

# Evaluation of human $\beta$ -defensins in the cerebrospinal fluid of suspected meningitis

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**Abstract.** Human  $\beta$ -defensins (HBDs) are an important class of antimicrobial peptides that have immunomodulatory functions; however, the role of HBDs have not been well explored in the pathogenesis of meningitis. A cross-sectional study was performed to explore the levels of HBD1, HBD2, HBD3, and HBD4 in the cerebrospinal fluid (CSF) of 176 suspected meningitis cases. CSF samples were first subjected to PCR analysis using a set of universal primers targeting a portion of the eubacteria 16S rRNA gene. The analysis demonstrated that 66 samples (37.5%) were PCR-positive, whilst 110 samples (62.5%) were PCR-negative. DNA sequence analysis of the PCR-positive products identified two broad categories of bacteria, Gram-negative (68.2%) and Gram-positive (31.8%). A total of 88 PCR-negative CSF samples showed abnormal leukocyte counts, glucose concentrations, and/or protein concentrations, and were considered abnormal (ABN). The remaining 22 CSF samples were considered normal (NOR). HBD1, HBD2, and HBD4 levels did not exhibit significant differences between PCR-positive, ABN, and NOR CSF samples. However, HBD3 levels were significantly higher in the ABN CSF samples than in the NOR CSF samples ( $P=0.005$ ). HBD3 levels were also elevated in the PCR-positive CSF samples compared with the NOR CSF samples, but the

difference was not significant ( $P=0.151$ ). HBD2, HBD3, and HBD4 were correlated with leukocyte counts, glucose concentration, and protein concentration. In conclusion, HBD3 levels were significantly elevated in the CSF of suspected meningitis cases regardless of the cause of meningitis. The CSF levels of certain HBDs were affected by specific diagnostic laboratory parameters for meningitis, including leukocyte counts, glucose concentration, and protein concentration.

## Introduction

Meningitis is a life-threatening disease associated with increased mortality rates amongst newborns, children, adolescents, and adults. Furthermore, survivors may be at risk of developing a permanent disability (1). Infectious and non-infectious processes can cause meningitis, and among the infectious agents involved are bacteria, viruses, and fungi (2). These agents can cause inflammation of the membranes (meninges) and/or cerebrospinal fluid (CSF) surrounding the brain and spinal cord (3). Bacteria are an important cause of meningitis and it is estimated that >1.2 million cases of bacterial meningitis occur annually worldwide, with incidence varying by region, country, age, and pathogen (4). A global and regional analysis of meningitis from 1990-2016 showed that incident meningitis cases increased from 2.5 million in 1990 to 2.82 million in 2016 and the overall incidence rate in 2016 varied from 0.5 cases per 100,000 individuals in Australia to 4.2 cases per 100,000 individuals in South Sudan (5). In Iraq, the annual incidence of laboratory-confirmed bacterial meningitis was 1.47 cases per 100,000 individuals (6).

Several risk factors associated with meningitis have been described including age, sex, otitis or sinusitis, neurosurgery, diabetes, splenectomy, pneumonitis, endocarditis, chronic hepatitis with cirrhosis, head trauma, and impaired consciousness (7). In addition, it has been indicated that inflammatory reactions in the CSF play an essential role in the pathogenesis of brain injury associated with various meningitis pathogens, and bacteria are among the most important pathogenic components that have been shown to stimulate the release of pro-inflammatory substances (8). Thus, the permeability of the blood-brain barrier increases, and leukocytes are attracted to the central nervous system (CNS), which is observed as

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**Abbreviations:** +ve, positive; -ve, negative; ABN, abnormal; AMP, antimicrobial peptide; AUC, area under the curve; CI, confidence interval; CNS, central nervous system; CSF, cerebrospinal fluid; G, gram; HBD, human  $\beta$ -defensin; IL, interleukin; IQR, interquartile range; NOR, normal; P, probability; PCR, polymerase chain reaction; ROC, receiver operating characteristic;  $r_s$ , Spearman's correlation coefficient; SD, standard deviation; YI, Youden index

**Key words:** meningitis, human  $\beta$ -defensin, cerebrospinal fluid, polymerase chain reaction, glucose, protein

pleocytosis in the CSF. Therefore, the CSF profile of inflammatory mediators, such as the cytokines tumor necrosis factor- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6, may predict the severity and consequences of meningitis (9). The secretion of cytokines can also be induced by components of the innate immune response, such as antimicrobial peptides (AMPs), which have been shown to play several potential roles in inflammatory responses, and their role in the pathogenesis of meningitis has also been proposed (10,11).

AMPs are essential components of innate immunity and play an important role in fending off invasive microbial pathogens. Most AMPs can directly kill microbial pathogens, whilst others act indirectly by modulating the immune defense mechanisms of the host (12). Based on their structure, AMPs are classified into four classes, which include linear  $\alpha$ -helical peptides,  $\beta$ -sheet peptides, or both, as well as a linear extension structure (13). The most common class is  $\beta$ -sheet peptides, and this class includes the largest group of AMPs, defensins, which in humans consist of two major types,  $\alpha$ - and  $\beta$ -defensins. Both types play a significant role in mediating antibacterial, antiviral, antifungal, immune, and anti-inflammatory responses (14).

Six human  $\beta$ -defensins (HBD1, HBD2, HBD3, HBD4, HBD5, and HBD6) have been described, although gene-based analysis indicates an additional 28 HBDs (15). The first three HBDs are primarily expressed by epithelial cells, but peripheral blood mononuclear cells, macrophages, and plasmacytoid dendritic cells also express them (16). HBD4 is expressed by neutrophils, the thyroid glands, testes, gastric antrum, uterus, lungs, and kidneys, whilst expression of HBD5 and HBD6 is restricted to the epididymis (17). The most widely studied HBDs are HBD1, HBD2, HBD3, and HBD4 (15,16). Recent evidence indicates that in addition to being components of innate antimicrobial immunity, HBDs also play a key role as pro-inflammatory mediators and immunostimulators that increase the response to infection (18-20). In meningitis, HBDs have not been well investigated, but their roles in neuroimmune function and neurodegeneration have been proposed (21). Additionally, insights have been provided to describe their function as part of the innate immune defense against pathogens in bacterial CNS infections (22). Recently, the expression of HBD2 was shown to be modulated by *Neisseria meningitidis*, a Gram-negative bacteria that causes meningitis (11,23).

The present study analyzed the levels of HBD1, HBD2, HBD3, and HBD4 in the CSF of suspected meningitis cases. These cases were divided into two groups based on the PCR assessment of bacterial CSF infection (PCR-positive and PCR-negative). Next, PCR-negative CSF samples were classified as abnormal (ABN) or normal (NOR) based on the leukocyte counts, and glucose and protein concentrations.

## Patients and methods

*Suspected meningitis cases.* A cross-sectional study was performed on 176 cases of suspected meningitis (mean age, 26.5 $\pm$ 20.8 years; minimum age, <1 year; maximum age, 81 years; 78 males, 98 females) following a diagnosis of hydrocephalus in infants and children and increased intracranial pressure in adults. Cases were admitted to two major neurological hospitals in Baghdad (Iraq), Neurosurgery Teaching

Hospital and Alwitri Neuroscience Teaching Hospital, during a period of 11 months (January to November 2020). Patients with hydrocephalus or intracranial pressure who agreed to participate were included in the present study. Excluded patients were those who did not provide written consent by themselves or through their guardian. Patients were also excluded if they had neurosurgical disease or traumatic lumbar puncture. From each participant, one CSF sample was obtained by lumbar puncture and transferred to a sterile tube. Information regarding age, sex, and antibiotic use was recorded. The Institutional Ethics Committee of the College of Science, University of Baghdad (Baghdad, Iraq) approved the study (approval no. CSEC/1022/0136) and written informed consent was obtained from patients or their legal guardian prior to sample collection.

*Leukocyte count.* The direct microscopic method was used to count the number of leukocytes in the CSF using a Neubauer chamber. Leukocyte counts are expressed as cells/mm<sup>3</sup>. CSF samples were classified as having either normal leukocyte counts (0-15 cells/mm<sup>3</sup> for infants  $\leq$ 28 days; 0-9 cells/mm<sup>3</sup> for infants 29-60 days of age; and 0-5 cells/mm<sup>3</sup> for children and adults) or pleocytosis as previously described (24).

*Measurement of glucose and protein concentrations.* Quantitative determination of glucose and protein concentrations in the CSF was performed using commercially available kits (cat. nos. MDBSIS46-P and MDBSIS29-I, respectively; Spinreact) according to the manufacturer's protocol. The normal ranges for glucose and protein concentrations in the CSF are 40-70 and 15-45 mg/dl, respectively (25,26).

*Immunoassay for HBDs.* ELISA kits were used to measure the CSF levels of HBD1, HBD2, and HBD3 (cat. nos. CSB-E14186h, CSB-E13201h, and CSB-E14187h, respectively; Cusabio Technology LLC), and HBD4 (cat. no. GWB-SKR005; GenWay Biotech) according to the manufacturer's protocol.

*PCR analysis of CSF.* DNA was isolated from CSF samples using a HiPurA *Mycobacterium tuberculosis* DNA purification kit (cat. no. MB545-250PR; Himedia Laboratories) according to the manufacturer's protocol. PCR analysis was performed following a previously developed universal PCR protocol to amplify a 996-bp DNA fragment of the eubacteria 16S rRNA gene in CSF samples (27). PCR amplification was performed using two primers: U1, 5'-CCAGCAGCCGCGTAATACG-3'; and U2, 5'-ATCGG[C/T]TACCTTGTTACGACTTC-3'. PCR products were separated by 1.5% agarose gel electrophoresis, and those that showed an amplified band (PCR-positive) were subjected to Sanger sequencing (performed by MacroGen, Inc.) using a Genetic Analyzer System (model ABI-310; MacroGen, Inc.). Two broad categories of bacteria, Gram-negative (G-ve) and Gram-positive (G+ve), were considered based on DNA sequence alignment with NCBI sequence databases using the BLAST function (<https://blast.ncbi.nlm.nih.gov>).

*Statistical analysis.* Statistical analysis was performed using GraphPad Prism version 8.0.0 (GraphPad Software, Inc.) and IBM SPSS Statistics 25.0 (IBM Corp.). Categorical

Table I. Baseline characteristics of suspected meningitis cases and cerebrospinal fluid laboratory data.

| Characteristics/laboratory data <sup>a</sup> |             | PCR-positive,<br>n=66 | PCR-negative CSF, n=110 |            | P-value          |
|--|-------------|-----------------------|-------------------------|------------|------------------|
|  |             |                       | ABN <sup>b</sup> , n=88 | NOR, n=22  |                  |
| Age, years                                   |             | 28.8±20.7             | 25.1±22.2               | 25.6±14.7  | 0.549            |
| Age group, years                             | <2          | 14 (21.2)             | 32 (36.4)               | 3 (13.6)   | 0.085            |
|  | 3-12        | 4 (6.1)               | 3 (3.4)                 | 1 (4.5)    |                  |
|  | 13-18       | 5 (7.6)               | 4 (4.5)                 | 3 (13.6)   |                  |
|  | 19-39       | 21 (31.8)             | 17 (19.3)               | 10 (45.5)  |                  |
|  | 40-59       | 17 (25.8)             | 29 (33.0)               | 5 (22.7)   |                  |
|  | ≥60         | 5 (7.6)               | 3 (3.4)                 | 0 (0.0)    |                  |
| Sex  | Male        | 27 (40.9)             | 44 (50.0)               | 7 (31.8)   | <b>&lt;0.001</b> |
|  | Female      | 39 (59.1)             | 44 (50.0)               | 15 (68.2)  |                  |
| Leukocyte count, cells/mm <sup>3</sup>       |             | 2.0 (0-62.5)          | 11.5 (1-74.3)           | 0.5 (0-2)  | <b>&lt;0.001</b> |
| Leukocyte count                              | Normal      | 43 (65.2)             | 42 (47.7)               | 22 (100.0) | <b>&lt;0.001</b> |
|  | Pleocytosis | 23 (34.8)             | 46 (52.3)               | 0 (0.0)    |                  |
| Glucose, mg/dl                               |             | 60±30                 | 58±34                   | 57±9       | 0.868            |
| Glucose percentile                           | ≤25         | 18 (27.3)             | 28 (31.8)               | 0 (0.0)    | <b>&lt;0.001</b> |
|  | 26-50       | 14 (21.2)             | 19 (21.6)               | 10 (45.5)  |                  |
|  | 51-75       | 13 (19.7)             | 19 (21.6)               | 12 (54.5)  |                  |
|  | >75         | 21 (31.8)             | 22 (25.0)               | 0 (0.0)    |                  |
| Protein, mg/dl                               |             | 106±137               | 128±151                 | 27±7       | <b>0.009</b>     |
| Protein percentile                           | ≤25         | 17 (25.8)             | 18 (20.7)               | 10 (45.5)  | <b>&lt;0.001</b> |
|  | 25-50       | 18 (27.3)             | 13 (14.9)               | 12 (54.5)  |                  |
|  | 51-75       | 15 (22.7)             | 29 (33.3)               | 0 (0.0)    |                  |
|  | >75         | 16 (24.2)             | 27 (31.0)               | 0 (0.0)    |                  |
| Broad categories of bacteria                 | Gram-ve     | 45 (68.2)             | NA                      | NA         |                  |
|  | Gram+ve     | 21 (31.8)             | NA                      | NA         |                  |
| Antibiotic medication                        | Yes         | 23 (34.8)             | 40 (45.5)               | 6 (27.3)   | 0.194            |
|  | No          | 43 (65.2)             | 48 (54.5)               | 16 (72.7)  |                  |

<sup>a</sup>Data are provided as the mean ± SD, median followed by interquartile range in parentheses, or number followed by percentage in parentheses.

<sup>b</sup>CSF exhibits pleocytosis, abnormal protein concentration and/or glucose concentration. Significant P-values are indicated in bold. PCR, polymerase chain reaction; +ve, positive; -ve, negative; CSF, cerebrospinal fluid; ABN, abnormal; NOR, normal; NA, not applicable; P, probability of one-way ANOVA (parametric variables), Kruskal-Wallis test (non-parametric variables) or Pearson's  $\chi^2$ -test (categorical variables).

variables are displayed by number and percentage, and significant differences were assessed using a Pearson's  $\chi^2$ -test. Continuous variables were subjected to two normality tests, Kolmogorov-Smirnov and Shapiro-Wilk tests. Normally distributed (parametric) variables are presented as the mean ± SD and significant differences and were compared using a one-way ANOVA (with Fisher LSD test post hoc). Variables that did not exhibit normal distribution (non-parametric) are expressed as the median and interquartile range (IQR), and significant differences were assessed using a Mann-Whitney U test (to compare two groups) or a Kruskal-Wallis test (to compare more than two groups). Receiver operating characteristic (ROC) curve analysis (Wilson/Brown method) was applied to calculate the area under the curve (AUC), the 95% confidence interval (CI), the cut-off value, and the sensitivity and specificity. The cut-off value was optimized using the Youden index (YI). Spearman's rank correlation analysis was used to estimate the correlation coefficient ( $r_s$ ) between

variables.  $P < 0.05$  was considered to indicate a statistically significant difference. In the case of multiple comparisons, the P-value was adjusted using a Dunn's test.

## Results

*Characteristics of participants.* Molecular analysis of 176 CSF samples demonstrated that 66 samples (37.5%) were PCR-positive (presence of a 996-bp band after agarose gel electrophoresis), whilst 110 samples (62.5%) showed no band (PCR-negative). DNA sequence analysis of PCR-positive products identified two broad categories of bacteria, G-ve (45/66; 68.2%) and G+ve (21/66; 31.8%). When PCR-negative CSF samples were explored for leukocyte counts, and glucose and protein concentrations, 88 samples exhibited pleocytosis, glucose concentrations lower or higher than normal, and/or protein concentrations lower or higher than normal. These CSF samples were considered ABN. The remaining 22 CSF

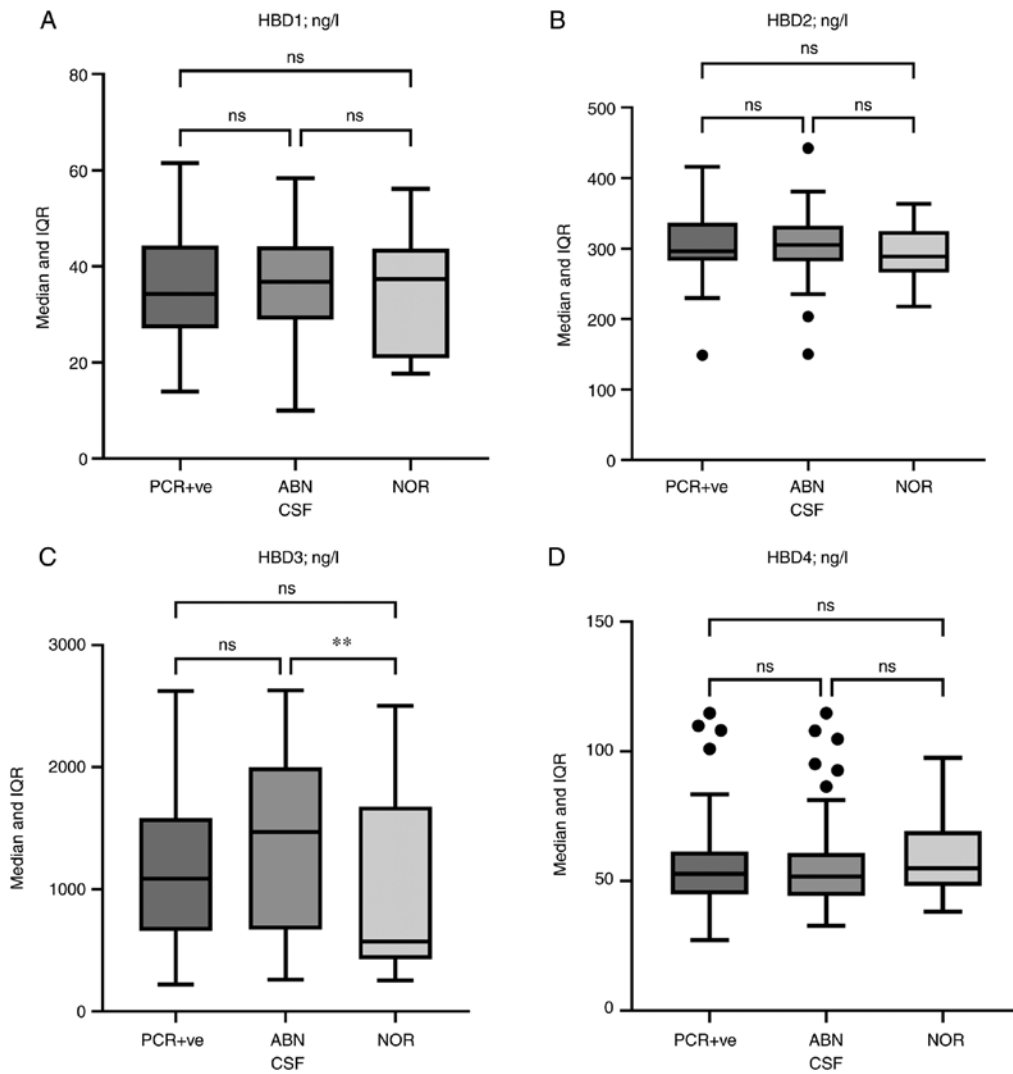


Figure 1. Box and whisker plots of HBD levels. (A) HBD1, (B) HBD2, (C) HBD3, and (D) HBD4 levels in the CSF of PCR-positive and PCR-negative cases, and in the ABN or NOR CSF cases. Horizontal lines inside the boxes indicate the median value, whilst the whiskers indicate the IQR. Outliers are presented as black circles. Only HBD3 levels showed differences between the three groups; the difference was significant between the ABN and NOR groups [1,470 (IQR: 671-2,001) vs. 572 (IQR: 427-1,679) ng/l;  $P=0.005$ ]. \*\* $P<0.01$ . HBD, human  $\beta$ -defensin; CSF, cerebrospinal fluid; ABN, abnormal; NOR, normal; IQR, interquartile range; ns, not significant.

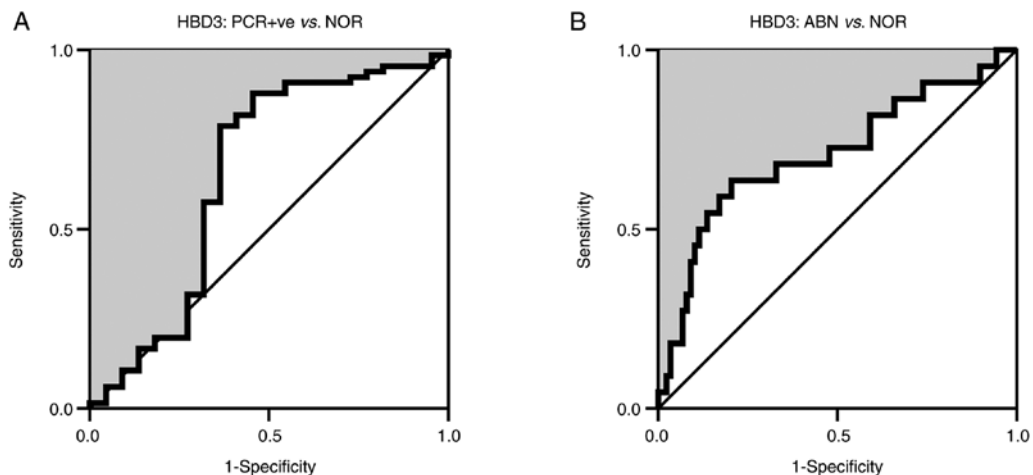


Figure 2. ROC curve analysis (Wilson/Brown method) of HBD3 in the CSF of (A) PCR-positive cases and (B) PCR-negative cases in the ABN vs. NOR CSF. In PCR-positive vs. NOR, the estimated AUC was 0.652 (95% CI=0.495-0.809;  $P=0.033$ ; cut-off value=652 ng/l;  $YI=0.42$ ; sensitivity=78.8%; specificity=63.3%). In ABN vs. NOR, the AUC was higher than in the PCR-positive vs. NOR (AUC=0.707; 95% CI=0.573-0.841;  $P=0.003$ ; cut-off value=650 ng/l;  $YI=0.44$ ; sensitivity=63.6%; specificity=80.0%). ROC, receiver operating characteristic; HBD, human  $\beta$ -defensin; CSF, cerebrospinal fluid; ABN, abnormal; NOR, normal; AUC, area under the curve; CI, confidence interval;  $YI$ , Youden index.

Table II. Cerebrospinal fluid levels of HBD1, HBD2, HBD3 and HBD4 classified by characteristics of PCR-positive cases.

| Characteristics       | Median (IQR), ng/l |            |               |                  |              |
|-----------------------|--------------------|------------|---------------|------------------|--------------|
|                       | HBD1               | HBD2       | HBD3          | HBD4             |              |
| Age group, years      | ≤2                 | 36 (29-49) | 300 (282-342) | 1660 (1310-2147) | 54 (46-75)   |
|                       | 3-12               | 36 (30-44) | 349 (319-380) | 1364 (751-2254)  | 54 (44-86)   |
|                       | 13-18              | 31 (27-53) | 287 (284-322) | 609 (271-711)    | 61 (59-62)   |
|                       | 19-39              | 33 (24-40) | 291 (283-336) | 770 (606-1206)   | 48 (44-58)   |
|                       | 40-59              | 30 (26-40) | 292 (274-306) | 1198 (803-1483)  | 52 (45-74)   |
|                       | ≥60                | 44 (42-46) | 304 (299-327) | 629 (432-1139)   | 48 (47-53)   |
|                       | P-value            | 0.435      | 0.315         | <b>&lt;0.001</b> | 0.543        |
| Sex                   | Male               | 30 (25-42) | 297 (282-348) | 1194 (770-1575)  | 58 (48-74)   |
|                       | Female             | 38 (28-44) | 296 (283-330) | 1033 (609-1626)  | 48 (45-57)   |
|                       | P-value            | 0.282      | 0.51          | 0.527            | <b>0.021</b> |
| Leukocyte count       | Normal             | 34 (27-44) | 292 (283-331) | 845 (629-1575)   | 53 (45-61)   |
|                       | Pleocytosis        | 35 (28-46) | 311 (281-343) | 1194 (773-1628)  | 51 (45-62)   |
|                       | P-value            | 0.691      | 0.278         | 0.261            | 0.732        |
| Glucose percentile    | ≤25                | 33 (27-49) | 300 (281-347) | 1488 (1194-1814) | 57 (46-61)   |
|                       | 26-50              | 39 (30-44) | 291 (280-324) | 869 (770-1256)   | 47 (40-62)   |
|                       | 51-75              | 36 (27-44) | 303 (288-354) | 684 (549-824)    | 52 (45-71)   |
|                       | >75                | 33 (27-42) | 296 (284-311) | 846 (629-1611)   | 52 (47-58)   |
|                       | P-value            | 0.835      | 0.608         | <b>0.013</b>     | 0.704        |
| Protein percentile    | ≤25                | 33 (27-42) | 295 (285-343) | 630 (561-782)    | 51 (47-69)   |
|                       | 26-50              | 40 (24-44) | 296 (283-331) | 1156 (660-1483)  | 55 (45-58)   |
|                       | 51-75              | 30 (23-40) | 282 (272-304) | 1229 (790-1814)  | 53 (40-62)   |
|                       | >75                | 36 (29-46) | 328 (294-351) | 1437 (1004-1620) | 53 (46-74)   |
|                       | P-value            | 0.504      | <b>0.012</b>  | <b>&lt;0.001</b> | 0.808        |
| Antibiotic medication | Yes                | 36 (29-45) | 311 (290-346) | 1310 (845-1882)  | 58 (47-75)   |
|                       | No                 | 31 (25-44) | 292 (280-329) | 794 (606-1494)   | 50 (45-59)   |
|                       | P-value            | 0.350      | 0.172         | <b>0.005</b>     | 0.086        |

Significant P-values are indicated in bold. HBD, human  $\beta$ -defensin; IQR, interquartile range; P, probability of Mann-Whitney U test (to compare two groups) or Kruskal-Wallis test (to compare more than two groups).

samples showed normal leukocyte counts, as well as glucose and protein concentrations, and were included in the NOR group. Accordingly, the 176 CSF samples were classified into three groups: PCR-positive (37.5%), ABN (50.0%), or NOR (12.5%) (Table I).

Mean age did not show a significant difference between participants in the three groups [28.8±20.7 (PCR-positive), 25.1±22.2 (ABN), and 25.6±14.7 years (NOR), P=0.549]. In addition, when participants were categorized into age groups (≤2, 3-12, 13-18, 19-39, 40-59, and ≥60 years), no significant differences were found with regard to the frequency of participants in each group (P=0.085). The median of the leukocyte counts was significantly higher in the PCR-positive and ABN CSFs compared to the NOR CSF [2.0 (IQR: 0-62.5) and 11.5 (IQR: 1-74.3) vs. 0.5 (IQR: 0-2) cell/mm<sup>3</sup>, respectively; P<0.001]. According to the leukocyte count, CSF samples were classified into two groups (normal and pleocytosis). It was found that 34.8 and 52.3% of PCR-positive and ABN CSFs were classed as pleocytotic, respectively, whilst none of the NOR CSFs were considered pleocytotic; these differences were significantly different (P<0.001). Glucose

concentrations exhibited no significant differences between PCR-positive, ABN, and NOR CSFs (60±30, 58±34, and 57±9 mg/dl, respectively; P=0.868). However, when these concentrations were ordered by percentiles (≤25, 26-50, 51-75, and >75%), significant differences were revealed (P<0.001). Low glucose concentrations (≤25 mg/dl) were observed in 27.3 and 31.8% of PCR-positive and ABN CSFs, respectively, compared with 0% in the NOR CSFs. Protein concentrations were significantly higher in the PCR-positive and ABN CSFs than in the NOR CSFs (106±137 and 128±151 vs. 27±7 mg/dl, respectively; P=0.009). A similar observation was made when protein concentrations were classified into percentiles, and >56% of PCR-positive and ABN CSFs were classified in the percentiles 51-75, and >75, whilst none of the NOR CSFs were placed in these percentiles; these differences were significant (P<0.001). Some participants were on antibiotic medication at the time of CSF collection, but their distribution in the three groups of participants did not show significant differences (34.8, 45.5, and 27.3% of PCR-positive, ABN, and NOR CSF groups, respectively; P=0.194; Table I).

Table III. Cerebrospinal fluid levels of HBD1, HBD2, HBD3 and HBD4 classified by characteristics of PCR-negative cases with abnormal cerebrospinal fluid.

| Characteristics       |             | Median (IQR), ng/l |               |                  |            |
|-----------------------|-------------|--------------------|---------------|------------------|------------|
|                       |             | HBD1               | HBD2          | HBD3             | HBD4       |
| Age group, years      | ≤2          | 40 (32-44)         | 316 (280-334) | 1763 (1342-2008) | 51 (44-60) |
|                       | 3-12        | 34 (24-44)         | 320 (298-381) | 1996 (343-2263)  | 47 (35-56) |
|                       | 13-18       | 38 (31-45)         | 294 (265-332) | 785 (478-1695)   | 77 (67-86) |
|                       | 19-39       | 40 (32-44)         | 320 (286-353) | 827 (598-2003)   | 50 (46-74) |
|                       | 40-59       | 34 (29-43)         | 298 (274-311) | 969 (704-1773)   | 51 (44-58) |
|                       | ≥60         | 52 (10-56)         | 348 (284-357) | 668 (614-2152)   | 55 (47-57) |
|                       | P-value     | 0.784              | 0.243         | 0.282            | 0.143      |
| Sex                   | Male        | 36 (30-44)         | 315 (278-335) | 1549 (654-2028)  | 50 (44-61) |
|                       | Female      | 41 (31-45)         | 303 (282-327) | 975 (687-1965)   | 53 (45-65) |
|                       | P-value     | 0.341              | 0.576         | 0.494            | 0.537      |
| Leukocyte count       | Normal      | 35 (27-44)         | 311 (284-341) | 858 (592-2003)   | 51 (44-62) |
|                       | Pleocytosis | 40 (32-44)         | 301 (276-332) | 1493 (887-1996)  | 53 (45-59) |
|                       | P-value     | 0.168              | 0.488         | 0.078            | 0.796      |
| Glucose percentile    | ≤25         | 42 (35-45)         | 316 (275-333) | 1813 (1360-2182) | 54 (44-61) |
|                       | 26-50       | 37 (32-44)         | 321 (297-357) | 1540 (745-2003)  | 57 (43-75) |
|                       | 51-75       | 36 (28-44)         | 310 (282-342) | 781 (574-2060)   | 48 (46-54) |
|                       | >75         | 33 (26-45)         | 292 (271-311) | 730 (598-1510)   | 49 (43-62) |
|                       | P-value     | 0.173              | 0.252         | <b>0.002</b>     | 0.577      |
| Protein percentile    | ≤25         | 37 (26-42)         | 278 (263-310) | 674 (574-950)    | 52 (44-61) |
|                       | 26-50       | 34 (30-47)         | 304 (293-338) | 781 (527-1510)   | 46 (44-49) |
|                       | 51-75       | 37 (32-44)         | 324 (303-348) | 1522 (766-1956)  | 51 (42-64) |
|                       | >75         | 42 (30-46)         | 300 (284-333) | 1850 (1244-2134) | 55 (47-59) |
|                       | P-value     | 0.584              | <b>0.006</b>  | <b>&lt;0.001</b> | 0.306      |
| Antibiotic medication | Yes         | 41 (32-44)         | 317 (291-332) | 1653 (984-2061)  | 51 (44-63) |
|                       | No          | 36 (28-44)         | 299 (272-340) | 803 (595-1954)   | 52 (45-58) |
|                       | P-value     | 0.185              | 0.283         | <b>0.004</b>     | 0.821      |

Significant P-values are indicated in bold. HBD, human  $\beta$ -defensin; IQR, interquartile range; P, probability of Mann-Whitney U test (to compare two groups) or Kruskal-Wallis test (to compare more than two groups).

*CSF levels of HBDs.* Median HBD1, HBD2, and HBD4 levels did not exhibit significant differences between PCR-positive, ABN, and NOR CSFs. Conversely, HBD3 levels were significantly higher in ABN CSFs than in NOR CSFs [1,470 (IQR: 671-2,001) vs. 572 (IQR: 427-1,679) ng/l; P=0.005]. HBD3 levels were also elevated in PCR-positive CSFs compared with the NOR CSFs, but the difference was not significant [1,086 (IQR: 659-1,584) vs. 572 (IQR: 427-1,679) ng/l; P=0.151]. Additionally, HBD3 levels showed no significant differences between PCR-positive and ABN CSFs (P=0.303, Fig. 1).

*ROC curve analysis of HBD3.* ROC curve analysis of HBD3 in the PCR-positive group vs. the NOR group revealed an AUC value of 0.652 (95% CI=0.495-0.809; P=0.033). The YI-adjusted cut-off value of HBD3 was 652 ng/l with sensitivity and specificity percentages of 78.8 and 63.3%, respectively. When the analysis was conducted on the ABN group vs. the NOR group, a higher discriminatory power was found for HBD3 (AUC=0.707; 95% CI=0.573-0.841;

P=0.003; cut-off value=650 ng/l, YI=0.44, sensitivity=63.6%, specificity=80.0%; Fig. 2).

*Stratification of HBD levels by characteristics.* The median levels of HBD1, HBD2, HBD3, and HBD4 were stratified by age group, sex, leukocyte counts (pleocytosis and normal), glucose and protein percentiles, and antibiotic medication in the PCR-positive and ABN groups. In the PCR-positive group, six significant differences were observed. Upregulated HBD2 levels were associated with the fourth percentile (>75) of protein concentration (P=0.012). HBD3 levels were increased in age groups ≤2, 3-12, and 40-59 years compared to age groups 13-18, 19-39, and ≥60 years (P<0.001). Upregulated HBD3 levels were associated with the first percentile (≤25) of glucose concentration (P=0.013), whilst downregulated levels were associated with the first percentile of protein concentration (P<0.001). Antibiotic medication was also associated with higher CSF levels of HBD3 (P=0.005). HBD4 levels were higher in males than in females (P=0.021, Table II).

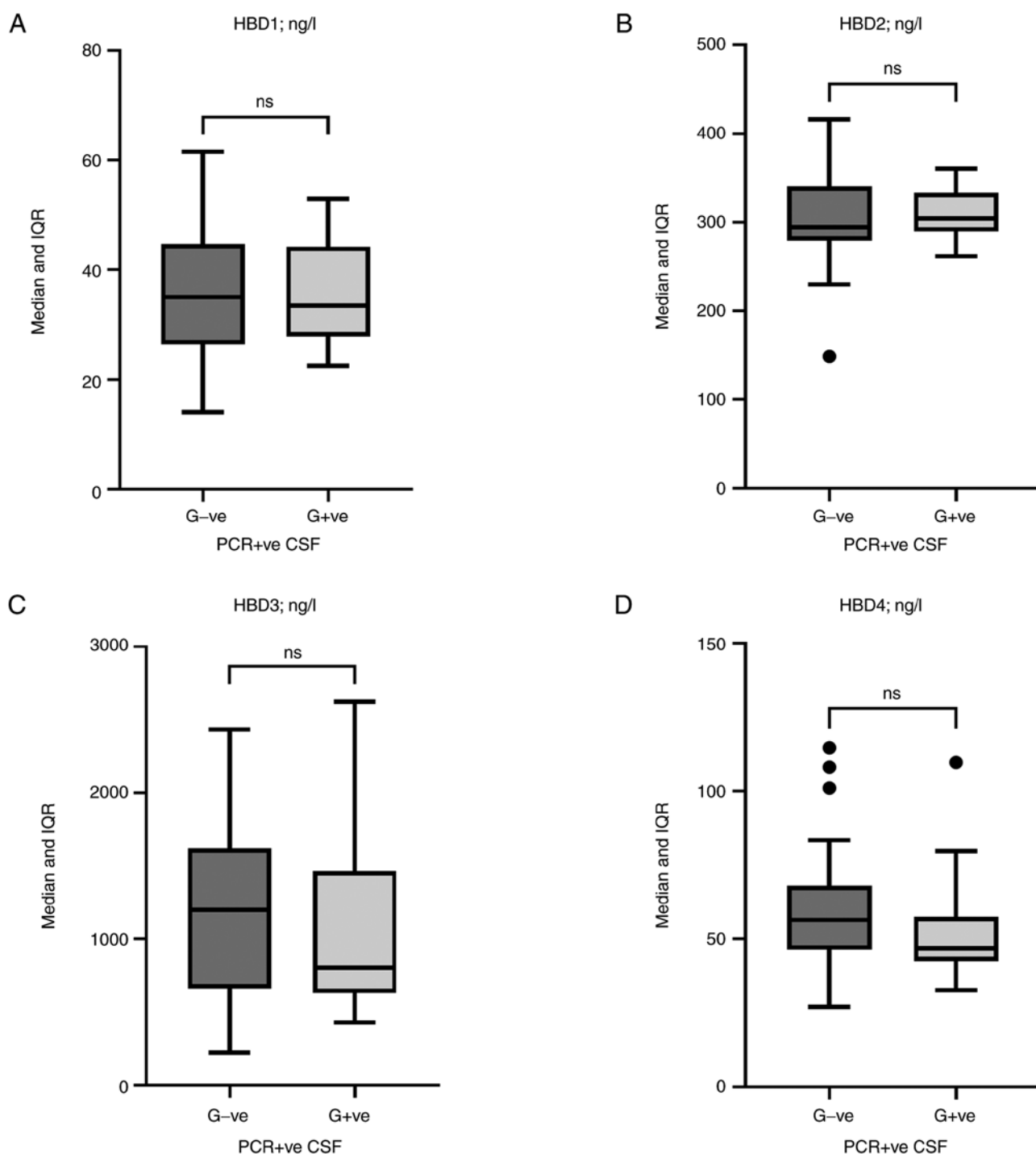


Figure 3. Box and whisker plots of human  $\beta$ -defensin levels. (A) HBD1, (B) HBD2, (C) HBD3, and (D) HBD4 levels in the CSF of PCR-positive cases (G-ve and G+ve bacteria). Horizontal lines inside the boxes indicate the median value, whilst the whiskers indicate the IQR. Outliers are presented as black circles. Although there were no significant differences, HBD3 [1,200 (IQR: 662-1,620) vs. 803 (IQR: 631-1,466) ng/l;  $P=0.28$ ] and HBD4 [56 (IQR: 46-68) vs. 47 (IQR: 43-57) ng/l;  $P=0.07$ ] tended to have higher levels in the G-ve than in the G+ve group. HBD, human  $\beta$ -defensin; CSF, cerebrospinal fluid; G-ve, Gram-negative; G+ve, Gram-positive; IQR, interquartile range.

Regarding the ABN group, four significant differences were found. HBD2 levels showed variations between the protein percentiles, and the lowest levels were associated with the first percentile ( $P=0.006$ ). Upregulated HBD3 levels were associated with the first and second percentiles ( $\leq 25$  and 26-50) of glucose concentration ( $P=0.002$ ), whilst the opposite was observed in protein concentrations, and these percentiles were associated with downregulated levels

( $P<0.001$ ). Finally, as in the PCR-positive group, upregulated HBD3 levels were associated with antibiotic medication ( $P=0.004$ , Table III).

*HBD levels stratified by G-ve and G+ve bacteria.* HBD1, HBD2, HBD3, and HBD4 levels were examined in the CSF of PCR-positive cases after being classified into two broad categories of bacteria, G-ve and G+ve. Although there were

Table IV. Spearman's rank correlation analysis of cerebrospinal fluid variables in all participating subjects.

| Variables  | Statistics | Age   | Leukocytes | Glucose          | Protein          | HBD1         | HBD2         | HBD3             | HBD4         |
|------------|------------|-------|------------|------------------|------------------|--------------|--------------|------------------|--------------|
| Age        | $r_s$      | 1.000 | -0.102     | <b>0.534</b>     | <b>-0.216</b>    | -0.120       | -0.083       | <b>-0.226</b>    | -0.014       |
|            | P-value    |       | 0.179      | <b>&lt;0.001</b> | <b>0.004</b>     | 0.113        | 0.275        | <b>0.003</b>     | 0.853        |
| Leukocytes | $r_s$      |       | 1.000      | <b>-0.344</b>    | <b>0.506</b>     | <b>0.164</b> | 0.112        | <b>0.258</b>     | 0.007        |
|            | P-value    |       |            | <b>&lt;0.001</b> | <b>&lt;0.001</b> | <b>0.031</b> | 0.140        | <b>&lt;0.001</b> | 0.931        |
| Glucose    | $r_s$      |       |            | 1.000            | <b>-0.452</b>    | -0.117       | -0.102       | <b>-0.329</b>    | -0.100       |
|            | P-value    |       |            |                  | <b>&lt;0.001</b> | 0.123        | 0.179        | <b>&lt;0.001</b> | 0.187        |
| Protein    | $r_s$      |       |            |                  | 1.000            | <b>0.162</b> | <b>0.186</b> | <b>0.457</b>     | 0.011        |
|            | P-value    |       |            |                  |                  | <b>0.033</b> | <b>0.014</b> | <b>&lt;0.001</b> | 0.883        |
| HBD1       | $r_s$      |       |            |                  |                  | 1.000        | 0.099        | <b>0.283</b>     | -0.124       |
|            | P-value    |       |            |                  |                  |              | 0.190        | <b>&lt;0.001</b> | 0.102        |
| HBD2       | $r_s$      |       |            |                  |                  |              | 1.000        | 0.048            | <b>0.202</b> |
|            | P-value    |       |            |                  |                  |              |              | 0.529            | <b>0.007</b> |
| HBD3       | $r_s$      |       |            |                  |                  |              |              | 1.000            | -0.133       |
|            | P-value    |       |            |                  |                  |              |              |                  | 0.079        |
| HBD4       | $r_s$      |       |            |                  |                  |              |              |                  | 1.000        |

Significant correlations are indicated in bold. HBD, human  $\beta$ -defensin;  $r_s$ , correlation coefficient; P, two-tailed probability.

no significant differences, HBD3 [1,200 (IQR: 662-1,620) vs. 803 (IQR: 631-1,466) ng/l; P=0.28] and HBD4 [56 (IQR: 46-68) vs. 47 (IQR: 43-57) ng/l; P=0.07] tended to have higher levels in the G-ve group than in the G+ve group (Fig. 3).

**Correlation analysis.** Spearman's rank correlation analysis was performed between age, leukocytes, glucose concentrations, protein concentrations, and HBD1, HBD2, HBD3, and HBD4 levels in the CSF of all participants. Age was positively correlated with glucose ( $r_s=0.534$ ; P<0.001) and negatively correlated with protein concentrations ( $r_s=-0.216$ ; P=0.004) and HBD3 levels ( $r_s=-0.226$ ; P=0.003). Leukocyte counts were negatively correlated with glucose concentrations ( $r_s=-0.344$ ; P<0.001), and positively correlated with protein concentrations ( $r_s=0.506$ ; P<0.001), HBD1 ( $r_s=0.164$ ; P=0.031) and HBD3 levels ( $r_s=0.258$ ; P<0.001). Glucose concentrations were negatively correlated with protein concentrations ( $r_s=-0.452$ ; P<0.001) and HBD3 levels ( $r_s=-0.329$ ; P<0.001). Protein concentrations were positively correlated with HBD1 ( $r_s=0.162$ ; P=0.033), HBD2 ( $r_s=0.186$ ; P=0.014) and HBD3 levels ( $r_s=0.457$ ; P<0.001). HBD1 was positively correlated with HBD3 levels ( $r_s=0.283$ ; P<0.001) Finally, HBD2 levels were positively correlated with HBD4 levels ( $r_s=0.202$ ; P=0.007) (Table IV).

## Discussion

The present study focused on four HBDs (HBD1, HBD2, HBD3, and HBD4), which represent an important class of innate immune modulators that act non-specifically against microbial challenges (12). The systemic profile of HBDs has been extensively studied in infectious, autoimmune, and inflammatory diseases, and dysregulated production has been associated with disease progression (18-20). In

the case of meningitis, the CSF profile of HBDs is the least studied and there have been limited data assessing their role in the development of meningitis. Meningitis is generally described as an inflammation-based disease associated with high mortality and significant morbidity rates (1). HBDs are also functionally linked to inflammation, and induction of HBD1, HBD2, HBD3, and HBD4 can occur due to exposure to microbial infection and inflammatory stimuli, as well as endogenous danger signals (15). Additionally, it has been hypothesized that abnormal expression and regulatory function of some antimicrobial peptides, such as HBDs, are associated with neuropathological changes due to chronic CNS diseases (21). Therefore, it is necessary to examine HBD levels in the CSF of patients with meningitis as information in this regard is scarce.

The present study included suspected cases of meningitis, where the cause of meningitis was not known. The initial interest was bacterial meningitis, thus a PCR-based method capable of detecting all types of bacteria using the eubacteria 16S rRNA gene as a target was adopted (27). The method successfully identified 66 meningitis cases (PCR-positive) and DNA sequence analysis grouped the cases into two broad categories, G-ve and G+ve. The remaining 110 CSF samples were PCR-negative, but analysis of leukocyte count and glucose and protein concentrations revealed that 88 CSF samples had abnormal results and were assigned to a group called ABN. The three parameters of analysis used in the present study are the most relevant for diagnosis of meningitis, and elevated leukocyte counts (pleocytosis) or protein concentrations, and lower glucose concentrations may be associated with viral, bacterial, and fungal infections (28,29). Since a bacterial cause was excluded in the ABN group, as indicated by the PCR analysis, a viral or fungal infection could not be ruled out. CSF samples with normal leukocyte counts, as well as normal glucose and protein concentrations, were



also encountered and were used as the control (NOR group). The rationale for adopting this classification for CSF samples (PCR-positive, ABN, and NOR CSFs) was to reduce causative differences between samples in each group. This may aid in better understanding the role of HBDs in the development of meningitis of various etiologies.

Among the four HBDs studied, HBD3 levels were significantly higher in the ABN CSF than in the NOR CSF, particularly in patients  $\leq 12$  years old. In addition, higher levels of HBD3 were associated with lower glucose concentrations and higher protein concentrations in the CSF. PCR-positive CSFs showed a nearly similar profile to ABN CSF, in addition, higher levels of HBD3 were associated with G-ve bacteria over G+ve bacteria. These findings suggest a role for HBD3 in meningitis regardless of the cause, bacterial or otherwise. Another interesting issue is the association of upregulated HBD3 levels with abnormal CSF concentrations of glucose and proteins, which are important diagnostic tests in meningitis (28,29). This may also highlight the diagnostic value of HBD3 in meningitis, and ROC analysis showed acceptable discriminatory power between ABN CSF and NOR CSF (AUC=0.707). HBD3 is an important AMP involved in protection against bacterial and viral infections, and is also known to have immunomodulatory functions (20). Regarding its antibacterial effects, studies have shown significant bactericidal activity of HBD3 against different G+ve and G-ve bacteria and this may be related to the cationic charges of HBD3 molecules (30,31). Additionally, high expression of HBD3 significantly enhanced wound closure in diabetic animal models infected with *Staphylococcus aureus* (32). The antiviral effects of HBD3 have also been demonstrated and experimental evidence has shown the effectiveness of HBD3 against various viruses, for example, West Nile virus and human immunodeficiency virus (33,34). Both effects (antibacterial and antiviral) are also potentiated by the immunomodulatory functions of HBD3. In this context, the role of HBD3 in innate immunity is well-recognized due to its antimicrobial activity. However, it has also been indicated that it contributes to the adaptive immune response, and immune-modulating properties of HBD3 such as chemotaxis to T lymphocytes, macrophages, neutrophils, and immature dendritic cells have been described (35). Therefore, HBD3 has been revealed to be associated with inflammatory diseases; for instance, dysregulated expression of HBD3 has been reported in inflammatory bowel disease, and periodontitis patients (36,37). Taken together, these findings suggest a role for HBD3 in inflammatory reactions associated with bacterial and viral infection, and this role may extend to the CNS and dysregulated expression of HBD3 in CSF could be expected.

The levels of HBD1, HBD2, and HBD4 in the CSF of the current suspected meningitis cases did not show significant differences between PCR-positive, ABN, and NOR CSF. However, CSF levels of HBD2 tended to parallel CSF protein concentrations. Significantly elevated levels of HBD2 were associated with protein concentrations  $>25\%$  in the PCR-positive and ABN CSFs. Elevated levels of CSF proteins are a reliable marker in diagnosing bacterial and viral meningitis (28). HBD2 is recognized as an AMP that integrates innate immune defenses against bacterial and

viral infections and may also be considered a marker of inflammation. Therapeutic administration of HBD2 has been suggested to maintain systemic homeostasis on the basis of an appropriate microbial composition (38). Thus, HBD2 may have a similar functional role in the CSF of meningitis patients. In addition to HBD2, HBD4 levels were significantly elevated in male patients with PCR-positive CSF compared to female patients. There is no supporting evidence for this observation, but in patients with allergic rhinitis, the opposite observation was made and serum HBD4 levels were significantly elevated in female patients compared to male patients (39). In COVID-19 patients, there were no significant differences between males and females regarding serum HBD4 levels (19). With these conflicting results, the association between HBD4 and sex remains uncertain, and further studies are warranted.

Correlation analysis showed that CSF levels of HBD1 and HBD3, as well as HBD2 and HBD4, were positively correlated. Similar positive correlations have been found in the serum of COVID-19 patients (19). Furthermore, a positive correlation between HBD2 and HBD4 has been reported in the serum of allergic rhinitis patients and healthy controls (39). This may indicate functional associations between these HBDs. In fact, it has been recognized that in addition to their common antimicrobial activity, HBDs in general enhance certain immune functions such as chemotaxis (20). Additional correlation findings included positive correlations between HBD1 with leukocyte counts and protein concentration, as well as between HBD2 and glucose concentration. In the case of HBD3, more correlations were found. It was negatively correlated with age and glucose concentration, and positively correlated with leukocyte counts and protein concentration. The most important of these correlations was with leukocyte counts, glucose concentration, and protein concentration, which are diagnostic parameters in the CSF for meningitis (28,29).

The present study has some limitations. First, a detailed clinical history of suspected meningitis cases was not obtained. Second, only one CSF sample was collected from each participant, and a second sample is necessary to confirm the results of the first. Third, molecular analysis of CSF included evaluation of only bacterial DNA, whilst viral and fungal presence was not assessed. Fourth, the plasma concentrations of glucose were not determined.

In conclusion, among the four HBDs studied, HBD3 levels were significantly elevated in the CSF of suspected meningitis cases regardless of the cause of meningitis. CSF levels of some HBDs were affected by specific diagnostic laboratory parameters for meningitis, including leukocyte counts, glucose concentrations, and protein concentrations.

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### Availability of data and materials

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

### Authors' contributions

All authors (LKJ, MAU, KBJK, and AHA) conceptualized the study and contributed to data curation, data analysis, the methodology, and validation of the results, as well as wrote the original draft, and revised and edited the manuscript. LKJ and AHA confirm the authenticity of the raw data. MAU and AHA supervised the study. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The Institutional Ethics Committee of the College of Science, University of Baghdad (Baghdad, Iraq) approved the study (approval no. CSEC/1022/0136) and written informed consent was obtained from patients or their legal guardian prior to sample collection.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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