Cerebrospinal fluid markers to distinguish bacterial meningitis from cerebral malaria in children [version 2; referees: 1 approved, 1 approved with reservations]

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Abstract

Background. Few hospitals in high malaria endemic countries in Africa have the diagnostic capacity for clinically distinguishing acute bacterial meningitis (ABM) from cerebral malaria (CM). As a result, empirical use of antibiotics is necessary. A biochemical marker of ABM would facilitate precise clinical diagnosis and management of these infections and enable rational use of antibiotics.

Methods. We used label-free protein quantification by mass spectrometry to identify cerebrospinal fluid (CSF) markers that distinguish ABM (n=37) from CM (n=22) in Kenyan children. Fold change (FC) and false discovery rates (FDR) were used to identify differentially expressed proteins. Subsequently, potential biomarkers were assessed for their ability to discriminate between ABM and CM using receiver operating characteristic (ROC) curves.

Results. The host CSF proteome response to ABM (Haemophilus influenzae and Streptococcus pneumoniae) is significantly different to CM. Fifty two proteins were differentially expressed (FDR<0.01, Log FC≥2), of which 83% (43/52) were upregulated in ABM compared to CM. Myeloperoxidase and lactotransferrin were present in 37 (100%) and 36 (97%) of ABM cases, respectively, but absent in CM (n=22). Area under the ROC curve (AUC), sensitivity, and specificity were assessed for myeloperoxidase (1, 1, and 1; 95% CI, 1-1) and lactotransferrin (0.98, 0.97, and 1; 95% CI, 0.96-1).

Conclusion. Myeloperoxidase and lactotransferrin have a high potential to distinguish ABM from CM and thereby improve clinical management. Their validation requires a larger cohort of samples that includes other bacterial aetiologies of ABM.
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Inteivection

The following text was added under limitations in the discussion section: “Further, in endemic areas, the definition of CM is often challenging and CM is often over-diagnosed. The WHO definition of CM may misclassify up to 25% of cases48 and its specificity is greatly improved by adding a clinical test for Malaria Retinopathy57-60. However, Retinal changes specific to CM require specialist examination techniques, are difficult to examine in conscious children, and such data was not available in this study. Samples analyzed in this study were left over specimens following normal microbiology and chemistry laboratory procedures. There is lacking data for the samples on the time taken from collection during lumbar puncture to storage at -80°C and therefore there is possibility that proteome changes occurred during that period49. Label-free proteomics are cost-efficient, offer higher proteome coverage and a higher dynamic range. However, as every sample is handled separately, variations that can bias the quantitative analysis may be introduced.” The percent of sample for which each protein was quantified in either ABM or CM has been added to Table S2.

See referee reports

Methods

Study participants

The study used archived CSF samples (n = 59) from paediatric admissions (2002–2011) at Kilifi County Hospital (Formerly Kilifi District Hospital), Kilifi, Kenya. All samples used in this study had been consented and approved for storage and research by the Kenya Medical Research Institute. Samples comprised two groups of children, based on clinical and laboratory findings. Acute bacterial meningitis (ABM; n=37) was defined as children who had peripheral asexual malarial parasitemia >2500 parasites/μl on blood film39; negative CSF cultures, CSF leukocyte count <10 cells/μl and no CSF biochemical feature of ABM. ABM was defined without respect to parasitemia, and therefore 5 children had Plasmodium falciparum coincidental infection.

Sample preparation and LC-MS/MS analysis

Aliquots of 10 μl of CSF were denatured in 50 mM ammonium bicarbonate (Fluka) containing 8 M urea (Sigma). Proteins were reduced with 20 mM dithiothreitol (Sigma) at room temperature with shaking for 1 hour (h) and subsequently alkylated in the dark for 1 h with 65 mM iodoacetamide (Sigma). Excess iodoacetamide was quenched using 65 mM dithiothreitol. Urea present in the sample was dialyzed out with 50mM ammonium bicarbonate, using 3 kDa amicon filters (Millipore). Proteins were digested with trypsin (Thermo Scientific) overnight (16 hours) and peptides obtained were desalted using C18 Spin columns (Thermo Scientific), according to manufacturer’s instructions, dried in a Speedvac concentrator (Thermo Scientific), and re-suspended in 50 μl loading solvent (97.05% H2O, 2% acetonitrile, 0.05% formic acid). Peptides (5 μl) were loaded using a Dionex Ultimate 3000 nano-flow ultra-high-pressure liquid chromatography system (Thermo
Data preparation

Mass spectrometer files (.Raw files) were analysed by MaxQuant software version 1.5.3.30 by searching against the human Uniprot FASTA database (downloaded February 2014) using the Andromeda search engine. Cysteine carbamidomethylation was set as a fixed modification and N-terminal acetylation and methionine oxidations as variable modifications. The false discovery rate (FDR) was set to 0.01 for both proteins and peptides with a minimum length of seven amino acids and was determined by searching a decoy database. A decoy FASTA database is generated from the target database, comprising sequences derived from the organism being studied, by switching the amino-carboxyl orientation of a protein’s amino acids to generate sequences that do not exist in nature, which are then concatenated with the target FASTA database. Enzyme specificity was set as C-terminal to arginine and lysine with trypsin as the protease. A maximum of two missed cleavages were allowed in the database search. Peptide identification was performed with an allowed initial precursor mass deviation of up to 7 ppm and an allowed fragment mass deviation of up to 20 ppm. The label free quantification (LFQ) algorithm in MaxQuant was used to obtain quantification intensity values.

Statistical analysis

a) Pre-processing and exploration. Study participants characteristics data was uploaded and analyzed in Stata version 13.1 and significance was tested using Wilcoxon rank-sum (Mann–Whitney) test for non-parametric variables, while two-sample t test with equal variances was used for parametric data. Chi square test was used for binary data.

Proteome data analysis was conducted in R version 3.3.2 and all samples were included in the analysis. We limited protein analysis to those identified and quantified in at least half of the samples in either ABM or CM. Proteins that were not detected in a sample were presumed to be on the lower limit of detection and their LFQ values were set at 0. Range and logarithmic normalization was performed to adjust protein quantities to a comparable scale. Unsupervised clustering using principal component analysis (PCA) and hierarchical clustering were applied to assess variation and determine group separation (i) among the ABM samples that comprised Haemophilus influenzae (n = 12) and Streptococcus pneumoniae (n = 25) and (ii) between ABM and CM. Group separation among ABM samples was carried out to determine whether the host response is bacterial-specific or generic. To visualize protein clustering patterns, a heatmap was generated with the Pearson correlation coefficients as the distance metric.

b) Biomarker extraction. Fold change (FC) and FDR were used to identify differentially expressed proteins. Selection of candidate biomarkers was performed through feature-importance assignment, based on variable importance, as implemented in the random forest (RF) algorithm version 4.6-12. Here, recursive feature elimination (RFE) resulted in a reduced subset of proteins whose ability to distinguish between ABM and CM groups was evaluated using the mean decrease in accuracy (MDA) scores. The Boruta R package version 5.2.0 is designed as a wrapper around RF facilitating RFE and MDA weight assignment. Subsequently, each protein was assessed for its ability to discriminate between ABM and CM by evaluating its receiver operating characteristic (ROC) curves. Potential biomarkers were thus identified from the differentially expressed proteins ranked according to the area under curve (AUC) and MDA scores.

Results

Characteristics of study participants

The study analysed 59 samples from two clinical groups, whose characteristics are presented in Table 1.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Acute bacterial meningitis (n=37)</th>
<th>Cerebral malaria (n=22)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, months</td>
<td>35 (9–90)</td>
<td>30.5 (13–37)</td>
<td>0.61</td>
</tr>
<tr>
<td>Sex, male, n (%)</td>
<td>21 (56.8)</td>
<td>8 (36.4)</td>
<td>0.1</td>
</tr>
<tr>
<td>Parasite density, iRBCs ×10^7/μL</td>
<td>0 (0–0)</td>
<td>230000 (100800–393600)</td>
<td>0.0001</td>
</tr>
<tr>
<td>CSF WBC count, cells/μL</td>
<td>3370 (288–5120)</td>
<td>2 (1–4)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total CSF protein, mg/dL</td>
<td>2.1 (1.3–2.49)</td>
<td>0.28 (0.22–0.37)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Blood glucose, mg/dL</td>
<td>5.4 (4–7.6)</td>
<td>5.3 (3.2–7.7)</td>
<td>0.9</td>
</tr>
<tr>
<td>CSF glucose, mg/dL</td>
<td>0.6 (0.3–0.9)</td>
<td>3.1 (2.6–3.7)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ratio of CSF to blood glucose</td>
<td>0.1 (0.06–0.16)</td>
<td>0.69 (0.42–1)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Outcome, dead, n (%)</td>
<td>13 (35)</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>MUAC, cm</td>
<td>13.95 (11.5–15.4)</td>
<td>13.95 (13.3–15.2)</td>
<td>0.3</td>
</tr>
<tr>
<td>Seizures, n (%)</td>
<td>9 (24)</td>
<td>10(46)</td>
<td>0.1</td>
</tr>
</tbody>
</table>
CSF proteomes of ABM and CM differ significantly

The LC-MS/MS analysis resulted in the quantification of a total of 708 non-redundant proteins, of which 183 proteins were commonly expressed in both ABM and CM (Figure 1A). One hundred and sixty proteins were quantified in >50% within each group and were selected for further analysis (Figure 1B). Of the 160 proteins, 32 proteins were not quantified in CM, while two proteins were not quantified in ABM, as shown in Figure 1B. Overall, ABM had a higher number of quantified proteins than CM. All quantified proteins and those included in subsequent analysis are listed in Table S1 and Table S2, respectively.

Normalized protein LFQ values revealed clear sample separation into ABM and CM groups (Figure 2). S100A8/S100A9 (calprotectin), lactotransferrin (LTF), myeloperoxidase (MPO), and myeloblastin (PRTN3) were the top five proteins driving the sample group separation observed in dimension 1 (data not visualised). The CM samples showed strong within-cluster connectivity, suggesting lower proteome variation compared to ABM samples, which showed greater cluster spread. The ABM group comprising Gram-negative *H. influenza* and Gram-positive *S. pneumoniae* did not exhibit any bacteria-specific clustering (Figure S1).

Figure 1. Cerebral spinal fluid (CSF) proteomes of acute bacterial meningitis (ABM) and cerebral malaria (CM) differ significantly. (A) Distribution of total proteins quantified (n = 708) between ABM and CM. (B) Distribution of proteins included in the biomarker analysis, where proteins had to be quantified in at least half of the samples in either ABM or CM. The CSF of ABM patients is characterized by a larger protein diversity compared to CM.

Figure 2. Host response acute bacterial meningitis (ABM) and cerebral malaria (CM) is pathogen specific. Unsupervised clustering using principal component analysis (PCA) was employed to determine clustering patterns of samples. The PCA score plot of the cerebral spinal fluid proteomes depicts clear group separation. Dimension (Dim) 1 of the PCA accounted for 41% of variation, while Dim 2 accounted for 8%.
In order to visualize clustering patterns of proteins based on normalized LFQ quantities, a heatmap with the rows, representing 160 proteins, and columns, representing 59 samples, was generated with the Pearson correlation coefficients as the distance metric (Figure 3). The protein expression profiles distinguished the two groups (Figure 3). It is notable that two ABM samples clustered with the CM group and this was similarly observed with the PCA analysis (Figure 2 and Figure 3).

Analysis revealed that 52 proteins were differentially expressed (FDR<0.01, Log FC≥2) (Table 2, Figure S2), of which 83% (43/52) were upregulated in ABM compared to CM (Figure S2). Proteins including MPO, LTF, PRTN3, PFN1, LCN2, MMP8, MMP9, RETN, PGLYRP1, and S100A8/S100A9 were among the significantly expressed proteins in ABM, while SPARC, CNTN1, CHGB, and SPARCL1 were upregulated in CM (Table 2).

MPO and LTF as the best biomarkers distinguishing ABM from CM
To identify top ranking biomarkers that distinguish ABM from CM, differentially expressed proteins were subjected to a feature-based weighting procedure where protein importance was assigned using the MDA scores. Higher scores imply increased ability of a protein to distinguish between ABM and CM (Figure S3). The proteins selected by the algorithm as the most important biomarkers are listed on Table 3, where MPO, SPARCL1, and LTF rank as the top three proteins.

Consistently, there was an overlap of differentially expressed proteins and the top ranking biomarker proteins. Additionally, ROC curves were generated independently for each of the biomarkers and the AUC determined. The top ranking biomarkers were selected based on an AUC >0.9 and MDA scores above those of

![Figure 3. Heatmap demonstrating sample clustering based on protein expression profiles from acute bacterial meningitis (ABM) and cerebral malaria (CM). The heatmap was generated using hierarchical clustering based on protein expression levels calculated from normalized label free quantification values (2 and -2). The Pearson correlation coefficients were used as the distance metric. Rows represent individual proteins, while columns represents samples. Red indicates upregulation and blue indicates downregulation. Distinct sample clustering based on protein expression levels was observed with clear separation between the ABM and CM, except for two ABM samples that clustered with the CM.](image-url)
Table 2. List of 52 quantified proteins that showed differential expression (FDR<0.01, and log FC≥2). FDR, false discovery rate; FC, fold change.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein name</th>
<th>Gene name</th>
<th>Log FC</th>
<th>P value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>P05164-2</td>
<td>Myeloperoxidase</td>
<td>MPO</td>
<td>-31.7</td>
<td>8.32E-77</td>
<td>1.33E-74</td>
</tr>
<tr>
<td>P02788</td>
<td>Lactotransferrin</td>
<td>LTF</td>
<td>-32.8</td>
<td>5.05E-66</td>
<td>4.04E-64</td>
</tr>
<tr>
<td>U3KPS2</td>
<td>Myeloblastin</td>
<td>PRTN3</td>
<td>-30.8</td>
<td>9.89E-42</td>
<td>5.27E-40</td>
</tr>
<tr>
<td>P07737</td>
<td>Profilin-1</td>
<td>PFN1</td>
<td>-30.7</td>
<td>1.21E-37</td>
<td>4.85E-36</td>
</tr>
<tr>
<td>P29401</td>
<td>Transketolase</td>
<td>TKT</td>
<td>-30.6</td>
<td>1.23E-33</td>
<td>3.94E-32</td>
</tr>
<tr>
<td>P08670</td>
<td>Vimentin</td>
<td>VIM</td>
<td>-32</td>
<td>1.7E-28</td>
<td>4.54E-27</td>
</tr>
<tr>
<td>P13796</td>
<td>Plastin-2</td>
<td>LCP1</td>
<td>-32</td>
<td>1.41E-27</td>
<td>3.22E-26</td>
</tr>
<tr>
<td>P12814</td>
<td>Alpha-actinin-1</td>
<td>ACTN1</td>
<td>-30.5</td>
<td>1.32E-22</td>
<td>2.65E-21</td>
</tr>
<tr>
<td>P31146</td>
<td>Coronin-1A; Coronin</td>
<td>CORO1A</td>
<td>-29.6</td>
<td>3.36E-22</td>
<td>5.98E-21</td>
</tr>
<tr>
<td>P80188</td>
<td>Neutrophil gelatinase-associated lipocalin</td>
<td>LCN2</td>
<td>-30.6</td>
<td>2.67E-19</td>
<td>4.27E-18</td>
</tr>
<tr>
<td>P22894</td>
<td>Neutrophil collagenase</td>
<td>MMP8</td>
<td>-29.4</td>
<td>8.23E-19</td>
<td>1.2E-17</td>
</tr>
<tr>
<td>P09486</td>
<td>SPARC</td>
<td>SPARC</td>
<td>28.5</td>
<td>5.41E-18</td>
<td>7.21E-17</td>
</tr>
<tr>
<td>Q12860</td>
<td>Contactin-1</td>
<td>CNTN1</td>
<td>28.6</td>
<td>5.91E-18</td>
<td>7.27E-17</td>
</tr>
<tr>
<td>Q9HD89</td>
<td>Resistin</td>
<td>RETN</td>
<td>-29.9</td>
<td>1.11E-17</td>
<td>1.27E-16</td>
</tr>
<tr>
<td>P06744</td>
<td>Glucose-6-phosphate isomerase</td>
<td>GPI</td>
<td>-29.8</td>
<td>1.57E-17</td>
<td>1.68E-16</td>
</tr>
<tr>
<td>P14780</td>
<td>Matrix metalloproteinase-9</td>
<td>MMP9</td>
<td>-29.4</td>
<td>2.95E-17</td>
<td>2.95E-16</td>
</tr>
<tr>
<td>P80511</td>
<td>Protein S100-A12</td>
<td>S100A12</td>
<td>-31.2</td>
<td>1.8E-15</td>
<td>1.7E-14</td>
</tr>
<tr>
<td>P62937</td>
<td>Peptidyl-prolyl cis-trans isomerase A</td>
<td>PPIA</td>
<td>-30.1</td>
<td>3.59E-15</td>
<td>3.19E-14</td>
</tr>
<tr>
<td>P31949</td>
<td>Protein S100-A11</td>
<td>S100A11</td>
<td>-28.9</td>
<td>2.6E-14</td>
<td>2.19E-13</td>
</tr>
<tr>
<td>P26038</td>
<td>Moesin</td>
<td>MSN</td>
<td>-29.4</td>
<td>4.99E-13</td>
<td>3.99E-12</td>
</tr>
<tr>
<td>P11142</td>
<td>Heat shock cognate 71 kDa protein</td>
<td>HSPA8</td>
<td>-29.3</td>
<td>6.65E-13</td>
<td>5.07E-12</td>
</tr>
<tr>
<td>P06753-2</td>
<td>Tropomyosin alpha-3 chain</td>
<td>TPM3</td>
<td>-29.4</td>
<td>3.48E-12</td>
<td>2.53E-11</td>
</tr>
<tr>
<td>P60600-2</td>
<td>Myosin light polypeptide 6</td>
<td>MYL6</td>
<td>-28.9</td>
<td>5.27E-12</td>
<td>3.67E-11</td>
</tr>
<tr>
<td>P62158</td>
<td>Calmodulin</td>
<td>CALM1</td>
<td>-29.3</td>
<td>1.94E-11</td>
<td>1.3E-10</td>
</tr>
<tr>
<td>P08107</td>
<td>Heat shock 70 kDa protein 1A/1B</td>
<td>HSPA1A</td>
<td>-29.5</td>
<td>3.34E-11</td>
<td>2.14E-10</td>
</tr>
<tr>
<td>P04003</td>
<td>C4b-binding protein alpha chain</td>
<td>C4BPA</td>
<td>-28.5</td>
<td>1.32E-10</td>
<td>8.12E-10</td>
</tr>
<tr>
<td>O75594</td>
<td>Peptidoglycan recognition protein 1</td>
<td>PGLYRP1</td>
<td>-28.1</td>
<td>1.68E-10</td>
<td>9.93E-10</td>
</tr>
<tr>
<td>Q93079</td>
<td>Histone H2B type 1-H</td>
<td>HIST1H2BH</td>
<td>-30</td>
<td>2.73E-10</td>
<td>1.56E-09</td>
</tr>
<tr>
<td>Q01518</td>
<td>Adenyl cyclase-associated protein 1</td>
<td>CAP1</td>
<td>-29.4</td>
<td>7.17E-10</td>
<td>3.95E-09</td>
</tr>
<tr>
<td>P04114</td>
<td>Apolipoprotein B-100; Apolipoprotein B-48</td>
<td>APOB</td>
<td>-28.3</td>
<td>1.07E-09</td>
<td>5.73E-09</td>
</tr>
<tr>
<td>P04083</td>
<td>Annexin A1</td>
<td>ANXA1</td>
<td>-29</td>
<td>3.69E-09</td>
<td>1.9E-08</td>
</tr>
<tr>
<td>P07900</td>
<td>Heat shock protein HSP 90-alpha</td>
<td>HSP90AA1</td>
<td>-28.9</td>
<td>4.54E-09</td>
<td>2.2E-08</td>
</tr>
<tr>
<td>P35579</td>
<td>Myosin-9</td>
<td>MYH9</td>
<td>-30.2</td>
<td>4.52E-09</td>
<td>2.2E-08</td>
</tr>
<tr>
<td>O43866</td>
<td>CD5 antigen-like</td>
<td>CD5L</td>
<td>-27.2</td>
<td>2.32E-08</td>
<td>1.07E-07</td>
</tr>
<tr>
<td>P00338</td>
<td>L-lactate dehydrogenase A chain</td>
<td>LDHA</td>
<td>-28.4</td>
<td>2.34E-08</td>
<td>1.07E-07</td>
</tr>
</tbody>
</table>
**Table 3.** Area under the curve (AUC), sensitivity (Sens.), specificity (Spec.), and mean decrease in accuracy (MDA) scores for the best performing biomarkers.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>AUC (95% CI)</th>
<th>Sens.</th>
<th>Spec.</th>
<th>Classified correctly %</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO</td>
<td>1.00 (1 to 1)</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>8.7</td>
</tr>
<tr>
<td>LTF</td>
<td>0.98 (0.96 to 1)</td>
<td>0.97</td>
<td>1</td>
<td>98</td>
<td>6.9</td>
</tr>
<tr>
<td>PRTN3</td>
<td>0.96 (0.91 to 1)</td>
<td>0.92</td>
<td>1</td>
<td>95</td>
<td>5.6</td>
</tr>
<tr>
<td>PFN1</td>
<td>0.96 (0.91 to 1)</td>
<td>0.92</td>
<td>1</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>TKT</td>
<td>0.95 (0.89 to 0.99)</td>
<td>0.89</td>
<td>1</td>
<td>93</td>
<td>4.1</td>
</tr>
<tr>
<td>VIM</td>
<td>0.91 (0.86 to 0.97)</td>
<td>0.83</td>
<td>1</td>
<td>90</td>
<td>2.98</td>
</tr>
<tr>
<td>LCP1</td>
<td>0.90 (0.84 to 0.97)</td>
<td>0.81</td>
<td>1</td>
<td>88</td>
<td>2.97</td>
</tr>
<tr>
<td>ACTN1</td>
<td>0.90 (0.84 to 0.97)</td>
<td>0.81</td>
<td>1</td>
<td>88</td>
<td>2.76</td>
</tr>
<tr>
<td>CORO1A</td>
<td>0.90 (0.84 to 0.97)</td>
<td>0.81</td>
<td>1</td>
<td>88</td>
<td>3.16</td>
</tr>
<tr>
<td>CSF Glucose (mg/dL) &lt;2.4*</td>
<td>0.88 (0.80 to 0.99)</td>
<td>0.91</td>
<td>0.87</td>
<td>88</td>
<td>-</td>
</tr>
<tr>
<td>CSF WBC count, cells/μL &gt;10*</td>
<td>0.95 (0.89 to 1)</td>
<td>1</td>
<td>0.90</td>
<td>97</td>
<td>-</td>
</tr>
<tr>
<td>Total CSF protein &gt;0.54*</td>
<td>0.94 (0.87 to 1)</td>
<td>0.97</td>
<td>0.90</td>
<td>95</td>
<td>-</td>
</tr>
</tbody>
</table>

*indicates the best cut-off that achieved high sensitivity and specificity.
shadow proteins (Table 3). However, among the biomarkers, MPO and LTF achieved high sensitivity (≥0.98) and specificity (1), depicting their predictive potential as biomarkers (Table 3).

Discussion

Clinical differentiation of ABM and CM is important as it dictates management and prognosis. A diagnostic test based on host proteins avoids heterogeneity in pathogen proteins between infecting bacterial species. We found significant proteome difference between ABM and CM, implying that the host responds differently to bacterial and Plasmodial infections. This is consistent with previous work indicating a differential host response in plasma and CSF of children with a diagnosis of CM compared to those with a malaria-slide-negative ABM17. In a previous study, Gitau et al. reported differentially expressed proteins in plasma and CSF from children with CM, ABM, and nonspecific encephalopathies17. The approach enhanced proteome coverage through pre-fractionation, but precluded relative quantification of proteins. Biomarker discovery has been enhanced by recent developments in mass spectrometry instrumentation25 and advanced computational and bioinformatics algorithms20,28. In the present study, we performed shotgun proteomics and quantitative differences in host proteins in CSF.

ABM was characterized by a higher CSF total protein concentration and higher host protein diversity. This likely results from protein infiltration following breakdown in the blood brain barrier and secretion from host cells including infiltrating neutrophils. Whilst in CM the blood brain barrier is mildly impaired with few morphological changes25–28 and Plasmodia parasites are usually restricted to the vascular compartment rather than the meninges or the parenchyma of the brain, unless haemorrhage occurs.

The aetiology of ABM comprised Gram-negative H. influenza and Gram-positive S. pneumoniae. However, there was no species-specific clustering, implying that host responses to bacterial infections is largely generic. This homogeneity supports using host biomarkers for distinguishing meningitis.

A large proportion of the proteins that were not consistently quantified were excluded from the main analysis where biomarker mining followed criterion to include only proteins quantified in more than half of the samples from either of the two groups. This increased the reliability of a selected biomarker. Reducing the dataset in this way resulted in a substantial increase in proteins shared between the two groups, with ABM retaining a higher proportion of unique proteins compared to CM.

MPO and LTF were the most promising proteins for consideration as biomarkers based on their expression, sensitivity and specificity, as judged by the AUC and also ranked among the top biomarkers using RF. MPO contributes to innate host defences through microbial killing and is stored in large quantities in the azurophilic granules of neutrophils and released upon cell activation31,32. MPO and LTF are also part of neutrophil extracellular traps; fibrillar matrices comprised of chromatin and antimicrobial proteins, released by activated neutrophils33. As a biomarker, MPO has previously been shown to be higher in the CSF of patients with infectious causes compared to those of non-infectious causes, and its levels have been shown to correlate well with neutrophil counts34. MPO has already been developed as a diagnostic tool for cardiovascular disease risk stratification35.

LTF is an antimicrobial polypeptide found in secondary granules of neutrophils and in human mucosal secretions, and plays a role in iron metabolism and inflammation16,37. LTF plays a bacteriostatic role in host defence due to its iron sequestering properties that inhibit bacterial proliferation. It is ineffective against bacteria able to acquire their iron from either LTF or transferrin36–40. Previously, LTF has been shown to be elevated in the CSF of patients with bacterial meningitis41–44. In a study in adults, CSF LTF showed diagnostic efficiency (AUC; 0.946, sensitivity; 96.6, specificity; 92.4) when distinguishing between bacterial and aseptic meningitis45. Such findings point to LTF as a molecular marker for ABM. Currently, LTF can be assayed using ELISA, but has the potential to be developed as a diagnostic test. It is notable that both MPO and LTF performed better than surrogate markers in the CSF, including glucose, pleocytosis, and total protein. Determination of pleocytosis requires microscopy and couldn’t be a point-of-care test. Bed-side glucose rapid tests are not currently accurate enough to reliably distinguish ABM from CM.

In resource limited settings in Africa, point-of-care diagnostics could considerably help in diagnosis. In malarious areas, a rapid test sensitive and specific for ABM could help in identification and timely management of comatose children suffering from ABM even though they may have a positive malaria slide or RDT. Such an approach would contribute to antibiotic stewardship in the face of increasing resistance and improve resource use. The markers identified in this study await validation and development as point-of-care diagnostics.

Our study had several limitations. For ethical reasons, we lacked CSF control samples without disease, as previously described17. The sample size used was relatively small, which could limit comparison between groups of syndromes, and so the results should be validated using an independent cohort of study participants. Further, in endemic areas, the definition of CM is often challenging and CM is often over-diagnosed. The WHO definition of CM may misclassify up to 25% of cases46 and its specificity is greatly improved by adding a clinical test for Malaria Retinopathy47,48. However, Retinal changes specific to CM require specialist examination techniques, are difficult to examine in conscious children, and such data was not available in this study. Samples analysed in this study were left over specimens following normal microbiology and chemistry laboratory procedures. There is lacking data for the samples on the time taken from collection during lumbar puncture to storage at -80°C and therefore there is possibility that proteome changes occurred during that period49.
Label-free proteomics are cost-efficient, offer higher proteome coverage and a higher dynamic range. However, as every sample is handled separately, variations that can bias the quantitative analysis may be introduced. The results for ABM need to be validated with other aetiologies of ABM and other age groups, such as neonates, since the two pathogens are now uncommon due to the introduction of conjugate vaccines.30

Conclusions
Children with ABM and CM have different CSF host proteomes.17 In the present study, two neutrophil proteins, MPO and LTF, were found to be the best biomarkers to distinguish ABM and CM. They have the potential to be developed as point-of-care diagnostics following validation in an independent cohort.

Ethical statement
The samples used in this study had been collected under a previous study examining the mechanisms of neurological damage in Kenyan children with cerebral malaria and acute bacterial meningitis with the approval of KEMRI Scientific Steering Committee (protocol No. 480; by Prof. Charles R. Newton). The parents or guardians of all study participants had provided written informed consent for sample use for future study during sample collection.

Data availability
The mass spectrometry raw files generated and analysed in the current study have been deposited to the ProteomeXchange Consortium (PXD006357), via the MassIVE partner repository (MSV000080979), under the following title: Cerebrospinal fluid markers to distinguish bacterial meningitis from cerebral malaria in children.

The FTP for the dataset is available here.

Competing interests
No competing interests were disclosed.

Grant information
This work was supported by the Wellcome Trust [084538], Strategic Award, [107769]; Strategic Award and Initiative to Develop African Research Leaders (IDeAL). JMN is a beneficiary of both Strategic Award and IDeAL early career post-doc, EG and MKR are beneficiaries of Strategic Award. NKK is a post-doc currently supported by IDeAL.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments
We thank the study participants, their families, and the clinical staff at the KEMRI-Wellcome Trust Programme. We would like to thank Prof. Philip Bejon and Dr. Abdirahman Abdi for critical reviews of the manuscript.

This paper is published with the permission of the Director of the Kenya Medical Research Institute.

Supplementary material
Table S1. List of quantified proteins.
Click here to access the data.

Table S2. List of proteins included in the biomarker analysis quantified proteins.
Click here to access the data.

Figure S1. Host response to *Haemophilus influenzae* and *Streptococcus pneumoniae* is generic. The principal component analysis score plot depicts overlap of the cerebral spinal fluid proteomes of *H. influenzae* and *S. pneumoniae*. HIN, *H. influenzae*; SPN, *S. pneumoniae*.
Click here to access the data.

Figure S2. A volcano plot showing differential protein expression between acute bacterial meningitis and cerebral malaria, with the latter as the reference. FDR < 0.01 (y axis) and Log FC≥2 (x axis) were set as the significance threshold values as shown with blue lines. FDR, false discovery rate; FC, fold change.
Click here to access the data.

Figure S3. Weight assignment of protein biomarkers using the Random Forests mean decrease in accuracy scores. “Important biomarkers” were extracted using a recursive feature elimination (Boruta wrapper package). Each box plot corresponds to mean decrease in accuracy (MDA) scores for 1000 iterations. The proteins are ranked according to their mean importance score. Briefly, MDA scores for actual proteins were compared to those of shadow proteins (randomly shuffled quantities).
Click here to access the data.
References


41. Dastych M, Gottwaldová J, Čermáková Z: Calprotectin and lactoferrin in the


Open Peer Review

Current Referee Status: ✓ ?

Version 2

Referee Report 27 September 2017

doi:10.21956/wellcomeopenres.13799.r26406

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I am satisfied with the changes made after my initial review. All concerns have been addressed appropriately and satisfactorily. I now approve this manuscript for publication without any reservations.

Competing Interests: No competing interests were disclosed.

Referee Expertise: Malaria Immunology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 14 August 2017

doi:10.21956/wellcomeopenres.12925.r24112

Clarissa Valim
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The article by Njunge et al. is a very well written report of a sound investigation about biomarkers to differentiate bacterial encephalitis from cerebral malaria in children. The findings of this study, set keystones in the research agenda to develop a so needed point-of-care test for pediatric encephalitis in Africa. We have two major comments and some minor ones.

Major
1. The study was conducted with samples collected during 9 years. During these years, were samples from only 59 patients collected and archived? Given the importance of selection bias in biomarker studies, it would be important for the authors to provide details of the criteria used to
select patients/samples for this study. Perhaps they could also provide a flowchart of enrollment in the supplementary material.

2. The authors may want to provide minimal details of the collection and storage of their study samples, since Mass Spec proteomics can be very sensitive to details in the collection process including elapsed time to centrifuge and refrigerate samples. If not many details are known, given the age of these samples, it would be helpful to add to the discussion a limitation specifying that some proteins could have been degraded and thus, could not be properly quantified.

To better understand the importance of selection bias to the author’s results, it would also be helpful to see a distribution of samples by the year they were collected. Hopefully, it can be shown that bacteria samples were collected contemporaneous to malaria samples.

3. The authors state that proteins with more than 50% of samples missing quantification were excluded from the analysis but those that could be quantified in up to 50% of samples a value set to 0 when they could not be quantified. That is fine but it would be helpful to know what was the percent of samples that could not be quantified in each of the two comparison groups for the most important markers (perhaps those included in Table S2). Have the authors considered that an alternative explanation for the non-quantification was that the samples were degraded?

Minor

1. Given the importance of the case classification criteria in this study, the authors may want to provide a few more details of the non-ABM group. They were classified as cerebral malaria but how strong was the evidence of the presence of cerebral malaria in this group? Could they have viral encephalitis, for instance, or a non-detected ABM? Could the authors provide their rational to state that this group represented patients with CM? Regardless of the presence of CM in the group without ABM, the results of the study are relevant since they show biomarkers that identified ABM and non-ABM (or unlikely ABM) patients.

2. The authors may want to include in Table 3, the optimal cut-off value of the proteins that achieve the reported sensitivity, specificity. It would also be helpful for the top 10 or so proteins to see the boxplots comparing the two diagnosis groups to have a better sense of the variability.

3. The authors may want to reconsider and exclude the ROC curve from Figure 4. The curve of an AUC-ROC of approximately 1 is not very illustrative. Also, although the actual accuracy of the identified proteins is probably very high, the AUC-ROC estimated by the authors was probably influenced by the fact that proteins were selected and had their accuracy estimated in the same samples. The curve in this paper is probably giving more weight to the estimated accuracy than it would be necessary.

4. We have a few statistical suggestions for a future study conducted by the authors. However, the authors shall feel free to ignore those suggestions in this study. These different analysis would be unlikely to change conclusions and we believe the results of the study shall be made public and not wait for any additional analysis. The suggestions are:

a) Wilcoxon and t-tests assuming equal variances were used to compare protein in the two comparison groups. Often the variance of proteins is very different across the two groups and none
of these two tests are adequate when the variances vary across comparison groups.

b) If the number of samples with zeros were substantial, a more appropriate approach would be using Zero inflated or Zero truncated models to compare proteins.

c) In the future, the authors may want to explore using multiple imputation if missingness is substantial

d) In the future the authors may want to explore additional feature selection procedures. Random forests are excellent algorithms to create classification signatures but may not be the most appropriate algorithm to select markers. Moreover, conducting a few different algorithms and evaluating consensus may help to decrease a bit the impact of overfitting in each one of these algorithms.

e) In future studies the authors may explore using re-sampling to estimate accuracy, for instance, using cross-validation. That may help to discount the overestimation in accuracy that occurs when there is neither a validation nor a test set.

5. We could not find any “phenotype” or metadata associated with the protein data. Without that, it would not be possible to reproduce the analysis presented in this paper. I do not consider any action is necessary to address this comment since the authors made available what nearly all investigators do.

6. Alternative explanations for the extremely high estimated AUC-ROC could be inserted in the discussion, such as issues with sample storage and selection that could have differentially affected some proteins (that have different degradation rates), and the impact of misclassification of patients in the CM group.

7. My understanding is that the study used a label-free Mass Spec. To prevent problems with the Mass Spec community, the authors may want to add a comment to the discussion about limitations of the technology used as opposed to a labelled quantitative approach. This is not terribly relevant and it is fine if the authors choose to ignore this comment.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Biomarkers in bacterial infection, Epidemiology of malaria, Biostatistics

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 21 Sep 2017

James Njunge, KEMRI-Wellcome Trust, Kenya

Major

1. The study was conducted with samples collected during 9 years. During these years, were samples from only 59 patients collected and archived? Given the importance of selection bias in biomarker studies, it would be important for the authors to provide details of the criteria used to select patients/samples for this study. Perhaps they could also provide a flowchart of enrollment in the supplementary material.

We provide below bar plots of sample distribution per year. This was a discovery study for biomarkers and we randomly selected samples that had left over CSF samples and fitting study inclusion. We selected samples from a database of approximately 3,420 CSF samples that includes ABM, CM, and unknown aetiology as well as others. Since the proteomics approach used was label free protein quantification, the number of sample that could be used for the discovery study was limited. The study used samples collected over 9 years, although the distribution of these samples for CM was in a span of 4 years (2003 – 2006: Figure 1). For ABM, samples included mainly originated from the years (2002 – 2005 and 2007) (Figure 1.).

2. The authors may want to provide minimal details of the collection and storage of their study samples, since Mass Spec proteomics can be very sensitive to details in the collection process including elapsed time to centrifuge and refrigerate samples. If not many details are known, given the age of these samples, it would be helpful to add to the discussion a limitation specifying that some proteins could have been degraded and thus, could not be properly quantified.

In the limitation section, the following statement has been included “Samples analyzed in this study were left over specimens following normal microbiology and chemistry laboratory procedures. There is lacking data for the samples on the time taken from collection during lumbar puncture to storage at -80°C and therefore there is possibility that proteome changes occurred during that period [1].”

To better understand the importance of selection bias to the author’s results, it would also be helpful to see a distribution of samples by the year they were collected. Hopefully, it can be shown that bacteria samples were collected contemporaneous to malaria samples.

**Figure 1:** Yearly distribution of ABM and CM samples included in the study
3. The authors state that proteins with more than 50% of samples missing quantification were excluded from the analysis but those that could be quantified in up to 50% of samples a value set to 0 when they could not be quantified. That is fine but it would be helpful to know what was the percent of samples that could not be quantified in each of the two comparison groups for the most important markers (perhaps those included in Table S2). Have the authors considered that an alternative explanation for the non-quantification was that the samples were degraded? The percentages of proteins quantified per group has been provided in Table S2 as requested.

In the limitation section, the following statement has been included “Samples analyzed in this study were left over specimens following normal microbiology and chemistry laboratory procedures. There is lacking data for the samples on the time taken from collection during lumbar puncture to storage at -80°C and therefore there is possibility that proteome changes occurred during that period [1].”

Minor

1. Given the importance of the case classification criteria in this study, the authors may want to provide a few more details of the non-ABM group. They were classified as cerebral malaria but how strong was the evidence of the presence of cerebral malaria in this group? Could they have viral encephalitis, for instance, or a non-detected ABM? Could the authors provide their rational to state that this group represented patients with CM? Regardless of the presence of CM in the group without ABM, the results of the study are relevant since they show biomarkers that identified ABM and non-ABM (or unlikely ABM) patients.

The following text has been included in the limitation section. “Further, in endemic areas, the definition of CM is often challenging and CM is often over-diagnosed. The WHO definition of CM may misclassify up to 25% of cases [2] and its specificity is greatly improved by adding a clinical test for Malaria Retinopathy [3, 4]. However, Retinal changes specific to CM require specialist examination techniques, are difficult to examine in conscious children, and such data was not available in this study.”

2. The authors may want to include in Table 3, the optimal cut-off value of the proteins that achieve the reported sensitivity, specificity. It would also be helpful for the top 10 or so proteins to see the boxplots comparing the two diagnosis groups to have a better sense of the variability.

Bar plots have been generated as below for review purposes

Figure 2: SPARC
Figure 3: PRTN3
Figure 4: PFN1
Figure 5: MPO
Figure 6: LTF
Figure 7: LCP1
Figure 8: CORO1A
Figure 9: ACTN1
Figure 10: SPARCL1

3. The authors may want to reconsider and exclude the ROC curve from Figure 4. The curve of an
AUC-ROC of approximately 1 is not very illustrative. Also, although the actual accuracy of the identified proteins is probably very high, the AUC-ROC estimated by the authors was probably influenced by the fact that proteins were selected and had their accuracy estimated in the same samples. The curve in this paper is probably giving more weight to the estimated accuracy than it would be necessary.

The AUR-ROC curve has been removed

4. We have a few statistical suggestions for a future study conducted by the authors. However, the authors shall feel free to ignore those suggestions in this study. These different analysis would be unlikely to change conclusions and we believe the results of the study shall be made public and not wait for any additional analysis. The suggestions are:

a) Wilcoxon and t-tests assuming equal variances were used to compare protein in the two comparison groups. Often the variance of proteins is very different across the two groups and none of these two tests are adequate when the variances vary across comparison groups.

b) If the number of samples with zeros were substantial, a more appropriate approach would be using Zero inflated or Zero truncated models to compare proteins.

c) In the future, the authors may want to explore using multiple imputation if missingness is substantial

d) In the future the authors may want to explore additional feature selection procedures. Random forests are excellent algorithms to create classification signatures but may not be the most appropriate algorithm to select markers. Moreover, conducting a few different algorithms and evaluating consensus may help to decrease a bit the impact of overfitting in each one of these algorithms.

e) In future studies the authors may explore using re-sampling to estimate accuracy, for instance, using cross-validation. That may help to discount the overestimation in accuracy that occurs when there is neither a validation nor a test set.

We will consider these suggestion which are very appropriate in our biomarker and related work.

5. We could not find any “phenotype” or metadata associated with the protein data. Without that, it would not be possible to reproduce the analysis presented in this paper. I do not consider any action is necessary to address this comment since the authors made available what nearly all investigators do.

6. Alternative explanations for the extremely high estimated AUC-ROC could be inserted in the discussion, such as issues with sample storage and selection that could have differentially affected some proteins (that have different degradation rates), and the impact of misclassification of patients in the CM group.

This has been addressed by excluding the AUC-ROC (Figure 4). We have included additional text in the limitation section of the discussion that address challenges (1) with the classification of CM and (2) sample storage.
7. My understanding is that the study used a label-free Mass Spec. To prevent problems with the Mass Spec community, the authors may want to add a comment to the discussion about limitations of the technology used as opposed to a labelled quantitative approach. This is not terribly relevant and it is fine if the authors choose to ignore this comment.

*We have included the following additional text in the limitation section “Label-free proteomics are cost-efficient, offer higher proteome coverage and a higher dynamic range. However, as every sample is handled separately, variations that can bias the quantitative analysis may be introduced.”*

**Competing Interests:** None
3. They may need to double check that in the Results section the number of Tables and Figures being referred to are correct.

References

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Malaria Immunology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 21 Sep 2017

James Njunge, KEMRI-Wellcome Trust, Kenya

**Methods section**

1. The study analysed archived CSF samples which had been collected over a period spanning almost nine years. The authors need to say more on how the samples were originally collected, how long it took before they were stored and at what temperature. They may also need to add this as a limitation in the Discussion section bearing in mind that the stability of CSF proteins is compromised if kept under different temperatures for some time (Ranganathan et al., 2006).

The following text has been included in the limitation section. “Samples analyzed in this study were left over specimens following normal microbiology and chemistry laboratory procedures. There is lacking data on the time taken from lumbar puncture to freezing at -80°C for the samples and therefore there is possibility for proteome changes before sample storage.”

2. Definition of CM: although archive CSF samples were used, I have strong reservation with the criteria used in defining CM cases. They may need to include this as a limitation and cite recent papers on CM classification in addition to Milner et al., 2014 (Beare et al., 2011, Severe Malaria, 2014, Seydel et al., 2015).

The following text has been included in the limitation section. “Further, in endemic areas, the definition of CM is often challenging and CM is often over-diagnosed. The WHO definition of CM may misclassify up to 25% of cases [2] and its specificity is greatly improved by adding a clinical test for Malaria Retinopathy [3, 4]. However, Retinal changes specific to CM require specialist examination techniques, are difficult to examine in conscious children, and such data was not available in this study.”

**Discussion section**

1. The statement "whilst in CM the blood brain barrier is MILDLY impaired....." may need to be revisited and supported by what has been covered in some recent reviews (Renia et al., 2012, Polimeni and Prato, 2014, Prato, 2014 and what has been published based on ECM in mice models (Poh et al., 2014).)

References that support mild impairment to the blood brain barrier during CM compared to bacterial and viral infections have been added as suggested

2. Minor: in Figs 2 and S1, there are some points that seem to overlap (i.e. especially for CM in
Fig2). Can these be explained in the Fig legends. For consistency it might also be better to keep the type of labels uniform in all Figs (i.e. if CM is red dot, then it stays that way in all Figs, easier to follow).

Overlap of the figures removed in Figure S1 – the CM group has been excluded for clarity

3. They may need to double check that in the Results section the number of Tables and Figures being referred to are correct.

This has been thoroughly checked

Competing Interests: No competing interests were disclosed.