The Journal of Infectious Diseases
MAJOR ARTICLE
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Cytokine Profile Distinguishes Children With *Plasmodium falciparum* Malaria From Those With Bacterial Blood Stream Infections

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Background. Malaria presents with unspecific clinical symptoms that frequently overlap with other infectious diseases and is also a risk factor for coinfections, such as non-Typhi *Salmonella*. Malaria rapid diagnostic tests are sensitive but unable to distinguish between an acute infection requiring treatment and asymptomatic malaria with a concomitant infection. We set out to test whether cytokine profiles could predict disease status and allow the differentiation between malaria and a bacterial bloodstream infection.

Methods. We created a classification model based on cytokine concentration levels of pediatric inpatients with either *Plasmodium falciparum* malaria or a bacterial bloodstream infection using the Luminex platform. Candidate markers were preselected using classification and regression trees, and the predictive strength was calculated through random forest modeling.

Results. Analyses revealed that a combination of 7–15 cytokines exhibited a median disease prediction accuracy of 88% (95th percentile interval, 73%–100%). Haptoglobin, soluble Fas-Ligand, and complement component C2 were the strongest single markers with median prediction accuracies of 82% (with 95th percentile intervals of 71%–94%, 62%–94%, and 62%–94%, respectively).

Conclusions. Cytokine profiles possess good median disease prediction accuracy and offer new possibilities for the development of innovative point-of-care tests to guide treatment decisions in malaria-endemic regions.

Keywords. biomarkers; cytokine profile; differential diagnosis; malaria.

Malaria remains a major global health problem with a substantial economic and social burden in sub-Saharan Africa. Despite decreasing case numbers, approximately 219 million cases and 435 000 deaths were reported in 2017, 92% of which occurred in the World Health Organization (WHO) African region [1]. Those most affected by malaria are children under the age of 5 years. Fever is a leading symptom of malaria, and, based on clinical symptoms alone, the presentation of malaria can resemble several different infectious diseases [2–6]. Developing countries often lack the financial means to maintain microbiology facilities to perform bacterial blood cultures, which is still the most common method to identify bacterial infections [7]. Even in settings where blood cultures are available, there is a limitation of diagnostic sensitivity to blood cultures due to low blood sample volumes in children. Therefore, the WHO recommends simultaneous treatment with antibiotics and antimalarials in severe cases [8]. As a result, rise in antibacterial as well as antimalarial drug pressure and ultimately drug resistance stand in contrast to failure to treat alternative causes of severe infection. A biomarker or combination of markers, representative for the underlying disease and true cause of illness, could improve

Received 31 July 2019; editorial decision 1 November 2019; accepted 5 November 2019; published online November 8, 2019.

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The Journal of Infectious Diseases® 2020;221:1098–106

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DOI: 10.1093/infdis/jiz587

diagnostic accuracy and help guide treatment decisions. This study aimed to test whether the cytokine profile of patients suffering from either clinical malaria (ie, malaria as a reason for hospital admission) or a bacterial blood stream infection (BSI) would allow a prediction of their disease status and therefore improve diagnostic accuracy in malaria-endemic settings.

METHODS

Study Area and Selection of Samples

Patient samples were collected in the framework of 2 previous studies [9, 10], which were approved by the Committee on Human Research, Publications and Ethics, School of Medical Science, Kwame Nkrumah University of Science and Technology, in Kumasi, Ghana, and the Ethics Committee of the Ärztekammer Hamburg, Germany. Patient samples were collected between 2010 and 2015 at the Agogo Presbyterian Hospital, a district hospital with 250 beds, situated in the Ashanti Region of Ghana. The study area is holoendemic for malaria with seasonal peaks for malaria transmission.

Samples from children aged ≤5 years with either *Plasmodium falciparum* malaria or a BSI were selected, whereas children with coinfections were excluded from the study to establish a distinct cytokine profile for each disease. Malaria case definitions were based on children admitted to the hospital with a fever (≥37.5°C), a *P. falciparum* positive slide, and a negative bacterial blood culture. Case definitions for BSI patients were children admitted to the hospital with a fever (≥37.5°C) and a positive blood culture in the absence of parasitemia (negative slide). The BSI patients were selected based on the pathogen detected in the respective blood culture; the distribution of commonly found pathogens in the population is proportionally represented in the study samples. Due to low amounts of available patient material, 8 malaria, 4 BSI, and 3 control samples had to be replaced by alternative samples that had been matched by sex. Altogether, 38 malaria and 30 BSI samples were analyzed, as well as 10 healthy control samples measured for their cytokine content but not included in the prediction model.

Experimental Procedure and Equipment Settings

Common markers of inflammation (eg, C-reactive protein [CRP], interleukin [IL]-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-27), endothelial activation (eg, soluble cell adhesion molecule [sICAM], soluble vascular cell adhesion molecule [sVCAM], RANTES), coagulation (eg D-Dimer), complement system (eg complement components C1, C2, C4b, C5, C5b, C9 and mannose binding protein [MBP]), mannose binding protein [MBP]), and others (eg, granzyme B, platelet-derived growth factor [PDGF], haptoglobin) were selected and matched against commercially available cytokine detection kits from the XMAP collection of Merck Millipore (Supplemental Data Table S2). Serum samples of patients were diluted as instructed in the manual. Beads were diluted 1:1.5. Working solutions were prepared according to the manufacturer's instructions. Ninety-six-well microtiter plates were loaded with patient serum and immunoassays were performed. The incubation step for binding of beads was performed overnight at 4°C with agitation on a plate shaker. Assays were measured using a Luminex 200 xPONENT 3.1 system [11]. Equipment calibration and performance verification were executed as required. The Luminex magnetic bead regions were programmed for each kit. The reaction volume was 50 µL (except for the complement panel, which was 100 µL), bead count was set to 30, gate settings were 8000–15000, reporter gain was left at the default setting (low PMT), as well as a 60-second measuring timeout. Myeloperoxidase (MPO), sICAM1, and sVCAM1 were measured in the "cardiovascular disease kit 2" (indicated as MPO^{CVD2}, $sICAM1^{CVD2}$, and $sVCAM1^{CVD2}$) as well as in the neurodegenerative disease kit (MPO^{NDD3}, $sICAM1^{NDD3}$, and $sVCAM1^{NDD3}$).

Data Preprocessing and Statistical Analyses

Median fluorescence intensity (MFI) was translated into concentration levels using a standard curve best-fit algorithm implemented in the MILLIPLEX Analyst 5.1 software and harmonized to the scale of nanograms/milliliter. Standard measurements that were not within the range indicated in the manual were excluded from the curve fitting. Out-of-range measurements of samples were replaced with the minimum or maximum threshold value of the curve, respectively. To account for a loss of information in this context, a "unique MFI value count" was applied: multiple out-of-range measurements resulted in identical MFI values; more than 50% of identical values for a single cytokine led to exclusion from subsequent analyses.

Categorical variables (eg, gender and pathogens) were described as frequencies and percentages. Continuous variables (eg, age) were presented as median with its interquartile range (IQR). Cytokine concentration levels were depicted as box and whisker plots. To visualize the relative differences in median concentration levels between malaria and BSI patients, we calculated the median ratio between these 2 groups, where the BSI group served as denominator. Ratios equal to 1 represent no difference in median expression, a ratio >1 reflects a higher median concentration in the malaria group, whereas a ratio of <1 implies a higher median concentration in the BSI group.

Candidate cytokines were preselected based on univariate classification and regression trees (CART). Markers achieving a prediction accuracy (PA) of ≥70%, in determining whether a patient had malaria or BSI, were considered for random forest (RF) modeling. The CARTs were run with 1 split per tree to avoid overfitting of the models. Spearman's rank correlation coefficient (r_s) was calculated for all possible cytokine pairs to explore possible correlations between markers.

Random forest models were performed to evaluate how well the selected set of cytokines predicted whether a patient had

malaria or a BSI. Each forest was based on 1000 CARTs. To fit RF models, the data set with the preselected biomarkers were randomly split into training (N = 51 [75%] of the observations) and testing (N = 17 [25%] of the observations) data sets, while keeping the proportions of malaria and BSI cases proportionate to the full data set. Training data were used to fit a model based on cytokine profiles, and the results were used to predict patients' diagnoses within the test data set. The PA is defined as the proportion of correctly identified malaria and BSI cases calculated for each model.

In a first step, univariate RF models for each cytokine (1 split per tree) and a full model containing all selected biomarkers with PAs ≥70% were established. A sequence of 1000 RF models was performed, each with randomly allocated test and training data sets. Model performance was reported as the median PA of each model, with its 95th percentile interval (PI).

To identify potential marker combinations with the highest PA, a backward elimination was applied, where a marker with the lowest variable importance (Gini index) was removed from the full RF model. This procedure was applied iteratively with the remaining cytokines until only 1 biomarker remained in the model. The cytokine selection process was performed 1000 times, each time with randomly allocated test and training data sets. The importance of respective variables varied across the 1000 selection sequences, depending on how the constellation of test and training data set fostered splitting on that specific cytokine. To gain insight into which cytokines contributed most to the PA (ie, was eliminated later in the process), we calculated the median elimination rank. Each rank reflects how many other cytokines were eliminated before the respective marker.

As previously mentioned, 15 samples had to be replaced to complete cytokine measurements. To test whether this had led to changes in the model estimates due to introduction of bias, we applied the classification algorithm described above to a data set without the replacement samples and compared model performances (Supplemental Data Figure S2_1–4).

All statistical preprocessing and analysis steps were done in R, version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria). The CARTs were constructed using the *rpart* package version 4.1–13, and RF modeling was performed using *randomForest* version 4.6–14 [12].

RESULTS

Demographics of Study Population

Malaria and BSI patients showed a similar age distribution with a median age of 24 (IQR, 13–38) and 29 months (IQR, 12–36), respectively. Sex was evenly distributed among malaria and BSI cases with 58% (22/38) and 50% (15/30) female participants. Among the 30 selected BSI patients, non-Typhi *Salmonella* was the most frequently identified pathogen (50%, 15/30) and the malaria group presented a median parasitemia of 206 500 (IQR, 44 900–477 975) parasites/µL (Table 1).

Cytokine Concentration Levels and Preselection

Of the initial selection of cytokines, 39 were considered for statistical analyses. Exclusion of analytes was based on insufficient (below 50%) unique values or insufficient (below 30) bead count.

Absolute concentration levels and demographics of healthy controls are shown in the Supplemental Data section (Supplemental Data Figure S1 and Table S1). Cytokine concentrations of patients varied widely within disease groups. Figure 1 shows a median cytokine concentration ratio plot according to disease group.

High concentrations (median ratio ≥2) of sCD137, granzyme B, sFas ligand ([sFasL] first apoptosis signal), D-dimer, and sVCAM-1 were observed for the malaria group, whereas concentration levels of complement component C2 and C4b, as well as P-selectin, CRP, acid glycoprotein (AGP), haptoglobin, PDGF-AA, and RANTES was more pronounced in BSI patients (median ratios ≤0.5).

Strong positive correlations were observed among 3 cytokine pairs: D-dimer and $sICAM1^{CVD2}$ (r_s = .86), A2M and AGP (r_s = .87), as well as BNDF and RANTES (r_s = .81). The CART analyses revealed PAs ranging from 56% to 85% of correctly classified diagnoses (listed below the box plots in Supplemental Data Figure S1). Applying an accuracy threshold of ≥70% resulted in selection of 15 candidates eligible for further modeling: platelet endothelial cell adhesion molecule (PECAM)-1 (71%), PDGF-AA

Table 1. Patient Demographics Stratified by Disease Group

Characteristics	Malaria (N = 38)	BSI (N = 30)
Age in months [median (IQR)]	24.0 (13.5–37.5)	28.5 (12.0–36.0)
Female sex [N (%)]	22 (58)	15 (50)
Parasite count/µL [median (IQR)]	206 500 (44 900–477 975)	NA
Bacterial Isolate [N (%)]		
Non-Typhi *Salmonella*	NA	15 (50)
Streptococcus pneumoniae	NA	7 (23)
Salmonella Typhi	NA	6 (20)
Staphylococcus aureus	NA	1 (3)
Streptococcus milleri	NA	1 (3)

Abbreviations: BSI, bacterial bloodstream infection; IQR, interquartile range; N, sample size; NA, not applicable.

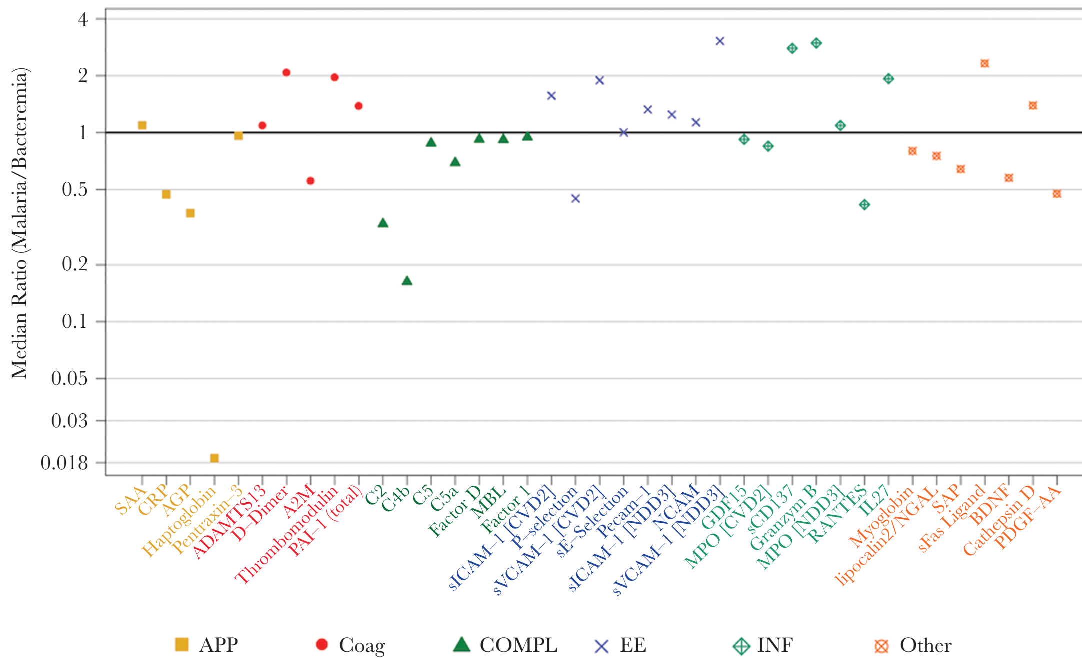

Figure 1. Median cytokine concentration ratio plot (log_2 scale). A ratio of 1 represents similar median cytokine concentration in both disease groups. A ratio of ≥1 indicates a higher median concentration in malaria patients, whereas ≤1 reflects a higher median concentration in bacterial bloodstream infection patients. APP, acute-phase proteins; Coag, coagulation markers; COMPL, complement components; EE, endothelial markers; INF, inflammation.

(71%), Il-27 (72%), sCD137 (75%), complement component C5a (75%), RANTES (75%), sVCAM1^{NDD3} (75%), sP-Selectin (76%), VCAM1^{CVD2} (76%), complement component C4b (78%), thrombomodulin (79%), granzyme B (82%), haptoglobin (84%), sFasL (84%), and complement component C2 (85%).

Modeling Disease Prediction

The RF models were performed to evaluate how well the selected cytokines can distinguish malaria from BSI. Univariable RF models were calculated to determine the PA for every single marker (Figure 2). Calculations were repeated without

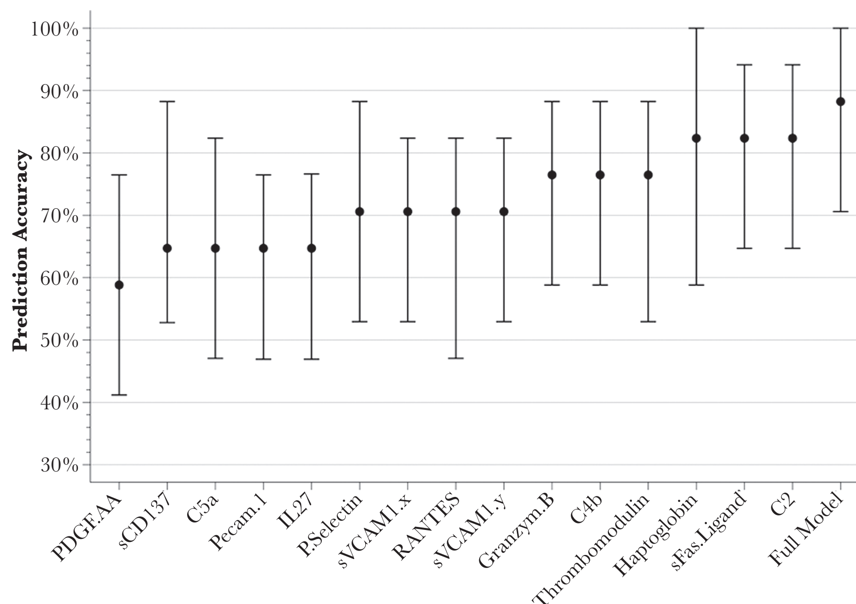

Figure 2. Prediction accuracy (PA) and 95% percentile interval (PI). Black circles represent the median PA of the random forest models, and whiskers represent the lower and upper end of the 95% PI, respectively. The full model included all 15 cytokines.

replacement samples, which resulted in comparable numbers (Supplementary Data Figure S2_1–4).

Of all markers, complement component C2 (PA = 82; 95% PI, 71%–94%), haptoglobin (PA = 82; 95% PI, 62%–94%), and sFasL (PA = 82; 95% PI, 62%–94%) had the highest predictive power; the 2 former markers had a higher concentration in patients with BSI, the latter was more pronounced in the malaria group. Patients of both disease groups showed a similar likelihood of being misclassified. A full model comprising all 15 candidate markers yielded a slightly higher median PA of 88% (95% PI, 73%–100%) compared with any single marker alone.

The backward elimination revealed 4 major segments (Figure 3), within which the median PA as well as the PI did not considerably change after a further elimination step. Model estimates remained stable down to 7 cytokines (PA of 88%; 95% PI, 64%–100%) before decreasing in predictive power. Reduced models containing 1 single cytokine contained the lowest PA with 76% (95% PI, 53%–94%).

We calculated the median elimination rank for each cytokine to estimate which marker played a major role in each of the selection sequences (Figure 4). Depending on each marker's contribution towards the predictive accuracy of the disease status, some markers were excluded earlier by backward elimination (PDGF-AA, RANTES, P-Selectin, sCD137, PECAM-1, IL-27, and C5a), whereas others contributed more to the correct classification and were eliminated later (thrombomodulin, haptoglobin, granzyme B, sFasL, and complement components C2 and C4b).

DISCUSSION

Accurate malaria diagnostics in endemic regions is difficult. The effectiveness of the current malaria case definition (fever + positive rapid diagnostic test or positive blood smear) for the diagnosis of malaria is known to be sensitive but not specific [8, 13]. It implies that, in a holoendemic malaria region, a high number of patients with asymptomatic parasitemia will be treated with antimalarial medication, although the fever may be caused by a concurrent infection [14, 15]. Studies in Gambia [16], Tanzania [17], and Niger [18] showed specificities for malaria diagnoses based on clinical symptoms [19] to be 61%, 52%, and 21%, respectively, even coming down to 0% in Niger during the wet season.

Our study aimed to test whether cytokine profiles of pediatric patients with acute malaria or BSI were able to differentiate between the 2 conditions and predict a patient's disease status, thereby laying the groundwork for the development of a new biomarker-based test in the future.

The amount of available patient sample did not allow testing of each marker in a single enzyme-linked immunosorbent assay. Therefore, commercially available multiplex panels were chosen to enable the largest possible information output from a small amount of sample material. A downside of multiplexing is that errors in 1 experimental setup (eg, obstruction of sample flow through the cuvette or readout failures) leads to missing data on multiple cytokines. Procalcitonin, for example, a marker of sepsis [20], was not available in a multiplex format and therefore not part of the analysis. On the other hand, IL-6, IL-8, IL-10, and IL-1β were part of the "CYTOMAG" panel, which as a total failed to reach a bead count of 30.

It is known that *P. falciparum* malaria infections lead to a strong proinflammatory immune response, endothelial cell activation, as well as activation of the coagulation and complement pathway. In accordance with that, we measured increased concentration levels of endothelial cell surface receptors (eg,

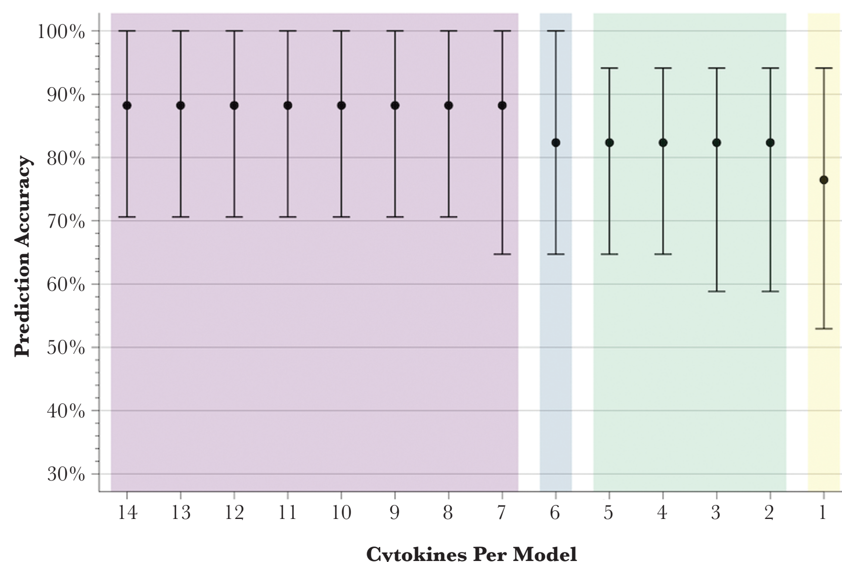

Figure 3. Backward elimination. Black circles represent the median prediction accuracy (PA), and whiskers represent the lower and upper limit of the 95% percentile interval (PI), respectively. The PA and PI are shown for each cytokine reduction step.

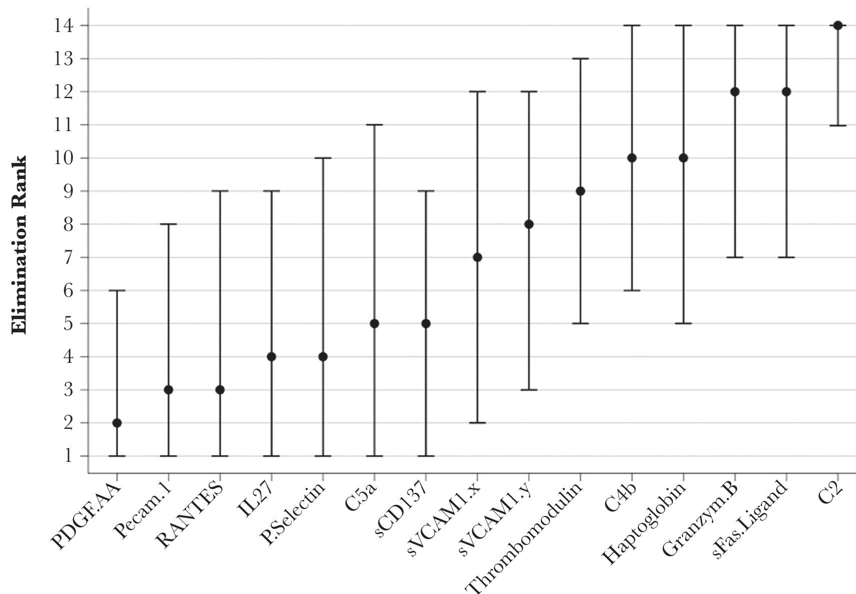

Figure 4. Elimination rank. Black circles reflect the median elimination rank, and whiskers reflect the lower and upper limit of the 95% percentile interval, respectively. Markers with higher ranks were excluded later in the modeling process, due to a higher contribution towards correctly predicting disease status.

VCAM1, P-selectin, E-selectin, and thrombomodulin), inflammatory cytokines as well as acute-phase proteins (eg, sCD137, pentraxin-3, granzyme B, GDF15, MPO, CRP, AGP), and coagulation markers (eg, plasminogen activator inhibitor type 1, thrombomodulin, and D-dimer) in malaria patients compared with healthy controls.

Although expression levels did not differ between patients with malaria or BSI, higher levels of sCD137, granzyme B, sFasL, D-Dimer, and VCAM1 were observed in children with malaria, while complement component C2 and C4b, as well as P-selectin, CRP, AGP, haptoglobin, PDGF-AA, and RANTES were more pronounced in BSI patients. However, a considerable difference in relative concentration in 1 of the 2 groups alone was not sufficient enough to accurately predict disease status; for example, CRP is an acute-phase protein and common marker of inflammation as well as tissue damage [21]. The median CRP concentration was notably higher in the BSI group (median concentration ratio lower than 0.5); nonetheless, a CART-based PA of 63% led to exclusion from the modeling process. A possible explanation as to why CRP is not an adequate marker for a differential diagnosis could be that CRP, as an acute-phase protein, is stimulated by any pathogen, inflammatory disease, trauma, or allergic complication [22].

Consistent with previous studies that showed elevated levels of fibrin degradation products in association with severe malaria [23], our results showed higher levels of D-dimer in malaria patients and a positive correlation with the endothelial marker sICAM. It has also been suggested that D-dimer levels increase with disease severity and might reflect the level of endothelial damage [24]. Likewise, one of the many functions of the endothelium includes maintenance of hemostatic processes. A crosstalk between players of coagulation and inflammation and endothelial activation seems plausible. Finally, endothelial cells are involved in all 3 pathways associated with coagulopathy in sepsis [25, 26]. Taken together, this might explain why D-dimer does not allow a differentiation between malaria and BSI (PA of 59%).

Haptoglobin, sFasL, and complement component C2 were the best disease predictors in our model (PA of 82%) in terms of accuracy for a single cytokine in our study. Haptoglobin binds free hemoglobin and plays a protective role whenever intravascular haemolysis occurs [27, 28]. The haptoglobin-hemoglobin complex is rapidly cleared by macrophages in the body, which leads to a decrease of haptoglobin concentrations in the plasma. Correspondingly, we found subdetectable levels of haptoglobin in the malaria group and considerably higher levels in the serum of BSI patients. It has a fairly good capacity to predict disease status, which makes haptoglobin a promising candidate marker for the differential diagnosis between malaria and BSI patients [29]. Likewise, sFasL showed a considerable difference in median concentration level between the 2 groups (>2 in the median ratio plot), and a similarly reasonable accuracy. Fas and its ligand FasL are part of the tumor necrosis factor superfamily of death receptors that, upon receptor-ligand binding, trigger apoptosis via the extrinsic pathway [30]. Studies have discovered an upregulation of apoptosis-related genes during acute uncomplicated malaria episodes [31], enhanced Fas/FasL serum concentration levels associated with malaria lymphopenia [32] and severe malarial anemia [33], which make it a well known mediator in the field of malaria and, according to our results, a

promising candidate marker for the differentiation of malaria from BSI.

Several studies have shown that malaria infections cause complement activation, which (similar to what is seen for haptoglobin) lead to the reduction of complement factors in the serum [34, 35]. Correspondingly, our results showed lower C2 concentrations in the malaria group compared with BSI patients. Furthermore, similar to haptoglobin and sFasL, this marker also showed a good disease prediction on its own. Because complement component C2 has never before been described in the context of malaria diagnostics and differential diagnostics, respectively, it will be crucial to evaluate our finding in another study and with a larger sample size.

Although all 3 markers were similarly accurate on their own, the set of 15 markers increased the predictive power up to 88%. Backward elimination revealed that precision and accuracy remained stable across declining number of markers until 7 cytokines were left in the model. Further reductions lead to a decrease in accuracy and precision. Previous work has also shown that the combination of more than 1 marker exhibits more accurate discriminatory power than 1 marker alone [29, 36]. This might be the case because the pathology of malaria as well as severe BSI involves systemic inflammatory responses, where no single marker could sufficiently reflect the entire "cytokine storm" but merely interpret a different angle of it [37–39].

A ranking based on a marker's particular contribution to the disease prediction model uncovered that, besides haptoglobin, sFasL, and C2, granzyme B and C4b were also strong contributors. Granzymes are serine proteases and soluble markers for the activation of cytotoxic T lymphocytes and natural killer cells. They are elevated in viral infections [40] as well as malaria [41] and appear to be associated with cerebral malaria in mice [42]. We measured considerably higher levels of granzyme B in the serum of malaria patients compared with BSI, which translated into a high median ratio (>2) but exhibited a moderate PA in our model (PA of <80%). However, granzyme B is among the 3 strongest contributors and probably a likely participant in our minimum set of markers albeit being a weaker marker on its own.

A limitation of the study is the small sample size, which had to be further split into training and test data set. This is most likely the reason for the varying calculated precisions of the models across the 1000 runs, because they strongly depend on the composition of the training and test data set. As already mentioned, the predictive performance of presented markers needs to be assessed in a larger study with a greater sample size to confirm these finding as well as increase robustness of model estimates. Bacterial isolates selected for the BSI disease group were chosen based on the most frequently identified species from the same population [9]. Patient samples had not been tested for possible viral infections before this study. Finally, an evaluation of our findings with a larger sample size should include a patient group with both a malaria infection and BSI. The study was conducted on children aged 5 years or younger. A predication as to how this translates onto adult patients is complex, because cytokine levels change in the course of (a lifetime) time and, in the case of malaria, exposure to infection. This is best answered by assessing older age groups in a separate study.

CONCLUSIONS

Unspecific clinical symptoms and a systemic dysregulated immune response make it difficult to differentiate malaria from other causes of infection. We could show that a minimum set of 7 cytokines were able to differentiate patients with malaria from BSI with good accuracy. Three single markers from our model produced slightly lower but still acceptable results in predicting disease status, which could be used for a biomarker-based point-of-care test in the future.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We express our gratitude to the dedicated staff of the Agogo Presbyterian Hospital for their continued efforts and commitment.

Financial support. The main study was funded by the German Center for Infection Research (800 880 3804). Sample collection was performed through the Typhoid Fever Surveillance in Africa (TSAP) program that was funded through a grant from the Bill and Melinda Gates Foundation (OPPGH5231), as well as the German Center for Infection Research (8000 201-3).

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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