Cerebrospinal fluid cytokines and chemokines exhibit distinct profiles in bacterial meningitis and viral meningitis

RAMONA CARAGHEORGHEOPOL[1,2*], CĂTĂLIN ȚUCUREANU[2*], VERONICA LAZĂR[1], SIMIN AYSEL FLORESCU[3,4], DRAGOȘ ȘTEFAN LAZĂR[3,5] and IULIANA CARAȘ[2]

[1]Department of Microbiology and Immunology, Faculty of Biology, University of Bucharest, Bucharest 77206; [2]Immunology Laboratory, 'Cantacuzino' National Institute for Medico-Military Research and Development, Bucharest 050096; [3]Infectious Diseases Department II, 'Carol Davila' University of Medicine and Pharmacy, Bucharest 050474; [4]Clinical Department A5 for Infectious and Tropical Diseases, 'Dr Victor Babes' Clinical Hospital for Infectious and Tropical Diseases; [5]Adults Department B2, 'Dr Victor Babes' Clinical Hospital for Infectious and Tropical Diseases, Bucharest 030303, Romania

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Abstract. Differential diagnosis of bacterial meningitis (BM) and viral meningitis (VM) is a critical clinical challenge, as the early and accurate identification of the causative agent determines the appropriate treatment regimen and markedly improves patient outcomes. Clinical and experimental studies have demonstrated that the pathogen and the host immune response contribute to mortality and neurological sequelae. As BM is associated with the activation of an inflammatory cascade, the patterns of pro- and anti-inflammatory cytokines/chemokines (CTs/CKs) present in the cerebrospinal fluid (CSF) in response to the immune assault may be useful as sensitive markers for differentiating BM from VM. In the present study, the ability of CTs/CKs in the CSF to differentiate between BM and VM was investigated. For this, biochemical markers and CT/CK profiles were analysed in 145 CSF samples, divided into three groups: BM (n=61), VM (n=58) and the control group (C; n=26) comprising patients with meningism. The CSF concentrations of monocyte chemoattractant protein-1, interleukin (IL)-8, IL-1β, IL-6, macrophage inflammatory protein-1α (MIP-1α), epithelial-neutrophil activating peptide, IL-10, tumour necrosis factor-α (TNF-α), proteins and white blood cells were significantly higher and the CSF glucose level was significantly lower in the BM group compared with the VM and C groups (P<0.01). Correlation analysis identified 28 significant correlations between various CTs/CKs in the BM group (P<0.01), with the strongest positive correlations being for TNF-α/IL-6 (r=0.75), TNF-α/MIP-1α (r=0.69), TNF-α/IL-1β (r=0.64) and IL-1β/MIP-1α (r=0.64). To identify the optimum CT/CK patterns for predicting and classifying BM and VM, a dataset of 119 BM and VM samples was divided into training (n=90) and testing (n=29) subsets for use as input for a Random Forest (RF) machine learning algorithm. For the 29 test samples (15 BM and 14 VM), the RF algorithm correctly classified 28 samples, with 92% sensitivity and 93% specificity. The results show that the patterns of CT/CK levels in the CSF can be used to aid discrimination of BM and VM.

Introduction

Meningitis is a life-threatening medical condition, in which the inflammatory response plays a key role in the pathogenesis of cerebral injury associated with different aetiologies (1). Viral meningitis (VM) is the most common form of the disease; it has a favourable prognosis, only requires supportive treatment to alleviate the symptoms, and resolves in 7-10 days (2,3). By contrast, the course of bacterial meningitis (BM) is much more severe. Despite marked improvements in intensive care therapy, powerful antibiotic therapy and large-scale vaccination, contracting BM is fatal in 5-10% of children and 20-30% of adults (4,5). In addition, ~40% of survivors suffer neurological sequelae (6,7). Classically, the diagnosis of meningitis involves a lumbar puncture (LP), and analysis of the collected cerebrospinal fluid (CSF) to determine its protein, glucose and lactate levels, cell counts, Gram staining results, cultures, supernatant colour and latex agglutination reactions, in addition to polymerase chain reaction (PCR) assays (8). However, these methods may fail to provide the required results in a timely manner. BM can be challenging to differentiate from aseptic meningitis/encephalitis as the clinical characteristics such as neck stiffness, fever and altered mental status are

Correspondence to: Mrs. Ramona Caragheorgheopol, Immunology Laboratory, 'Cantacuzino' National Institute for Medico-Military Research and Development, 103 Splaiul Independentei, Bucharest 050096, Romania
E-mail: ramona_pit@yahoo.com

*Contributed equally

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common to both conditions but are observed in only 41% of patients with BM (3,9). As the delayed initiation of treatment of BM is strongly associated with poor outcomes (10), empiric antibiotic therapy and adjunctive corticosteroids are usually started early (8,11), leading to frequent cases of improper treatment with associated health and economic impacts (12). Therefore, the identification of early infection biomarkers to help discriminate BM from non-BM would be highly important.

In recent years, increasing importance has been ascribed to the cytokine/chemokine (CT/CK) levels of patients with meningitis to identify particularities of the host immune response that could represent a 'host signature' of infection (13-18). CTs/CKs released during the host immune response have an important role in the recruitment of innate and adaptive immune cells, but they also promote inflammation, thus playing an essential role in the pathogenesis of meningitis. Therefore, it may be hypothesised that the invading bacteria influence CT/CK concentrations and profiles, affecting disease severity and patient outcomes, including long-term sequelae and survival (19). In meningitis, CTs/CKs have been found to contribute to the loss of integrity of the blood-brain barrier (BBB) (20) and favour pleocytosis (21). They can even induce neuronal cell death, either by increasing the secretion of neurotoxic products by microglia (22) or by the direct activation of apoptotic pathways (23-25).

In this context, CTs/CKs have been the subject of numerous studies regarding the pathogenesis of meningitis. For example, data from clinical observations and animal studies have frequently demonstrated that CTs/CKs including tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and IL-8 are involved in the inflammatory cascades in VM and BM, and mediate neuronal damage (4,13,19). Also, the intrathecal injection of TNF-α has been shown to reproduce some of the pathological features of meningitis (26).

In one study, higher levels of IL-6, interferon-γ (IFN-γ) and IL-10 were detected in the CSF of children with BM compared with those without BM (15). In another study, it was found that epithelial-neutrophil activating peptide-78 (ENA-78) was undetectable in patients with aseptic meningitis or control subjects but was present at high concentrations in patients with BM (27). In an analysis of IL-1β, IL-6, TNF-α, IFN-γ and IL-10 in CSF samples to distinguish between various pathogens causing BM in patients of all ages (28), the authors found that only IFN-γ was significantly higher in patients with *Streptococcus pneumoniae* than in those with *Neisseria meningitidis*. IL-6 has been demonstrated to be an important tool for the diagnosis of BM (29,30). The authors of one study (31) suggested that the CSF/blood IL-6 ratio could be a promising biomarker for the discrimination of BM. In addition, another study found that IL-1β and IL-18 levels in the CSF of patients with BM were associated with systemic complications and the survival rate (29). Furthermore, in a study of BM in infants, the authors found that IL-6 and IL-10 were valuable tools for planning the course of treatment for culture-negative but antibiotic-pretreated subjects (30).

Previous studies have shown that the analysis of biomarkers in the CSF is promising for the discrimination of patients with BM or VM, or without meningitis, while they have not reached a consensus on what biomarkers are most appropriate (27-29,31). Therefore, reliable CT/CK tests for the discrimination of BM from VM are not yet available to clinicians. In the present study, to further explore the discriminating power of various CTs/CKs, the concentrations of nine CTs/CKs relevant to the pathophysiology of BM were assessed in CSF samples from patients with BM, VM and meningism, and the correlations of defined CT/CK profiles with other CSF parameters were investigated. Instead of focusing on specific threshold values for specific CTs/CKs, the approach was to evaluate whether a pattern of CTs/CKs could be robustly and accurately powerful for discriminating between BM and VM. In this respect, the study sought to predict BM using a machine learning approach, namely the Random Forest (RF) algorithm.

Materials and methods

Study design. Residual CSF samples from the diagnostic LP of 145 patients of all ages who were admitted with a suspicion of meningitis from October 2014 to July 2017 to the Clinical Hospital for Infectious and Tropical Diseases 'Dr Victor Babes' were used in the present study. The study followed the ethics policies on human subject research of the Clinical Hospital for Infectious and Tropical Diseases 'Dr Victor Babes', and was approved by the Medical Ethics Committee of the hospital (approval no. 5105). Written informed consent forms were signed by the patients or their legal representatives before the samples requested by the attending clinician were collected and analysed. Only the CSF specimens that remained after the completion of routine analysis and diagnostic procedures were used in the current research. Under no circumstances were samples collected for any purpose other than standard analysis for diagnosis. Data were gathered and kept in a coded and securely stored electronic database.

The criteria for meningitis diagnosis at hospital admission were the presence of two or more of the following clinical signs: Fever, neck stiffness, meningeal irritation signs, headache, altered consciousness and vomiting. The epidemiological criteria were: Contact with other known cases, community origin such as in an orphanage, nursery or army, recent travel to areas in which meningitis is endemic (Africa and Asia) and VM epidemic.

According to the CSF cytochemical profile and bacterial or viral detection results, patients were divided into the BM group, VM group and non-meningitis control group (C group). The CSF characteristics for the definition of BM according to the clinical protocol were pleocytosis (100-5,000 leucocytes/mm^3), the predominance of neutrophils, turbid CSF, CSF glucose <50 mg/dl, CSF protein 0.5-2 g/l, positive bacterial CSF culture or Gram stain, positive CSF latex agglutination assay or PCR assay for a bacterial pathogen. The CSF findings for the definition of VM were clear CSF, 50-100 leucocytes/mm^3, CSF glucose >50 mg/dl, CSF protein 0.4-0.8 g/l and positive PCR, GenExpert or BioFire FilmArray results.

For the patients considered in the study design, the following inclusion and exclusion criteria were employed: Only samples with positive culture or Gram stain, CSF latex agglutination or PCR confirmation were included in the BM group; for VM, the inclusion criteria comprised positive PCR, GenExpert or BioFire FilmArray results; the C group included patients admitted for meningitis who did not test positive in

any of the aforementioned tests. Exclusion criteria were as follows: Patients with brain tumours, tuberculous meningitis, fungal meningitis, skull fractures and human immunodeficiency virus infection.

Sample collection and detection. Samples were collected under full aseptic precautions by LP at the time of admission to the hospital and were analysed by the standard routine for the diagnosis of meningitis. After undergoing routine analysis, the remaining CSF specimens were centrifuged to remove cellular debris (715 x g for 20 min at 4˚C). The supernatants were divided into aliquots to optimise multiple freeze-thaw cycles and stored at -80˚C until assayed. Each sample was measured on the first thaw. The protein and glucose levels were evaluated in the CSF samples, and the white blood cell (WBC) count was determined. CT/CK contents in the CSF were measured simultaneously using Human Multianalyte Profiling Base Kit A (R&D Systems, Inc.). A panel of nine CTs/CKs, comprising monocyte chemoattractant protein-1 (MCP-1), IL-8, IL-1β, IL-6, macrophage inflammatory protein-1α (MIP-1α), ENA-78, IFN-γ, IL-10 and TNF-α, was selected and assessed according to the manufacturer's protocol. Briefly, the standards and 4-fold diluted samples were incubated with antibody-coated fluorescent microspheres overnight at 4˚C. After washing, the samples were incubated with biotinylated antibodies. Following another wash, a streptavidin-phycoerythrin conjugate was added. Following the final wash, the fluorescent microspheres were resuspended in the assay buffer and analysed using a Luminex 200™ detection platform (Luminex Corporation). Data were processed with Luminex 200 IS 2.3 Star Station software (Applied Cytometry). A 5-parameter regression formula weighted with reciprocal y (1/y) was used to calculate the sample concentrations from the standard curves.

Statistical analysis. Statistical analyses were performed using the R programming language version 3.6.3 (https://www.r-project.org) and R Studio version 1.4.1106 (http://www.rstudio.com/). CSF cytochemical parameters, including proteins, WBC count, glucose, age and CT/CK levels were compared overall among groups using the Kruskal-Wallis rank-sum test in R. P-values adjusted for multiple pairwise comparisons were calculated using Dunn's test (dunn.test version 1.3.5 in R) with Bonferroni corrections. Two-sided unpaired two-sample Wilcoxon test in R was used to determine if statistically significant differences existed between two groups when the BM group was split into gram-positive and -negative groups. Differences in sex distribution were assessed using Pearson's Chi-square test in R. Samples with CT/CK levels <1 pg/ml were arbitrarily assigned as 1 pg/ml, and the CT/CK levels in CSF specimens were compared after the $\log_{10}$ transformation of data. Categorical variables are presented as proportions or percentages. Continuous variables are expressed as the median and interquartile range (IQR). The ggplot2 R package version 3.3.5 was used for data visualisation.

Correlation matrices were obtained using the corrplot library in R (https://www.rdocumentation.org/packages/corrplot/versions/0.92), and displayed as schematic correlograms. Spearman's correlation coefficient (r) was used to evaluate the correlation between specific variables. The package also provided P-values and confidence intervals for the correlations.

Hierarchical clustering of the log-transformed data matrix was performed to classify patient groups according to different CT/CK types and the heatmap.2 library in R was used to produce heatmaps (https://www.rdocumentation.org/packages/gplots/versions/2.3.0/topics/heatmap.2). For all statistical tests, P<0.01 was considered to indicate a statistically significant result.

Prediction of the BM cases was achieved with a machine learning approach, via RF analysis, using the package randomForest in R (https://www.rdocumentation.org/packages/randomForest/versions/4.7-1.1/topics/randomForest). The method was applied to the dataset comprising all 119 BM and VM samples from the 145 CSF samples. The samples were randomly split into a training set (n=90) and a testing set (n=29). The samples from the testing set were not used for training the prediction model; they were only used to evaluate its performance. The training set was used to build the model with the RF method optimised using 10-fold cross-validation, repeated 10 times.

Results

Demographic, clinical and laboratory findings. CSF samples were collected at the time of hospital admission from 162 patients. These patients included 17 individuals (10.5%) who had a clinical diagnosis of BM based on clinical symptoms, CSF cytochemical findings and therapeutic response to the antibiotic treatment but for whom no causative agent could be identified. Therefore, these patients were not included in the study. Of the remaining 145 patients, 85 (59%) were males. BM was confirmed in 61 (42%) patients and VM in 58 (40%) patients. As the collection of CSF is an invasive procedure that is only performed in emergencies, it was not possible to include healthy controls. The controls (C group) comprised 26 (18%) patients with symptoms who tested negative for meningitis (Table I). The median (IQR) age of the patients with BM was 40 (12-57) years as compared with 14 (9-36) years for the patients with VM and 11 (5-29) years for the C group. While the median age varied among the groups, the difference did not reach statistical significance (P>0.01). Among the patients with BM, 35 (57%) were males; the VM group comprised 35 (60%) males and the C group comprised 15 (58%) males (Table I). The sex distribution was not significantly different among the three groups (P>0.01).

Of the 61 BM samples, gram-positive bacteria were identified in 38 samples, which included *Streptococcus pneumoniae* (n=31, 51%), *Listeria monocytogenes* (n=4, 7%), *Streptococcus beta haemolyticus* (n=1, 2%), *Staphylococcus haemolyticus* (n=1,2%) and *Streptococcus suis* (n=1,2%), whereas gram-negative bacteria were identified in 23 samples, and included *Neisseria meningitidis* (n=16, 26%), *Haemophylus influenzae* (n=4, 7%) and *Escherichia coli* (n=3, 5%).

CT/CK profiles of the BM, VM and C groups. The levels of IL-1β, IL-6, IL-8, IL-10, TNF-α, MIP-1α, MCP-1, IFN-γ and ENA-78 in the CSF samples were measured using a multiplex CT assay. The matrix of $\log_{10}$-transformed data of individual CT/CK concentrations for each patient was

Table I. Characteristics of patients included in the study.

Characteristics	Overall cohort	Bacterial meningitis	Viral meningitis	Controls
Samples, n (%)	145	61 (42)	58 (40)	26 (18)
Adults, n (%)	74 (51)	44 (72)	22 (38)	8 (31)
Age, median (IQR), years	21 (8-47)	40 (12-57)	14 (9-36)	11(5-29)
Male sex, n (%)	85 (59)	35 (57)	35 (60)	15 (58)

IQR, interquartile range.

used for hierarchical clustering to examine if inflammatory response patterns could be defined in the 145 patients. A heatmap (Fig. 1) was built using an agglomeration algorithm to visualise the automatic grouping of samples based on the CT/CK pattern determined in the CSF. In this figure, the segregation of samples with the same clinical diagnostics can be observed in clusters characterised by a particular CT/CK profile. The hierarchical clustering of the 145 samples in the heatmap, based on Euclidean distance in CT/CK secretion, showed moderate grouping according to aetiology, with cases of BM mainly being grouped in a separate branch. However, this analysis clustered numerous BM samples with those of the VM and C groups.

To better illustrate the differences between groups, the median levels and IQR of the CSF cytochemical parameters WBC count, glucose and proteins (Table II) and the nine measured CTs/CKs (Table III) were compared between the BM and C groups, VM and C groups and BM and VM groups. The comparison revealed that the levels of all CTs/CKs were significantly higher in the BM group compared with the C group ($P<0.01$). As compared with CT/CK levels in the VM group, those in the BM group were significantly higher ($P<0.01$), except for IFN-γ ($P=0.192$). While the CT/CK levels were generally higher in the VM group than in the C group, the differences did not reach statistical significance for IL-1β, MIP-1α, ENA-78, IL-10 and TNF-α. However, significantly higher levels of MCP-1, IL-8, IL-6 and IFN-γ were measured in samples from the VM group compared with those from the C group (Table III, Fig. 2).

When the BM group was split into gram-positive and -negative samples, the comparison showed no significant difference between the two categories, except for IFN-γ levels (Table SI), which were higher in the patients with pneumococcal meningitis ($P=0.01$).

Data for WBC, protein and glucose values in CSF were available for 54 BM cases, 27 VM cases and 26 controls. The median CSF WBC count and protein levels were significantly elevated and the median CSF glucose level was significantly decreased in the group with confirmed BM compared with the other two groups ($P<0.01$; Table II).

Correlation between CT/CK concentrations and CSF parameters. The correlations between different CSF CT/CK levels and between CTs/CKs and CSF WBC counts, protein and glucose levels were examined. All the correlations were performed using Spearman's correlation analysis. The correlation matrices are displayed as schematic correlograms and only the correlations with a statistical significance of $P<0.01$ are shown (Fig. 3). Correlations between CSF cytochemical markers and CT/CK values in the BM group showed that glucose negatively correlated with the proteins ENA-78, IL-10, IL-6 and TNF-α while IL-1β positively correlated with the WBC count. In the VM group, a negative correlation was detected between glucose and protein levels and a positive correlation was identified between WBC counts and IL-6. However, as data for CSF cytochemical markers were not available for all the patients in the study, only the correlations among CTs/CKs are shown.

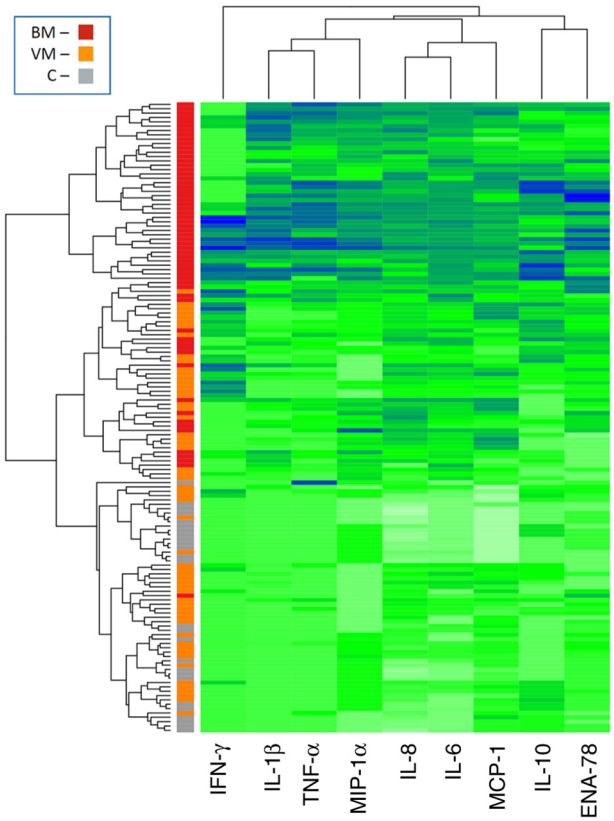

Figure 1. Hierarchical clustering of 145 cerebrospinal fluid samples based on CT/CK levels. The spectrum from white to green to blue corresponds to the increasing gradient of log10-transformed CT/CK concentrations. Each patient group is reported as a colour code on the left column, where red represents the BM group, orange represents the VM group and grey represents the C group. CT, cytokine; CK, chemokine; BM, bacterial meningitis; VM, viral meningitis; C, control; IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor-α; MIP-1α, macrophage inflammatory protein-1α; MCP-1, monocyte chemoattractant protein-1; ENA-78, epithelial-neutrophil activating peptide-78.

Table II. Laboratory findings for the CSF samples.

	Aetiological group				[b]P-value		
CSF variable	BM, median (IQR)	VM, median (IQR)	C, median (IQR)	[a]P-value	BM vs. VM	BM vs. C	VM vs. C
WBC, cells/mm^3	3,750 (1,360-12,985)	156 (74-261)	2 (1-4)	<0.01	<0.01	<0.01	<0.01
Glucose, mg/dl	25 (24-39)	61 (51-77)	66 (53-76)	<0.01	<0.01	<0.01	1
Protein, mg/dl	260 (158-401)	57 (33-87)	21 (15-32)	<0.01	<0.01	<0.01	0.015

[a]P-values determined by the Kruskal-Wallis rank-sum test; [b]P-values for pairwise comparisons adjusted for multiple comparisons (using Dunn's test with Bonferroni post hoc test). CSF, cerebrospinal fluid; BM, bacterial meningitis; VM, viral meningitis; C, control; IQR, interquartile range; WBC, white blood cell.

Table III. CT/CK levels in the cerebrospinal fluid samples of all studied patients.

	Aetiological group				[b]P-value		
CT/CK	BM, median (IQR), pg/ml	VM, median (IQR), pg/ml	C, median (IQR), pg/ml	[a]P-value	BM vs. VM	BM vs. C	VM vs. C
MCP-1	1,840 (286-5,007)	174 (86-714)	21.6 (1-80)	<0.01	<0.01	<0.01	<0.01
IL-8	3,233 (1,055-6,365)	196 (72-674)	4.6 (2.4-8.7)	<0.01	<0.01	<0.01	<0.01
IL-1β	235 (19-1,364)	1 (1-2.7)	1 (1-1)	<0.01	<0.01	<0.01	0.074
IL-6	13,585 (3,622-20,000)	117 (23-728)	1 (1-1)	<0.01	<0.01	<0.01	<0.01
MIP-1α	1,079 (224-2,531)	48 (91-82)	48 (1-48)	<0.01	<0.01	<0.01	0.717
ENA-78	119 (44-374)	14 (8-29)	8 (8-8)	<0.01	<0.01	<0.01	0.017
IFN-γ	9 (2-58)	2 (2-18)	2 (2-2)	<0.01	0.192	<0.01	<0.01
IL-10	180 (22-1,579)	16 (1-116)	1 (1-16)	<0.01	<0.01	<0.01	0.094
TNF-α	70 (11-650)	1 (1-3.7)	1 (1-1)	<0.01	<0.01	<0.01	0.145

[a]P-values determined by the Kruskal-Wallis rank-sum test. [b]P-values for pairwise comparisons adjusted for multiple comparisons (using Dunn's test with Bonferroni post hoc test). CT, cytokine; CK, chemokine; BM, bacterial meningitis; VM, viral meningitis; C, control; IQR, interquartile range; MCP-1, monocyte chemoattractant protein-1; IL, interleukin; MIP-1α, macrophage inflammatory protein-1α; ENA-78, epithelial-neutrophil activating peptide-78; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

Regarding the correlations in the BM and VM groups, distinct patterns can be observed for the two aetiologies. In the BM group, 28 significant correlations were identified, with the strongest positive correlations occurring among TNF-α, IL-6, IL-1β and MIP-1α (Fig. 3A, Table SIIA). Fewer correlations with statistical significance were found in the VM group (Fig. 3B). However, the strength of correlation was high for MCP-1 with IL-8 and IL-6, and IL-6 was also correlated with IL-1β (Fig. 3B, Table SIIB).

RF variable importance analysis. The hierarchical clustering (Fig. 1) grouped numerous BM samples with VM and C samples. Therefore, to identify the CT/CK patterns that best predict and classify BM and VM, a dataset comprising the CT/CK concentrations of 119 BM and VM patient samples (90 for training and 29 for testing) was used as input for a machine learning algorithm. A variable importance plot was thereby generated to rank the utility of each CT/CK as a predictor (Fig. 4). The predictors with the highest importance were IL-1β and IL-6 (Fig. 4, Table SIIIA). Due to IFN-γ inter-individual variability and the lack of significant differences in the levels of this CT between BM and VM (P=0.154), IFN-γ was removed from the dataset for further analysis.

Of the 29 testing samples (15 BM and 14 VM), the RF algorithm correctly classified 28, with 93% specificity and 92% sensitivity. When used for classifying BM vs. C (Fig. S1, Table SIIIB), the sensitivity and specificity were both 100%, while the comparison of VM vs. C had 90% specificity and a lower sensitivity of 77% (Fig. S2, Table SIIIC).

Discussion

The present study investigated nine CT/CK levels in the CSF samples of 145 patients with BM, VM and meningism. The results demonstrated that the patients with BM had significantly higher CSF levels of IL-1β, IL-6, ENA-78, MCP-1, TNF-α, MIP-1α, IL-10, and IL-8 than the patients with VM or the controls. Using RF analysis, the usefulness of these parameters in predicting BM was assessed. This analysis identified a CT/CK signature that was able to differentiate BM from VM

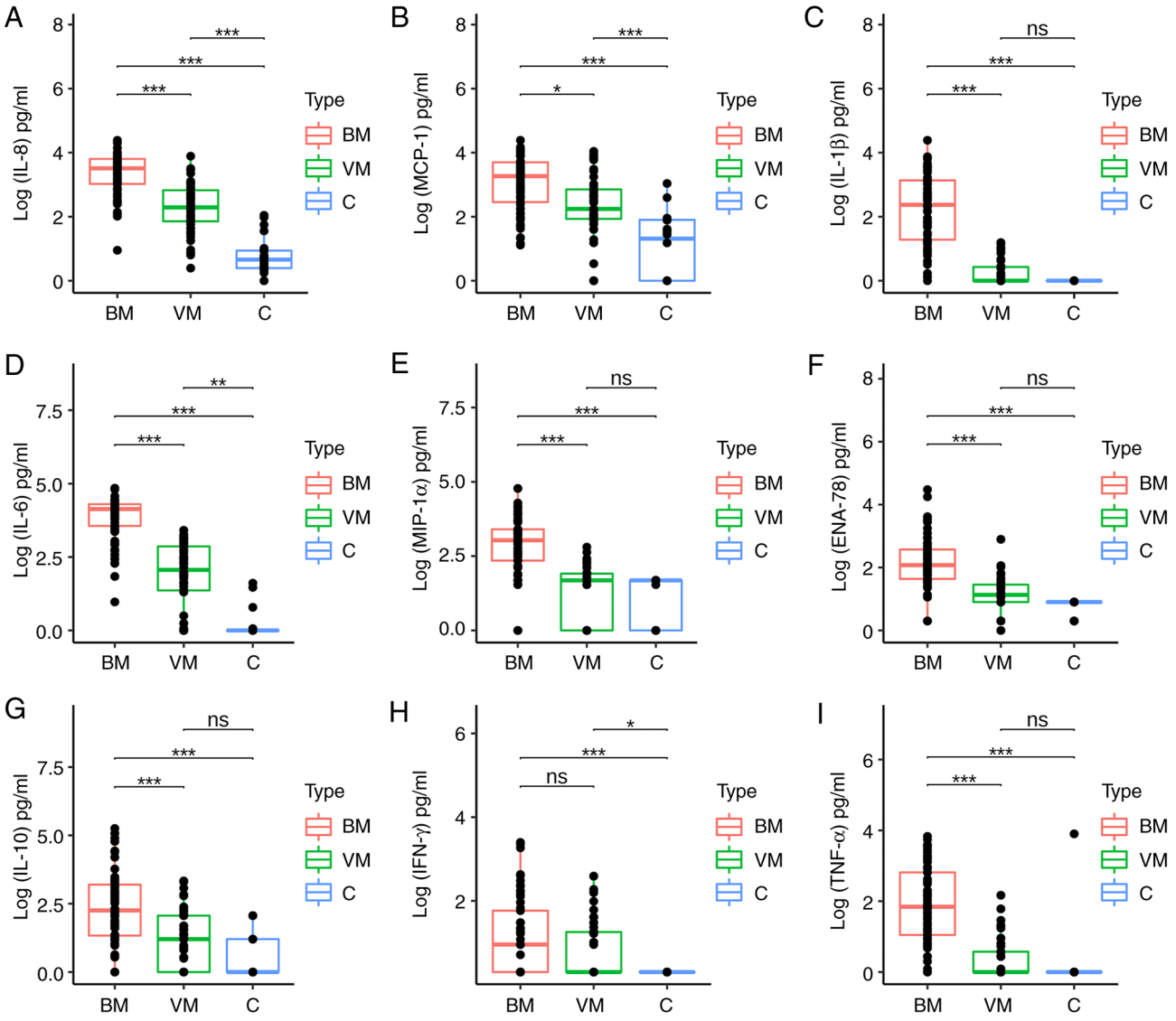

Figure 2. Comparison of $\log_{10}$-transformed CT/CK concentrations among the study groups, BM, VM and C. Concentrations of (A) IL-8, (B) MCP-1, (C) IL-1β, (D) IL-6, (E) MIP-1α, (F) ENA-78, (G) IL-10, (H) IFN-γ and (I) TNF-α. The boxes represent the IQR, the horizontal lines inside each box represent the median, and the dots represent individual measurements. Significance was calculated using Dunn's test with Bonferroni post hoc tests, following Kruskal-Wallis testing. P-values adjusted for multiple comparisons are indicated above each plot. ns, P>0.01 (not significant), *P<0.01, **P<0.001, ***P<0.0001. CT, cytokine; CK, chemokine; BM, bacterial meningitis; VM, viral meningitis; C, control; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; ENA-78, epithelial-neutrophil activating peptide-78; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

with 93% specificity and 92% sensitivity. IL-1β and IL-6 were identified as the variables with the highest importance score, followed by ENA-78.

The host inflammatory reaction caused by pathogens in the CSF includes the increased intrathecal production of soluble mediators, such as CTs/CKs. Pathogen-associated molecular patterns (PAMPs) are evolutionary conserved pathogen components, including the lipopolysaccharides (LPS) of gram-negative bacteria, peptidoglycans, lipoteichoic acid from gram-positive bacteria, and the DNA and RNA of bacteria and viruses. They are detected by a wide array of innate sensors, using pattern recognition receptors (PRRs), such as the Toll-like receptor, Nod-like receptor and RIG-like receptor families (32). The detection of PRRs by PAMPs triggers the activation of I-κB kinase NF-κB pathways and mitogen-activated protein kinase pathways, resulting in the secretion of signalling molecules that interact to orchestrate the early host response to infection and later adaptive immunity (33). Among these signalling molecules, CTs/CKs are the driving force in shaping a plethora of host responses, with CT signalling being indispensable to disease resolution but also responsible for numerous deleterious effects of dysregulated inflammatory responses.

Perivascular macrophages and resident cells in the central nervous system react to LPS, peptidoglycans and nucleic acids released by bacteria, producing the early response inflammatory CKs TNF-α, IL-1β and IL-6. Although they are generally considered to be necessary for active protection against BM (34), TNF-α and IL-1β also initiate meningeal inflammation (26) and stimulate the recruitment of monocytes and neutrophils to infection sites and activate them. Several studies have demonstrated that TNF-α, IL-1β and IL-6 are

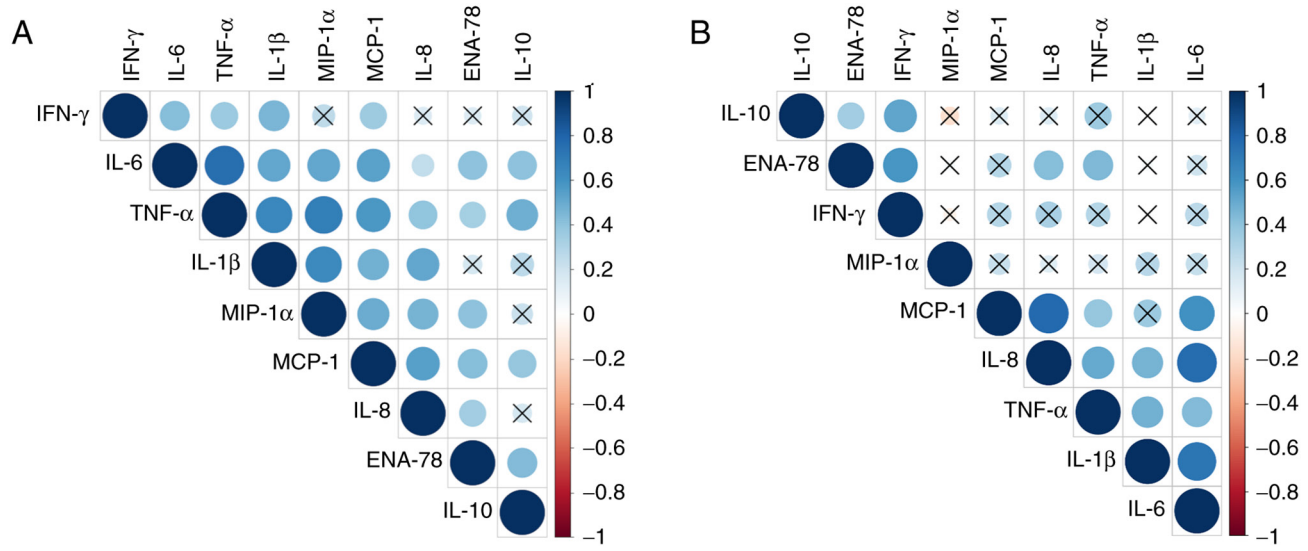

Figure 3. Correlograms representing the correlations among the CT/CK concentrations in patients with BM and VM. Correlograms for (A) BM and (B) VM are presented. The dark blue colour represents the highest positive correlation between the concentrations of two parameters (r=1), and the darkest red colour represents the strongest negative correlation (r=-1). Correlations with a significance level >0.01 are marked with an 'X'. (A) In the BM group 28 significant correlations among the CTs/CKs analysed were detected and (B) in the VM group, 14 significant correlations were detected. CT, cytokine; CK, chemokine; BM, bacterial meningitis; VM, viral meningitis; IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor-α; MIP-1α, macrophage inflammatory protein-1α; MCP-1, monocyte chemoattractant protein-1; ENA-78, epithelial-neutrophil activating peptide-78.

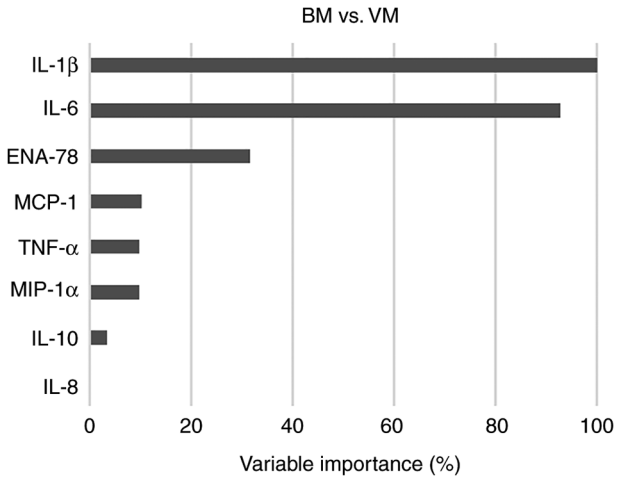

Figure 4. Variable importance plot of each cytokine or chemokine in the discrimination of BM from VM as determined by the Random Forest algorithm. BM, bacterial meningitis; VM, viral meningitis; IL, interleukin; ENA-78, epithelial-neutrophil activating peptide-78; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; MIP-1α, macrophage inflammatory protein-1α.

upregulated in BM, and demonstrate good sensitivity and specificity for the diagnosis of this disease (4,13,19). The data in the present study reveal three orders of magnitude difference in the IL-1β and IL-6 concentrations of patients with BM compared with those with VM, which supports these findings. In previous studies, higher levels of TNF-α and IL-1β CSF were found to be associated with complications and unfavourable disease outcomes when the relationship between inflammatory mediators and disease outcome was investigated (29,35). Also, the administration of TNF-α into the CSF was shown to result in pathophysiological changes characteristic of BM (26), while the blocking of IL-1β in a murine model of BM led to a reduction in disease severity (29). In the present study, the median TNF-α was significantly higher in patients with BM compared with patients with VM and the controls. However, in the present research, there were patients with BM who had low TNF-α levels, which might be explained by the timing of the LP. A previous study of patients with BM observed that TNF-α levels were significantly elevated in the CSF when the LP was performed ≤48 h from the onset of symptoms as compared with >48h, and the elevated TNF-α levels were not maintained because of the rapid decline of TNF-α during BM (28). Furthermore, in an animal model, the kinetics of TNF-α release showed that TNF-α reached its maximal level 1-2 h after LPS injection and was not detectable after 24 h (36).

In the present study, the early response CKs significantly correlated with each other (P<0.01): TNF-α/IL-6 (r=0.75), TNF-α/IL-1β (r=0.64) and IL-1β/IL-6 (r=0.52). The high levels of proinflammatory CKs in BM stimulate the secretion of CKs such as IL-8, ENA-78, MCP-1 and MIP-1α. CKs play an essential role in the recruitment and trafficking of leukocytes. The strong inflammatory response contributes to the loss of integrity of the BBB due to the recruitment of leukocytes, alteration of the meningeal vasculature and upregulation of various adhesion molecules on the endothelial cells, including selectins, intercellular adhesion molecules and vascular endothelial adhesion molecules. In parallel, proteins, complement factors and immunoglobulins leak into the CSF (37). While MCP-1 and MIP-1α are the major chemoattractants for monocytes during inflammatory responses, IL-8 and ENA-78 are the major chemoattractants for neutrophils that pass between the activated endothelial cells entering the subarachnoid space. It has been reported that IL-8 plays an important role in the pathogenesis of pneumococcal disease (19), pneumolysin is a factor influencing IL-8 levels (38) and the neuraminidase expressed by *S. pneumoniae*, NanA, mediates changes in IL-8 release (39).

ENA-78, also known as C-X-C motif chemokine 5 is a small CK secreted by immune and vascular endothelial cells which, like other CKs, facilitates chemotaxis and leucocyte recruitment and is involved in BBB dysfunction (40-42). ENA-78 is considered an important biomarker of neuroinflammation and neurodegeneration. Its role is currently being studied in Alzheimer's disease (42,43), multiple sclerosis relapse (40) and other neuroinflammatory diseases, such as HTLV-1-associated myelopathy (37), juvenile gangliosidoses diseases (44) and primary progressive aphasia (45). In a study using a machine learning approach, this CK and three other markers showed high discriminatory performance between patients with Alzheimer's disease and controls (42). However, studies on the expression of this CK in BM cases are scarce. The only study on this topic (27), to the best of our knowledge, showed that ENA-78 and IL-8 had profoundly elevated concentrations in the CSF of patients with BM and were not detectable in patients with VM. Confirming these results, the present study also found significantly higher levels of ENA-78 and IL-8 in patients with BM compared with patients with VM and the controls.

MIP-1α and MCP-1 are the other two important mediators of chemoattraction evaluated in the present study that were upregulated in the BM group compared with the VM and/or C groups. In line with these results, MIP-1α and MCP-1 levels have been observed to be elevated in the CSF of patients with pneumococcal and meningococcal meningitis in previous studies (28,35), with MCP-1 significantly higher in cases of pneumococcal meningitis compared with meningococcal meningitis (35). In the present study, MIP-1α and MCP-1 correlated with proinflammatory CKs but not with the WBC. Other authors have shown similar results, where no correlation was found between IL-8, MIP-1α and MCP-1 levels and the WBC in the CSF during BM (28).

Inflammatory markers in the CSF that have previously been reported to help diagnose BM include the WBC count, glucose and protein levels (13,30). To better understand the pathophysiology of meningitis, these parameters were analysed in the present study alongside CSF CTs/CKs. Confirming the findings of other studies (13,28,30), the CSF protein levels and WBC count in the present study were significantly higher in the BM group compared with the VM and C groups, whereas the glucose level was significantly lower. Other authors considered these parameters as having only modest sensitivity and specificity for meningitis detection (30,46).

In the present study, the protein and glucose levels and WBC count were not found to correlate well with the other parameters. Furthermore, as the data for CSF protein levels, glucose levels and WBC count were not available for all 145 patients, these correlations are not shown.

IFN-γ was the only CK with similar concentrations in the three studied groups. However, when the BM group was split into gram-positive and -negative samples, IFN-γ was the only CK to differentiate between the two groups, with a higher concentration in patients with gram-positive BM, confirming the results of a previous study (28).

Following the above analysis of how the expression of CSF biomarkers differs in various conditions, the effectiveness of this information for distinguishing the three groups (BM, VM and C) to help facilitate early-stage diagnosis requires consideration. Although all the studied CTs/CKs, with the exception of IFN-γ, were expressed at significantly higher levels in the BM group than in the other two groups, the goal of the study was to identify CT/CK patterns that best predict and classify BM and VM. Combining the expression of a number of the CTs/CKs would be expected to lead to more accurate and robust predictions. Other studies have mainly explored the expression dynamics of specific CKs in different forms of meningitis (1,4,16,31), as well as how they correlate with parameters such as CSF glucose, protein and WBC count (13,30). Attempts have been made to combine such results for early diagnostic purposes with promising results (15,34,46). In the present study, the RF state-of-the-art machine learning algorithm was employ for this purpose. This algorithm has been demonstrated to be useful, for instance, in the differentiation of the CK signatures of SARS-CoV-2 and influenza (47), classification of the risk of coronary artery disease using plasma CKs (48), and identification of CK profiles for distinguishing children with *Plasmodium falciparum* malaria from those with bacterial bloodstream infections (49). RF is considered to outperform numerous other known techniques for this type of data and predictions (48,50).

As an ensemble method, RF is based on the concept that a group of weak learners can work together and perform as one strong learner. In this case, the weak learners are decision trees, which, taken individually, are prone to overfitting and bias. However, RF uses bagging, also known as bootstrap aggregation, which entails choosing random samples from the training dataset (with replacement) and generating independent decision trees from these samples. A 'forest' of decision trees generated via this method is used to classify the data. Each tree votes for a class, and the majority decides. As an important detail, when generating a decision tree, in the present study, only two-thirds of the data samples were used for building the classification model, while one-third were set aside as 'out-of-bag' samples for data evaluation. To avoid the issue of correlated features (predictors) in the dataset, RF uses random subsets of features in each node to guide the node split. This results in uncorrelated decision trees and, together with the bagging technique, ensures a more robust classification method with a reduced risk of overfitting or bias (48,51,52). The RF method is also optimised using k-fold cross-validation, and in the present study, 10-fold cross-validation was used.

Due to the stochastic character of the process of growing decision trees, it can be challenging to interpret the results of the RF algorithm. Therefore, the RF algorithm evaluates the so-called variable importance of each predictor in the dataset. To that end, the increase in the mean squared error of the prediction is evaluated when a feature/predictor is removed from the model, allowing the importance of the variable to be estimated. In the present study, the most important predictors for discriminating BM and VM identified by the RF algorithm were IL-1β and IL-6. The patients with BM were correctly classified in 14 out of 15 cases, with very good specificity (93%) and sensitivity (92%), making RF a promising tool in the differentiation of BM from VM.

Regarding the strengths of the present study, using the sophisticated RF machine-learning algorithm, eight measured CT/CK levels were employed for classifying patients in the BM, VM and C groups. To assess classification strength, 29

random and completely independent out-of-bag test samples were used to establish the classification model. However, a limitation of the study was that CSF cytochemical marker data were not available for all the patients included in the study. Therefore, it was decided to conduct the classification with only CTs/CKs. This enabled the number of patients to be kept reasonably high. Despite using only eight CTs/CKs, only one of the 29 test samples was misclassified when distinguishing BM from VM. Another limitation was that the CSF samples were collected during hospital admission. While this is the clinically relevant time point, it may be a different moment in the disease for each patient, and the level of early inflammatory markers may have already declined for some patients.

In conclusion, in the present study, the concentrations of nine relevant CTs/CKs from CSF samples were assessed in the pathophysiology of BM, VM and meningism, and the correlation of defined CT/CK profiles with other CSF parameters was further investigated. Finally, whether a pattern of CTs/CKs has good discriminating power between BM and VM was studied using the RF machine learning algorithm. With 28 of 29 test samples correctly classified, the results suggest that CTs/CKs measured in the CSF, directly sampled when meningitis is suspected, can accurately classify BM and VM. Furthermore, based on these results, it is likely that improved patient care could be obtained in the future with a rapid test developed to enable the prompt measurement of these biomarkers.

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Availability of data and materials

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

Authors' contributions

The study was designed by IC, CT and VL. Clinical diagnosis, sample collection and management, and clinical data collection were performed by SAF and DSL. Data were measured by IC, CT and RC, while data analysis was performed by RC and CT. RC wrote the manuscript, and RC, CT, IC, VL, SAF and DSL reviewed the manuscript. VL and IC supervised the study. RC, IC and CT confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of the Clinical Hospital for Infectious and Tropical Diseases 'Dr Victor Babes' (approval no. 5105). All samples were collected with written informed consent from all participants or parents/guardians in the case of children under 18 years old and was conducted based on the principles expressed in the 1975 Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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