029	221	1715
31	EBV	Pos	2540	27314	64126	0	0	22213	2029	221	1715
32	CMV	Pos	2670000	957	80067	0	146	136	290	30	482
32	EBV	Pos	10500	957	80067	0	0	136	290	30	482
32	HIV1	Pos	940	957	80067	0	0	136	290	30	482
33	VZV	Pos	9050	4388	85014	0	0	2357	944	162	898
34	HIV1	Pos	10485	3765	43946	0	0	1164	1284	22	1270
35	HSV2	Pos	157000	4693	12485	0	0	1641	1604	76	1274
36	HSV2	Pos	4420	3008	8618	0	0	612	966	59	1357
37	EV	Pos	70104	5714	103412	22	14	689	2841	264	1838
38	HIV1	Pos	225930	1869	4777	6	16	235	694	77	688
38	EBV	Pos	7190	1869	4777	0	0	235	694	77	688
39	VZV	Pos	92900000	25495	111578	25	101	8072	3344	5024	4673
39	TTV	NT		25495	111578	44	33	8072	3344	5024	4673
40	EV	Pos	2129	9982	61362	0	0	2708	3997	298	2943
41	HSV1/2	Pos	6520	29228	71830	0	0	14096	4312	435	6948
42	EV	Pos	2953	4386	7949	0	0	945	1413	207	1609
43	EV	Pos	8328	28999	97887	1	0	13367	3130	2765	7135
44	EV	Pos	7298	37912	92529	12	0	5761	10937	1303	4678
44	HHV7	Pos	2740	37912	92529	12	0	5761	10937	1303	4678
45	EV	Pos	8924	4518	14263	2	5	1662	1484	159	1205

[1]Total sequence reads per sample for VIDISCA-NGS
[2]Total sequence reads per sample for DNase-free VIDISCA-NGS
[3]Number of detected viral reads per virus by VIDISCA-NGS
[4]Number of detected viral reads per virus by DNase-free VIDISCA-NGS

CHAPTER 6

DIAGNOSTIC ACCURACY OF VIDISCA-NGS IN PATIENTS WITH SUSPECTED CENTRAL NERVOUS SYSTEM INFECTIONS

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ABSTRACT

Objectives

Confirming the diagnosis in viral central nervous system (CNS) infections can be difficult with current available diagnostic tools. Virus discovery cDNA-AFLP next generation sequencing (VIDISCA-NGS) is a promising viral metagenomic technique, which enables detection of all viruses in a single assay. We performed a retrospective study on diagnostic accuracy of VIDISCA-NGS in cerebrospinal fluid (CSF) of patients with suspected CNS infections.

Methods

Consecutive adult patients presenting to the Emergency Department or inpatients, who underwent a lumbar puncture for the suspicion of a CNS infection, were included if 1) they were diagnosed with a viral CNS infection, or 2) a viral CNS infection was initially suspected but eventually a different diagnosis was made. A qPCR panel of the most common causative viruses was performed on CSF of these patients as reference standard and compared to the results of VIDISCA-NGS, the index test.

Results

We included 38 patients with viral CNS infections and 35 presenting with suspected CNS infection for whom an alternative aetiology was finally established. Overall sensitivity and specificity was 52% (95% CI 31-73%) and 100% (95% CI 91-100%), respectively. One enterovirus, detected by VIDISCA-NGS, was only identified by qPCR upon retesting. Additional viruses identified by VIDISCA-NGS consisted of GB virus C, human papilloma virus, human mastadenovirus C, Merkel cell polyoma virus and anelloviruses.

Conclusion

In patients for whom routine diagnostics do not yield a causative pathogen, VIDISCA-NGS can be of additional value as it can detect a broader range of viruses, but it does not perform well enough to replace qPCR.

INTRODUCTION

Viral central nervous system (CNS) infections have been associated with substantial morbidity and mortality depending on the causative pathogen.[1] Confirming the diagnosis can be difficult in patients suspected of a CNS infection because the differential diagnosis is broad. A substantial proportion of patients who are initially suspected of a CNS infection turns out to have a different diagnosis, including inflammatory diseases, epilepsy or stroke.[2] In patients with a clinical diagnosis of a viral CNS infection, no causative virus can be identified in 35- 42%.[1-3] As part of routine diagnostics for CNS infections, a selection of viruses is tested using quantitative PCR (qPCR), currently the gold standard for the majority of the most common viruses causing CNS infection.[4] The limitation of common diagnostic qPCR techniques is that they only target specific viruses and therefore other viruses are missed. Therefore, alternative diagnostic assays, which allow detection of a broader range of viruses, are desirable, yet currently not routinely available.[4]

Viral metagenomics has emerged as promising method to detect viruses hypothesis-free.[5] In theory, it should be able to detect all viruses, including unknown viruses, also in samples with low viral loads.[6] Virus discovery cDNA-amplified fragment length polymorphism (cDNA-AFLP) next generation sequencing (VIDISCA-NGS) is one of these methods. VIDISCA-NGS uses restriction enzyme digestion to generate the library for NGS, which has the advantage that a relatively low sequence depth is needed and thereby increases the number of samples that can be processed and drastically reduces costs and runtime per sample.[7,8] Multiple viruses have been discovered with VIDISCA-NGS, including one in cerebrospinal fluid (CSF).[8-11] We have previously optimised this method for CSF and analysed its performance from a viral perspective on a selection of CSF samples with a known viral load.[6] In the current study we examined the diagnostic accuracy of VIDISCA-NGS on a cohort of patients with suspected CNS infections.[2]

METHODS

Patients

To assess the diagnostic accuracy of VIDISCA-NGS, the index test, we selected patients from our previously described cohort[2]. Between 2012-2015, all consecutive, adult (≥16 years) inpatients or patients presenting to the emergency department of an academic hospital in Amsterdam, The Netherlands, were prospectively included in this cohort if they underwent a lumbar puncture for suspicion of a CNS infection. Patients were identified during morning rounds and through an overview of CSF samples received by

the laboratory. Clinical data and CSF were collected. Patients in this cohort were divided into five diagnosis categories (Figure 1), classified independently by two neurologists. Disagreements between these neurologists were resolved by discussion with a third (kappa 0.76). Episodes were classified as CNS infection if there was microbiological evidence of infection or when the neurologists independently classified the episodes as being due to bacterial, viral or other CNS infection based on all available clinical parameters.

For the current study we selected two groups of patients from this cohort. The first group included all consecutive patients with a final diagnosis of viral CNS infections as determined by the neurologists, to test the sensitivity of VIDISCA-NGS. The second group was a convenience series of patients to evaluate the specificity, in whom doubt about the final diagnosis was present for ≥2 days after lumbar puncture, but eventually a different diagnosis than CNS infection was made. This was retrospectively checked in the patient files. The reason for this was that, even though a different diagnosis was made, an undetected virus still could have played a role in their disease. We used CSF, obtained during the first lumbar puncture performed at presentation or during admission, which was frozen after the performance of regular diagnostics. As reference standard, the routine panel of the most common causative viruses was tested on the CSF for all patients by qPCR, including herpes simplex virus (HSV-1 and 2), cytomegalovirus (CMV), Epstein Barr virus (EBV), varicella zoster virus (VZV), enteroviruses and parechoviruses. In some of the patients, CSF was tested for human herpes virus (HHV) 6 and 7, John Cunningham virus (JCV) or human immunodeficiency virus (HIV-1) during regular diagnostics, if an indication existed. Subsequently, all the same CSF specimens were tested using VIDISCA-NGS. The investigators who performed the VIDISCA-NGS (AE, MD, LH) were blinded to clinical data and PCR results.

qPCR

100 µl CSF was used for nucleic acid extraction by automated extraction (MagnaPure, Roche Diagnostics). Real time PCR was performed as an internally controlled multiplex PCR for HSV-1 and 2, VZV, EBV, CMV, JCV and HHV-6 and 7. Reverse transcription using random hexamers was performed and 5 µl of reverse transcription reaction was subsequently used to detect enterovirus and human parechovirus by real time PCR.[12] Isolation, amplification and detection of HIV-1 in CSF was done by Abbot Real Time PCR (M2000, Abbott Diagnostics, Hoofddorp, NL).

VIDISCA-NGS

VIDISCA library preparation to detect RNA and DNA viruses was performed as previously described.[6,13] In brief, 110 µL of CSF was centrifuged and the supernatant was treated

with TURBO™ DNase (Thermo Fisher Scientific). Extraction of nucleic acids was done using Boom's method[14], followed by reverse transcription with non-ribosomal random hexanucleotides[15] and synthesis of second strands. DNA was digested with MseI (TˆTAA; New England Biolabs) and ligated to adapters containing a sample identifier sequence. Next, DNA-strands with a length of ~100-400 nucleotides were selected with AMPure XP beads (Agencourt), followed by a 28-cycle PCR using adaptor-annealing primers. Libraries were analysed using the Bioanalyzer (High Sensitivity Kit, Agilent Genomics) and Qubit (dsDNA HS Assay Kit, Thermo Fisher Scientific) kits to assess DNA length and concentration. Seventy sample libraries were pooled at equimolar concentration. Fifty pM DNA of the pool was clonally amplified on beads with the Ion Chef System (Thermo Fisher Scientific), and sequencing was performed on the Ion PGM™ System (Thermo Fisher Scientific), using the ION 316 Chip (400 bp lengths and 2 million sequences per run).

All reads from VIDISCA-NGS were translated into sequences of proteins and aligned to a local database of the NCBI eukaryotic viral Identical Protein Groups (downloaded March 2018) using UBLAST[16], the VIDISCA bioinformatics workflow[7], and an online metagenomic profiler (Taxonomer)[17] for identification of viral reads and background sequence classification.

We considered a sample VIDISCA-NGS-positive when at least one VIDISCA-read could be aligned to a reference sequence of a specific virus strain using CodonCode Aligner (version 6.0.2) with a nucleotide identity of at least 80%.

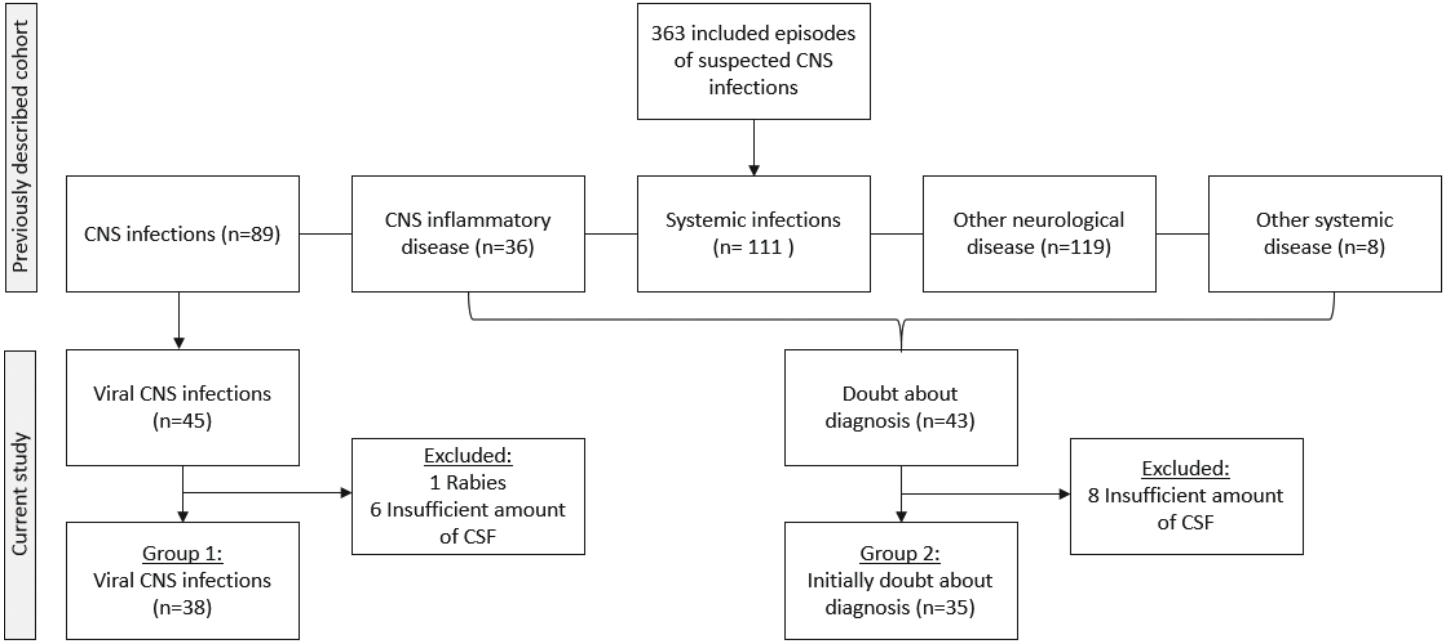
Statistical analysis

Diagnostic accuracy of the index test (VIDISCA-NGS) was evaluated by calculating sensitivity and specificity. We used both the reference test (qPCR) and clinical diagnosis as gold standard and calculated the sensitivity and specificity for both of them with 95% confidence intervals (CI). We had no missing data on the index test and reference standard. As no prior data of the test characteristic of the index tests was available, no power calculation could be performed and the study is considered an exploratory diagnostic accuracy study.

Ethics

This study was approved by the Biobank Ethical Review Committee of the Amsterdam UMC (number METC 2014_290).

Figure 1. Included patients



RESULTS

Of the 363 episodes included in the cohort, (Figure 1), 45 were clinically diagnosed as viral CNS infection. One patient with rabies was excluded from VIDISCA-NGS analysis for safety reasons. For six of the patients an insufficient amount of CSF was available for this analysis, leaving 38 patients with viral CNS infections. We identified 43 patients in whom doubt about the diagnosis was present for ≥2 days, but eventually a different diagnosis was made. A sufficient amount of leftover CSF was available for 35 patients.

The median age in patients with viral CNS infections was 40 years (IQR 30-56; Table 1). Viral meningitis was diagnosed in 27 patients, encephalitis in 8 and meningo-encephalitis in 1 patient. Two patients were diagnosed with progressive multifocal leukoencephalopathy. Acyclovir treatment was started in 18 patients (47%).

Table 1. Patient characteristics

Clinical characteristics at presentation	Viral CNS infection (n= 38)	Other diagnosis (n=35)
Age (years)	40 (IQR 30-56)	51 (IQR 38-75)
Male	21 (55%)	15 (43%)
Immunocompromised	16 (42%)	9 (26%)
Headache	29 (76%)	20/34 (59%)
Seizures	4 (11%)	8/34 (24%)
Temperature (Celsius)	37.8 (IQR 37.1-38.7)	37.0 (IQR 36.5-37.9)
Fever	19 (50%)	7/34 (21%)
Altered mental status (GCS <14)	6 (16%)	12 (34%)
Focal neurological abnormalities	8 (21%)	11 (31%)
Laboratory findings at presentation		
Blood leucocyte count (10^9/L)	8.7 (IQR 6.1-11.3)	9.8 (IQR 6.9-11.1)
Blood C-reactive protein (mg/L)	13 (IQR 3-36)	8 (IQR 2-26)
CSF opening pressure (cm H_2O)	25 (IQR 15-31)	25 (IQR 19-34)
CSF leucocyte count (10^6/L)	94 (IQR 14-224)	10 (IQR 3-38)
CSF protein count (g/L)	0.6 (IQR 0.37-0.78)	0.52 (IQR 0.33-0.84)
Admission		
Admitted to hospital	37 (97%)	34 (97%)
Lumbar puncture repeated	5 (13%)	16 (46%)
Duration of admission (days)	7 (IQR 2-13)	10 (IQR 2-29)
Treatment		
Antibiotic treatment	20 (53%)	15 (43%)
Acyclovir treatment	18 (47%)	13 (37%)
Outcome		
Unfavourable outcome at discharge (GOS of 1-4)	8 (21%)	14 (40%)
Dead at discharge (GOS = 1)	3 (8%)	2 (6%)

CNS= central nervous system, GCS= Glasgow Coma Scale score, GOS= Glasgow Outcome Scale score

The reference test – qPCR – identified 36 viruses in 23 patients (61%) with viral CNS infections. In the 15 remaining patients diagnosis was made based on clinical parameters. Viruses identified by qPCR included EBV (n=9), HSV (n=7), VZV (n=5), enteroviruses (n=6), HIV-1/2 (n=5), JCV (n=2), CMV (n=1), and human herpesvirus 7 (HHV-7; n=1). In two patients with EBV in their CSF, EBV was considered the cause of disease. In seven cases it was considered an incidental detection. Multiple viruses per patient were identified mainly in patients with HIV-infections. VIDISCA-NGS, the index test, was able to detect viruses in 12 patients (32%), of which 8 RNA viruses and 4 DNA viruses (Table 2). Additionally, VIDISCA-NGS showed human papilloma virus (HPV) and an anellovirus in one sample each (Table 3). One enterovirus identified by VIDISCA-NGS was initially not identified by qPCR. Following detection of the enterovirus by VIDISCA-NGS qPCR was repeated, which upon retesting was positive for enterovirus.

Table 2. Viruses identified during routine diagnostic work-up, sensitivity and specificity

	qPCR	VIDISCA-NGS	Sensitivity (95% CI)	Specificity (95% CI)
Positive[a]	23	12	52% (31-73%)	100% (91-100%)
EBV	9	0	0% (0-37%)	100% (93-100%)
HSV	7	0	0% (0-44%)	100% (93-100%)
Enterovirus	6[b]	4	67% (24-94%)	100% (93-100%)
VZV	5	2	40% (7-83%)	100% (93-100%)
HIV-1/2	5	4[c]	40% (7-83%)	NA[d]
JCV	2	1	50% (3-97%)	100% (68-100%)
CMV	1	1	100% (5-100%)	100% (94-100%)
HHV7	1	0	0% (0-95%)	100% (60-100%)

[a] Number of patients with one or more positive results for the viruses from the diagnostic qPCR panel
[b] First qPCR negative for one CSF sample, test repeated after positive VIDSCA-NGS result
[c] One CSF sample was positive for HIV-1 and one for HIV-2 using VIDISCA-NGS, but were not tested for HIV by qPCR
[d] No qPCR for HIV was performed with negative test result

In the second group, final diagnoses consisted of epilepsy (n=9), neurosarcoidosis (n=2), inflammatory (n=6) or paraneoplastic encephalitis (n=1), recurrent aseptic meningitis (n=5) and chronic meningitis (n=5). In this group no viruses were identified by qPCR. VIDISCA-NGS did detect a number of additional viruses (Table 3). The initial suspicion of viral CNS infections was high enough to start treatment with acyclovir in 13 patients (37%).

The overall sensitivity and specificity of VIDISCA-NGS for detecting a virus that is commonly tested by qPCR were 52% (95% CI 31-73%) and 100% (95% CI 91-100%), respectively, but differed per virus (Table 2). When using the clinical diagnosis as gold

standard, the sensitivity of VIDISCA-NGS for detecting a virus was 32% (95% CI 18-49%) with a specificity of 100% (95% CI 88-100%).

A weak correlation was found between the number of leucocytes in CSF and total number of sequence reads (Spearman correlation coefficient 0.31, p<0.01). We found no other correlations between any of the clinical parameters and the amount of reads that could be generated by VIDISCA-NGS.

Table 3. Additional viruses detected by VIDISCA-NGS

	Virus	Causative virus	Final diagnosis	Clinical presentation
1	HPV	HSV1/2	Viral meningitis	26yo female, no medical history. Headache and photophobia since 1 day. Temperature of 38.0 degrees Celsius, CSF 432/3µl leucocytes. Good recovery.
2	TTV	VZV	Disseminated varicella zoster with meningitis	53yo female, immunosuppressive therapy for systemic lupus erythomatodes. Fever, headache and diarrhoea for few days, fever 39.4 degrees Celsius. CSF 2001/3µl leucocytes. Good recovery.
3	TTV	x	(Chronic) meningitis, unknown pathogen	64yo male, multiple myeloma, immunocompromised. Altered consciousness, headache, nausea, neck stiffness. CSF 174 leucocytes/µl. Hydrocephalus, placement of EVD. Complicated by intraventricular haemorrhage, died.
4	HPV	x	Neurosarcoidosis	56yo female with hypertension. Presentation with headache, papilledema, dural enhancement on imaging. CSF 77 leucocytes/µl. Effectively treated with prednisone.
	Human mastadenovirus C	x		
5	GB virus C	x	Encephalitis e.c.i., possible vasculitis	74yo female with diabetes mellitus. Presented with cognitive problems, 39 cells/µl in CSF. Diagnosed with antiphospholipid syndrome, probably the cause of the cognitive problems.
6	Merkel cell polyoma virus	x	Paraneoplastic encephalitis	72yo female presenting with aphasia, bradyphrenia, perseverence. Small cell lung carcinoma discovered during admission. Start of chemotherapy, moved to nursing home.
	HPV	x		

CNS= central nervous system, HPV= human papilloma virus, HSV= human herpes virus, yo= years old, CSF= cerebrospinal fluid, TTV= Torque Teno virus, VZV= varicella zoster virus, EVD= external ventricular drain, CRP= C-reactive protein, MS= multiple sclerosis, HaNDL= Headache and Neurological Deficit with cerebrospinal fluid Lymphocytosis

DISCUSSION

VIDISCA-NGS was able to detect a virus in 52% of the patients with a qPCR confirmed virus in the CSF and in 32% of the patients with a clinical diagnosis of viral CNS infection. VIDISCA-NGS identified one enterovirus, which was initially missed by qPCR. A possible explanation for this could be the different types of methods used for nucleic acid extraction.[18,19] As expected, false negative results mainly occured in patients with HSV and EBV detected by qPCR, as VIDSCA-NGS is known to have difficulty detecting herpesviruses because herpesviral DNA in clinical specimens is often degraded and non-encapsidated.[6] No false positives were observed among viruses commonly tested during diagnostics, yielding a specificity of 100%. False positives due to contamination with remnant DNA are a known risk in metagenomics.[20] VIDISCA-NGS uses the Ion-Torrent platform, with emulsion PCR for clonal amplification, known for a lower chance of sequencing remnant DNA.

Metagenomic NGS is being studied extensively in CSF of patients with suspected CNS infections in recent years, with about half of the published studies concerning virus detection.[5] Most of these studies include only cases or case series, although recently a large prospective study was published on therapeutical consequences of results of metagenomic sequencing in a clinical setting.[21] In this study, 204 patients with idiopathic meningitis, encephalitis or myelitis were included, of which 30 were eventually diagnosed with viral CNS infections. Of these 30 viral infections, 6 viruses were identified solely by metagenomic NGS of the CSF. These viruses were either not considered by the treating physician (St. Louis encephalitis virus, hepatitis E virus, enteroviruses), were indicative of lymphoma (EBV), or had an unclear clinical significance (MW polyomavirus). This number is higher than in our cohort, but due to a different work-up including routine enterovirus and EBV testing in all patients these viruses would have been detected prior to NGS in our cohort.

As in many papers, we also found viruses of uncertain clinical relevance. To prove causality in these cases is difficult. A review on metagenomic sequencing for diagnosing encephalitis showed that in only 2/44 cases the researchers were able to demonstrate seroconversion to the pathogen in the patient, indicative of aetiological significance of the detected virus, and 8 more demonstrated presence of specific antibodies without information on the sero-status prior to the disease.[5] For some additional viruses we identified using VIDISCA-NGS, like human mastadenovirus C, it is possible that these were contaminants from the reagents used, as adenoviruses are often used as DNA vectors to produce enzymes. Anelloviruses are highly prevalent in blood in humans but relatively uncommon in CSF.[22] No association was described between HPV or MCPyV

and CNS infections, and for all three patients it is most likely that they are contaminants from skin when lumbar punction is performed.[23,24]

The fact that there is a large proportion of patients with a clinical diagnosis of viral CNS infection, where neither qPCR, nor VIDISCA-NGS found any virus in the CSF, may suggest that some viruses are no longer present in the CSF at the moment of clinical symptoms. For instance, enteroviruses have been shown to be detectable for a longer period in stool and respiratory specimens than in CSF of patients with enteroviral CNS infections.[25] Metagenomic assays, like qPCR assays, rely on the presence of the genomic material of a pathogen to be detected. Thereforeit can be important to analyse other patient samples like serum, stool, and respiratory secretions. Alternatively, as is the standard for some viruses (e.g. flaviviruses), one could determine the infection by analysing specific antibodies, which can be detected for a much longer time after infection. Recently, microarrays containing epitopes to all known human viruses or even the entire human epitome have been developed[26], which allow for the detection of all viruses at once. Nonetheless, detection of (the genome of) a pathogen is often preferred, as serological assays often lack diagnostic accuracy due to high cross-reacticity and ambiguous cut-off criteria.

Our study has several limitations. First, the retrospective design. Although clinical data and CSF were collected prospectively and analysed by qPCR, VIDISCA-NGS was performed at a later timepoint for which the majority of samples underwent one addtional freeze-thaw cycle. Second, the limited number of patients influences the study power. Since VIDISCA-NGS was only performed on a selection from the entire cohort, we were not able to calculate sensitivity and specificity based on all 363 patients. Third, our index test, VIDISCA-NGS, is not available in routine practice. To achieve this, a larger prospective trial should be conducted, evaluating changes in therapeutic decision-making and cost-effectiveness. Fourth, our study showed some technical limitations for VIDISCA-NGS. We were not able to reach a number of >10,000 total sequence reads in all of the analysed samples, which we have previously shown to be a lower cut-off to detect even low load viruses with high sensitivity.[6] This could explain a lower number of viruses detected by VIDISCA-NGS than by qPCR. Furthermore, as mentioned above, VIDISCA-NGS appears to have difficulty detecting herpesviruses. This is probably because of lack of intact viral particles in CSF from patients with a herpesviral CNS infection as previously described[6], whereas these herpesviruses will be identified by qPCR.

In conclusion, in patients where routine diagnostic work-up does not yield a causative pathogen, VIDISCA-NGS can be of additional value, but it currently does not perform well enough to replace routine PCR.

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Conflict of interest

No potential conflicts of interest relevant to this article exist. No support was given by the industry in the last three years.

Contribution

IZ contributed to data gathering, data analyses, data interpretation and writing the first draft of the manuscript. AE contributed to data gathering, data interpretation, review and critique of the report. DB contributed to review and critique of the report. MD contributed to data gathering and data interpretation. SK contributed to data gathering and data interpretation. KW contributed to data gathering, interpretation, review and critique of the report. LH contributed to study design, data gathering, data interpretation, review, and critique of the report. MB contributed to study design, data gathering, data analysis, data interpretation, review, and critique of the report.

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CHAPTER 7

BACTERIAL RIBOSOMAL RNA DETECTION IN CEREBROSPINAL FLUID USING A VIROMICS APPROACH

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ABSTRACT

Background

In patients with central nervous system (CNS) infections identification of the causative pathogen is important for treatment. Metagenomic next-generation sequencing techniques are increasingly being applied to identify causes of CNS infections, as they can detect any pathogen nucleic acid sequences present. Viromic techniques that enrich samples for virus particles prior to sequencing may simultaneously enrich ribosomes from bacterial pathogens, which are similar in size to small viruses.

Methods

We studied the performance of a viromic library preparation technique (VIDISCA) combined with low-depth IonTorrent sequencing (median ~25,000 reads per sample) for detection of ribosomal RNA from common pathogens, analyzing 89 cerebrospinal fluid samples from patients with culture proven bacterial meningitis.

Results

Sensitivity and specificity to *Streptococcus pneumoniae* (n = 24) before and after optimizing threshold parameters were 79% and 52%, then 88% and 90%. Corresponding values for *Neisseria meningitidis* (n = 22) were 73% and 93%, then 67% and 100%, *Listeria monocytogenes* (n = 24) 21% and 100%, then 27% and 100%, and *Haemophilus influenzae* (n = 18) 56% and 100%, then 71% and 100%. A higher total sequencing depth, no antibiotic treatment prior to lumbar puncture, increased disease severity, and higher c-reactive protein levels were associated with pathogen detection.

Conclusion

We provide proof of principle that a viromic approach can be used to correctly identify bacterial ribosomal RNA in patients with bacterial meningitis. Further work should focus on increasing assay sensitivity, especially for problematic species (e.g. *L. monocytogenes*), as well as profiling additional pathogens. The technique is most suited to research settings and examination of idiopathic cases, rather than an acute clinical setting.

INTRODUCTION

In patients with central nervous system (CNS) infections, rapid identification of the causative pathogen is essential to inform treatment and improve prognosis.[1,2] The differential diagnoses in these patients may include auto-immune disease, non-infectious neurological disease, or non-neurological infection.[3,4] Clinical characteristics fail to adequately differentiate between potential causes, therefore microbiological testing on cerebrospinal fluid (CSF) is the cornerstone of diagnosing CNS infections.[5] Currently available diagnostics include antigen/antibody detection assays, direct microscopy, culture techniques, and quantitative polymerase chain reaction (qPCR). Despite the availability of these tests, in a substantial proportion of patients with a high suspicion of CNS infection, no infectious organism can be identified. Because conventional assays often target specific, common pathogens, uncommon or unknown pathogens may be missed.[6,7] Metagenomic next-generation sequencing (mNGS) is an emerging technique to diagnose CNS infection without targeting specific pathogens[8,9], and is theoretically capable of identifying any pathogen RNA or DNA in samples. As sensitivity and specificity of mNGS assays have yet to match conventional testing, further development is warranted.[10,11]

For detection of viral pathogens, specialized 'viromic' mNGS methods have been developed in recent years. Viromic techniques apply mNGS to clinical samples enriched for virus-like particles, minimizing sequencing of host and background nucleic acids in order to maximize sensitivity to viruses. Virus discovery cDNA-amplified fragment length polymorphism (VIDISCA) is one such viromic assay that enables broad detection of known viruses, and has also been applied in the discovery of many novel eukaryotic viruses.[12-16] Viromic assays begin with centrifugation to remove cellular material while retaining virions in supernatant. VIDISCA then treats supernatant with DNase enzymes to remove residual genomic DNA (gDNA), which is unprotected - unlike most viral DNA. Neither step will remove residual mRNA or ribosomes, the latter of which are equivalent in size to small viruses and often highly abundant, depending on the sample type. Consequently, a high proportion of sequence data from clinical specimens can consist of ribosomal RNA (rRNA), which hinders virus detection via competition. To avoid this, VIDISCA incorporates a reverse transcription step using custom hexamer primers that mostly cannot anneal to mammalian rRNA[17], reducing human rRNA sequence reads by over 90%.[18] We previously observed these hexamers still bind to rRNA of some eukaryotic parasites[15], increasing the diagnostic capacity of VIDISCA. So far, the detection of prokaryotic pathogens has not been described. Here, we evaluated the performance of VIDISCA in detection of bacterial rRNA in CSF samples from patients with culture proven bacterial meningitis.

METHODS

Sample description

Patients participated in the MeninGene study, a nationwide prospective cohort study of community-acquired bacterial meningitis in the Netherlands, methods of which have been described elsewhere.[19,20] Briefly, patients with a positive CSF culture were identified by the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM), which receives the cultured pathogen from 85% of bacterial meningitis patients in the Netherlands. The NRLBM notified the researchers, who contacted the treating physician, who subsequently informed patients or their legal representative about the study. Patients could also be included by their treating physician without notification by the NRLBM. All patients or representatives gave written informed consent, and the study was approved by the Medical Ethics Committee of the Amsterdam UMC (METC2013_043). Clinical data were collected using an online case record form and patient outcome was recorded using the Glasgow Outcome Scale.[21] Leftover CSF was stored at treatment centers at -80 °C and transferred to the Amsterdam UMC biobank facility. For this study, 89 CSF samples with sufficient residual material were selected. Researchers performing library preparation, sequencing and metagenomic analysis were blinded to patient clinical information and the diagnosed pathogen. Subsequently, data were unblinded for optimization of threshold parameters. For controls, previously generated[22] sequencing data from 74 patient CSF samples tested negative in culture for bacteria were included in analysis, approved by a separate decision of the Medical Ethics Committee of the Amsterdam UMC (METC 2014_290). These samples were from patients undergoing lumbar puncture for suspected CNS infection, and either viral CNS infection was diagnosed or CNS infection was eventually ruled out.

Library preparation and sequencing

VIDISCA library preparation was performed on CSF as previously described.[14] Briefly, 110 µl of CSF was centrifuged for 10 minutes at 5,000 g, and supernatant was treated with TURBO DNase (Thermo Fisher Scientific) for 30 minutes at 37 °C. Nucleic acids were extracted using the Boom method[23], followed by reverse transcription primed with non-ribosomal hexamers[17] and second strand synthesis using Klenow fragment (3'→5' Exo-, NEB). After clean-up by phenol/chloroform extraction and ethanol precipitation, dsDNA was digested with Mse1 (NEB) and ligated to sample specific adapters. Size selection with AMPure XP beads (Agencourt) was done to remove small DNA fragments. After a 28-cycle PCR, further size selection was done to retain fragments 200-600 bp long. Library concentrations were analysed using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), and pooled at equimolar concentration. Pool concentration and fragment length distribution were analysed using the Qubit and Bioanalyzer (High

Sensitivity Kit, Agilent Genomics) instruments respectively. Sequencing was carried out on the Ion S5 System with the Ion 510 Chip Kit (Thermo Fisher Scientific).

Metagenomic analyses

For bacterial rRNA identification, reads were mapped to the SILVA 138.1 SSU and LSU NR99 rRNA databases[24] using BWA MEM v0.7.17-r1188.[25] Outputs were processed using the PathoID module of PathoScope v2.0.7[26], and hits to phylum Chordata were removed. Remaining reads were realigned to the GenBank nt database using BLASTn[27], and hits to the five bacterial pathogens included in this study were counted. Blinded diagnostic predictive ability was explored by selecting the pathogen with the highest read count per sample as the predicted species, and those with equal counts as indeterminate. Unblinded detection performance per pathogen was then measured via sensitivity and specificity calculations. This was repeated varying several threshold parameters to understand their impact and optimize detection performance; parameters were minimum pathogen specific read count (≥1 read versus ≥10 reads), pathogen read identity to reference (≥97% versus 100%), and sample sequencing depth (all samples versus samples ≥10,000 reads). The level of human background per sample was estimated by mapping reads to a human rRNA database, subsetted from the aforementioned SILVA database. Hits were realigned to the same database using BLASTn, with reads retained and counted if they matched with 100% nucleotide identity for at least 100 bp.

Sources of bacterial reads (rRNA versus non-rRNA) were assessed by mapping them to genome assemblies of *Streptococcus pneumoniae* (GCF_002076835.1), *Neisseria meningitidis* (GCF_008330805.1), *Listeria monocytogenes* (GCF_000196035.1), *Haemophilus influenzae* (GCF_004802225.1), and *Klebsiella pneumoniae* (GCF_000240185.1). This was done first using original assemblies, and then versions with rRNA genes masked by RepeatMasker v4.1.1.[28] Reads were curated by realignment to the respective original reference using BLASTn (requiring 100% identity for ≥100 bp). The rRNA count was calculated by subtracting the masked assembly read count from the original assembly read count. To determine if non-rRNA was predominately from mRNA or intact gDNA, the proportion mapping to reference coding sequences from each pathogen was calculated, with quality filtration as above. The proportion of coding sequence reads was compared with the gene density of each genome (coding sequence length/total genome length), since this represents the expected proportion of coding sequence reads derived from randomly sequenced pure gDNA.

Detection of viruses was done using a simplified version of a previously published workflow.[29] Reads were aligned to a database of viral proteins, with hits realigned to

the GenBank nt database using BLASTn. Those aligning to non-viral sequences were removed as false positives, while those aligning either to viruses or to no reference were retained for manual examination. Reads from viruses of interest were aligned to respective reference genomes (Enterovirus D68: AY426531.1, HIV-1: AF286365.1) in Geneious v4.8.5 (https://www.geneious.com) to visualize genomic position and coverage. HIV-1 reads were subtyped using the Los Alamos National Laboratory HIV BLAST tool (www.hiv.lanl.gov).

Statistical testing

We explored whether clinical characteristics were associated with detection of bacteria using Fisher's exact tests for dichotomous data or Mann-Whitney U tests for continuous data. Sequencing depth was correlated to clinical characteristics using Spearman's rank correlation. A p-value of <0.05 was considered statistically significant.

RESULTS

Patient characteristics

Between 2006 and 2022, 2705 patients with bacterial meningitis were included in the MeninGene study. We selected 89 patient samples from this cohort for VIDISCA analysis (Table S1). Culture based pathogen diagnoses were *S. pneumoniae* (n = 24), *N. meningitidis* (n = 22), *L. monocytogenes* (n = 24), *H. influenzae* (n = 18), and *K. pneumoniae* (n = 1). Thirty-nine patients were female (44%) and the median age was 58 years (interquartile range (IQR) 34-67). Nine patients (10%) were being treated with antibiotics at the moment of presentation. The most frequent symptoms on presentation were fever in 72 patients from 81 case record reports (89%) and headache in 65 patients from 81 reports (80%). An altered mental status, defined as a Glasgow Coma Scale (GCS) score <14, was seen in 47 patients (53%). Ten patients (11%) were in a comatose state (GCS <8) upon presentation. Median number of CSF leukocytes was 2,560/mm^3 (IQR 768-5,680) with a median CSF total protein concentration of 3.1g/L (IQR 1.8-5.5). Seventy-three patients (82%) had a favorable outcome, defined as a score of five on the Glasgow Outcome Scale at discharge, and six died (7%). The negative controls consisted of 38 patients with viral CNS infection and 36 patients with initial suspicion of CNS infection, eventually ruled out.

VIDISCA performance in bacterial diagnostics

All 89 CSF samples successfully yielded VIDISCA sequencing reads, with a median of 24,706 (IQR 10,151-40,320). From this we took the lower quartile value (rounded to 10,000 reads) as a threshold sequencing depth, to assess whether bacterial detection

was affected by total sequencing depth. Under permissive parameters (any read depth, ≥1 pathogen specific read, and ≥97% read identity) bacterial pathogen reads were identified in 65 of 89 samples (73%), though only 43 contained reads from a single species. Under strict parameters (sample total read depth ≥10,000, ≥10 pathogen specific reads, and 100% read identity) 37 of 67 samples (55%) met the pathogen read threshold. Selecting the pathogen with the highest read count per sample, for permissive parameters the culture diagnosed pathogen was correctly predicted for 45 of 65 (69%) samples with bacterial reads, or 51% of all 89 samples. In 19 samples an incorrect pathogen was predicted (21% of 89), all of which were predicted as *S. pneumoniae*, showing this pathogen carries a particular risk of false positive identification. The final sample with bacterial reads was indeterminate. Under strict parameters the culture diagnosed pathogen was correctly predicted in 34 of 37 (92%) samples, or 38% of all 89, with incorrect prediction for 3 of 89 (3%) samples.

We next explored the impact of threshold parameters on overall and per pathogen diagnostic performance. Alignment identity cut-off, sequencing depth, and pathogen specific read count impacted test diagnostic accuracy, with overall sensitivity to any pathogen ranging from 40% (30-51% CI) to 69% (56-79% CI), and specificity from 87% (82-90% CI) to 99% (97-100% CI; Figure 1, Table S2). For individual pathogens, there was high sensitivity to *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* across parameters, and poor sensitivity to *L. monocytogenes* (Figure 1, Table 1). The single *K. pneumoniae* positive sample was successfully detected across all parameter thresholds. Specificity was high across pathogens, with the exception of certain parameter thresholds for *S. pneumoniae*. Diagnostic performance was higher when only samples with ≥10,000 reads were considered, driven by increased sensitivity at low specificity cost, though the impact varied from minimal (e.g. *N. meningitidis*) to large (e.g. *S. pneumoniae*) (Figure 1). For minimum pathogen read count, requiring ≥10 reads reduced false positive rates for *S. pneumoniae* from ~50% to ~10% when compared to ≥1 read (Figure 1). The impact was lower for *L. monocytogenes, H. influenzae,* and *N. meningitidis,* which already had low false positivity rates at the ≥1 read threshold (≤11%). A ≥1 read threshold increased sensitivity for all pathogens compared with ≥10 reads, particularly for *L. monocytogenes* and *H. influenzae*. Alignment identity requirement had minimal overall impact, though in some cases the ≥97% cut-off increased sensitivity compared to the 100% cut-off at low to no specificity cost (Figure 1), leading us to select this as the universal cut-off.

Figure 1. Receiver operating characteristic curves showing VIDISCA diagnostic performance with various parameters set. All 89 samples are analysed together (top left), and then separately by pathogen. *K. pneumoniae* is not shown, because n = 1. Subset key refers to total sequencing depth required for sample inclusion, and minimum read alignment identity to a bacterial reference sequence (for ≥100 nt). Numbers within charts refer to minimum number of pathogen reads identified for a sample to be called positive

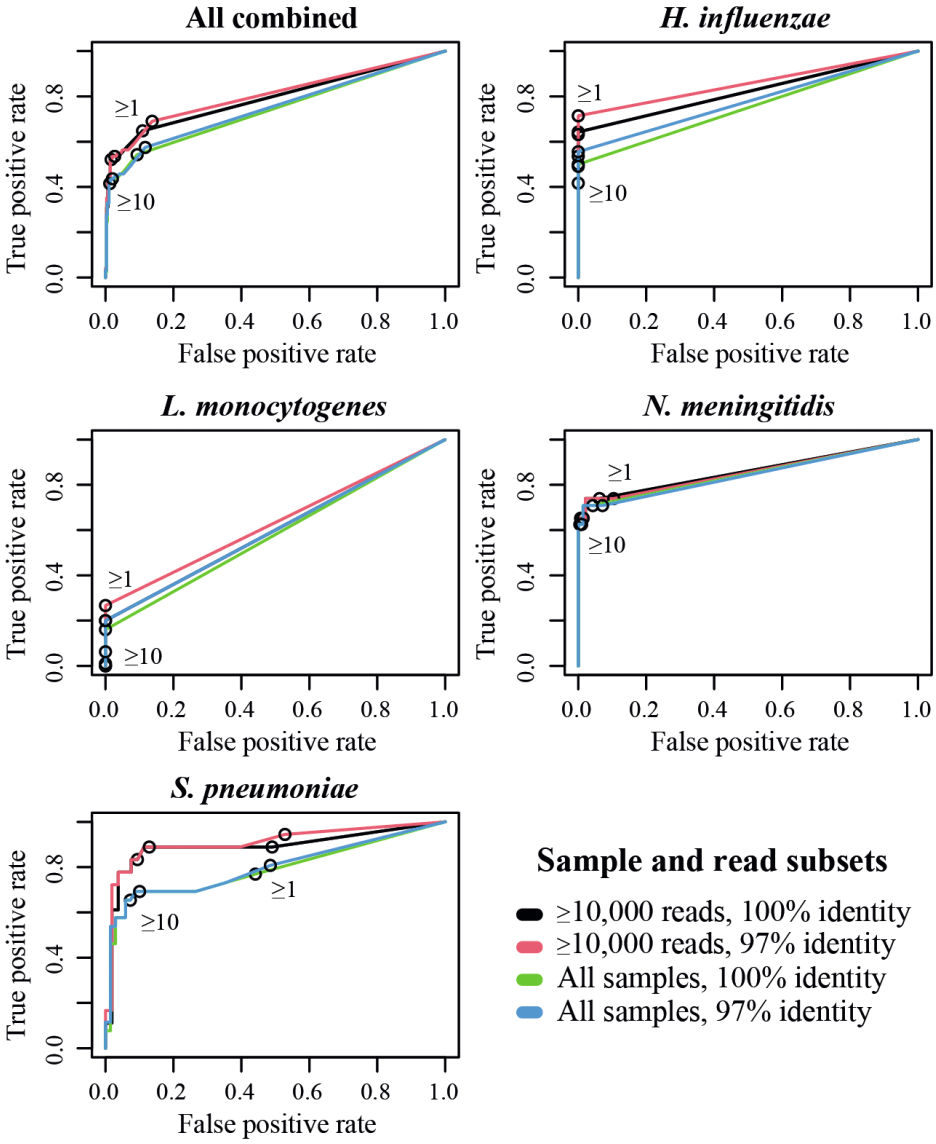


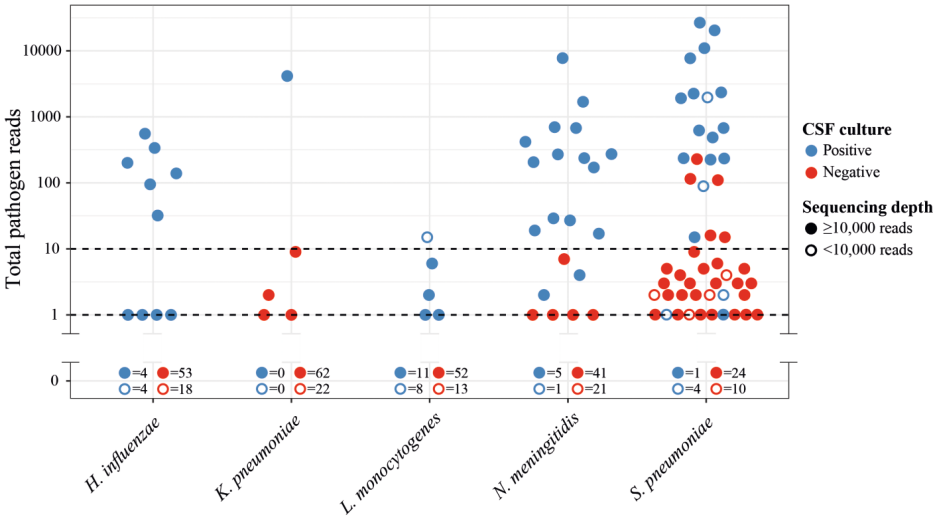
Table 1. Diagnostic performance per pathogen

Pathogen	Pathogen specific reads[a]	Total sample read depth (N)[b]	Sensitivity	Specificity
H. influenzae	≥1 read	All (18)	56%	100%
		≥10,000 reads (14)	71%	100%
K. pneumoniae	≥10 reads	All (1)	100%	100%
		≥10,000 reads (1)	100%	100%
N. meningitidis	≥10 reads	All (23)	64%	100%
		≥10,000 reads (22)	67%	100%
S. pneumoniae	≥10 reads	All (24)	67%	92%
		≥10,000 reads (16)	88%	90%
L. monocytogenes	≥1 read	All (24)	21%	100%
		≥10,000 reads (15)	27%	100%

[a]For each pathogen, the minimum pathogen read count with the best diagnostic performance is reported. [b]Performance was compared between all samples and the subset with total read count ≥10,000. The 97% read identity dataset was used. See Table S2 for performance at alternative threshold parameters.

To further understand the differences in diagnostic performance between pathogens, we produced a scatterplot of individual rRNA read counts for each, including all samples (Figure 2). This highlighted a universally low pathogen read count for *L. monocytogenes* with a maximum of 15 reads, which likely contributes to the 21% sensitivity we observed (5 of 24 positive samples detected). Notably, 37% of *L. monocytogenes* culture positive samples were low-depth (<10,000 reads), compared to a cohort average of 25%. Both *L. monocytogenes* and *H. influenzae* had zero false positive detections, while both *K. pneumoniae* and *N. meningitidis* had five, all with <10 reads (Figure 2). *S. pneumoniae* was frequently detected in samples from patients with bacterial meningitis caused by different pathogens, although 26 of 31 (84%) of these had <10 reads and all were <250 reads. Analysis of control CSF samples revealed similar false positive patterns (Figure S1), with no *L. monocytogenes* false positives, though this time four false *H. influenzae* detections were made, all with <10 reads. Six and two *N. meningitis* and *K. pneumoniae* false positives were found respectively (all <10 reads). Again *S. pneumoniae* carried the highest false positive risk, with 28 false positives, 26 of which had <10 reads, and all of which had <19.

Figure 2. Detection of pathogen rRNA reads by VIDISCA. For each pathogen, read detection across all 89 samples is plotted (reads required ≥97% alignment identity to a reference sequence). Dotted lines denote the pathogen read count thresholds examined



Associations between clinical data and VIDISCA results

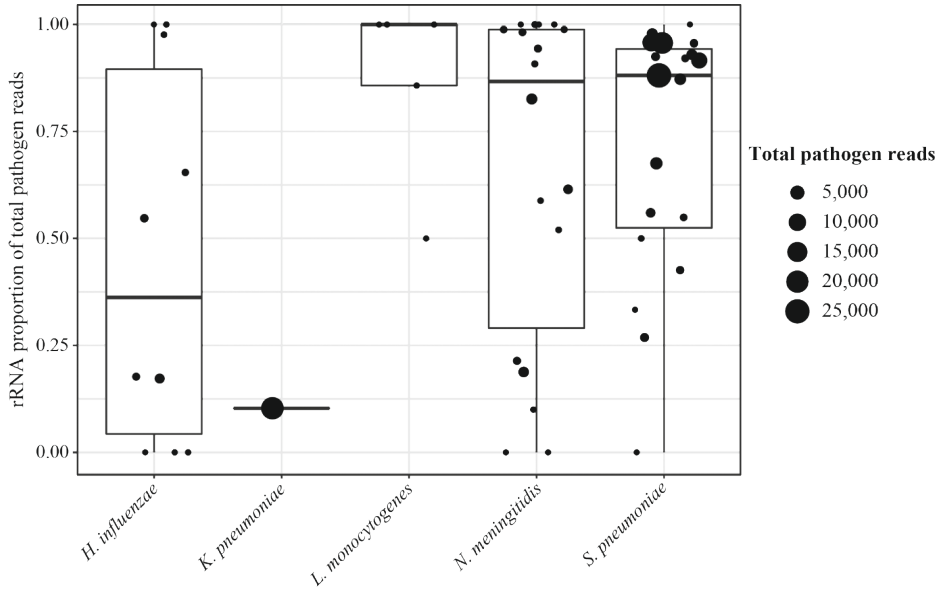
From exploratory testing, we found detection of the CSF culture diagnosed bacterium by VIDISCA was associated with no antibiotic treatment prior to presentation (49 of 78 (63%) versus 2 of 9 (22%); p = 0.03). Detection by VIDISCA was lower in patients on immunosuppressive therapy (2 of 12 (17%) versus 48 of 79 (61%); p = 0.004), and higher in patients presenting with an altered mental status (GCS score <14; 32 of 47 (68%) versus 19 of 42 (45%)) or a comatose state (GCS score <8; 9 of 10 (90%) versus 42 of 79 (53%); p = 0.04). GCS score was lower in cases accurately detected by VIDISCA, with 12 (IQR 9-15) versus 15 (IQR 12-15; p = 0.05). The level of C-reactive protein (CRP) was higher in cases detected by VIDISCA, with 206 mg/L (IQR 101-353) versus 91 mg/L (IQR 41-148; p = 0.003). The number of CSF leukocytes did not differ between groups, but CSF protein was higher in VIDISCA detected cases, with 4.1 g/L (IQR 2.9-6.0) versus 2.1 g/L (0.9-3.7; p < 0.001). No correlations between raw read count and clinical variables were found. Raw read count also did not correlate with human rRNA read count as a percentage of the total (Spearman's rho = 0.16, p = 0.14), but the latter was weakly correlated with CSF leukocyte count (Spearman's rho = 0.29; p = 0.006).

Source of bacterial nucleic acids

We hypothesized bacterial reads would primarily derive from rRNA and not residual gDNA, due to our library preparation methods. Generally, rRNA did make up the major fraction of detected pathogen reads (Figure 3), especially for *L. monocytogenes*, *N.*

meningitidis, and *S. pneumoniae* samples (all with median values ≥87%). *S. pneumoniae* was also notable in that some samples had very high rRNA counts, which were not seen in samples containing other bacteria. Across species however, substantial read fractions from bacterial-non-rRNA were also found. In particular, *H. influenzae* had a low median value of 36% rRNA, and the single *K. pneumoniae* sample had only 10% rRNA, with a high overall bacterial-read count. To determine the likeliest source of non-rRNA reads (mRNA versus residual gDNA) we determined the proportion of non-rRNA aligning to coding sequences of respective pathogen genomes. In most cases this proportion was consistent with a predominately mRNA source, being higher than the expected value for randomly sequenced gDNA (Figure S2). For *H. influenzae* however, the median proportion was precisely the expected value for gDNA, suggesting DNase treatment or centrifugation may have underperformed in CSF samples containing this species.

Figure 3. Source of bacterial reads detected by whole genome mapping. CSF culture positive samples with at least one filtered read from the respective pathogen are plotted. See also Figure S2



Detection of viruses in CSF

Viral metagenomic analysis identified six CSF samples positive for at least one human virus (Table 2). These belonged to four families (*Flaviviridae*, *Anelloviridae*, *Picornaviridae*, and *Retroviridae*). Reads from Enterovirus D68 (EV-D68, family *Picornaviridae*) were identified in one sample, from three regions of the viral genome (Figure S3A). The patient, who was taking prednisone, presented reporting gastrointestinal symptoms for several days and confusion on the day of presentation, followed by an epileptic seizure,

and was diagnosed with *L. monocytogenes* meningitis. Separately, two distinct HIV-1 reads were detected in the CSF sample of a patient with meningococcal meningitis, both overlapping regions of the *Env* gene (Figure S3B). Alignment to subtyped reference genomes showed the reads belonged to HIV-1 subtype B. HIV-1 infection in this patient had been discovered just prior to their admission due to meningitis, and antiretroviral therapy had not yet been started. The patient's CD4 count was 160 cells/mm^3, with a serum viral load of 150,000 copies/mL.

Table 2. Human viruses detected in patient CSF samples

Pathogen diagnosis	Virus	Read count
L. monocytogenes	Pegivirus A	4
L. monocytogenes	*Anelloviridae*	252
L. monocytogenes	Enterovirus D68	31
N. meningitidis	HIV-1 *Anelloviridae*	2 322
H. influenzae	*Anelloviridae*	8
S. pneumoniae	Pegivirus C	3

DISCUSSION

In patients with meningitis, no cause is found in approximately 42% of cases[30], demanding improved and broadly sensitive diagnostic methods. We demonstrate that the unmodified VIDISCA viromic method, and low-depth sequencing, can detect bacterial pathogens in CSF of patients with bacterial meningitis. Unoptimized predictive accuracy of the culture diagnosed species was low, between 38-51% depending on parameters, with inaccurate prediction in 3-21%. Overall sensitivity to bacterial reads was between 40% and 69% depending on threshold parameters, similar to a previous mNGS study[31], suggesting some utility as a diagnostic aid, though more suited to follow-up of undiagnosed meningitis rather than in an acute clinical setting. The performance varied substantially between pathogens, and species-specific parameter optimization improved sensitivity and specificity outcomes. Setting the threshold at a minimum of 10 pathogen reads eliminated all *K. pneumoniae* and *N. meningitidis* false positive read detections, and 26 of 31 for *S. pneumoniae*, while even one *H. influenzae* or *L. monocytogenes* read was always specific (though four negative control samples did contain up to two *H. influenzae* reads). This suggests universal cut-off criteria for diagnosis of pathogens in mNGS assays are suboptimal. Optimization by characterization of individual pathogen mNGS profiles improves the performance

and utility of mNGS; however, while this is possible for common etiological agents, it is less feasible for uncommon pathogens.

A lower GCS score on presentation, no use of antibiotics, higher CSF protein levels, and higher blood CRP levels were associated with correct identification of the pathogen by VIDISCA. Several of these variables have previously been associated with increased disease severity.[19,32] Likewise, in both clinical studies and experimental meningitis models higher bacterial loads have been shown to tightly correlate with disease severity.[33] In our study, a higher concentration of bacterial genomic material clearly influenced the likelihood of detection by mNGS. Because CSF is relatively low in genomic and protein background, we hypothesized that higher levels of background nucleic acids (proxied by CSF leukocyte count) would also increase diagnostic success by providing carrier for nucleic acid extraction. However, while CSF leukocyte count was weakly correlated with raw read count, it did not influence diagnostic success.

Apart from bacteria, we were able to identify viruses in a number of CSF samples. Some common and non-pathogenic ones were pegiviruses and members of the *Anelloviridae*.[34,35] Viruses known to cause CNS infections were found in two samples (EV-D68 and HIV-1), from patients diagnosed with *Listeria* meningitis and meningococcal meningitis respectively. *L. monocytogenes* infection of the CNS is commonly preceded by gastrointestinal infection or colonization[36,37], before the pathogen invades the blood and eventually crosses the blood brain barrier.[38] Although EV-D68 is primarily a respiratory virus, it may also present with gastrointestinal symptoms.[39,40] The patient in this case was taking prednisone, and presented reporting gastrointestinal symptoms for several days. The patient appears to have been co-infected by EV-D68 and *L. monocytogenes*, although which pathogen caused the gastroenteritis is unclear. The clinical significance of HIV-1 detection in one patient is also uncertain, as the virus can often be detected in untreated HIV-1 infection without clinical signs of CNS infection[41], though it also increases the risk of bacterial meningitis by as much as eightfold compared with uninfected individuals.[42]

This study has limitations. We only studied patients with bacterial meningitis confirmed by positive CSF culture, and thus the performance of VIDISCA to discriminate between different causes of infection (viral, bacterial, etc.) cannot be determined in this population. To address this, further studies in patients with suspected CNS infections should be performed. In testing the relationship between clinical variables and pathogen detection, we adopted an exploratory approach using clinical variables already known to correlate with bacterial loads, and therefore did not apply correction for multiple tests. Using data from clinically validated qPCRs would have been preferable, since this would

avoid the risks of using both proxy variables and multiple statistical tests. Further, we only studied detection performance for five common pathogens in meningitis. Inclusion of additional pathogens, especially those not found in currently applied clinical rapid tests could be of particular value. Other limitations of this study may not be specific to VIDISCA, for example, the substantial number of false positive detections of *S. pneumonia* has also been observed in multiplex PCR panels[43], probably reflecting a high carriage rate in the population.[44]

In conclusion, we have shown that VIDISCA is capable of detecting bacterial pathogens in CSF, mainly via rRNA. Selective depletion of human rRNA sequences enhances viral detection by VIDISCA[18], and our result implies the same effect likely applies to non-viral pathogens too. This requires rRNA sequences sufficiently distant in sequence to human rRNA, so that reverse transcription primers can anneal. VIDISCA was not developed primarily to detect bacteria, but rather as a low-depth screening method for viruses. As such, the detection rates imply that substantial improvement could be made with optimization, for example by increasing sample sequencing depth. Since VIDISCA selectively depletes gDNA, it is possible DNA-only libraries could be constructed that would enable detection of more bacterial genomic material, as has been done in other mNGS studies.[8,9] The high overall specificity indicates that bacterial analysis of VIDISCA data is applicable in certain circumstances, for example CSF samples already being processed for viral detection and discovery, or for follow-up of idiopathic meningitis cases. The large number of samples processed (40 to 70) is suited to research and perhaps outbreak settings. However, current sensitivity rates and the turnaround time of between five days to two weeks make it unsuited to the acute clinical setting, where single samples will often require processing. Here, rapid and high performance multiplex assays for common pathogens are more desirable.[45]

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Author contributions

Conceptualization, LvdH, MCB, CMK, AWDE; Methodology, AWDE, CMK, IEvZ, LvdH, MCB; Software, CMK; Clinical data and sample collection, MCB, DvdB, IEvZ; Formal analysis, AWDE, CMK, IEvZ; Investigation, AWDE, CMK, IEvZ, MD; Resources, MCB, LvdH, DvdB; Writing - Original Draft, CMK, LvdH, MCB, AWDE, IEvZ; Writing - Review & Editing, all authors; Visualization, AWDE, CMK; Supervision, LvdH, MCB; Funding acquisition, LvdH, MCB, DvdB.

Declaration of interests

The authors declare no competing interests.

Ethical statement

This study was approved by the Medical Ethical Committee of the Amsterdam UMC (METC2013_043). All patients or their legal representatives gave written informed consent.

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SUPPLEMENTARY TABLES

Table S1. Baseline characteristics of included patients

	All (N = 89)	*S. pneumoniae* (N = 24)	*N. meningitidis* (N= 22)	*L. monocytogenes* (N = 24)	*H. influenzae* (N = 18)	*K. pneumoniae* (N = 1)
Female	39/89 (44)	14/24 (58)	10/22 (45)	6/24 (25)	8/18 (44)	1/1 (100)
Age (years)	58 (34-67)	63 (41-69)	21 (18-42)	61 (47-73)	59 (42-66)	-
Predisposing factors						
Otitis/sinusitis	22/82 (27)	14/20 (71)	0/21 (0)	0/22 (0)	8/18 (44)	0/1 (0)
CSF leak	4/87 (5)	1/23 (4)	0/21 (0)	0/24 (0)	3/18 (17)	0/1 (0)
Cancer	10/89 (11)	4/24 (17)	0/22 (0)	5/24 (21)	1/18 (6)	0/1 (0)
Immunosuppressive treatment	12/88 (14)	1/23 (4)	0/22 (0)	11/24 (46)	0/18 (0)	0/1 (0)
Pretreatment with antibiotics	9/87 (10)	2/24 (8)	0/22 (0)	4/23 (17)	3/17 (18)	0/1 (0)
Symptoms						
Headache	65/81 (80)	16/21 (76)	17/19 (89)	16/22 (73)	15/18 (83)	1/1 (100)
Fever (>38ºC)	72/81 (89)	17/20 (85)	16/20 (80)	23/24 (96)	15/16 (94)	1/1 (100)
Seizures	2/86 (2)	1/23 (4)	1/20 (5)	0/24 (0)	0/18 (0)	0/1 (0)
GCS score	13 (10-15)	11 (9-13)	15 (11-15)	13 (11-15)	15 (10-15)	-
GCS <14 (altered)	47/89 (53)	19/24 (79)	8/22 (36)	12/24 (50)	7/18 (39)	1/1 (100)
GCS <8 (coma)	10/89 (11)	4/24 (17)	2/22 (9)	1/24 (4)	3/18 (17)	0/1 (100)
Laboratory results						
Blood leukocytes (•10^9/L)	17 (13-21)	20 (12-24)	18 (17-21)	14 (12-17)	14 (12-19)	-
CRP (mg/L)	135 (74-257)	179 (101-312)	230 (133-370)	86 (41-158)	79 (42-126)	-
CSF leukocytes/µl	2560 (768-5680)	2300 (849-7003)	5199 (976-13425)	1250 (324-2355)	3930 (1875-6157)	-
CSF protein (g/L)	3.1 (1.8-5.5)	6.0 (2.8-7.1)	3.4 (1.1-5.2)	2.4 (1.8-4.2)	3.1 (1.3-4.5)	-
CSF/blood glucose ratio	0.09 (0.01-0.31)	0.02 (0.01-0.17)	0.08 (0.01-0.37)	0.26 (0.12-0.39)	0.08 (0.02-0.31)	-
Outcome						
Favourable (GOS of 5)	73/89 (82)	21/24 (88)	20/22 (91)	16/24 (67)	16/18 (89)	0/1 (0)
Death	6/89 (7)	2/24 (8)	0/22 (0)	3/24 (13)	0/18 (0)	1/1 (100)

Values are n/N (%) or median (interquartile range). CSF = cerebrospinal fluid, GCS = Glasgow Coma Scale, CRP = C-reactive protein, GOS = Glasgow Outcome Scale.

Table S2. VIDISCA performance for bacterial diagnostics

Sample depth	**All**	**All**	**All**	**All**	**≥10,000**	**≥10,000**	**≥10,000**	**≥10,000**
Read identity cutoff	**≥97%**	**≥97%**	**≥97%**	**≥97%**	**≥97%**	**≥97%**	**≥97%**	**≥97%**
Minimum pathogen reads	**1**	**1**	**10**	**10**	**1**	**1**	**10**	**10**
Test	**Specificity**	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**	**Sensitivity**
All pathogens	0.89 (0.85-0.92)	0.57 (0.46-0.68)	0.99 (0.97-1.00)	0.43 (0.32-0.54)	0.87 (0.82-0.90)	0.69 (0.56-0.79)	0.98 (0.96-0.99)	0.52 (0.40-0.65)
H. influenzae	1.00 (0.95-1.00)	0.56 (0.31-0.78)	1.00 (0.95-1.00)	0.33 (0.13-0.59)	1.00 (0.93-1.00)	0.71 (0.42-0.92)	1.00 (0.93-1.00)	0.43 (0.18-0.71)
K. pneumoniae	0.95 (0.89-0.99)	1.00 (0.03-1.00)	1.00 (0.96-1.00)	1.00 (0.03-1.00)	0.94 (0.85-0.98)	1.00 (0.03-1.00)	1.00 (0.95-1.00)	1.00 (0.03-1.00)
N. meningitidis	0.93 (0.83-0.98)	0.73 (0.50-0.89)	1.00 (0.95-1.00)	0.64 (0.41-0.83)	0.89 (0.76-0.96)	0.76 (0.53-0.92)	1.00 (0.92-1.00)	0.67 (0.43-0.85)
S. pneumoniae	0.52 (0.40-0.65)	0.79 (0.58-0.93)	0.92 (0.83-0.97)	0.67 (0.45-0.84)	0.47 (0.33-0.62)	0.94 (0.70-1.00)	0.90 (0.79-0.97)	0.88 (0.62-0.98)
L. monocytogenes	1.00 (0.94-1.00)	0.21 (0.07-0.42)	1.00 (0.94-1.00)	0.04 (0.00-0.21)	1.00 (0.93-1.00)	0.27 (0.08-0.55)	1.00 (0.93-1.00)	0.00 (0.00-0.22)
Sample depth	**All**	**All**	**All**	**All**	**≥10,000**	**≥10,000**	**≥10,000**	**≥10,000**
Read identity cutoff	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**
Minimum pathogen reads	**1**	**1**	**10**	**10**	**1**	**1**	**10**	**10**
Test	**Specificity**	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**	**Sensitivity**
All pathogens	0.91 (0.87-0.94)	0.54 (0.43-0.65)	0.99 (0.98-1.00)	0.40 (0.30-0.51)	0.89 (0.84-0.92)	0.64 (0.52-0.76)	0.99 (0.97-1.00)	0.51 (0.38-0.63)
H. influenzae	1.00 (0.95-1.00)	0.50 (0.26-0.74)	1.00 (0.95-1.00)	0.33 (0.13-0.59)	1.00 (0.93-1.00)	0.64 (0.35-0.87)	1.00 (0.93-1.00)	0.43 (0.18-0.71)
K. pneumoniae	0.98 (0.92-1.00)	1.00 (0.03-1.00)	1.00 (0.96-1.00)	1.00 (0.03-1.00)	0.97 (0.89-1.00)	1.00 (0.03-1.00)	1.00 (0.95-1.00)	1.00 (0.03-1.00)
N. meningitidis	0.96 (0.87-0.99)	0.73 (0.50-0.89)	1.00 (0.95-1.00)	0.64 (0.41-0.83)	0.93 (0.82-0.99)	0.76 (0.53-0.92)	1.00 (0.92-1.00)	0.67 (0.43-0.85)
S. pneumoniae	0.57 (0.44-0.69)	0.75 (0.53-0.90)	0.95 (0.87-0.99)	0.62 (0.41-0.81)	0.51 (0.37-0.65)	0.88 (0.62-0.98)	0.94 (0.84-0.99)	0.81 (0.54-0.96)
L. monocytogenes	1.00 (0.94-1.00)	0.17 (0.05-0.37)	1.00 (0.94-1.00)	0.00 (0.00-0.14)	1.00 (0.93-1.00)	0.20 (0.04-0.48)	1.00 (0.93-1.00)	0.00 (0.00-0.22)

Values are proportions (95% confidence intervals).

SUPPLEMENTARY FIGURES

Figure S1. Detection of false positive reads in control CSF samples. For each pathogen, read detection across all 74 samples is plotted. Dotted lines denote the pathogen read count thresholds utilised in this study

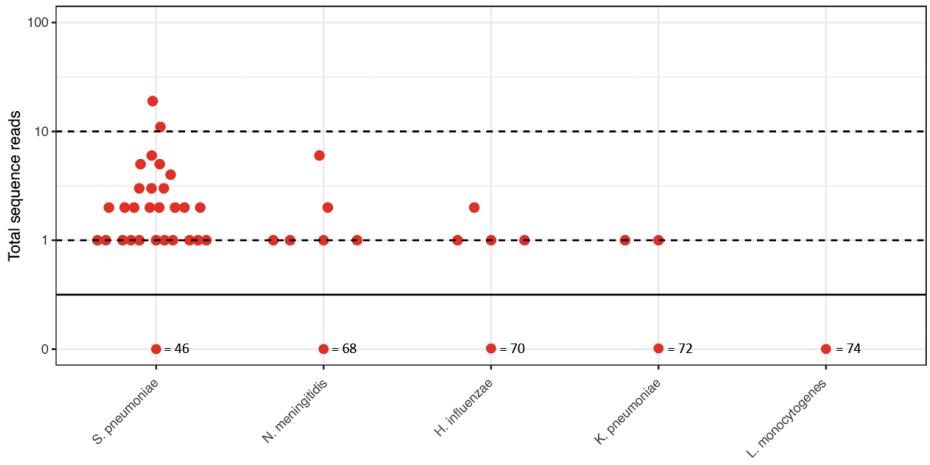


Figure S2. Source of bacterial non-rRNA reads detected by whole genome mapping. The proportion of non-rRNA reads derived from bacterial coding sequence (CDS) is plotted per sample. CSF culture positive samples with at least one filtered non-rRNA read from the respective pathogen are plotted. Dotted lines represent the expected proportion of CDS reads if pure gDNA were randomly sequenced (gene density in the genome expressed as a proportion). In contrast, the expected proportion of CDS reads if pure mRNA were randomly sequenced is 1.00. Low outlier samples all had <10 total non-rRNA reads

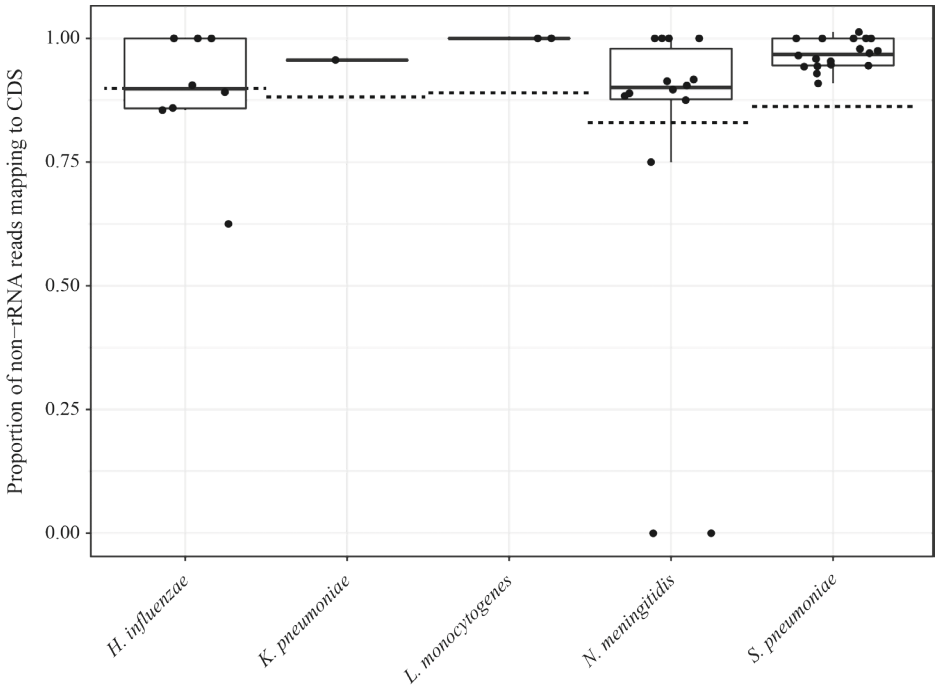
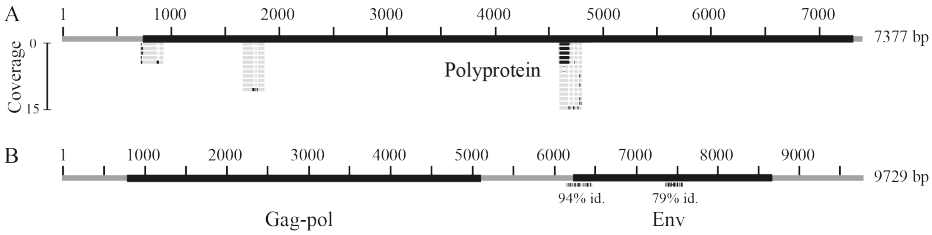


Figure S3. CSF detection of neuropathogenic viruses. A) Enterovirus D68 (EV-D68) in a patient with CSF culture confirmed *Listeria* meningitis. Reads were aligned to EV-D68 strain Fermon (AY426531.1), with the position of the polyprotein open reading frame shown in black. Read matches to references are light grey, while mismatches are black. B) HIV-1 subtype B in a patient with CSF culture confirmed *N. meningitidis* infection. Reads were aligned to HIV-1 isolate WR27 (AF286365.1), with the positions of structural genes shown in black. Read matches to references are light grey, while mismatches are black



CHAPTER 8

DIAGNOSTIC ACCURACY OF CLINICAL AND LABORATORY CHARACTERISTICS IN SUSPECTED NON-SURGICAL NOSOCOMIAL CENTRAL NERVOUS SYSTEM INFECTIONS

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ABSTRACT

Background

The diagnosis of meningitis in non-surgical hospitalised patients is often difficult and diagnostic accuracy of clinical, laboratory, and radiological characteristics is unknown.

Aim

To assess diagnostic accuracy for individual clinical characteristics of patients suspected of non-surgical nosocomial central nervous system (CNS) infections.

Methods

In a prospective multi-centre cohort study in the Netherlands with adults suspected of CNS infections, consecutive patients who underwent a lumbar puncture for the suspicion of a non-surgical nosocomial CNS infection were included. All episodes were categorized into five final clinical diagnosis categories, as reference standard: CNS infection, CNS inflammatory disease, systemic infection, other neurological disease, or non-systemic, non-neurological disease.

Results

Between 2012-2022, 114 out of 1275(9%) patients included in the cohort had suspected non-surgical nosocomial CNS infection: 16n(14%) had a confirmed diagnosis, including four (25%) patients with bacterial meningitis, nine (56%) viral CNS infections, two (13%) fungal meningitis, and one (6%) parasitic meningitis. Diagnostic accuracy of individual clinical characteristics was generally low. Elevated CSF leukocyte count had the highest sensitivity (81%; 95% CI 54-96) and negative predictive value (NPV) (96%; 95% CI 90-99). When combining the presence of abnormalities in neurological or CSF examination, sensitivity for diagnosing a CNS infection was 100% (95% CI 79-100) and NPV 100% (95% CI 78-100). CSF examination changed clinical management in 47% of patients.

Conclusion

Diagnostic accuracy for individual clinical characteristics was low, with elevated CSF leukocyte count having the highest sensitivity and NPV.

INTRODUCTION

Hospitalized patients are at risk for nosocomial central nervous system (CNS) infections, which may be related to neurosurgery, immunosuppression, or other comorbidities.[1,2] Nosocomial CNS infections that are not related to neurosurgery are relatively infrequent but often pose a diagnostic challenge.[3,4] A non-surgical nosocomial CNS infection may, for instance, be suspected in patients in a medical intensive care unit (ICU) with persistent mental status changes or in immunocompromised patients with fever of unknown origin.[5] Often, the diagnosis is difficult to rule out without cerebrospinal fluid (CSF) examination, even though the yield of lumbar puncture is reported to be low in specific patient categories.[5] For those with a non-surgical nosocomial CNS infection, adequate and timely treatment is important to improve the prognosis. So far, few studies have assessed clinical, radiological, and laboratory characteristics of this population and analysed their diagnostic accuracy for identifying CNS infections.

Our objective was to determine the diagnostic accuracy of clinical and laboratory characteristics for the diagnosis of non-surgical nosocomial CNS infections. We hypothesized that these characteristics would enable differentiation between patients with and without non-surgical nosocomial CNS infections.

METHODS

Patients

For this study, we used patient data from two studies including consecutive patients who underwent a lumbar puncture for the suspicion of a CNS infection. The first study was a prospective observational single-centre cohort study in the Amsterdam University Medical Center, AMC location, between 2012 and 2015. Methods have been described in detail previously.[6] This first study functioned as a pilot study for the second study, which is now an ongoing prospective multi-centre cohort study in multiple hospitals in The Netherlands since 2017. In both studies, inpatients or patients presenting to the emergency department (age ≥ 16 years) were prospectively included if they underwent a lumbar puncture for the suspicion of a CNS infection. Patients were excluded if they recently underwent neurosurgery or severe traumatic brain injury (≤ one month prior to the suspected infection), and/or had a neurosurgical device *in situ*. For this current study, we selected all patients with a suspected non-surgical nosocomial CNS infection from these cohorts, defined as the suspicion of a CNS infection occurring more than 48 hours after admission or within one week after discharge from the hospital.[7-9]

Data on clinical, laboratory and radiological characteristics at presentation (the index tests) and outcome were collected using an online case-record form. Patients were considered to be immunocompromised if they were taking immunosuppressant drugs, had a splenectomy, cancer, diabetes mellitus, organ transplantation, or HIV infection. Outcome at discharge was classified according to the Glasgow Outcome Scale score (GOS).[10] A favourable outcome was defined as a GOS score of 5, and an unfavourable outcome was defined as a GOS score ranging from 1 to 4.

Diagnostic categorization

As reference standard, the final clinical diagnoses of all included episodes were classified as being due to either 1) CNS infection, 2) CNS inflammation without infection, 3) systemic infection without CNS involvement, 4) other neurological disease, and 5) non-infectious, non- neurological disorder[6]. Two investigators independently classified the episode based on all available clinical, laboratory, and follow-up data. If there was no consensus, a third investigator was consulted. Inter-rater agreement was assessed by calculating the kappa coefficient with a kappa of 0.76 in the first cohort and 0.64 in the second cohort. A CNS infection was diagnosed if there was microbiological proof of infection by Gram stain, culture, or polymerase chain reaction (PCR) of the CSF or in blood in combination with CSF leucocytosis. Without microbiological confirmation, a CNS infection could be diagnosed based on the investigators' classification. In case of a CNS infection, subcategories were made according to the pathogen: bacterial, viral, fungal, or parasitic.

Statistical analysis

Data were analysed with SPSS software version 28 (Armonk, NY: IBM Corp.). Continuous variables were compared using the Mann-Whitney *U*-test. Categorical data were compared using χ^2-test or Fisher's exact test. For the diagnostic accuracy the following were calculated: sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), and the area under the curve (AUC) of the receiver operator characteristics (ROC) curve. $P < 0.05$ was considered statistically significant.

RESULTS

Patients

In the periods 2012-2015 and 2017-2022, at total of 1275 patient episodes of suspected CNS infections were included (363 and 912 episodes, respectively) in the two cohorts. Of those, 114 (9%) patients were suspected of a non-surgical nosocomial CNS infection. Median age of these patients was 63 years (interquartile range [IQR] 50-70) and 32% were female (n=37; Table 1). Predisposing conditions were present in 88 (77%) cases, the

majority (84) representing immunocompromised states(Table 1). Of the 114 included patients, 108 (95%) were included in a tertiary care facility. Forty-two (37%) were on an Intensive Care Unit, 45 (39%) on an Internal Medicine ward, nine (8%) on other wards, including Neurology, and 18 (16%) were seen on the emergency department. The latter group consisted of patients who were discharged from the hospital within the preceding week. Of these patients, three (17%) were admitted to the ICU, one to a Medium Care Unit (6%), four (22%) to the neurology ward, seven (39%) to other wards, and three (17%) were not admitted. The commonest reasons for suspecting that patients may have CNS infection were a severely immunocompromised state such as chemotherapy, immunosuppressive drugs, or primary immunodeficiency (59 patients) and signs of infection where CNS infection could not be ruled out (30 patients).

The most frequent recorded symptoms and signs were headache (32 out of 84 [38%]) and fever (45 out of 112 [40%]). An altered mental status (defined as Glasgow Coma Scale [GCS] <14) was observed in 68 (60%) patients, including 34 who were comatose (GCS score <8; Table 1). In 48 (42%) patients, two or more key symptoms (headache, fever, neck stiffness, or change in mental status) were present. Only six (5%) patients had the classical triad of bacterial meningitis symptoms (fever, neck stiffness, and altered mental status). Focal neurological deficits were present in 27 (33%) patients. The median blood leukocyte count was 9 per 10^9/L (IQR 4-15), and the median C-reactive protein concentration was 40 mg/L (IQR 14-104; Table 1).

In 81 out of 90 (90%) patients, cranial imaging was performed at first consultation. Computed tomography (CT) of one patient with bacterial meningitis showed subarachnoid blood, probably due to cerebral (cortical) venous thrombosis. In one patient with bacterial meningitis, cranial CT scan showed opacification of the mastoid and sinuses. No other relevant radiological findings were reported in the group of CNS infections. Imaging was performed prior to the lumbar puncture in 95 out of 99 patients (96%).

CSF examination showed a median leucocyte count of $2x10^6$L (IQR 1-7), and 39 patients (34%) had an elevated leucocyte count (>$4x10^6$/L leucocytes; Table 1). The median CSF protein concentration was 0.5 g/L (IQR 0.3-0.7), with 39 (34%) having an elevated protein level (≥0.6 g/L). CSF leucocyte and protein count were completely normal in 57 (50%) episodes. Gram stain was performed in 90 (79%) patients, and bacteria were reported in only three patients. One of these had pneumococcal meningitis; the other two reports were considered to represent false positives.

Treatment with antibiotics according to bacterial meningitis regimen was initiated prior to lumbar puncture in 51 (81%) out of the 63 patients for which data were available.

Table 1. Clinical characteristics at presentation

	All suspected of non-surgical nosocomial CNS infections (n=114)	Non-surgical nosocomial CNS infections (n=16)	Other final diagnosis (n=98)	P-value
Age (years)	63 (50-70)	64 (46-68)	63 (50-71)	0.95
Female sex	37/114 (32)	8/16 (50)	29/98 (30)	0.09
Using antimicrobial treatment at presentation[a]	88/111 (79)	13/16 (81)	75/95 (79)	0.09
Predisposing conditions	88/114 (77)	15/16 (94)	73/98 (74)	0.11
Immunocompromised state	84/111 (76)	13/15 (87)	71/96 (74)	0.35
Cancer	44/114 (39)	4/16 (25)	40/98 (41)	0.28
Diabetes Mellitus	31/114 (27)	5/16 (31)	26/98 (27)	0.76
Immunosuppressive therapy	53/114 (46)	10/16 (63)	43/98 (44)	0.19
HIV positive	6/114 (5)	1/16 (6)	5/98 (5)	1.00
Other infection[b]	21/114 (18)	5/16 (31)	16/98 (16)	0.17
Symptoms at presentation				
Headache	32/84 (38)	7/14 (50)	25/70 (36)	0.22
Seizures	12/105 (11)	2/15 (13)	10/90 (11)	0.94
Physical examination				
Fever >38 °C	45/112 (40)	6/16 (38)	39/96 (41)	1.00
Neck stiffness	10/89 (11)	3/12 (25)	7/77 (9)	0.43
Neurological examination				
Focal neurological deficits	27/81 (33)	1/13 (13)	26/68 (38)	0.05
GCS at presentation	13 (7-15)	14 (10-15)	13 (6-15)	0.23
GCS <14	68/114 (60)	9/16 (56)	59/98 (60)	0.79
GCS <8	34/114 (30)	3/16 (19)	31/98 (32)	0.39
Blood laboratory test				
Leukocytes (per 10^9/L)	9 (4-15)	7 (1-10)	9 (4-15)	0.12
C-reactive Protein (mg/L)	40 (14-104)	43 (19-115)	40 (12-104)	0.95
CSF parameters				
Leukocytes (per 10^6/L)	2 (1-7)	51 (16-110)	2 (1-5)	<0.01
Leukocytes >4x10^6/L	39/114 (34)	13/16 (81)	26/98 (24)	<0.01
Protein (g/L)	0.5 (0.3-0.7)	0.8 (0.5-1.4)	0.4 (0.3-0.7)	<0.01
Protein ≥0.6 g/L	39/114 (34)	10/16 (63)	29/98 (30)	<0.01

Data are n/N (%) or median (interquartile range); CNS= central nervous system, ICU= intensive care unit, GCS= Glasgow Coma Scale score, CSF= cerebrospinal fluid.

[a] Antibiotics in 62 (56%), antiviral medication in 7 (6%), both in 19 patients (17%), [b] Included pneumonia, otitis, sinusitis

Continuous variables: age, GCS at presentation, CSF leukocytes and protein available in all episodes, blood leukocytes in 112 episodes and C-reactive protein in 71 episodes.

Final diagnosis – reference standard

Of the 114 episodes suspected of non-surgical nosocomial CNS infections, 16 episodes were classified as CNS infection (14%), five as CNS inflammation (4%), 37 as systemic infection (32%), 52 as other neurological disease (46%) and four as non-infectious, non-neurological disease (4%; Figure 1 and Figure 2). In 11 out of 16 (69%) episodes classified as CNS infection, a pathogen was found through CSF or blood culture, or by CSF PCR. Four (25%) had bacterial meningitis, nine (56%) had a viral CNS infection, two (13%) had fungal meningitis, and there was one (6%) of parasitic meningitis. Seven of 45 (16%) of patients admitted to an Internal Medicine ward, were diagnosed with a CNS infection, and, of the patients in the ICU, three of 42 (7%) were diagnosed with a CNS infection. Ten out of 59 patients (17%) with severe immunocompromised state were diagnosed with a CNS infection, whereas three out of 30 (10%) in the group of patients with fever of unknown cause were diagnosed with a CNS infection.

Figure 1. Flow chart of diagnostic categories

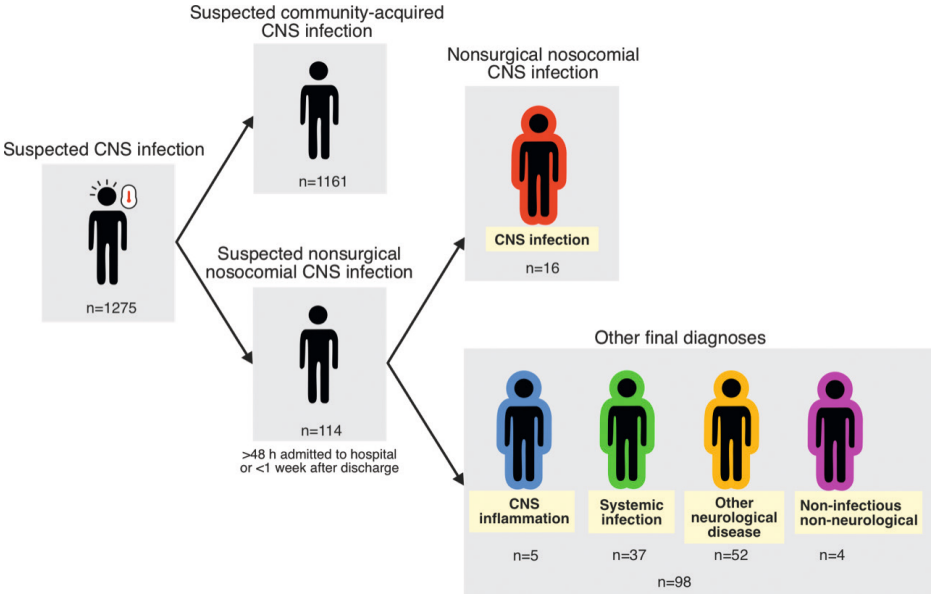

Figure 2. Final clinical diagnosis in patients initially suspected of non-surgical nosocomial CNS infections

CNS infection (n= 16, 14%)	
4 Bacterial meningitis	25%
9 Viral encephalitis/ meningitis	56%
1 Cryptococcal meningitis	6%
1 Cerebral toxoplasmosis	6%
1 Rhino-Orbito-Cerebral Mucormycosis	6%

CNS inflammation (n= 5, 4%)	
1 Guillain-Barré syndrome	20%
1 Neurosarcoidosis	20%
2 Paraneoplastic encephalitis[a]	40%
1 Inflammatory encephalitis	20%

Systemic infection (n= 37, 32%)	
14 Respiratory tract infection	38%
6 GI-tract infection	16%
4 Urinary tract infection	11%
3 Systemic viral infection	8%
2 Bacteremia	5%
2 Skin/soft tissue infection	5%
6 Other[b]	16%

Other neurological disease (n= 52, 46%)	
24 Metabolic or toxic encephalopathy	46%
7 Headache syndrome	13%
5 Stroke	10%
2 Epilepsy	6%
5 Delirium	4%
9 Other[c]	17%

Non-neurological, non-infectious (n= 4, 4%)	
1 Systemic disease	25%
3 Psychiatric condition	75%

CNS= central nervous system, GI=gastrointestinal. [a] In one of these patients there was a differential diagnosis of cerebral graft-versus-host disease [b] Other systemic infections included sepsis eci, ear-nose-throat infection, line sepsis and a malaria infection [c] Other included intracranial hypertension, intracerebral tumor, post-COVID encephalopathy and encephalopathy in systemic disease/postanoxia.

CSF cultures were performed in 93 episodes (82%) and were positive in three episodes (Table 2). Six viruses were identified in five CSF samples (Table 2). Another patient was categorized as having viral meningitis based on detection of influenza A by PCR on a pharyngeal swab. T. gondii was detected by PCR in one case, and another patient was diagnosed with rhino-orbitocerebral mucormycosis associated vasculopathy and had zygomycetes and *Beauveria* sp. identified by PCR of ethmoid sinus biopsy material.

In three immunocompromised patients a CNS infection was observed with a normal CSF leucocyte count. One patient with VZV encephalitis had an elevated CSF protein concentration; the other two patients (HHV6 encephalitis and rhino-orbitocerebral mucormycosis) had completely normal CSF cell counts and biochemistry.

Table 2. Distribution of final clinical diagnoses of patients with non-surgical nosocomial CNS infections

Diagnosis	n/N (%)	Predisposing factors
CNS infection	16/114 (14%)	
Bacterial meningitis	4/16 (25%)	
CSF culture positive	2/4 (40%)	
	R. mucilaginosa,	- Immunocompromised patient (acute myeloid leukemia)
	S. pneumoniae	- Immunocompromised patient (panhypopituarism, steroid use), recently discharged after COVID-pneumonia
Post mortem brain culture	*E. faecalis*[a]	- Patient with *S. bovis* endocarditis, perianal abscess
Viral meningitis/encephalitis	9/16 (56%)	
CSF PCR positive[b]	5/9 (56%)	
	VZV (n=2), EBV (n=1), HHV6 (n=1), HSV2 (n=2)	- Immunocompromised patients (HIV, AML, immunosuppressive drugs)
Pharyngeal swab PCR	Influenza A (n=1)	- Pneumonia
Fungal infection	2/16 (13%)	
Identified Fungi	*C. neoformans* (n=1; culture) *Zygomycete* and *Beauveria* sp (n=1)[c]	- Immunocompromised (kidney transplant) - ROCM patient
Parasitic meningitis	1/16 (6%) *Toxoplasma gondii* (PCR)	- Immunocompromised (lymphoma)

Data are n/N (%); CNS= central nervous system, CSF= cerebrospinal fluid, PCR= polymerase chain reaction, EBV= Epstein Barr Virus, CMV= cytomegalovirus, AML= acute myeloid leukemia. [a] *E. faecalis* was only identified in brain culture at autopsy, in vivo blood culture revealed *S. bovis* in patient with endocarditis [b] PCR was positive for 2 viruses in 1 patients (EBV and VZV), [c] PCR of the sinus area showed Zygomycetes and *B. caledonica* or *B. bassiana* or *B. brongniartii*, found in the Rhino-orbito-cerebral mucormycosis patient

Management and outcome

In 52 (47%) of 110 cases, the lumbar puncture caused a direct change in management. In 18 patients antibiotics according to bacterial meningitis regimen or acyclovir in encephalitis dosage were initiated after the lumbar puncture. In 28 patients antibiotics for bacterial meningitis or acyclovir for encephalitis were discontinued based on CSF results. In two cases pre-existing antibiotic therapy was modified, and in four cases other treatment (e.g. corticosteroids or immunoglobulins) were commenced. Outcome was unfavourable in 71 (62%) episodes and 33 (29%) patients died. Of the deceased patients, five (15%) had been diagnosed with a CNS infection. Nine (56%) of the patients with a confirmed CNS infection had an unfavourable outcom.

Diagnostic accuracy

Characteristics that were present in most patients with non-surgical nosocomial CNS infections were a predisposing factor at presentation, abnormalities in neurological examination, elevated CSF leucocyte count and CSF protein levels (Table 1). Elevated CSF leucocyte count had the highest sensitivity of 81% (95% CI 54-96) and negative predictive value of 96% (95% CI 90-99; Supplementary Table A1). An abnormal neurological examination had a sensitivity of 56% (95% CI 30-80) and a specificity of 27% (95% CI 19-37). The AUC of CSF leucocyte count for the diagnosis of a CNS infection was 0.88 (95% CI 0.77-0.99). For CSF protein level, the AUC was 0.72 (95% CI 0.57-0.87). The combination of an abnormal neurological examination, CSF pleiocytosis, and high CSF protein level had a sensitivity of 100% (95% CI 79-100) and a NPV of 100% (95% CI 78-100). However, only in 15 out of 97 (15%) patients a CNS infection could be ruled out using this combination.

DISCUSSION

This study shows that a CNS infection can be confirmed in one in seven patients with suspected non-neurosurgical nosocomial CNS infections. Non-neurosurgical nosocomial CNS infection is a severe disease with high rate of unfavourable disease outcome (56%). The diagnosis may be difficult to predict because the diagnostic accuracy of clinical and other laboratory parameters was poor. The best predictor of CNS infection was the CSF leucocyte count, with an AUC of 0.88, and therefore, CSF analysis is indicated in all patients with a suspected infection.

The rate of patients eventually diagnosed with a non-surgical nosocomial CNS infection in our study (14%) is comparable to the rate reported in a retrospective US study including 31 ICU patients with suspected non-neurosurgical CNS infections.[5] Interestingly, in our study, CSF results clearly changed patient management in 47% of cases. This in contrary to two retrospective US studies , involving a total of 121 patients, which found that CSF results did not clearly change patient management.[11,12] However, one of these studies did not include immunocompromised patients.[11] Moreover, microbiological diagnostics have advanced since these earlies studies; no routine testing for viruses was performed in previous publications.[5,11,12] In addition, the number of immunosuppressed patients has steadily increased over the years, with improved prognosis of cancer and transplant patients but a higher long-term risk of opportunistic CNS infections.[13-16] Finally, previous studies did not clearly define changes in medical management.[5,11] We observed a change of medical management in almost half of the cases, which included either initiating or ending a certain treatment because

of the results from CSF examination. Continuation of a previously initiated therapy when lumbar puncture confirms a diagnosis has not been included this percentage. Overall, our results support a low threshold for CSF examination in patients with suspected non-surgical nosocomial CNS infections, as it frequently changes management and identifies a CNS infection in one in seven patients.

We identified a large variety in causative pathogens in non-surgical nosocomial CNS infections, the distribution of both bacterial and viral pathogens differing substantially from those in community-acquired CNS infections.[6,17] Likewise, the pathogens in our patient cohort differed from those in neurosurgery-related nosocomial CNS infections.[4,9,18] The clear distinction in diagnostic work-up and empiric treatment between post neurosurgical and non-surgical nosocomial CNS infections is emphasized.

Based on CSF abnormalities alone, it was not possible to rule out CNS infections with confidence. Of note, two patients with CNS infection have neither elevated CSF leucocyte counts nor elevated CSF protein concentrations. These patients had rhinoorbital- cerebral mucormycosis and HHV6 encephalitis,

Conditions that have previously been reported as being associated with normal CSF parameters.[19,20] These infections primarily invade the brain, resulting in encephalitis or cerebritis without causing meningitis and CSF changes. If such diagnoses are suspected, combining cranial imaging (magnetic resonance) and CSF microbiological examination will often lead to the correct diagnosis. Low or normal CSF leucocyte count has been described in patients with bacterial meningitis, tuberculous meningitis, and depressed cell-mediated immunity, like elderly and patients with HIV.[21-23] In herpes simplex encephalitis, normal CSF parameters have been described in both immunocompetent and immunocompromised patients.[24] Therefore, if the clinical suspicion of a non-surgical nosocomial CNS infection remains high, especially in immunocompromised patients, repeated microbiological tests on CSF and/or radiological investigations should be considered despite a normal CSF leucocyte count. Awaiting further testing, empiric antibiotic treatment covering bacterial and viral causes should be considered.

Even though infection is confirmed in a substantial proportion of patients suspected of having non-surgical nosocomial CNS infections, most patients have alternative diagnoses, including systemic infection without CNS involvement arising from other sites, and non-infective neurological conditions such as stroke, metabolic encephalopathy, or epilepsy. These numbers seem comparable to those of suspected community-acquired

CNS infections.[6] Other sources of infection should therefore be investigated during diagnostic work-up, as well as other neurological diseases depending on the course of the disease.

Our study has several limitations. First, as this was an observational study, not all patients underwent the exact same diagnostic work-up (apart from the lumbar puncture), which could have led to missing diagnoses; however, this does reflect clinical practice. Also, since the final diagnosis was scored by physicians, patients might have been misclassified. Furthermore, for some of the CNS infections in this cohort, we cannot exclude the possibility of being late-diagnosed or chronic community-acquired CNS infections, for example in cerebral toxoplasmosis. However, the definition of nosocomial meningitis we used was based on previous research, and again reflects real-life clinical practice. Finally, 95% of the included patients were from a tertiary care facility, representing a complex patient population. Generalizability of our results to secondary care facilities should therefore be investigated in future research.

In conclusion, of all patients suspected of non-surgical nosocomial CNS infections, 14% were eventually diagnosed as non-surgical nosocomial CNS infection. The differential diagnosis is broad, and within the group diagnosed with CNS infections, various pathogens were detected. Furthermore, we found that CSF examination changes clinical management in 47% of patients. Therefore, lumbar puncture, together with extensive investigation of CSF samples, should be undertaken in all patients with suspected non-surgical nosocomial CNS infections.

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Conflicts of interest

All authors declare that they have no conflict of interest.

Ethics statement

This study was approved by the Biobank Ethical Review Committee of the Amsterdam UMC (number METC 2014_290). Written informed consent was obtained from all participants or their representatives.

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Author contributions

IvZ, LtH, and CP contributed to data gathering. IvZ, CP, and MB contributed to the methodology, data analyses, data interpretation, and writing the first draft of the manuscript. DvdB and MB contributed to study design, data gathering, review of the report, and interpretation of data.

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SUPPLEMENTARY DATA

Table A1. Diagnostic accuracy of clinical parameters for the diagnosis of non-surgical nosocomial CNS infections

	Non-surgical nosocomial CNS infections (n=16)		Other final diagnosis (n=98)		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
	Present	Absent	Present	Absent				
1. Abnormalities in NE [a]	9/16	7/16	70/96	26/96	56% (30-80)	27% (19-37)	11% (8-17)	79% (66-88)
2. CSF abnormalities [b]	14/16	2/16	43/98	55/98	88% (62-98)	56% (46-66)	25% (20-30)	96% (88-99)
3. CSF leukocytosis [c]	13/16	3/16	26/98	72/98	81% (54-96)	73% (64-82)	33% (25-43)	96% (90-99)
4. CSF elevated protein [d]	10/16	6/16	29/98	69/98	63% (35-85)	70% (60-79)	26% (17-36)	92% (86-96)
1 or 2	16/16	0/16	82/97	15/97	100% (79-100)	15% (9-24)	16% (15-18)	100% (78-100)

PPV= positive predictive value, NPV= negative predictive value. [a] Neurological examination (NE); scored as abnormal in case of altered consciousness or behavior, focal abnormalities (aphasia, ataxia, cranial nerve palsies or paresis) or Babinski signs. [b] Pleiocytosis or elevated protein. [c] CSF pleiocytosis >4x10^6/L; [d] CSF elevated protein ≥0.6 g/L.

CHAPTER 9

SEIZURES IN ADULTS WITH SUSPECTED CENTRAL NERVOUS SYSTEM INFECTION

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ABSTRACT

Background

Seizures can be part of the clinical presentation of central nervous system (CNS) infections. We describe patients suspected of a neurological infection who present with a seizure and study diagnostic accuracy of clinical and laboratory features predictive of CNS infection in this population.

Methods

We analyzed all consecutive patients presenting with a seizure from two prospective Dutch cohort studies, in which patients were included who underwent cerebrospinal fluid (CSF) examination because of the suspicion of a CNS infection.

Results

Of 900 episodes of suspected CNS infection, 124 (14%) presented with a seizure. The median age in these 124 episodes was 60 years (IQR 45-71) and 53% of patients was female. CSF examination showed a leukocyte count ≥ 5/mm^3 in 41% of episodes. A CNS infection was diagnosed in 27 of 124 episodes (22%), a CNS inflammatory disorder in 8 (6%) episodes, a systemic infection in 10 (8%), other neurological disease in 77 (62%) and in 2 (2%) episodes another systemic disease was diagnosed. Diagnostic accuracy of clinical and laboratory characteristics for the diagnosis of CNS infection in this population was low. CSF leukocyte count was the best predictor for CNS infection in patients with suspected CNS infection presenting with a seizure (area under the curve 0.94, [95% CI 0.88 – 1.00]).

Conclusions

Clinical and laboratory features fail to distinguish CNS infections from other causes of seizures in patients with a suspected CNS infection. CSF leukocyte count is the best predictor for the diagnosis of CNS infection in this population.

BACKGROUND

Patients suspected of a central nervous system (CNS) infection often pose a diagnostic dilemma.[1] The differential diagnosis can be broad, and the diagnostic accuracy of clinical and laboratory features in this group is insufficient to differentiate between neurological infections and other diagnoses.[2] Seizures can be part of the clinical presentation of a CNS infection and have been described in approximately a quarter of all patients[3], with frequencies ranging from 7 to 28% in bacterial meningitis[4-8] and from 40 to 75% in herpes simplex virus (HSV) encephalitis.[9-12] Pediatric studies have focused on how to identify patients with a CNS infection from cohorts of patients presenting with a first seizure and fever.[13,14] A meta-analysis of 1996 patients showed that the risk of bacterial meningitis in this population is low (2.6%).[15] However, characteristics predictive for bacterial meningitis could not be identified. Studies also show that an elevated CSF leukocyte count, previously identified as the strongest predictor of CNS infections, can be found in 10% of children presenting with seizures and no CNS infections.[16] In this study we aim to identify the diagnostic accuracy of clinical and laboratory characteristics for the diagnosis of CNS infection in patients suspected of a CNS infection who present with a seizure.

METHODS

Patient inclusion and data collection

We included adult patients (≥16 years of age) with a clinically suspected CNS infection who underwent CSF examination. Patients were included in two prospective cohort studies. The first study (September 2012 – February 2015) was a single center pilot study. The second study is an ongoing (September 2017 – now) multicenter cohort study in the Netherlands. Patients who were eligible for inclusion were reported to the investigators by the treating physician or identified during morning rounds. We obtained written informed consent from all participating patients or their legal representatives. We excluded patients with recent (≤ 1 month) head injury or neurosurgery, and patients with neurosurgical devices. Online case record forms (CRF) were used to collect data on patients' characteristics and medical history, symptoms at presentation, laboratory results, radiological imaging, antibiotic or antiviral treatment, and outcome. The CRF included a standard question on the presence or absence of seizures on admission, as well as the type of seizure.

All patient data was rendered anonymous and the study was carried out in accordance with Dutch privacy legislation. The study was approved by the biobank ethics committee

of the Amsterdam UMC, location AMC, Amsterdam, The Netherlands (number BTC AMC2014_290).

Procedures and definitions

Seizures were classified according to seizure type into focal onset, generalized onset or unknown onset using the International League Against Epilepsy classification.[17] Seizures without an identifiable cause were defined as seizures of uncertain etiology, in literature also known as idiopathic seizures, cryptogenic seizures or unprovoked seizures. Hospital-acquired disease was defined as an episode of (suspected) CNS infection occurring during admission (>48h after presentation) or within one week after discharge. Other episodes were considered community-acquired. Patients were considered to be immunocompromised if they were using immunosuppressive drugs or had a medical history of diabetes mellitus, auto-immune disease, alcoholism, human immunodeficiency virus (HIV) infection or splenectomy. The Glasgow Coma Scale (GCS) score was used to assess level of consciousness at presentation.[18] Patients with a GCS score of ≤14 were considered to have an altered mental status, and a GCS score of ≤8 indicated coma. In patients who underwent cranial imaging, modality (CT or MRI) and cranial abnormalities were documented in the CRF. Glasgow Outcome Scale (GOS) was used to score the outcome at time of discharge, with scores ranging from 1 to 5, indicating the following outcome: 1 death; 2 persistent vegetative state; 3 severe disability; 4 moderate disability and 5 good recovery. A score from 1-4 on the GOS was defined as an unfavorable outcome and a score of 5 was defined as a favorable outcome.[19]

Diagnostic categorization

The final diagnosis of all episodes was classified according to the following five categories, 1) CNS infection, 2) CNS inflammation, 3) systemic infection, 4) other neurological disease, 5) other systemic disease. The rationale and methods of this categorization have been described previously.[2] Two clinicians independently categorized all episodes and differences were resolved by consultation of a third clinician. Inter-rater agreement was assessed by calculation of the kappa coefficient with a Kappa of 0.76 in the first study and 0.64 in the second study.

Statistical analysis

Statistical analyses were conducted with the use of SPSS statistical software, version 26 (SPSS, Inc.). We used descriptive statistics for baseline characteristics with medians and interquartile range (IQR). Continuous data were compared with the Mann-Whitney U test. For categorical data the Fisher's exact test was used. The area under the curve (AUC) of receiver operator characteristics (ROC), sensitivity, specificity, positive

predictive value (PPV) and negative predictive value (NPV) were used to evaluate diagnostic accuracy of clinical and laboratory characteristics. All tests were 2-tailed, and P <0.05 was considered significant.

RESULTS

We included a total of 900 episodes with suspected CNS infection. Of these episodes, 124 (14%) presented with a seizure of whom 93 of 121 (77%) were evaluated at the emergency department, 12 (10%) in the intensive care unit and 16 episodes (13%) in a hospital ward. Community acquired CNS infection was suspected in 112 of 124 episodes (90%), and a nosocomial CNS infection in 12 out of 124 (10%). The median age was 60 years (IQR [45-71]) and 66 (50%) of the patients were female (Table 1). Of all episodes, 53 (43%) were immunocompromised, most often due to diabetes mellitus (23 episodes, 19%) and due to the use of immunosuppressive medication (17 episodes, 14%). A history of epilepsy was present in 31 episodes (25%), of which 11 (35%) were previously diagnosed with epileptic seizures of uncertain etiology.

Symptoms were present for less than 24 hours in 83 out of 124 episodes (67%). The most common presenting feature was an altered mental status (107 of 123 [86%]). Headache was reported in 33 of 91 (36%) episodes, fever in 41 of 122 (33%) and neck stiffness in 11 of 96 (11%). Focal neurological deficits were present in 53 of 124 (43%) episodes and included aphasia (15 episodes, 12%), cranial nerve palsy (11 episodes, 8%), paresis (46 episodes, 37%), ataxia (1 episode, 1%) and pathological reflexes (20 episodes, 16%).

Ancillary investigations

Cranial imaging (Computed Tomography [CT] or Magnetic resonance imaging [MRI]) at presentation was performed in 118 out of 124 (95%) episodes and showed abnormalities in 69 of 117 (59%) scans. Non recent vascular lesions were the most common abnormality and were found in 25 of 69 (36%) scans. Other abnormalities included (semi) recent infarction (6 episodes, 9%), mastoid and sinus opacification (5 episodes, 7%), generalized edema (3 episodes, 4%) and hydrocephalus (2 episodes, 3%). Electroencephalogram (EEG) was performed during or after admission in 54 of 124 (44%) of episodes and showed abnormalities consistent with epilepsy in 24 (44%) episodes.

Table 1. Clinical characteristics, laboratory parameters and outcome of 124 patients with suspected neurological infections presenting with seizures [†]

Characteristic	n/N (%)	Characteristic	n/N (%)	Characteristic	n/N (%)
Median age (IQR), years	60 (45-71)	Score Glasgow Coma Scale[‡]	11 (7-14)	Outcome	
Immunocompromised state	53/124 (43)	Altered mental status ≤14	107/123 (86)	Death	17/124 (14)
Diabetes	23/124 (19)	Coma ≤8	42/123 (34)	Unfavorable	63/124 (51)
Alcoholism	14/124 (11)	Neck stiffness	11/96 (11)	Good recovery	61/124 (49)
Immunosuppressive therapy	17/124 (14)	Type of seizure		Final diagnostic category	
HIV positive	7/124 (6)	Generalized	70/104 (67)	CNS infection	27/124 (22)
History of epilepsy	31/124 (25)	Focal	20/104 (19)	CNS inflammatory disease	8/124 (6)
Symptomatic epilepsy	20/31 (65)	Both	14/104 (13)	Systemic infection	10/124 (8)
Duration of symptoms		Blood chemistry[§]		Other neurological disease	77/124 (62)
< 24 hours	83/115 (72)	C-reactive protein (CRP)	10 (3 – 49)	Other systemic disease	2/124 (2)
		>5 mg/L	72/113 (64)		
Presenting symptoms		>40 mg/L	32/113 (26)		
Headache	33/91 (36)	Leukocytes	11.6 (7.7 – 15.2)		
Vomiting or nausea	26/94 (28)	>10.5 x 10^9/L	71/123 (57)		
Diarrhea	4/78 (5)	CSF examination[¶]			
Clinical signs		Opening pressure (cm H_2O)	19 (15-26)		
Fever (>38.0)	41/122 (33)	CSF leukocytes (/mm3)	3 (3-11)		
Hypotension (diastolic BP <50 mm Hg)	10/122 (8)	CSF leukocytes ≥ 5/mm3	51/123 (41)		
Tachycardia (HF >120 bpm)	17/122 (14)	CSF leukocytes > 100/mm3	16/123 (13)		
		CSF protein (g/L)	0.43 (0.33-0.78)		
		Blood to CSF glucose ratio	0.55 (0.44-0.65)		

[†] Data are n/N (%) or median (interquartile range)

[‡] Glasgow Coma scale score was known for 123 patients

[§] CRP was known for 114 episodes, blood leukocytes for 123 episodes.

[¶] Lumbar puncture opening pressure was known for 80 episodes, CSF leukocyte count for 123 episodes, CSF protein concentration for 122 episodes, CSF to blood glucose ratio for 117 episodes.

Lumbar puncture was performed in all patients. The opening pressure was measured in 80 of 124 episodes (65%) and showed a median pressure of 19 cm H_2O (IQR 15-26). An opening pressure of ≥ 20 cm H_2O was observed in 39 (49%) episodes, and in 3 (4%) episodes a pressure of ≥40 cm H_2O was measured. Median CSF leukocyte count was 3/mm^3 (IQR 3-11). Elevated leukocyte count (≥ 5mm^3) was present in 51 of 123 (41%) episodes and 16 of 123 (13%) episodes showed a leukocyte count of >100/mm^3. CSF protein levels of >0.6 g/L were present in 43 of 122 (35%) episodes and a decreased CSF to blood glucose ratio (<0.6) was found in 72 of 117 (62%) episodes. Of all patients, 23 had a final diagnosis of epileptic seizures of uncertain etiology (19%) of which 2 (9%) had a leukocyte count ≥ 5/mm^3, presenting with a CSF leukocyte count of 6 and 16/mm^3 (Table 2). These elevated counts could be explained by an elevated blood leukocyte count of 28.9 x 10^9/L, and blood admixture during the lumbar puncture resulting in an red blood cell count of 17,000/mm^3, respectively.

Table 2. CSF examination in 23 patients with epileptic seizures of uncertain etiology[†]

Characteristics	n/N(%)
Opening pressure (cm H_2O) [‡]	16 (11-19)
CSF leukocytes (per mm^3)	1 (1-2)
CSF leukocytes ≥ 5/mm^3	2/23 (9)
CSF leukocytes >100/mm^3	0/23 (0)
CSF protein (g/L)	0.37 (0.33-0.42)
CSF protein > 0.6 g/L	4/23 (17)
Blood to CSF glucose ratio	0.55 (0.50-0.60)
Ratio < 0.6	16/23 (70)

[†] Data are n/N (%) or median (interquartile range)
[‡] Lumbar puncture opening pressure was known for 14 episodes, CSF leukocyte count, CSF protein and CSF to blood glucose ratio for 23 episodes

CSF culture was performed in 92 of 124 (74%) episodes and was positive in 5 episodes (5%). Polymerase chain reaction (PCR) for viral and bacterial DNA in CSF was performed in 92 of 124 (74%) and was positive in 12 episodes (13%) of which 2 positive Epstein-Barr virus PCRs were judged to be clinically not relevant. CSF Cultures and PCR were not performed if the suspicion of a CNS infection was no longer present after the lumbar puncture, i.e. if an alternate condition was diagnosed or if the suspicion was low prior to the lumbar puncture and the CSF examination showed no leukocytosis.

Antiviral or antibiotic treatment was started in 97 out of 124 episodes (78%). Of these, 59 received (61%) antibiotics according to bacterial meningitis protocol and 16 (16%) patients received monotherapy of acyclovir. In 25 episodes, (20%), the patients received

both acyclovir and antibiotics. For 92 of 122 (75%) episodes, patients were treated with anti-epileptic drugs during admission, of which 30 out of 92 (33%) had been using antiepileptic drugs before admission.

Final diagnosis and outcome

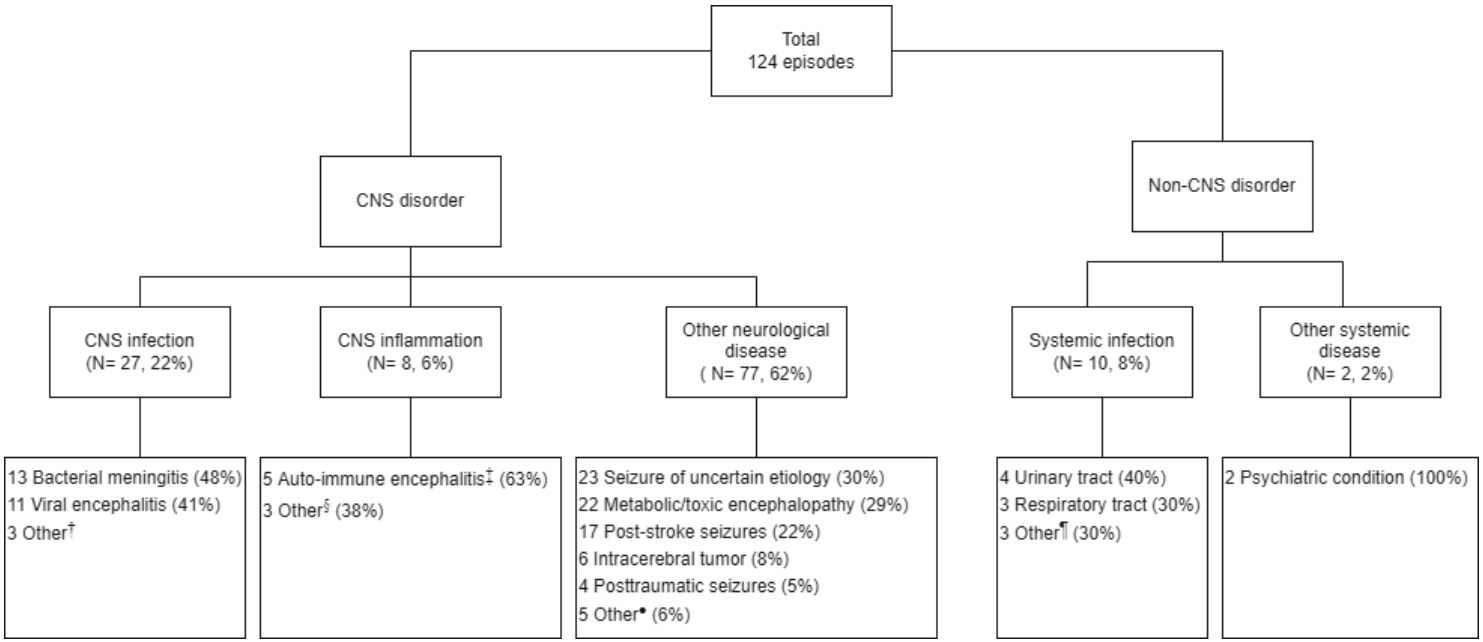
A CNS infection was diagnosed in 27 of 124 episodes (22%; Table 1), most commonly bacterial meningitis (13 episodes, 10%) and viral encephalitis (11 episodes, 9%; Fig 1). Overall, the causative pathogen was found in 17 out of 27 episodes (63%) of CNS infections. In bacterial meningitis the causative bacteria were identified in CSF (culture or PCR) or blood in 6 of 13 episodes (46%): *Streptococcus pneumoniae* in 5 episodes (38%) and *Streptococcus anginosus* in 1 episode (8%). The causative virus in viral encephalitis was found in 7 out of 11 episodes (64%), HSV in 4 episodes (50%), varicella zoster (VZV) in 2 episodes (25%) and John Cunningham (JC) virus in 1 episode (13%).

CNS inflammation was diagnosed in 8 episodes (6%), of which 5 (4%) were diagnosed with auto-immune encephalitis. Other neurological diagnosis were made in 77 (62%) episodes, most commonly seizures of uncertain etiology (23 episodes, 19%), metabolic or toxic encephalopathy (22 episodes, 18%), post-stroke seizures (17 episodes, 7%), and intracerebral tumors (6 episodes, 5%). In 10 episodes (8%) a systemic infection was diagnosed, most often urinary tract infections (4 episodes, 3%) and respiratory tract infections (3 episodes, 2%). Two episodes (2%) were diagnosed with another systemic disease. These patients initially presented with an episode highly suspicious for an epileptic seizure, but were both ultimately diagnosed with pseudo-epilepsy.

Outcome was known for all episodes: 63 patients (51%) had an unfavorable outcome, of which 17 (14%) died during admission. (Table 1, 3) An unfavorable outcome most commonly occurred in patients with CNS inflammation (8 of 8 episodes, 100%). Outcome in patients diagnosed with CNS infection did not differ from patients with another final diagnosis (p=0.39).

Patients presenting with a new-onset seizure were more often diagnosed with CNS infection or inflammation (31 of 93 [33%]) than patients with a history of seizures (4 of 31 episodes [13%], p=0.04) (Table 4).

Figure 1. Diagnosis in patients with suspected neurological infections presenting with seizures



† 1 Neurocysticercosis, 1 cerebral toxoplasmosis, 1 cerebral Whipple's disease, ‡ 1 anti-N-methyl-D-aspartate (NMDA), 1 anti-Leucine-Rich Glioma-Inactivated1 (LGI1) and 3 seronegative, § 1 cerebral vasculitis, 1 neuro systemic lupus erythematosus (SLE), 1 Acute disseminated encephalomyelitis (ADEM), • 3 status post CNS infection, 1 hypertensive encephalopathy, 1 encephalopathy due to thrombotic thrombocytopenic purpura. ¶ 1 skin/soft tissue infection, 1 abdominal infection, 1 bacteremia.

Table 3. Clinical presentation, laboratory characteristics and outcome per disease category[†]

Characteristic	CNS infection (N = 27)	CNS inflammation (N = 8)	Systemic infection (N = 10)	Other neurological disease (N = 77)	Other systemic disease (N = 2)
Headache	10/20 (50)	5/6 (83)	2/6 (33)	15/57 (26)	1/2 (50)
Neck stiffness	5/20 (25)	3/7 (43)	2/9 (22%)	1/58 (2%)	0
Fever	15/27 (56)	2/7 (29)	5/10 (50)	19/76 (25)	0
Predisposing infection	5/27 (19)	0	2/10 (20)	4/77 (52)	0
Altered mental status	24/27 (89)	5/8 (63)	7/10 (70)	69/76 (91)	2/2 (100)
Coma	12/27 (44)	1/8 (13)	1/10 (10)	28/76 (37)	0
Blood leukocytosis (>10.5)	15/27 (56)	4/8 (50)	7/10 (70)	44/76 (58)	1/2 (50)
CRP, median	54 (4-270)	3 (1-27)	41 (9-192)	7 (2-33)	4 (-)
CRP > 4	19/26 (73)	3/7 (43)	7/9 (78)	43/70 (61)	0
CRP > 40	13/26 (50)	1/7 (14)	5/9 (56)	13/70 (19)	0
CSF leukocytes count, median	112 (36-684)	7 (2-37)	1 (1-2)	2 (1-5)	2 (-)
≥ 5/mm3	24/26 (92)	4/8 (50)	0	23/77 (30)	0
>100/mm3	15/26 (60)	1/8 (13)	0	0	0
Unfavorable outcome	16/27 (59)	8/8 (100)	5/10 (50)	33/77 (43)	1/2 (50)
Death	8/27 (30)	1/8 (13)	2/10 (20)	6/77 (8)	0

[†] Data are n/N (%) or median (interquartile range).

Table 4. Clinical and laboratory features, diagnostic category and outcome in 93 patients with a first seizure and 31 patients with a history of seizures[†]

Characteristics	First seizure (N=93)	History of seizures (N=31)	p-value
Age	59 (57-61)	63 (60-66)	0.954
Immunocompromised state	41/93 (44)	12/31 (39)	0.678
Duration of symptoms <24h	63/93 (68)	20/31 (65)	0.443
Focal neurologic deficits	32/93 (34)	21/31 (68)	0.002**
CSF leukocytes ≥ 5/mm3	28/92 (30)	13/31 (42)	1.000
Final diagnosis of CNS infection or inflammation	31/93 (33)	4/31 (13)	0.037*
Unfavorable outcome	43/93 (46)	20/31 (65)	0.098

[†] Data are n/N (%) or median (interquartile range), * P ≤ 0.05, ** P ≤ 0.01

Prediction of diagnosis – diagnostic accuracy

There were no distinctive differences between diagnostic groups with regard to clinical, laboratory and radiological features (Table 3, 5). Of all CNS infection episodes, 10 out of 20 (50%) presented with headache. Neck stiffness was found in 5 of 20 (25%) episodes of CNS infection, but was also found in CNS inflammation, systemic infections and other neurological diseases. In 15 of 27 (56%) episodes of CNS infection there was a fever upon presentation.

CSF leukocytosis ≥ 5/mm^3 was present in 24 of 26 (92%) episodes of CNS infection, 4 of 8 (50%) in CNS inflammation and in 23 of 77 (30%) of other neurological disease episodes. CSF leukocytosis ≥ 5/mm^3 was not present in patients with a systemic infection or other systemic disease. The specificity of CSF leukocytosis >5/mm^3 for distinguishing all CNS disorders (CNS infection, CNS inflammation and other neurological diseases) from all non-CNS disorders (systemic infection and other systemic disease) was high, but with low sensitivity (sensitivity 46%, 95% CI 36-56%; specificity 100%, 95% CI 74-100%). CSF leukocytosis >100/mm^3 had a high specificity but low sensitivity for differentiating CNS infections from other diagnosis (sensitivity 58%, 95% CI 37- 77%; specificity 99%, 95% CI 94-100%; Table 5). CSF leukocytosis >100/mm^3 was present in 15 of 26 (60%) episodes of CNS infection, and in 1 of 8 (13%) episodes in de CNS inflammation group. CSF leukocytosis >100 mm^3 was not present in any of the other diagnostic groups. Both patients who were diagnosed with a CNS infection but had a CSF leukocyte below the threshold of 5/mm^3 were HIV positive, and suffered from cerebral toxoplasmosis and progressive multifocal leukoencephalopathy (CD4 count respectively 120 and 34 x10^6/l, viral load respectively 1984 and 17600 copies/ml).

Table 5. Test characteristics of clinical and laboratory characteristics

	Neurological infection		Other diagnoses		Sens (95%CI)	Spec (95%CI)	PPV (95%CI)	NPV (95%CI)
	Present	Absent	Present	Absent				
Headache	10	10	23	48	50% (27% - 73%)	68% (55% – 78%)	30% (20% - 43%)	83 (75% - 88%)
Nausea/vomiting	6	15	20	53	29% (11% - 52%)	73% (61% - 82%)	23% (12% - 40%)	78% (72% - 83%)
Immunocompromised	10	17	43	54	37 (19% – 57%)	56 (45% - 66%)	19% (12% - 29%)	76% (69% - 82%)
Altered mental status (GCS ≤ 14)	24	3	83	13	89% (71% - 98%)	14% (7% - 22%)	22% (20% - 25%)	81% (57% - 94%)
Coma (GCS ≤ 8)	12	15	30	66	44 % (25% - 65%)	69% (58% - 78%)	29% (19% - 40%)	81% (75% - 86%)
Neck stiffness	5	15	6	70	25% (8% - 49%)	92% (84% - 97%)	45% (22% - 71%)	82% (78% - 85%)
Generalized seizure	16	8	54	35	67% (45%-84%)	39% (29% - 59%)	23% (18% - 29%)	81% (70% - 89%)
Diast BP < 50 mmHg	3	24	7	88	11% (2% - 29%)	93% (85% - 97%)	30% (11% - 61%)	79% (66% - 82%)
Tachycardia	7	20	10	85	26% (11% - 46%)	89% (81% - 95%)	41% (23% - 62%)	81% (77% - 84%)
Fever > 38.5 ˚C	15	12	26	69	56% (35% - 75%)	73% (63% - 81%)	37% (27% - 48%)	85% (79% - 89%)
Focal neurological abnormalities	8	19	45	52	30% (14% - 50%)	54% (43% - 64%)	15% (9% - 25%)	73% (67% - 79%)
Blood leukocytose (≥ 10.5)	15	12	56	40	56% (36% - 75%)	42% (32% - 52%)	21% (16% - 28%)	77% (67% - 84%)
CRP > 5 mg/L	19	7	53	34	73% (52% - 88%)	39% (29% - 50%)	26% (21% - 32%)	83% (71% - 91%)
CRP > 40 mg/L	13	13	19	68	50% (30% - 70%)	78% (68% - 86%)	41% (28% - 54%)	84% (78% - 89%)
CSF leukocytes ≥ 5/mm^3	24	2	27	70	92% (75% - 99%)	72% (62% - 81%)	47% (39% - 56%)	97% (90% - 99%)
CSF leukocytes > 100/mm^3	15	11	1	96	58% (37% - 77%)	99% (94% - 100%)	94% (67% - 99%)	90% (85% - 93%)
CSF protein > 0.6 g/L	18	9	25	72	67% (46% - 83%)	74% (74% - 83%)	42% (32% - 53%)	89% (82% - 93%)
CSF protein > 2g/L	8	19	3	94	30% (14% - 50%)	97% (91% - 99%)	72% (43% - 90%)	83% (79% - 86%)
CSF pressure > 22 mm H_2O	7	3	21	49	70% (35% - 93%)	70% (58% - 80%)	25% (16% - 36%)	94% (86% - 98%)
CSF:blood glucose ratio < 0.6	21	4	53	37	84% (64% - 95%)	41% (31% - 52%)	28% (24% - 34%)	90% (78% - 95%)

For single predictors, the AUC for predicting CNS infection was 0.94 (95% CI 0.88 – 1.00) for CSF leukocytes, 0.81 (95% CI 0.70 – 0.91) for CSF total protein and 0.74 (95% CI 0.63 – 0.85) for CSF-blood glucose ratio. Combining these individual predictors did not substantially increase the diagnostic accuracy compared to CSF leukocyte count (AUC 0.96 [95% CI 0.93 – 1.00]).

DISCUSSION

Our study showed that 22% of episodes with suspected CNS infections presenting with a seizure was diagnosed with a CNS infection. The incidence of CNS infection as cause of acute symptomatic seizures has not been well established and has only been studied in retrospective cohorts studying acute symptomatic seizures. In these cohorts the proportion of patients in whom CNS infection was the cause of the seizure ranged from 15 to 28%, with a higher incidence in countries where neurotuberculosis and neurocysticercosis are endemic.[20,21] Other common causes of acute symptomatic seizures are alcohol/drugs use or abstinence, brain tumors, neuroinflammatory diseases, traumatic head injury and cerebrovascular disease.[20-23] Differentiating between these causes can pose a diagnostic challenge. Our study shows that the diagnostic accuracy of most clinical characteristics and laboratory features for the diagnosis of CNS infection was low.

CSF leukocyte count was the best predictor for CNS infections with an AUC of 0.94, but lacked specificity. CSF leukocytosis was present in 92% of episodes with a CNS infection, but in 28% of other diagnosis as well. Only 2 patients without an elevated CSF leukocyte count were finally diagnosed with a CNS infection. Both patients were HIV infected and suffered from HIV-associated opportunistic infections. The patients in our study were diagnosed with a cerebral *Toxoplasma gondii* infection and progressive multifocal leukoencephalopathy (PML). As these infections are primarily located intracerebrally, CSF examination is often not diagnostic for these diseases as CSF parameters can be within normal limits.[24-26] A normal CSF leukocyte count in non-HIV patients with a CNS infection was not encountered, and ruled out CNS infection in our study population.

One third of patients presenting with seizures but without CNS infection had an elevated CSF leukocyte count. These patients were diagnosed with a range of different disorders, such as post-stroke epilepsy, seizures due to intracerebral tumors or metabolic disturbances. CSF leukocytosis has been reported in these conditions, independently of the presence of epileptic seizures.[27-29] In the current study, CSF changes in epileptic seizures of uncertain etiology were uncommon. Only 2 of 23 episodes with a final

diagnosis of epileptic seizures of uncertain etiology had an elevated CSF leukocyte count, both of which could be explained by external factors (blood leukocytosis and blood admixture). The hypothesis that epileptic seizures of uncertain etiology cause CSF leukocytosis due to ictal activity alone has been mostly supported by studies conducted in the 1980s.[30-33] These studies found an incidence ranging from 11%-30% of CSF leukocytosis in epileptic seizures of uncertain etiology. More recent studies have shown that CSF leukocytosis in this group is very rare and that in most cases an underlying cause for the elevated leukocyte count is found.[34-38] This difference can be explained by a number of factors. First, diagnostic options when the initial studies were conducted were limited compared to today. MRI and PCR were not or only scarcely available, which might have led to an incorrect diagnosis of epileptic seizure of uncertain etiology. Furthermore, the definition of leukocytosis differed. Some studies regarded a CSF polymorphonuclear leukocyte count of > 0 as leukocytosis,[30,31,33] while in current practice a leukocyte count of ≥5 is generally defined as leukocytosis, regardless of leukocyte type. [39] This has led to an overestimation of the proportion of patients with seizure of uncertain etiology and CSF leukocytosis. Finally, inclusion and exclusion criteria were not always clear and some of the less recent studies excluded patients with symptomatic seizures, caused by infection, stroke or trauma.[31,32] Our results confirm the more recent studies, and therefore CSF leukocytosis in patients with seizures and suspected CNS infection should prompt further search for the underlying cause as it cannot be attributed to seizure activity alone.

There were several limitations to our study. First, in our study we only included patients who underwent CSF examination. Patients who presented with a seizure and where cranial imaging revealed a probable cause of the seizure are unlikely to undergo a lumbar puncture and were therefore not included in our study. Also, in patients presenting with a seizure without other signs of a CNS infection a lumbar puncture is not routinely performed. This means that CNS infections could have been missed. Furthermore, the presence of an epileptic seizure was diagnosed by the treating physician by a compatible anamnesis or observation of a seizure. Previous studies showed that 8-29 % of patients presenting to the emergency room with clinically suspected seizures are eventually classified as having Psychogenic Non-epileptic Seizures (PNES).[40-42] In our study only two patients received a final diagnosis of PNES. Potentially, more patients were misclassified as having a seizure. However, as an altered mental status was present in a large proportion of patients (post-ictal phase) which is more common in epileptic seizures than in PNES[43,44], it is unlikely that this considers a substantial number of patients. Lastly, in this study approximately 5-10% of all patients eligible for inclusion did not give consent for participation. Considering this small proportion, we assume that selection bias did not influence results.

CONCLUSIONS

In conclusion, in patients suspected of a CNS infection presenting with a seizure, approximately one in five was diagnosed with a CNS infection, and almost half showed elevated CSF leukocyte count. CSF changes in epileptic seizures of uncertain etiology were uncommon and could not be attributed to ictal activity alone. The best predictor for CNS infection in this population was CSF leukocyte count, and diagnostic accuracy of other clinical and laboratory features was low. Therefore, these characteristics cannot be used to rule out CNS infection.

Ethics approval and consent to participate

The study was approved by the biobank ethics committee of the Amsterdam UMC, location AMC, Amsterdam, The Netherlands (number BTC AMC2014_290). We obtained written informed consent from all participating patients or their legal representatives. All patient data was rendered anonymous and the study was carried out in accordance with Dutch privacy legislation.

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SO contributed to data collection, data analysis, data interpretation and writing the first draft of the manuscript. IvZ contributed to study design, data gathering and critique of the report. LtH contributed to data gathering and critique of the report. DvdB contributed to review and critique of the report. MB contributed to study design, data interpretation, review and critique of the report.

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CHAPTER 10

PREDICTORS OF UNFAVOURABLE OUTCOME IN ADULTS WITH SUSPECTED CENTRAL NERVOUS SYSTEM INFECTIONS: A PROSPECTIVE COHORT STUDY

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ABSTRACT

Suspected central nervous system (CNS) infections may pose a diagnostic challenge, and often concern severely ill patients. We aim to identify predictors of unfavourable outcome to prioritize diagnostics and treatment improvements. Unfavourable outcome was assessed on the Glasgow Outcome Scale at hospital discharge, defined by a score of 1 to 4. Of the 1152 episodes with suspected CNS infection, from two Dutch prospective cohorts, the median age was 54 (IQR 37-67), and 563 episodes (49%) occurred in women. The final diagnoses were categorized as CNS infection (N=358 episodes, 31%), CNS inflammatory disease (N=113, 10%), non-infectious non-inflammatory neurological disorder (N=388, 34%), non-neurological infection (N=252, 22%), and other systemic disorder (N=41, 4%). Unfavourable outcome occurred in 412 of 1152 (36%), and 99 died (9%). Predictors for unfavourable outcomes included advanced age, absence of headache, tachycardia, altered mental state, focal cerebral deficits, cranial nerve palsies, low thrombocytes, high CSF protein, and the final diagnosis of CNS inflammatory disease (odds ratio 4.5 [95% confidence interval 1.5-12.6]). Episodes suspected of having a CNS infection face high risk of experiencing unfavourable outcome, stressing the urgent need for rapid and accurate diagnostics. Amongst the suspected CNS infection group, those diagnosed with CNS inflammatory disease have the highest risk.

INTRODUCTION

Patients suspected of a central nervous system (CNS) infection often present with severe illness, including decreased consciousness, neurological deficits and hemodynamic instability.[1] Diagnosing these patients frequently poses a challenge due to the wide range of possible conditions, ranging from life-threatening diseases such as bacterial meningitis or septic encephalopathy to more benign and sometimes self-limiting diseases such as migraine or systemic viral infections.[1] Previous studies have reported an overall mortality of 10% and incomplete recovery in an additional 17% in this population.[1,2] Prompt diagnostic work-up, identification of the cause-specific diagnosis, and early targeted treatment have been shown to be crucial in improving outcome, particularly in patients with bacterial meningitis.[2-7] However, clinical characteristics and ancillary investigations often lack sensitivity and/or specificity to differentiate between these various causes, although cerebrospinal fluid (CSF) leukocyte count differentiated best between bacterial meningitis and other diagnoses in this population.[1] Difficulty in making the diagnosis may lead to delayed or unnecessary treatment with antibiotics and antiviral drugs. To improve outcome in this patient population, it is essential to recognize high-risk categories for unfavourable outcome. This prospective study aims to determine predictors for an unfavourable outcome to identify subgroups for enhancing diagnostics and treatment.

METHODS

Patient inclusion and data collection

We included episodes from two prospective cohort studies performed between 2012 and 2015 and between 2017 to 2022. The first study (PACEM – Paediatrics and Adult Causes of Encephalitis and Meningitis) was a single-centre study, and a pilot study for the second study (I-PACE – Improving Prognosis by using innovative methods to diAgnose Causes of Encephalitis), which is an ongoing multi-centre study running in 11 Dutch hospitals.[1] Both studies included adult patients aged 16 years or older with suspected CNS infection presenting to the emergency department or inpatients who underwent CSF examination. Episodes were identified during morning rounds or reported to the investigators by the treating physician. Physicians could contact the investigators 24/7 to include patients. Episodes of suspected CNS infections within three months after head trauma or neurosurgery, and those with a neurosurgical device in situ, were excluded.

Data on patient characteristics, medical history, symptoms and signs on admission, laboratory results, radiological examination, treatment and outcome were collected in online case record forms. All patients and/or their legal representatives have given written informed consent for this study after receiving written information about the study. All patient data were rendered anonymous, and the study was carried out in accordance with Dutch privacy legislation.

Procedures and definitions

Episodes were classified as suspected nosocomial CNS infection if the suspicion occurred during hospital admission (>48 hours after presentation) or within one week after discharge.[8,9] All other episodes were classified as community-acquired. Neurological examination was performed upon admission and at discharge. The level of consciousness was scored using the Glasgow Coma Scale (GCS).[10] An altered mental state was defined as a GCS score of <14 and coma as a GCS score of ≤8. Patients were considered immunocompromised if they were using immunosuppressive drugs or had a medical history of diabetes mellitus, alcoholism, HIV infection or a splenectomy.

Outcome at discharge was scored according to the Glasgow Outcome Scale (GOS), a well-validated scale ranging from 1 to 5. A score of 1 indicates death, 2 vegetative survival, 3 severe disability, 4 moderate disability, and 5 indicates mild or no disability.[10] A score of 5 was considered a favourable outcome. If pre-existing conditions were the cause of the outcome score below 5 on the GOS, and the patient's condition did not worsen due to the current episode, we classified the outcome as favourable.

Diagnostic categorization

The final diagnosis of all included episodes was classified into five categories, as previously described.[1] The categories were; 1) CNS infection, 2) CNS inflammatory disease, 3) non-infectious non-inflammatory neurological disorder, 4) non-neurological infection, and 5) other systemic disorders. Two clinicians independently classified the final diagnoses in the five categories based on all available clinical, laboratory and follow-up data. If there was no consensus, a third investigator was consulted. Inter-rater agreement between the first assessors was assessed by calculating the kappa coefficient, which was 0.76 in cohort 1 and 0.64 in cohort 2.

Statistical analysis

Statistical analyses were conducted using SPSS statistical software, version 28 (SPSS Inc.) and R studio version 4.0.3. We used descriptive statistics for baseline characteristics, with medians and interquartile range (IQR, describing their 25th to 75th percentile). Comparisons were made with the Mann-Whitney U test used for continuous data, and

the Fisher exact test was used for categorical data. All tests were 2-tailed, and P <0.05 was considered significant. We chose possible predictors of an unfavourable outcome based on previous research and availability to examine the predictor early upon disease presentation.[11] We investigated the association between these predictors and outcomes with logistic regression, providing odds ratios (ORs) and 95% CIs. Univariable and multivariable binary logistic regression models assessed prognostic factors for discharge outcomes. For these multivariable logistic models, missing values in the selected prognostic factors were imputed (median 2.1% per prognostic factor [IQR 0.33 – 8.8%]). Non-normally distributed continuous variables were transformed into categorical variables.

Standard protocol approvals, registrations and patient consents

The two studies were approved by the Biobank Ethics Assessment Committee of the Amsterdam UMC; number AMC 2014_290. Written informed consent was obtained from all participants or their representatives. All methods were performed in accordance with this approval.

Data availability

Anonymized data not published within this article will be made available by request from any qualified investigator. Proposals can be directed to the corresponding author, Matthijs Brouwer, by sending an email to ipace@amc.nl.

RESULTS

A total of 1165 episodes were included: 363 episodes in the PACEM study and 802 in the I-PACE study. Of these, 13 episodes (1%) were excluded based on exclusion criteria or missing outcome data (Figure 1), resulting in 1152 episodes in 1127 patients. Patients were evaluated at the emergency department in 861 of 1140 episodes (76%), at the intensive care in 59 (5%), and 220 (19%) at other clinical departments. The episode was classified as nosocomial in 106 of 1137 (9%).[8,9]

The median age was 54 years (IQR 37-67), and 563 episodes (49%) occurred in women (Table 1). An immunocompromising condition was present in 450 of 1151 episodes (39%), which was due to HIV infection in 74 of 1150 (6%), the use of immunosuppressive drugs in 208 of 1149 (18%), and diabetes mellitus in 188 of 1151 (16%; Table 1). In 417 of 1111 episodes (38%), symptoms were present for less than 24 hours. The most common symptoms included headache in 639 of 998 episodes (64%), fever in 466 of 1051 episodes (44%), and neck stiffness in 188 of 892 episodes (21%). An altered mental state was present in 364 of 1143 episodes (32%) and neurological deficits in 347 of 754 (46%).

Figure 1. Selection of patients

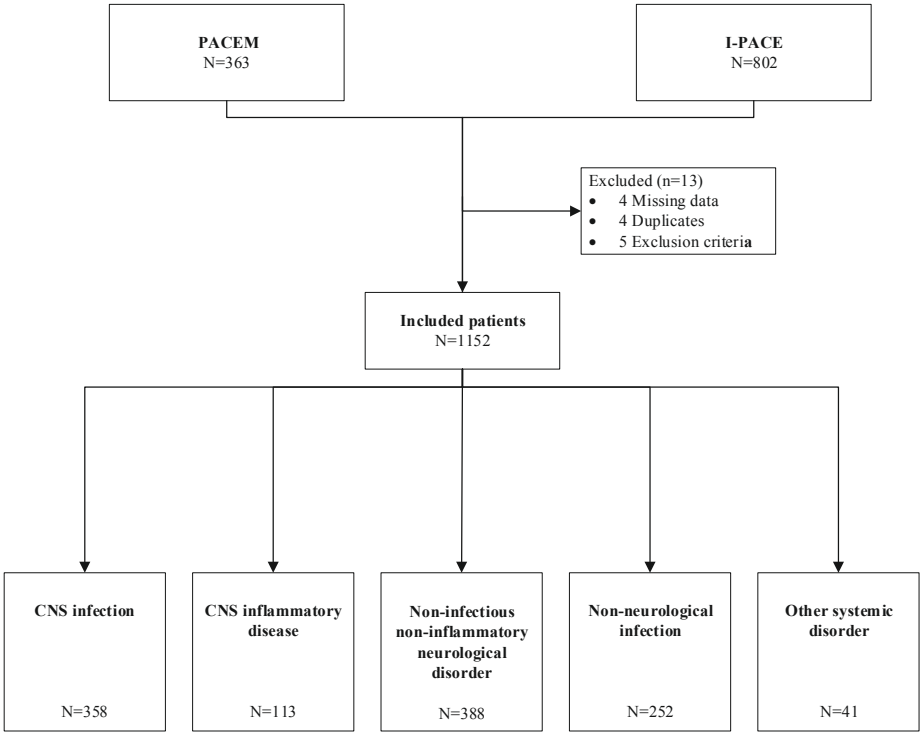

Table 1. Characteristics of all episodes with Suspected Central Nervous System infections (n=1152)

Characteristic	1152 patients	Characteristic	1152 patients
Age	54.0 (37-67)	Heart rate	90 (76-105)
Female sex	563/1152 (49)	Diastolic blood pressure	78 (68-89)
Medical history		Aphasia or Paresis	249/896 (28)
Immunocompromised state	450 /1151 (39)	Seizures on admission	159/1078 (15)
HIV	74/1150 (6)	Cranial nerve palsy	155/1052 (15)
Splenectomy	6/1148 (1)	**Laboratory results**	
Immunosuppressive treatment	208/1149 (18)	Thrombocytes $\times 10^{12}$/L	230 (168-287)
Diabetes	188/1151 (16)	C-reactive protein mg/L	16 (3-70)
Alcoholism	62/1023 (6)	Blood leukocyte count x 10^9/L	9.3 (6.6-13.2)
Other focus of infection*	112/1152 (10)	CSF leukocytes /mm^3	5 (1-60)
Symptoms on presentation		CSF leukocytes ≥4 cells/mm^3	517/1139 (45)
Location of neurology presentation		CSF protein <0.60	463/1149 (40)
Emergency department	861/1140 (76)	**Glasgow Outcome Scale score**	
Inpatient departments	220/1140 (19)	1 - Dead	99/1152 (9)
Intensive care unit	59/1140 (5)	2 - Vegetative survival	2/1152 (0.2)
Symptoms <24h	417/1111 (38)	3 - Severely disabled	91/1152 (8)
Glasgow Coma Scale score		4 - Moderately disabled	220/1152 (19)
Median (IQR)	15 (13-15)	5 - Good recovery	740/1152 (64)
GCS <14	364/1143 (32)		
GCS ≤ 8	123/1143 (11)		
Neck stiffness	188/892 (21)		
Headache	639/998 (64)		
Temperature ≥ 38.5˚C	466/1051 (44)		

Data are median (IQR) or n/N (%). Abbreviations: GCS = Glasgow Coma Scale; CSF = cerebrospinal fluid; DBP = diastolic blood pressure. * otitis and/or sinusitis and/or pneumonia. [a] Age known in all episodes. [b] Glasgow Coma Scale score was known for 1143 episodes. [c] Heart rate was known for 1112 episodes. [d] Diastolic blood pressure was known for 1117 episodes. [e] Thrombocytes was known for 1094 episodes. [f] C-reactive protein was known for 1039 episodes. [g] Blood leukocyte count was known for 1119 episodes. [h] CSF leukocyte count was known for 1139 episodes.

A lumbar puncture was performed in all episodes, and CSF examination showed an elevated leukocyte count (≥4 cells/mm^3) in 622 of 1139 episodes (55%). The CSF leukocyte count was between 4 – 99 cells/mm^3 in 378 (33%) episodes, between 100 – 999 cells/mm in 147 (13%), and more than 1000 cells/mm^3 in 97 (9%) episodes. During the clinical course, antibiotics according to bacterial meningitis regime or antiviral treatment were started in 695 of 1150 episodes (60%).

A final clinical diagnosis was available for all episodes. CNS infection was diagnosed in 358 (31%), CNS inflammatory disease in 113 (10%), non-infectious non-inflammatory neurological disorder in 388 (34%), non-neurological infection in 252 (22%), and other systemic disorder in 41 (4%, Table 2). Of the 358 CNS infections, the diagnosis was microbiologically confirmed in 236 episodes (66%). CSF culture was positive in 79 of 236 (33%) episodes, CSF PCR in 117 (50%), CSF antigen testing in 19 (5%), blood culture in 92 episodes (39%), and blood PCR in 17 (7%).

The outcome was unfavourable in 412 episodes (36%), and in 99 of 1152 episodes (9%), the patient died (Table 3). Neurological sequelae were present in 352 of 1015 (35%) surviving patients. The rate of unfavourable outcome varied per disease category and was 118 out of 358 episodes (33%) diagnosed with CNS infections, 74 out of 113 (65%) with CNS inflammatory diseases, 150 out of 388 (39%) with non-infectious non-inflammatory neurological disorders, 54 out of 252 (21%) with non-neurological infections, and in 16 out of 41 (39%) with other systemic disorders (Table 2). The mortality rate was 36 of 358 (10%) in episodes with CNS infections, eight out of 113 (7%) with CNS inflammatory disease, 28 out of 388 (7%) with non-infectious non-inflammatory neurological disorders, 25 out of 252 (10%) with non-neurological infections two of 41 (5%) with other systemic disorders. To analyse changes in time period and outcome between cohort 1 and cohort 2, we found an unfavourable outcome in 91 of 363 episodes (25%) in cohort 1 versus 321 of 793 episodes (41%) in cohort 2, $P<0.001$.

Table 2. Final diagnoses in 1152 episodes

	Number of episodes N=1152	Unfavourable outcome N=412	Favourable outcome N=740	P-value
Central Nervous System Infection	358 (31)	118/358 (33)	240/358 (67)	
Bacterial meningitis	138/358 (39)	51/138 (37)	87/138 (63)	
Viral meningitis	108/358 (30)	11/108 (10)	97/108 (89)	
Viral encephalitis	54/358 (15)	32/54 (59)	22/54 (41)	
Other CNS infections	58/358 (16)	24/58 (41)	34/58 (59)	
Central Nervous Inflammatory Disease	113 (10)	74/113 (65)	39/113 (34)	<0.001
Confirmed Autoimmune Encephalitis	10/113 (9)	8/10 (80)	2/10 (20)	
Paraneoplastic encephalitis	2/113 (2)	1/412 (0)	1/740 (0)	
AIE of unknown cause	26/113 (23)	18/26 (69)	8/26 (31)	
Myelitis/myelopathy	9/113 (9)	8/9 (89)	1/9 (11)	
Chronic meningitis	16/113 (14)	4/16 (25)	12/16 (75)	
Inflammatory polyneuropathy	3/113 (3)	3 (100)	0 (0)	
HaNDL syndrome	4/113 (4)	0 (0)	4 (100)	
Other CNS autoimmune diseases *	43/113 (37)	32 (74)	11 (26)	
Non-neurological infection	252 (22)	54/252 (21)	198/252 (79)	<0.001
Non-infectious non-inflammatory -neurological disorder	388 (34)	10/388 (39)	238/388 (61)	0.15
Other systemic disorder	41 (4)	16/41 (39)	25/41 (61)	0.74

Data are in n/N (%). HaNDL= Headache with neurological deficits and CSF lymphocytosis; CNS= Central Nervous System. *Other CNS autoimmune diseases; Guillain-Barré Syndrome (10), Vasculitis (10), Neurosarcoidosis (9), Acute disseminated encephalomyelitis (4), Other CN autoimmune disease of unknown cause (7), Neuro SLE (2), Immune Reconstitution Inflammatory Syndrome (1).

Table 3. Clinical characteristics and outcome

	Outcome		P-value
	Unfavourable 412 patients	**Favourable 740 patients**	
Age, median	62 (49-72)	48 (32-63)	<0.001
Female Sex	181/412 (44)	382/740 (52)	0.007
Predisposing factors			
Immunocompromised state	179/412 (43)	271/739 (37)	0.01
HIV	26/411 (6)	48/739 (7)	0.51
Immunosuppressive therapy	82/410 (20)	126/738 (17)	0.12
Diabetes	85/412 (21)	103/739 (14)	0.002
Alcoholism	26/364 (7)	36/659 (6)	0.17
Symptoms on presentation			
Symptoms <24h	129/385 (34)	288/726 (40)	0.03
GCS <14	176/408 (43)	188/735 (26)	<0.001
GCS <8	76/408 (19)	47/735 (6)	<0.001
Neck stiffness	62/274 (23)	126/618 (20)	0.25
Headache	132/315 (42)	507/683 (74)	<0.001
Fever >38 ˚C	119/398 (30)	291/719 (41)	<0.001
Tachycardia >120 beats/min	43/399 (11)	48/713 (7)	0.01
Diastolic blood pressure, mmHg	80 (69-92)	77 (67-88)	0.01
Aphasia or Paresis	140/346 (41)	109/702 (16)	<0.001
Seizures on admission	74/376 (20)	85/702 (12)	<0.001
Cranial nerve palsy	90/355 (25)	65/697 (9)	<0.001
Thrombocytes $\times 10^{12}$/L	233 (160-301)	230 (175-283)	0.97
C-reactive protein >40 mg/L	120/342 (35)	241/697 (35)	<0.001
CSF leukocytes, cells/mm3	6 (2-59)	4 (1-61)	0.13
CSF leukocytes ≥4 cells/mm3	242/407 (60)	380/732 (52)	0.008
CSF protein >0.6	210/408 (52)	257/733 (35)	<0.001

Data are median (IQR) or n/N (%). Abbreviations: GCS = Glasgow Coma Scale, CSF = cerebrospinal fluid. [a] Age was known in all episodes. [b] Glasgow Coma Scale score was known for 1143 episodes. [c] Diastolic blood pressure was known for 1117 episodes. [d] Thrombocytes was known for 1094 episodes. [e] CSF leukocyte count was known for 1139 episodes.

In the multivariable analysis, predictors for unfavourable outcome were advanced age, the absence of headache, tachycardia, GCS score <14, focal cerebral deficits (aphasia or paresis), cranial nerve palsies, thrombocyte count <150 $\times 10^{12}$/L, CSF protein count >0.60 g/L, and the final diagnosis of a CNS inflammatory disease (Table 4).

Table 4. Predictive characteristics for unfavourable outcome

Characteristic	Univariable OR (95% CI)	Multivariable OR (95% CI)	P-value
Age 16 to 39	Reference	Reference	
Age 40 to 70	3.28 (2.34-4.59)	2.01 (1.35-2.99)	<0.001
Age >70	6.67 (4.48-9.93)	3.46 (2.14-5.59)	<0.001
Female sex	0.74 (0.58-0.94)	0.95 (0.71-1.28)	0.74
Predisposing factors			
Immunocompromised state	1.33 (1.04-1.70)	0.99 (0.73-1.34)	0.93
Other focus of infection	0.99 (0.66-1.50)	-	
Symptoms on presentation			
Symptoms <24h	0.78 (0.60-1.01)	-	
GCS score	0.87 (0.83-0.90)	0.94 (0.89-1.00)	0.04
Neck stiffness	1.08 (0.73-1.60)	-	
Headache	0.24 (0.18-0.33)	0.39 (0.27-0.57)	<0.001
Tachycardia >120 beats/min	1.68 (1.09-2.58)	1.89 (1.08-3.32)	0.03
Fever ≥ 38˚C	0.62 (0.48-0.80)	0.81 (0.58-1.14)	0.23
Diastolic blood pressure <60 mmHg	1.29 (0.85-1.95)	1.29 (0.78-2.14)	0.33
Diastolic blood pressure 60-80 mmHg	Reference	Reference	
Diastolic blood pressure >80 mmHg	1.56 (1.20-2.02)	1.33 (0.97-1.80)	0.07
Aphasia or Paresis	3.84 (2.83-5.21)	2.01 (1.32-3.04)	
Seizures on admission	1.75 (1.26-2.45)	0.80 (0.51-1.25)	
Cranial nerve palsy	3.11 (2.17-4.46)	2.24 (1.48-3.38)	<0.001
Thrombocytes <150 $\times 10^{12}$/L	1.42 (1.04-1.94)	1.69 (1.15-2.47)	0.008
Thrombocytes 150 to 450 $\times 10^{12}$/L	Reference	Reference	
Thrombocytes >450 $\times 10^{12}$/L	1.98 (0.98-4.01)	1.46 (0.67-3.20)	0.34
CRP <40 mg/dL	Reference	-	
CRP 40-150 mg/dL	0.93 (0.64-1.35)	-	
CRP >150 mg/dL	1.35 (0.94-1.94)	-	
Blood leukocytosis	1.01 (0.79-1.31)	-	
CSF leukocytes <4 cells/mm^3	Reference	Reference	
CSF leukocytes 4 to 100 cells/mm^3	1.54 (1.17-2.02)	1.13 (0.78-1.65)	0.53
CSF leukocytes 100 to 1000 cells/mm3	1.21 (0.82-1.77)	1.39 (0.74-2.61)	0.31
CSF leukocytes >1000 cells/mm3	0.89 (0.56-1.43)	0.70 (0.33-1.50)	0.36
CSF protein >0.60 g/dL	1.97 (1.54-2.53)	1.57 (1.08-2.29)	0.02
Final diagnosis			
CNS infection	Reference	Reference	
CNS inflammatory disease	3.98 (2.54-6.23)	3.97 (2.28-6.93)	<0.001
Systemic infection	0.57 (0.39-0.83)	0.55 (0.31-1.00)	0.05
Other neurological disease	1.28 (0.95-1.74)	1.03 (0.64-1.68)	0.90
Non-neurological non-infectious disease	1.31 (0.67-2.54)	1.32 (0.56-3.15)	0.53

The multivariable analysis used an imputed dataset with 5 imputation rounds**,** all variables in the table were entered in the multivariable logistic regression model simultaneously. Abbreviations: GCS= Glasgow Coma Scale; CRP = C-reactive protein; CSF= Cerebrospinal fluid; CNS= Central Nervous System.

Predictors for death were advanced age (>70 years old), an immunocompromised state, GCS score <14, the absence of headache, diastolic blood pressure <60 mm Hg, thrombocyte count <150 $\times 10^{12}$/L, CRP of 40 to 150 mg/dL, and CSF protein concentration >0.60 g/L (Table 5).

The group of CNS inflammatory diseases consisted of 113 of 1152 episodes (10%). The rate of unfavourable outcome differed between the definitive diagnoses included in this category. Eight out of ten (80%) episodes with confirmed autoimmune encephalitis (AE) had an unfavourable outcome, 18 out of 26 (69%) with possible AE of unknown cause, eight out of nine (89%) with myelitis, and 32 out of 43 (74%) with other neurological autoimmune disorders (Table 2). Unfavourable outcome was due to residual neurological sequelae in 62 out of 74 (84%).Twenty-three of 113 episodes (20%) with CNS inflammatory disease were initially treated with antibiotics consisting of amoxicillin and ceftriaxone according to bacterial meningitis protocol. Aciclovir was given in 35 episodes (31%). When probable CNS inflammation was diagnosed, first line immunosuppressive therapy (e.g., methylprednisolone (MPS), prednisone, intravenous immunoglobulins [IVIg]), was started in 87 of 113 episodes (77%) and escalation to second-line therapy (e.g., plasma exchange [PLEX], azathioprine, rituximab, cyclophosphamide, and mycophenolate mofetil [MMF], methotrexate) was required in 31 of 87 episodes (36%). First line therapy was commenced during initial admission, in 71 of 87 episodes (82%), with escalation to 2nd line therapy during this admission in 19 of 71 episodes (27%). Escalation to 2nd line therapy at a later point in the outpatient clinic or when readmitted was done in 10 of 71 episodes (14%). For 16 of 87 episodes (18%), first line treatment was only started after admission with escalation to 2nd line immunosuppressive therapy in 2 of 16 episodes (13%).

The time between presentation to immunosuppressive treatment was known in 84 of 87 (96%), with a median time to treatment of 5 days (IQR 1- 30). A univariate analysis for time to treatment and outcome showed no association (odds ratio 0.83 [0.51-1.35], P=0.45). Immunosuppressive treatment was not administered in the remaining 26 episodes for various reasons, including spontaneous recovery occurred in 6 episodes (26%), mild symptoms well-manageable with symptom relief medication in 6 episodes (26%), a self-limiting disorder in 4 (15%), and one patient died before commencing immunosuppressants (4%).

Table 5. Predictive characteristics for mortality

Characteristics	Univariable OR (95% CI)	Multivariable OR (95% CI)	P-value
Age 16 to 39	Reference	Reference	Reference
Age 40 to 70	2.53 (1.26-5.08)	1.30 (0.57-2.93)	0.53
Age >70	7.99 (3.92-16.3)	3.40 (1.44-8.06)	0.005
Female sex	0.79 (0.52-1.19)	-	
Predisposing factors			
Immunocompromised state	2.39 (1.56-3.65)	1.91 (1.16-3.16)	0.01
Other focus of infection	2.45 (1.42-4.22)	1.51 (0.76-3.00)	0.24
Symptoms on presentation			
Symptoms <24h	1.21 (0.77-1.89)	-	
GCS score	0.80 (0.76-0.84)	0.86 (0.80-0.93)	<0.001
Neck stiffness	1.33 (0.75-2.34)	-	
Headache	0.24 (0.14-0.43)	0.50 (0.27-0.94	0.03
Tachycardia	3.85 (2.25-6.59)	1.73 (0.87-3.43)	0.12
Fever	0.1.04 (0.68-1.59)	-	
Diastolic blood pressure <60 mmhg	2.65 (1.49-4.71)	2.22 (1.12-4.40)	0.02
Diastolic blood pressure 60-80 mmhg	Reference	Reference	Reference
Diastolic blood pressure >80 mmhg	1.07 (0.67-1.70)	1.17 (0.69-1.99)	0.56
Aphasia or Paresis	3.88 (2.18-6.92)	1.43 (0.80-2.56)	0.23
Seizures on admission	1.68 (1.003-2.82)	0.78 (0.41-1.51)	0.47
Cranial nerve palsy	2.21 (1.28-3.81)	1.72 (0.93-3.17)	0.08
Thrombocytes <150	2.42 (1.51-3.87)	1.88 (1.06-3.34)	0.03
Thrombocytes 150 to 450	Reference	Reference	Reference
Thrombocytes >450	4.06 (1.77-9.29	2.85 (1.00-8.14)	0.05
CRP <40 mg/dL	Reference	Reference	Reference
CRP 40-150 mg/dL	2.31 (1.38-3.86)	2.04 (1.13-3.69)	0.02
CRP >150 mg/dL	3.54 (1.83-6.86)	2.00 (0.88-4.57)	0.01
Blood leukocyte count	1.56 (1.02-2.37)	0.81 (0.60-1.10)	0.17
CSF leukocytes <4 cells/mm3	Reference	-	
CSF leukocytes 4 to 100 cells/mm3	1.33 (0.83-2.12)	-	
CSF leukocytes 100 to 1000 cells/mm3	1.31 (0.69-2.49)	-	
CSF leukocytes >1000 cells/mm3	1.17 (0.53-2.55)	-	
CSF protein >0.60	2.39 (1.57-3.65)	2.29 (1.40-3.75)	<0.001
CNS infection	Reference	-	
CNS inflammatory disease	0.69 (0.31-1.5)	-	
Systemic infection	1.00 (0.59-1.72)	-	
Other neurological disease	0.70 (0.42-1.18)	-	
Non-neurological non-infectious disease	0.46 (0.11-1.99)	-	

The multivariable analysis used an imputed dataset with 5 imputation rounds**,** all variables in the table were entered in the multivariable logistic regression model simultaneously. Abbreviations: GCS= Glasgow Coma Scale; CRP = C-reactive protein; CSF= Cerebrospinal fluid; CNS= Central Nervous System

DISCUSSION

Our study shows that patients presenting with an episode of suspected CNS infection have a high risk (36%) of experiencing an unfavourable outcome. Consistent with previous studies, advanced age was found to be an independent predictor of unfavourable outcome.[12-14] The association between outcome and focal cerebral deficits, an altered mental state, and elevated CSF protein count and outcome is likely to reflect the severity of neurological damage, while thrombocytopenia and tachycardia are associated with sepsis.[15-19]

Patients who were eventually diagnosed with CNS inflammatory disease showed the poorest prognosis. This association can be explained by various factors, including the severity of the conditions. Unfavourable outcome was most prevalent in confirmed cases with autoimmune encephalitis (80%) or suspected autoimmune encephalitis (69%). These rates are relatively high compared to previous studies on autoimmune encephalitis, which reported rates ranging from 13-80%, depending on the follow-up duration, associated antibodies, and aetiology of the autoimmune encephalitis episode.[20-26] The difference in outcome between our cohort and the literature may be due to the limited follow-up time in our study, as most studies provided an extensive follow-up time of up to 33 months, with outcomes that continued to improve for up to 18 months after symptom onset.[21,22,25,27] Moreover, our cohort consisted of a relatively small group of autoimmune encephalitis cases, most of whom were admitted to a tertiary hospital. Furthermore, our observation that other inflammatory conditions, like inflammatory myelitis, vasculitis, Guillain Barre syndrome, neurosarcoidosis, are associated with an unfavourable outcome aligns with existing literature.[28-31]

Contrary to previous studies on predictors for unfavourable outcome in CNS infections, the presence of seizures or an immunocompromised state, e.g., diabetes mellitus, did not show an association in our cohort.[35,36] This can be explained due to the heterogeneity in diagnoses in the cohort. Notably, for these variables, the odds ratios shifted from indicating a higher likelihood to suggesting a lower likelihood of an unfavourable outcome between the univariate and the multivariate analyses. This change could be caused by interactions with a covariate, such as final diagnosis associated with diabetes or an immunocompromised state, although this is speculative.

In CNS inflammatory diseases, treatment choice frequently rely on expert opinions rather than on randomized controlled trials for comparing treatments. Although our study did not find an association between treatment delay and outcome in CNS inflammatory episodes, it is generally accepted that time to treatment is a modifiable

risk factor for poor outcome. Moreover, accumulating evidence and recent guidelines point to the beneficial effects of early diagnosis and treatment on outcome.[32-34,37-39]

Currently, diagnostic methods only establish the etiologic cause in 50% of encephalitis cases, with at least 10% being diagnosed as autoimmune encephalitis, of which causative anti-neuronal antibodies could only be detected in 35% .[1,26,40] The median time to treatment initiation for a CNS inflammatory disease was 5 days, and treatment was started only after 30 days in 25% of the cases. This can be attributed to an insidious onset of the disease, as well as the lengthy duration of diagnostic tests for autoimmune encephalitis, such as anti-neuronal antibody testing. Such episodes can initially be suspected of infectious meningoencephalitis, but after microbiological tests return negative, diagnostic tests for autoimmune encephalitis are ordered and generally take several weeks to generate results. Unfortunately, empirical treatment for autoimmune disorders is often not initiated while waiting for these tests.[41]

Prompt immunotherapy has been associated with a favourable outcome for all types of autoimmune encephalitis, as spontaneous clinical improvement is infrequent.[21] Various treatment options are available, including corticosteroids, TPE, IVIG, and immunosuppressant drugs. Treatment choice depends on the pathophysiology of the specific type of autoimmune encephalitis and the patients' comorbidity.[26,42] A recent study concluded that more aggressive treatment regimens in autoimmune encephalitis patients improved the 2-year outcome. However, a comment on this study suggested that first-line immunotherapy's effect was underestimated while second-line immunotherapy's effect was overestimated.[26,43] Based on our study, early treatment with anti-inflammatory drugs should be considered to minimize the risk of an unfavourable outcome in cases of CNS inflammatory diseases.

Our study had several limitations. First, episodes could only be included when a lumbar puncture was performed, and the researchers identified the patients. This may have resulted in missed inclusions. Second, in some episodes, the final diagnosis was based on the clinical picture rather than microbiological evidence, demonstrated antibodies or radiological features, and thus may have led to misclassification. To solve this, we scored the final clinical diagnoses with two independent investigators and a third to solve discrepancies representing a proper classification process. Third, patients were predominantly admitted to a tertiary hospital and were inherently more complex than those in a general hospital, potentially causing selection bias. However, the majority of patients presented at the emergency department, reducing this risk of bias. Fourth, we did not analyse predictors for outcome for each diagnostic category separately. Instead, our focus was on evaluating all adults presenting with a suspected CNS

infection, aiming to aid physicians in the acute setting, particularly when patients are still undifferentiated. This approach allowed us to gain insights into which patient subgroup requires more targeted investigation on diagnostics and treatment in future research.

In conclusion, patients suspected of having a CNS infection are at high risk of experiencing an unfavourable outcome, stressing the urgent need for improving rapid and accurate diagnostics. Amongst this suspected CNS infection group, those eventually diagnosed with CNS inflammatory disease have the highest risk of an unfavourable outcome. Our findings underscore the importance of prioritizing diagnostic and treatment improvements in this population. Based on our study, early treatment with immunosuppressive drugs may be considered to reduce the risk of an unfavourable outcome in cases of CNS inflammatory diseases.

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Contributors

LtH: methodology, data collection, data analysis, data interpretation, and writing the original draft of the manuscript; IvZ; study design, data collection, review and editing of the report; SO: data collection, review and editing of the report; DvdB: review, editing, and supervision of the report; MB: methodology, study design, data interpretation, review, editing and supervision of the report, and funding acquisition. All authors read and approved the final manuscript.

Declaration of interests

All authors declare no competing interests.

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CHAPTER 11

GENERAL DISCUSSION

GENERAL DISCUSSION

The aim of this thesis was to assess and improve diagnostic accuracy of clinical predictors, biomarkers and pathogen discovery sequencing for diagnosing CNS infections.

Clinical predictors

Differentiation between acute bacterial and viral CNS infections or no CNS infection at all, based on clinical or laboratory results, remains difficult in many patients. Individual clinical or laboratory characteristics show limited diagnostic accuracy for the diagnosis of CNS infections.[1-3] Various diagnostic prediction models have been developed to evaluate combinations of clinical predictors for the diagnosis of bacterial meningitis. We validated sixteen diagnostic prediction models for the diagnosis of bacterial meningitis in adults and twenty-six models in children.[Chapter 2 and 3] These models included combinations of at least three clinical and/or laboratory variables to predict the probability of bacterial meningitis. In conclusion, none of the validated models performed well enough to recommend routine use in clinical practice based on measures for diagnostic accuracy. Since missing a diagnosis of bacterial meningitis will have devastating consequences, an acceptable prediction model would require a sensitivity of no less than a 100%, combined with a high specificity. However, clinical judgement of a physician might not reach this percentage either. Because of this, a more reasonable question might be whether or not a model adds any value to, or outperforms, clinical judgement of the physician. For prediction models for bacterial meningitis this has only been evaluated for the model of Hoen et al.[4] They demonstrated that the model could be a helpful tool in clinical decision-making, especially in the decision to refrain from administration of antibiotics. However, since in a few cases physicians rightfully made a different decision than the model, it was advised to never use the model in its own. Similar results have been described in, for instance, children with traumatic brain injury. One study assessed the performance of three clinical decision rules compared to physician accuracy and showed comparable sensitivities but higher specificities in physicians.[5] Therefore, the potential of these rules to increase accuracy of detection of clinically relevant traumatic brain injuries seems limited and they potentially do increase the number of CT scans being performed. For almost all diagnostic prediction models for CNS infections it is so far unclear to what extent they add any value, or whether they increase the amount of unnecessary ancillary investigations or treatment in clinical practice.

Although some of the models previously mentioned are designed to differentiate between bacterial and viral CNS infections (mostly meningitis), no specific diagnostic prediction rules for the diagnosis of acute viral encephalitis have been developed. This might be explained by the fact that clinical symptoms largely depend on the location of

inflammation in the brain and are therefore too heterogeneous to include in a model. Moreover, CSF abnormalities are usually less pronounced than in bacterial meningitis. The International Encephalitis Consortium has, however, proposed a case definition for encephalitis in general, and suggestions have been made on different clinical syndromes that are more likely to be caused by certain viruses or autoantibodies.[3,6]

Reliable clinical predictors are specifically warranted in doubtful cases. Recently, a new risk score was proposed in patients with an elevated number of leukocytes in CSF and a negative Gram stain.[7] First validation of this score revealed a sensitivity of 99.6% and a specificity of 41.2%, but further external validation has yet to be performed. Simultaneously, a German research group evaluated consecutive in-patients with an elevated CSF leukocyte count, and developed the CHANCE score to differentiate bacterial meningitis from other causes of pleocytosis.[8] Sensitivity turned out to be 88% with a specificity of 87% in the first validation cohort, the area under the curve (AUC) was 0.96. Calculation of this score in our cohort lead to similar numbers in adults: sensitivity was 90% (95% CI 84-95%) and specificity 76% (95% CI 70-80%).[9] However, when applying the criterion of CSF leukocytes >100 cells/µl individually, sensitivity was also 90% (95% CI 84-95%), with a specificity of 63% (95% CI 57-68%) in adults.[9] Additional diagnostic value to CSF leukocytes alone therefore seems limited.

Another subgroup that can pose a diagnostic dilemma are patients suspected of nosocomial CNS infections (not related to trauma or neurosurgery). In our cohort, these were mostly patients with an immunocompromised state (76%), and therefore prone to all kinds of infections.[Chapter 8] The complexity in these patients lies in the fact that some of them suffer from leukopenia, making the interpretation of CSF leukocytes more complicated due to potentially false-negative results. Despite of this, CSF leukocyte count still had the highest sensitivity of all individual predictors to predict a CNS infection in this group, although no more than 81%. Of the three patients diagnosed with CNS infections and normal leukocyte counts in the CSF, one of them with VZV meningitis did have an elevated protein count. The other two, who were diagnosed with HHV6 encephalitis and rhino-orbital-cerebral-mucormycosis, had completely normal CSF inflammatory parameters. For both diseases, normal CSF parameters have been described.[10,11] Despite this, in case of the suspicion of such diagnoses, combining neurological examination, microbiological CSF examination and cranial imaging with MRI will often lead to the diagnosis.

Interpretation of CSF leukocyte count in patients suspected of CNS infections and presenting with seizures can be challenging as well, although in that case because of allegedly false-positive results. A meta-analysis on studies in children, focusing on the

differentiation between CNS infections and febrile seizures without CNS infection, showed a low risk of 2.6% for bacterial meningitis in that population.[12] However, an elevated CSF leukocyte count of >5 leukocytes/mm3 was described in up to 10% of children with complex febrile seizures and 12% in nonfebrile seizures.[13] In our study, 22% of the patients initially suspected of a CNS infection and presenting with a seizure, was diagnosed with a CNS infection.[Chapter 9] CSF leukocyte count was again the best predictor for diagnosing CNS infections, with an AUC of 0.94, but low specificity. One third of all patients in this cohort not diagnosed with a CNS infection, had an elevated CSF leukocyte count. However, changes in CSF in patients with epileptic seizures of unknown cause were not common. This is in line with more recent studies on this topic[14-18], whereas research supporting the hypothesis that CSF leukocytosis can be caused by ictal activity alone comes from earlier dates.[19-22] Therefore, in patients presenting with seizures and CSF leukocytosis, additional investigation for the underlying cause should be performed.

Besides prediction of the diagnosis, clinical predictors can be used to predict outcome as well. The population of patients, in whom a CNS infection is in the differential diagnosis, frequently presents with severe illness, reflected by neurological deficits, impaired consciousness and hemodynamic instability.[2] Mortality in these patients has been reported up to 10%, and we observed an overall unfavorable outcome of 27-36%.[2,23, Chapter 10] In order to improve outcome in this population, recognition of patients at risk for an unfavorable outcome is essential. Therefore, we conducted a study on prognostic clinical predictors in our cohort of patients with suspicion of a CNS infection, revealing several independent predictors for poor outcome.[Chapter 10] Advanced age was one of them, which is consistent with previous research, and also tachycardia and a low thrombocyte count were found to be independently predictive of unfavorable outcome, most likely due to their association with sepsis.[24-27] Additionally, an altered mental status, focal neurological deficits, cranial nerve palsies, and a high CSF protein level were revealed as predictors, probably reflecting the severity of neurological damage.[28-30] Patients in our cohort who were eventually diagnosed with CNS inflammatory disorders, showed the worst prognosis. This was mainly attributable to the cases of confirmed and suspected autoimmune encephalitis in the cohort, showing an unfavorable outcome in 80%. This relatively high percentage might be explained by several factors[31]: first, the short period of follow-up we had in these patients, whereas we know that recovery can continue up to 18 months after symptom-onset.[31,32] Moreover, our number of patients with auto-immune encephalitis is relatively small. On the other hand, delayed diagnosis and treatment could potentially have contributed to poor outcome in these patients, either caused by a clinical picture that leads to a broad differential diagnosis and/or by lengthy duration of diagnostic trajectories. Timely start of treatment with immunotherapy and removal of the immunologic trigger, such as tumors, are associated

with favorable outcome in patients with autoimmune encephalitis.[31] Therefore, our data stress the need for improved strategies to be able to establish a timely diagnosis in all patients suspected of CNS infection and inflammation.

In recent years, artificial intelligence based prediction models have been showing promising results. In children with traumatic brain injuries, diagnostic machine-learned models showed higher specificities, positive predictive values and positive likelihood ratios compared to standard prediction rules.[33] Also in patients with stroke, prognostic machine learning models performed slightly better than a standard prognostic model, although differences were small.[34] In young febrile children with suspected serious bacterial infections, machine-learned based models would have reduced the number of performed lumbar punctures with 69%.[35] This was, however, not structurally compared to any standard model or judgement of the physician. A model to differentiate between tuberculous and viral meningitis showed higher AUC's than all four participating residents and one of the two infectious specialists it was compared to.[36] Another artificial intelligence model that differentiates between etiologies of meningitis or encephalitis did outperform all three physicians it was compared to.[37] None of these models was yet prospectively evaluated.

Future directions

So far, no individual clinical predictor gives us enough certainty to confirm or exclude a diagnosis of a CNS infection, especially not in the most doubtful cases. Combinations of several clinical predictors have been analyzed in various diagnostic prediction models. However, if we want to be able to use any of these models in clinical practice, prospective evaluation of their additional value to clinicians accuracy should take place. Also, evaluation of additional ancillary investigations and use of antimicrobial treatment, hospital stay length and overall costs should be taken into account in this analysis.

Nevertheless, up to this date, various diagnostic prediction rules have been developed resulting in limited diagnostic accuracy. We might conclude that future research should focus on different strategies to improve diagnostic accuracy in diagnosing CNS infections, rather than developing any more prediction models.

Biomarkers

Besides attempts to improve the interpretation of clinical predictors and currently available diagnostic markers, the search for novel biomarkers is ongoing. Various diagnostic markers for the diagnosis of CNS infections have been studied, but up to this date no marker with significant additional value in clinical practice has been discovered. Chapter 1 We conducted a study on the diagnostic accuracy of neurofilament light chain

(NfL) in CSF, which is a known marker of neuronal damage in a variety of CNS diseases.[38, Chapter 4] We hypothesized that the level of NfL in CSF might differ between different disease categories in our cohort. However, we found no differences in the concentration of NfL in CSF, resulting in poor diagnostic accuracy for the diagnosis of CNS infections. [Chapter 4] Higher concentrations were, however, associated with mortality and unfavorable outcome, which was confirmed in another recent study that found NfL in CSF to be an independent predictor for unfavorable outcome in bacterial meningitis.[39, Chapter 4] Additionally, in our study, the level of NfL was related to an altered mental status, consistent with NfL being a marker for axonal loss, in which high concentrations are probably due to the generalized brain damage in these cases.

Several studies on the diagnostic accuracy of NfL in non-infectious neurological diseases found differences in concentrations between samples of patients with and without neurodegenerative diseases, but discrimination between different types of neurodegenerative diseases remains less accurate.[40-42] Therefore, it seems likely that NfL works best as a measure to discriminate between diseases with varying degrees of neuronal damage, rather than between disease categories that are clinically more similar.[38,43] Usefulness of NfL as a diagnostic marker in patients suspected of a CNS infection remains limited.

Future directions

The best predictor for CNS infections thus far is the CSF leukocyte count, however, half of patients with an alternate diagnosis have elevated leukocytes in their CSF as well.[2] Therefore, alternative biomarkers are necessary, preferably markers that can be rapidly determined. Various inflammatory markers that are being routinely performed in clinical laboratories, like C-reactive protein (CRP), procalcitonin and interleukin 6, 12 and 1β, have been studied as markers for acute bacterial meningitis, with promising results.[44-46] Sample sizes were, however, small, and often only patients with confirmed CNS infections and negative controls were analyzed.[44-48] Therefore, future research in measurements of inflammatory markers, performed in the entire group of patients suspected of CNS infections, might give us more insights.

Besides cytokines, chemokines and acute phase reactants, other molecules like metabolites and lipids can be measured in both blood and CSF. Lactate and glucose are metabolites that have been studied extensively in the context of CNS infections[49,50], but in recent years, due to technical advances in mass spectrometry and bioinformatics tools, metabolomics and lipidomics have emerged in the search for biomarkers. In metabolomics and lipidomics the metabolome or lipidome is being measured by means of nuclear magnetic resonance spectroscopy or mass spectrometry, in order

to identify and quantify metabolite or lipid profiles in samples at a specific time point. It has yet provided information on disease specific metabolic pathways in for instance tuberculous meningitis and Alzheimer's disease.[51-53] Also, several metabolites and lipids have been found to distinguish between bacterial and viral CNS infections or non-infectious cases in both infants and adults.[54-56] However, none of them have so far been evaluated in all consecutive patients suspected of a CNS infection.

Another next step in the improvement of the diagnostic process in CNS infections could be the determination of RNA expression profiles in blood and CSF of the host. Few studies demonstrated significant up- and downregulation of several (mainly immunological) pathways in peripheral blood of patients with bacterial meningitis compared to patients without bacterial meningitis.[57,58] Knowledge on specific profiles per disease might give us more insight in (patho)physiological processes and could possibly be beneficial for both the diagnostic process, as well as for determining prognosis or specific treatment targets. A disadvantage of this type of transciptome analysis is that it requires prior knowledge about the cell populations, in order to determine the origin of the transcripts. Single-cell RNA sequencing is a technique which makes it possible to study gene-expression on a cellular level, in which for instance clustering or expression of specific immune cells can be studied. It has yet yielded several (patho)physiological insights in multiple sclerosis, Lewy body dementia, HIV and COVID-19 with neurological symptoms.[59-62] No data in patients with bacterial meningitis or other CNS infections is available thus far.

Pathogen discovery sequencing

Ultimately, clinical predictors and biomarkers are just circumstantial evidence. Theoretically, the most definite proof of a CNS infection would be detection of the causative pathogen - with a quick, reliable test. In recent years, metagenomic next-generation sequencing (mNGS) has emerged as a promising tool to detect pathogens (Introduction). In the last decade, mNGS of the CSF has led to a diagnosis of a CNS infection in numerous cases, including infections caused by novel detected species.[63-66] More recently, even several prospective clinical studies on the use of mNGS of for the diagnosis of CNS infections were performed, with promising results.[67-69]

We evaluated the performance of VIDISCA-NGS, a viral metagenomic technique that was developed in our institution, in CSF of patients from the PACEM and IPACE study. Chapter 6,7,8 This technique is characterized by its fragmentation of ds(c)DNA by a frequent-cutting restriction enzyme, which differs from most viral metagenomic essays. Because of restriction enzyme digestion, it has the advantage that a relatively low sequence depth is needed which reduces costs and runtime per sample. We optimized this

method for CSF and determined its performance in CSF. It turned out that VIDISCA-NGS has difficulty detecting herpes viruses.[Chapter 5,6] This is probably due to degraded and non-encapsidated herpesviral DNA in clinical specimens, which is therefore not protected by a virus particle during DNase treatment.[Chapter 5,6] Subsequently, we assessed the ability of VIDISCA-NGS to identify bacteria, by means of bacterial ribosomal RNA detection.[Chapter 7] Overall sensitivity appeared to be 40-69% depending on the threshold of several parameters, such as sequencing depth, percentage of alignment and number of pathogen specific reads.[Chapter 7] Besides these parameters, sensitivity and specificity varied greatly between pathogens, with the highest number of false positives for *S. pneumoniae.*[Chapter 7] In conclusion, at this point, VIDISCA-NGS might be useful in addition to conventional microbiological testing, in cases where no pathogen can be identified.

Future directions

Even though VIDISCA-NGS is not capable of replacing conventional microbiological tests for the diagnosis of CNS infections at this moment, its results are promising. Further optimization and prospective evaluation alongside regular ancillary investigations should be performed. Also, since VIDISCA-NGS was primarily developed for the detection of viruses, we should continue with the development, improvement and validation of metagenomic sequencing methods which can detect all types of pathogens in the CSF.

It is likely that we are not going to be able to identify a causative pathogen in the CSF in 100% of the patients who are clinically suspected of a CNS infection. One of the reasons for this, is that in some viral CNS infections, the virus might only be present in the nervous system during the first few hours or days of the disease. For instance, in West Nile Virus the peak viremia occurs 3-5 days prior to symptom onset.[70] In enteroviruses, the virus remains detectable in stool and respiratory samples for a longer period than in CSF.[71] Because of this, for the I-PACE study we collected pharyngeal and rectal swabs from included patients. In future research we should analyze these samples for the presence of viruses, to determine the human 'virome' and distinguish those viruses from potentially clinically relevant viruses.

Finally, there might be a part of the patients in who we, as physicians, are wrong about the clinical diagnosis. As we know, various diagnoses are made in patients who are initially suspected of a CNS infection.[2] Potentially, there are patients with clinical diagnoses of CNS infections who in fact suffer from inflammatory CNS diseases, and vice versa. In future research, we should analyze patient samples for neuronal antibodies and see if we missed any diagnosis of auto-immune encephalitis. Caution is, however, warranted, since misdiagnosing of auto-immune encephalitis can be harmful.[72] Also,

auto-immune encephalitis after viral encephalitis is a well-known phenomenon, but even the presence of neuronal antibodies does not necessarily mean that a patient develops auto-immune encephalitis.[73]

In conclusion, we made progress in our aim to improve the diagnostic accuracy of several methods to diagnose CNS infections, but we still have a lot to do. The best clinical predictor for the diagnosis of a CNS infection, thus far, is the CSF leukocyte count, but specificity is poor. In the future, we should focus on metabolomics and lipidomics, RNA expression profiles and the optimization of metagenomic sequencing techniques for the diagnosis of CNS infections.

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APPENDICES

ENGLISH SUMMARY

DUTCH SUMMARY (NEDERLANDSE SAMENVATTING)

LIST OF ABBREVIATIONS

CONTRIBUTING AUTHORS AND AFFILIATIONS

PHD PORTFOLIO

LIST OF PUBLICATIONS

ACKNOWLEDGEMENTS (DANKWOORD)

ABOUT THE AUTHOR

SUMMARY

Central nervous system (CNS) infections are associated with high morbidity and mortality, depending on the causative pathogen. Virtually all pathogens can infect the brain or meninges surrounding it, including viruses, bacteria, fungi and parasites. The differential diagnosis in patients suspected of CNS infections is broad, and the diagnosis can be difficult to establish. Also, in a substantial part of the patients in which the diagnosis of a CNS infection is extremely likely, no pathogen can be identified by currently available diagnostic methods. Treatment and prognosis of patients with CNS infections do, however, depend on the type of CNS infection. Also, empirical treatment without confirmation of the pathogen has its drawbacks, like antimicrobial resistance or kidney damage caused by acyclovir. For this reason, development and improvement of diagnostic methods to diagnose CNS infections is needed to improve prognosis in these patients. The aim of this thesis was to assess and improve diagnostic accuracy of clinical predictors, biomarkers and pathogen discovery sequencing techniques to diagnose CNS infections. To address this aim, several methods were studied or validated in patients included in the PACEM and/or IPACE studies, which included consecutive patients in whom a lumbar puncture was performed because of the suspicion of a CNS infection.

First, in **Chapter 2** an external validation of existing diagnostic prediction models for bacterial meningitis was conducted. We performed a search of the literature and systematic review of the identified prediction models, and validated them in adults from the PACEM cohort. We found an excellent discrimination in all models but one. Calibration, however, showed over- or underestimation of the risk of bacterial meningitis by every model. There was no model that showed both a good sensitivity and specificity, which raises the question whether these models add any value in clinical practice. Since a substantial part of the identified prediction models in **Chapter 2** was originally developed in cohorts of children, **Chapter 3** presents the results of a validation of diagnostic prediction models for bacterial meningitis in children from the PACEM study. A systematic review of the literature yielded several additional models since the publication of **Chapter 2**. Validation of all these models in children lead to similar results in terms of discrimination and calibration, and again none of the models showed few false-negatives and false-positives.

Then, in the search for new biomarkers, **Chapter 4** presents a study on the diagnostic accuracy of neurofilament light chain (NfL) as diagnostic marker for CNS infections. NfL is a component of the axonal skeleton and identified as marker for axonal damage in several CNS diseases. NfL was measured in the cerebrospinal fluid (CSF) of adult patients

from the PACEM cohort, initially suspected of a CNS infection. Diagnostic accuracy of NfL in CSF for the diagnosis of CNS infections was poor. We did find associations between NfL and severe neurological symptoms (reflected by an altered mental status), as well as with mortality and unfavorable outcome. Therefore, the use of NfL in CSF for diagnostic purposes is not advised and prognostic accuracy in patients with CNS infections should be investigated in future research.

Chapter 5, 6 and 7 describe the use of a viral metagenomic sequencing technique called virus discovery cDNA amplified fragment length polymorphism next generation sequencing (VIDISCA-NGS), in patients with suspected CNS infections. In **Chapter 5** the performance of VIDISCA-NGS is evaluated in CSF samples, in which previously a virus was detected by quantitative polymerase chain reaction (qPCR) to test the reproducibility of the method. VIDISCA-NGS detected an RNA virus in all medium to high viral load samples and in 67% of the low viral load samples. Herpes viruses were particularly difficult to detect with VIDISCA-NGS. In **Chapter 6** we assessed the diagnostic accuracy of VIDSCA-NGS in CSF of patients with and without proven viral CNS infections. For this study we selected patients with a clinical diagnosis of a viral CNS infection and patients in whom finally a different diagnosis was established. Overall sensitivity and specificity were 52% and 100%, respectively. The difficulty to detect herpes viruses was confirmed in this study. One enterovirus, detected by VIDISCA NGS, was only identified by qPCR upon retesting. Several additional viruses were detected by VIDISCA-NGS, including GB virus C, human papillomavirus, human mastadenovirus C, Merkel cell polyoma virus and anelloviruses, but clinical relevance seems limited. Subsequently, in **Chapter 7** we explored the possibility of VIDISCA-NGS being able to detect bacterial RNA in CSF in addition to viruses. Overall sensitivity turned out to be 40-69% depending on the threshold of several parameters, such as sequencing depth, percentage of alignment and number of pathogen specific reads. Besides these parameters, sensitivity and specificity varied greatly between pathogens, with the highest number of false positives for *S. pneumoniae*. Furthermore, we found that a higher total sequencing depth, no antibiotics prior to CSF examination, increased disease severity, and higher C-reactive protein levels were associated with detection of pathogens.

Finally, **Chapter 8 and 9** describe clinical and laboratory characteristics in specific subgroups of patients with the initial suspicion of a CNS infection, whereas **Chapter 10** focused on outcome. One specific subgroup consisted of patients with the suspicion of a nosocomial CNS infection, not related to trauma or neurosurgery. **Chapter 8** gives an overview of their clinical characteristics, the diagnostic accuracy of these characteristics and what final diagnoses were established in this group of non-surgical nosocomial

CNS infections. We found that of all patients suspected of a non-surgical nosocomial CNS infection, 14% is eventually diagnosed with a CNS infection. Causative pathogens included bacteria, viruses, fungi and parasites. Combining the presence of abnormalities in neurological or CSF examination, a sensitivity of 100% was reached for the diagnosis of a CNS infection. The performance of a lumbar puncture changed medical management in 47% of the patients. A different subgroup was studied in **Chapter 9**, where we studied patients who present with a seizure and receive a lumbar puncture for the suspicion of a CNS infection. CSF leukocyte count was the best individual clinical predictor, with an area under the curve of 0.94. One-third of the patients not diagnosed with a CNS infection in this cohort, had elevated CSF leukocytes. However, abnormalities in CSF of patients with epileptic seizures of unknown cause were uncommon. The study in **Chapter 10** focused on outcome of the entire cohort of patients in the PACEM and I-PACE cohort, which seems poor: mortality rate was 9%, and 36% of the patients had an unfavorable outcome. Multivariable regression revealed age, focal neurological deficits, an altered mental status, cranial nerve palsies, low thrombocyte count, tachycardia, and a high CSF protein level as independent predictors for poor outcome. Also, patients who were eventually diagnosed with inflammatory CNS disorders showed the worst prognosis. These data emphasize the need for improved methods to be able to make a timely diagnosis in all patients suspected of CNS infections and inflammation.

NEDERLANDSE SAMENVATTING

Centraal zenuwstelsel (CZS) infecties zijn geassocieerd met een hoge morbiditeit en mortaliteit, afhankelijk van de verwekker. In principe kunnen alle pathogenen de hersenen of de hersenvliezen daaromheen infecteren, waaronder virussen, bacteriën, schimmels en parasieten. De differentiële diagnose bij patiënten die verdacht worden van een CZS infectie is breed, en de diagnose kan lastig zijn om definitief te stellen. Daarbij kan in een substantieel deel van de patiënten waarin de diagnose van een CZS infectie zeer waarschijnlijk lijkt, geen verwekker worden aangetoond met de huidige beschikbare diagnostische methoden. Behandeling en prognose van patiënten met een CZS infectie zijn echter wel afhankelijk van de specifieke verwekker. Ook heeft empirische behandeling zonder bevestiging van de specifieke verwekker zijn nadelen, zoals bijvoorbeeld antimicrobiële resistentie en nierschade als bijwerking van aciclovir. Om deze redenen is de ontwikkeling en verbetering van diagnostische methoden om CZS infecties te diagnosticeren noodzakelijk. Het doel van dit proefschrift was om de diagnostische accuratesse van klinische voorspellers, biomarkers en 'pathogen discovery sequencing' technieken, voor de diagnose van CZS infecties, vast te stellen en te verbeteren. Om dit te bereiken hebben we verschillende methoden bestudeerd en gevalideerd onder patiënten uit de PACEM en I-PACE studies, waarin patiënten zijn geïncludeerd die een lumbaalpunctie hebben ondergaan vanwege de verdenking op een CZS infectie.

Allereerst hebben we in **Hoofdstuk 2** een externe validatie van bestaande voorspelmodellen voor bacteriële hersenvliesontsteking verricht. We hebben de literatuur doorzocht en een systematic review verricht op de geïdentificeerde artikelen over voorspelmodellen, en die vervolgens gevalideerd in volwassen patiënten uit de PACEM studie. We stelden een excellente discriminatie vast in alle modellen, op één na. Calibratie liet echter over- of onderschatting van het risico op bacteriële hersenvliesontsteking zien in alle modellen. Er was geen model wat zowel een goede sensitiviteit als specificiteit liet zien, waardoor je je af moet vragen of deze modellen iets toevoegen in de klinische praktijk. Aangezien een substantieel deel van de voorspelmodellen in **Hoofdstuk 2** oorspronkelijk ontwikkeld is in cohorten met kinderen, beschrijft **Hoofdstuk 3** de resultaten van validatie van deze modellen in kinderen uit de PACEM studie. Een nieuwe systematic review van de literatuur leverde nog een aantal aanvullende modellen sinds de publicatie van **Hoofdstuk 2** op. Validatie van al deze modellen in kinderen leidde tot vergelijkbare resultaten wat betreft discriminatie en calibratie, en ook in dit geval was er geen model dat zowel weinig vals-positieven als vals-negatieven liet zien.

In de zoektocht naar nieuwe biomarkers wordt in **Hoofdstuk 4** vervolgens een studie gepresenteerd naar de diagnostische accuratesse van neurofilament light chain (NfL), als diagnostische marker voor CZS infecties. NfL is een component van het axonale skelet en is in verschillende CZS aandoeningen geïdentificeerd als marker voor axonale schade. NfL werd gemeten in het hersenvocht van volwassenen uit de PACEM studie die in eerste instantie werden verdacht van een CZS infectie. De diagnostische accuratesse van NfL in hersenvocht voor de diagnose van CZS infecties was slecht. We vonden wel associaties tussen NfL en zowel ernstige neurologische symptomen (weerspiegeld door een verlaagd bewustzijn), als mortaliteit en ongunstige uitkomst. Het gebruik van NfL in hersenvocht voor diagnostische doeleinden is daarom niet aan te bevelen, en prognostische accuratesse voor patiënten met CZS infecties moet verder onderzocht worden in toekomstig onderzoek.

Hoofdstuk 5,6, en 7 beschrijven het gebruik van een metagenomic sequencing techniek voor detectie van virussen in hersenvocht van patiënten met de verdenking op CZS infecties, genaamd VIDISCA-NGS (virus discovery cDNA amplified fragment length polymorphism next generation sequencing). In **Hoofdstuk 5** evalueerden we de mate van prestatie van VIDISCA-NGS in hersenvocht van patiënten waar eerder al een virus was gedetecteerd door kwantitatieve polymerase kettingreactie (qPCR), om zo de reproduceerbaarheid van de methode te testen. VIDISCA-NGS detecteerde een RNA virus in alle monsters met een medium tot hoge virale load en in 67% van de monsters met een lage virale load. Herpesvirussen bleken moeilijk te detecteren met VIDISCA-NGS. In **Hoofdstuk 6** hebben we de diagnostische accuratesse van VIDISCA-NGS in hersenvocht bepaald bij patiënten met en zonder bewezen virale CZS infecties. Voor dit onderzoek hebben we patiënten geselecteerd met een klinische diagnose van een virale CZS infectie en patiënten waarbij uiteindelijk een andere diagnose is gesteld. Over het geheel bleek de sensitiviteit en specificiteit, respectievelijk, 52% en 100%. Ook nu weer bleek het detecteren van herpesvirussen moeilijk. Één enterovirus dat gedecteerd werd door VIDISCA-NGS, werd door qPCR pas gevonden na opnieuw testen van het monster. Er werden een aantal aanvullende virussen gevonden, namelijk GB virus C, human papillomavirus, human mastadenovirus C, Merkel cell polyoma virus en anelloviruses, maar de klinische relevantie hiervan lijkt beperkt. Vervolgens hebben we in **Hoofdstuk 7** de mogelijkheid van VIDISCA-NGS om, naast virussen, ook bacterieel RNA in hersenvocht te kunnen detecteren verkend. De sensitiviteit daarvoor bleek 40-69% te zijn, afhankelijk van de drempelwaarde van verschillende parameters, zoals sequencing diepte, percentage alignment en het aantal pathogeen specifieke reads. Afgezien van deze parameters verschilden de sensitiviteit en specificiteit ook nogal per pathogeen, waarbij het hoogste aantal vals-positieven werd gezien bij de *S. pneumoniae*. Ook vonden we dat een hogere totale sequencing diepte, geen gebruik van antibiotica

voorafgaand aan de lumbaalpunctie, ernstiger ziek zijn en een hogere CRP-waarde geassocieerd waren met detectie van een verwekker.

Tenslotte beschrijven **Hoofdstuk 8 en 9** de klinische en laboratorium karakteristieken van specifieke subgroepen van patiënten met de initiële verdenking op een CZS infectie, en kijken we in **Hoofdstuk 10** vooral naar de uitkomst van deze patiënten. Een specifieke subgroep zijn de patiënten die ervan verdacht worden een CZS infectie te hebben opgelopen in het ziekenhuis (niet gerelateerd aan recent trauma of neurochirurgische interventie). **Hoofdstuk 8** geeft een overzicht van hun klinische karakteristieken en de diagnostische accuratesse daarvan, alsmede van de uiteindelijke diagnoses die gesteld zijn in deze groep. We zagen dat van alle patiënten die van zo'n niet-chirurgische, nosocomiale CZS infectie werden verdacht, 14% uiteindelijk een CZS infectie blijkt te hebben. Verwekkers van deze infecties bestonden uit bacteriën, virussen, schimmels en parasieten. Het combineren van de aanwezigheid van afwijkingen bij het neurologisch onderzoek en in het hersenvocht leidde tot een sensitiviteit van 100% voor het diagnosticeren van een CZS infectie. De resultaten van het hersenvochtonderzoek zorgden in 47% van de gevallen voor een verandering in het medisch beleid. Een andere subgroep werd bestudeerd in **Hoofdstuk 9**, namelijk patiënten die zich presenteren met een epileptische aanval en een lumbaalpunctie ondergaan om een CZS infectie aan te tonen of uit te sluiten. Het aantal leukocyten in het hersenvocht bleek de beste individuele klinische voorspeller voor het hebben van een CZS infectie, met een 'area under the curve' van 0.94. Een derde van de patiënten die niet gediagnosticeerd werden met een CZS infectie hadden wel verhoogde leukocyten in het hersenvocht. Het bleek echter ongebruikelijk dat patiënten met epileptische aanvallen zonder duidelijke oorzaak afwijkende waarden hadden in het hersenvocht. Het onderzoek in **Hoofdstuk 10** heeft zich gericht op de uitkomst van het gehele cohort van de PACEM en de I-PACE studies, die slecht blijkt te zijn: er was sprake van 9% mortaliteit en 36% van de patiënten had een ongunstige uitkomst. Multivariabele regressie liet zien dat leeftijd, focale neurologische uitval, een verlaagd bewustzijn, hersenzenuwuitval, een laag aantal trombocyten, tachycardie en een hoog eiwit in het hersenvocht onafhankelijke voorspellers voor een slechte uitkomst zijn. Daarbij hadden patiënten die uiteindelijk gediagnosticeerd werden met een inflammatoire CZS aandoening de slechtste uitkomst. Deze resultaten benadrukken de noodzaak voor verbeterde methoden om zo snel mogelijk de juiste diagnose te stellen bij patiënten die verdacht worden van CZS infecties of inflammatoire aandoeningen.

LIST OF ABBREVIATIONS

(A)BM	(Acute) bacterial meningitis
AE	Autoimmune encephalitis
AUC	Area under the ROC curve
AUMC	Amsterdam University Medical Centers
(A)VM	(Acute) viral meningitis
(c/g/ds)DNA	(complementary/genomic/double stranded) Deoxyribonucleic acid
CI	Confidence interval
C. neoformans	*Cryptococcus neoformans*
CRP	C-reactive protein
CNS	Central nervous system
CMV	Cytomegalovirus
CSF	Cerebrospinal fluid
CT	Computed tomography
EBV	Epstein-Barr virus
EEG	Electroencephalogram
E. faecalis	*Enterococcus faecalis*
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EV-D68	Enterovirus D68
GCS	Glasgow Coma Scale
GOS	Glasgow Outcome Scale
HBV	Hepatitis B virus
HHV	Human herpes virus
H. influenzae	*Haemophilus influenzae*
HIV	Human immunodeficiency virus
HL	Hosmer-Lemeshow
HPV	Human papilloma virus
HSV	Herpes simplex virus
ICU	Intensive care unit
I-PACE	Improving prognosis using innovative methods to diagnose causes of encephalitis
IQR	Interquartile range
IvIg	Intravenous immunoglobulins
JCV	John Cunningham virus

K. pneumoniae	*Klebsiella pneumoniae*
L. monocytogenes	*Listeria monocytogenes*
LP	Lumbar puncture
LR	Likelihood ratio
MeSH	Medical Subject Heading
mNGS	Metagenomic next generation sequencing
MP	Methylprednisolone
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NA	Not applicable
NfL	Neurofilament light chain
N. meningitidis	*Neisseria meningitidis*
NPV	Negative predictive value
NRLBM	Netherlands Reference Laboratory for Bacterial Meningitis
OR	Odds ratio
PACEM	Pediatric and adults causes of encephalitis and meningitis
(q)PCR	(quantitative) Polymerase chain reaction
PNES	Psychogenic non-epileptic seizures
PPV	Positive predictive value
R. mucilaginosa	*Rothia mucilaginosa*
(r)RNA	(ribosomal) Ribonucleic acid
ROC	Receiver operating characteristic
ROCM	Rhino-orbito-cerebral mucormycosis
S. bovis	*Streptococcus bovis*
sCJD	Sporadic Creutzfeldt-Jakob's disease
Sens	Sensitivity
Spec	Specificity
S. pneumoniae	*Streptococcus pneumoniae*
TPE	Total plasma exchange
TTV	Torque Teno virus
VIDISCA-NGS	Virus discovery cDNA amplified fragment length polymorphism next generation sequencing
VZV	Varicella zoster virus

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PhD PORTFOLIO

1. PhD training

General courses	Year	ECTS
eBROK – NFU/UvA, Amsterdam	2017	1.0
Re-registration BROK – NFU/UvA, Amsterdam	2021	0.2
Practical Biostatistics – Graduate School, UvA, Amsterdam	2017	1.4
The AMC World of Science - Graduate School, UvA, Amsterdam	2018	0.7
Scientific writing in English - Graduate School, UvA, Amsterdam	2018	1.5
EndNote - Graduate School, UvA, Amsterdam	2018	0.1
Systematic Reviews - Graduate School, UvA, Amsterdam	2019	0.7
Computing in R - Graduate School, UvA, Amsterdam	2019	0.7
Specific courses		
ESCMID Course 'Omics of Host and Pathogens During Infections', Grenoble, France	2017	1.0
ESCMID Course 'Acute CNS infections of the brain', München, Germany	2018	1.0
ESCMID Course 'Encephalitis', Grenoble, France	2019	1.0
Infectious Diseases Course - Graduate School, UvA, Amsterdam	2019	1.3
ESCMID Course 'Therapeutic Approach to patients with CNS infections', Amsterdam	2023	1.0
Seminars, workshops and master classes		
Weekly department research meetings	2017-2020, 2023	5.0
Clinical and research seminars ANV, Amsterdam	2017-2024	1.4
(Inter)national conferences and presentations		
4th European Academy for Neurology congress, Lisbon, Portugal. Poster presentation.	2018	1.5
Amsterdam Neuroscience Annual Meeting, Amsterdam. Pecha kucha presentation.	2018	1.0
29th European Congress for Clinical Microbiology and Infectious Diseases, Amsterdam. Poster presentation.	2019	1.0
5th European Academy for Neurology congress, Oslo, Norway. Oral presentation.	2019	1.5
Nederlandse Vereniging voor Neurologie Wetenschapsdagen, Nunspeet. Oral presentation.	2019	0.8
32nd European Congress for Clinical Microbiology and Infectious Diseases, Lisbon, Portugal. Poster presentation.	2022	1.5

2. Teaching

Lecturing	**Year**	**ECTS**
Neurological infectious diseases – Nurse training Amstel Academy, Amsterdam	2018, 2019	0.5
Teacher Interprofessional Education, UvA/HvA, Amsterdam	2018, 2019	1.5
Tutoring, Mentoring		
Mentoring medical bachelor students	2018-2020	2.0
Supervising		
Master thesis C.J. Pennartz, 'Diagnostic accuracy of clinical and laboratory characteristics in suspected non-surgical nosocomial central nervous system infections'	2019, 2020	1.0

3. Parameters of Esteem

Grants	**Year**
Young scientist grant ESCMID Postgraduate Education Course	2017, 2018, 2019
Travel grant for young scientists European Academy of neurology	2018, 2019

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ABOUT THE AUTHOR

Ingeborg van Zeggeren was born in Hoorn on January 31st, 1990. In 2008 she graduated from secondary school at the Murmellius Gymnasium in Alkmaar, and that same year she started medical school at the University of Amsterdam. Her interest in neurology arose during her neurology internship in the Flevoziekenhuis in Almere. In her final years of medical training she did her senior internship on the neurology department in the Slotervaart Hospital in Amsterdam, and an elective internship on the emergency department in Paramaribo, Surinam. After obtaining her medical degree in 2016, she returned to the Slotervaart Hospital as a neurology resident not in training. She worked there until she started her PhD trajectory in 2017, about diagnostic methods in central nervous system infections, supervised by prof. dr. D. van de Beek en prof. dr. M.C. Brouwer. In 2021, Ingeborg started her neurology residency in the Amsterdam University Medical Centers, under the supervision of prof. dr. Y.B.W.E.M. Roos, dr. V.J.J. Odekerken, prof. dr. H.W. Berendse and dr. J.H.T.M. Koelman. Ingeborg lives in Amsterdam with her partner, Rainier, and their son, Stijn.