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MAJOR ARTICLE

Elevated Levels of the Endothelial Molecules ICAM-1, VEGF-A, and VEGFR2 in Microscopic Asymptomatic Malaria

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Background. In malaria, clinical disease has been associated with increased levels of endothelial activation due to the sequestration of infected erythrocytes. However, the levels and impact of endothelial activation and pro-angiogenic molecules such as vascular endothelial growth factor (VEGF)–A and its receptor vascular endothelial growth factor receptor 2 (VEGFR2) in asymptomatic malaria have not been well characterized.

Methods. Blood samples were obtained from community children for malaria diagnosis using microscopy and polymerase chain reaction. A multiplex immunoassay was used to determine the levels of intracellular adhesion molecule (ICAM)–1, vascular endothelial growth factor (VEGF)–A, and VEGFR2 in the plasma of children with microscopic or submicroscopic asymptomatic parasitemia and compared with levels in uninfected controls.

Results. Levels of ICAM-1, VEGF-A, and VEGFR2 were significantly increased in children with microscopic asymptomatic parasitemia compared with uninfected controls. Also, levels of VEGF-A were found to be inversely associated with age. Additionally, a receiver operating characteristic analysis revealed that plasma levels of ICAM-1 (area under the curve [AUC], 0.72) showed a moderate potential in discriminating between children with microscopic malaria from uninfected controls when compared with VEGF-A (AUC, 0.67) and VEGFR2 (AUC, 0.69).

Conclusions. These data imply that endothelial activation and pro-angiogenic growth factors could be one of the early host responders during microscopic asymptomatic malaria and may play a significant role in disease pathogenesis.

Keywords. asymptomatic; ICAM-1; malaria; microscopic; submicroscopic; VEGF-A; VEGFR2.

Even though malaria cases continue to decline globally due to concerted control efforts, malaria burden remains high, with ~409 000 deaths in 2019, and most of these deaths occurred in the World Health Organization (WHO) African region [1]. Infection with *Plasmodium* spp. such as *P. falciparum* and *P. vivax* is associated with most malaria-related morbidity and mortality [2, 3]. Protective immunity to malaria may manifest as asymptomatic infection, which is characterized by the absence of clinical symptoms despite the presence of parasites in the blood. Individuals with asymptomatic malaria are rarely diagnosed and treated, making them silent reservoirs that sustain malaria transmission [4, 5]. Whether these asymptomatic infections may be responsible for the development of any other health complications remains unclear [6, 7].

Previous studies have identified that children with microscopic asymptomatic parasitemia have increased levels of C-reactive protein and endothelial activation molecules, such as platelet factor–4 and intercellular adhesion molecule (ICAM)–1 [8, 9]. For instance, Park et al. observed that the serum levels of ICAM-1, vascular adhesion molecule-1 (VCAM-1), and von Willebrand factor (vWF) were elevated in children with microscopic asymptomatic parasitemia compared with those with submicroscopic infections [9]. Also, other studies have reported increased thrombocytopenia and lymphocytopenia in individuals with asymptomatic malaria compared with uninfected controls [10, 11]. The results of these studies imply that asymptomatic malaria may have a significant impact on the development of other health complications.

Inflammation, which is the body's natural response to foreign antigens, precedes angiogenesis, the process whereby new red blood vessels develop from preexisting ones. Inflammation

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may lead to tissue damage through the upregulation of adhesion molecules such as ICAM-1. ICAM-1 enables the infiltration of immune cells to the site of inflammation, leading to the secretion of mediators such as chemokines and cytokines [12–14]. Tissue damage due to inflammation leads to the release of angiogenic growth factors such as vascular endothelial growth factor (VEGF)–A and VEGF receptor (R) 2 [15, 16]. VEGF-A, an isoform of VEGF, is important for controlling angiogenesis as well as for regulating the expression of VEGFR2 to maintain endothelial cell homoeostasis [17–19]. It is essential for endothelial cell proliferation and survival during endothelial cell activation and interacts with VEGFR1 and VEGFR2 [20, 21]. However, most signaling activities for VEGF-A are mediated through VEGFR2, making their biological interactions intertwined [22, 23]. The pathophysiology of infectious diseases such as sepsis and malaria that are driven by inflammation has been associated with increased levels of VEGF [24–27]. During severe malaria, VEGF levels were found to be increased in both blood and brain tissues in naïve populations and children exposed to malaria in an endemic region [28, 29]. ICAM-1 has also been shown to be important for the cytoadherence of infected erythrocytes to the host endothelium and to contribute to the exacerbation of disease in both human [30, 31] and murine models [32, 33].

P. falciparum can cytoadhere to the endothelium by binding to receptors such as CD36 in the vasculature and ICAM-1 and endothelial protein C receptor (EPCR) in the brain, leading to endothelial activation [34–36]. Sequestration enables the parasite to evade splenic clearance, resulting in tissue damage, which is repaired by endothelial growth factors that tightly regulate the endothelial function [37, 38]. While upregulation of these factors has mostly been demonstrated in mild and severe malaria cases [9, 35, 38], it is not clear whether endothelial activation occurs during asymptomatic malaria and can result in dysregulated endothelial activity [8, 9]. Therefore, we hypothesized that asymptomatic malaria results in dysregulated endothelial function, which leads to increased expression of angiogenic molecules such as VEGF-A and VEGFR2. This study, therefore, aimed to compare the profiles of ICAM-1, VEGF-A, and VEGFR2 in children with microscopic or submicroscopic asymptomatic malaria and to determine if any of the molecules could help differentiate the study populations.

METHODS

Patient Consent

The study was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, University of Ghana, with approval number 089/14–15. Written informed consent was obtained from parents/guardians before recruitment, and assent was received from children of appropriate age. All methods were performed following relevant guidelines and regulations.

Study Design and Participants

This was a cross-sectional study that recruited infected asymptomatic and aparasitemic children living in Obom, an area with stable malaria transmission. Obom is a farming community located in the Ga South Municipality in the Greater Accra Region of Ghana, and peak malaria transmission occurs between June and August. Samples were collected at 1 time point in December. The malaria parasite prevalence in Obom, estimated by microscopy during the rainy and dry season is 65% and 28% respectively. [39]. Children who qualified for this study were those ≤15 years of age. Asymptomatic cases were defined by the presence of parasitemia, having an axillary temperature of <37.5°C, and not having taken any antimalarial medication within the 2 weeks before sampling. Parasitemia levels below the detection limit of microscopy but picked up by PCR were categorized as submicroscopic, while those detectable by microscopy were referred to as microscopic. Uninfected controls were defined by the absence of parasite by both microscopy and PCR, axillary temperature <37.5°C, and not having been on any antimalarial medication within the 2 weeks before sampling. The study was explained to the community, parents, guardians, and children before commencement.

Sample Collection

Venous blood (5 mL) was collected from each volunteer into EDTA tubes. The collected blood samples were used to prepare both thick and thin blood films on microscope slides to define the microscopic asymptomatic group. The remaining blood samples were processed into blood cells, some of which were used for genomic DNA (gDNA) extraction and plasma preparation. Plasma was stored in Eppendorf tubes at −80°C until it was used for experiments.

Determination of Parasitemia by Microscopy and Polymerase Chain Reaction

The prepared thick films were processed and Giemsa-stained to estimate parasite density by counting the number of parasites per 200 white blood cells on the film according to WHO standards with a limit of detection of about 40 parasites/μL of blood. Blood films were read by 2 independent microscopists. Blood films that were negative for microscopy were further assessed by PCR to determine whether they were positive for submicroscopic infections or negative. Briefly, gDNA was extracted from 100 μL of whole blood using the Zymo DNA Kit (Zymo Research, Irvine, CA, USA) as per the manufacturer's instructions. The *P. falciparum* 18S ribosomal RNA gene was amplified from extracted DNA and gDNA from the culture-adapted 3D7 parasite strain (positive control) and a no template negative control using a nested PCR. This nested PCR has a limit of detection of 1-0.1 parasites/μL [40]. The amplified products were visualized on a 2% agarose gel stained with 0.5 μg/mL of ethidium bromide. After electrophoresis, gels were viewed under ultraviolet light and images captured using the Vilber

Lourmat Gel Dock System (Vilber Wielandstrasse, Germany). Children who were positive for malaria parasites by PCR but negative by microscopy were categorized as having submicroscopic infections; those who were negative by both microscopy and PCR were categorized as uninfected.

Multiplex Immunoassay

Plasma samples were retrieved and defrosted on ice. A magnetic bead-based multiplex assay was used for the quantification of soluble ICAM-1, VEGF-A, and VEGFR2 using a 3-plex assay in a 96-well plate format (R & D Systems, Minneapolis, MN, USA). Sample dilutions, reagents, and standards were all prepared according to the manufacturer's instructions. The blanks and standards used in the assay were prepared in duplicate on each plate to ensure uniformity. Likewise, for each individual, the plasma sample was run in duplicate. Plates were read using the LUMINEX 200 system, running on Xponent 3.1. The levels of ICAM-1, VEGF-A, and VEGFR2 were reported as median fluorescent intensity (MFI).

Statistical Analysis

All data analyses were performed using Prism, version 6.01 (GraphPad Software, Inc.), and R Software, version 3.5.2. Categorical data were analyzed using the chi-square test. Continuous variables that were not normally distributed were analyzed using the Mann-Whitney *U* test and Kruskal-Wallis test, followed by a Dunn's post hoc test when necessary, whereas normally distributed data were analyzed using 1-way analysis of variance followed by a post hoc test. Correlation between endothelial molecules was determined using Spearman's rank correlation. Additionally, a generalized additive model that provides a very robust tool to assess the association between predictors and dependent variables was used. This GAM-based analysis was used to determine (i) the effect of age on the levels of the endothelial activation molecule (ICAM-1) and angiogenic molecules (VEGF-A and VEGFR2) and (ii) the effect of parasitemia on ICAM-1, VEGF-A, and VEGFR2. The estimates of these effects were modeled using smoothing splines in the mgcv package in R [41]. The Medcalc, version 19.4.1, was used for the receiver operator characteristics (ROC) curve analysis of the endothelial molecules ICAM-1, VEGF-A, and VEGFR2 for children with microscopic asymptomatic malaria. Statistical significance was set at $P < .05$.

RESULTS

Characteristics of the Study Participants

This was a cross-sectional study in which a total of 78 children were recruited: 20 uninfected controls, 22 with submicroscopic parasitemia, and 36 with microscopic parasitemia. There was no significant difference in the proportions of males to females ($P = .93$). Likewise, age and hemoglobin levels did not differ between children with either microscopic or submicroscopic parasitemia and uninfected controls (Table 1).

Levels of ICAM-1 Are Increased in Children With Microscopic Asymptomatic Infection

Levels of sICAM-1 were significantly increased in children with microscopic parasitemia compared with uninfected controls ($P = .01$) but were not significantly different when compared with children with submicroscopic parasitemia ($P = .53$). Also, ICAM-1 levels were not significantly different between children with submicroscopic parasites and uninfected controls ($P = .48$) (Figure 1A). Additionally, in a generalized additive model ICAM-1 did not have any significant association with parasitemia ($P = .27$) (Table 2) or age ($P = .35$) (Table 3).

VEGF-A and its Receptor VEGFR2 Are Upregulated in Children With Microscopic Asymptomatic Infection

Levels of VEGF-A and VEGFR2 were found to be significantly higher in children with microscopic parasitemia compared with uninfected controls ($P = .03$ and $P = .048$, respectively). However, levels between children with submicroscopic infections and uninfected controls were comparable for both VEGF-A and VEGFR2 ($P > .05$) (Figure 1B and C). Additionally, levels of VEGF-A and VEGFR2 were positively correlated ($r = 0.43$; $P = .009$, Spearman rank correlation).

Table 1. Clinical Characteristics of the Study Participants

Characteristics	Controls	Submicroscopic	Microscopic	*P* Value
Sample size, No.	20	22	36	
Sex, No.				
Male, No. (%)	8 (40)	10 (45.5)	16 (44.4)	.93[a]
Age, mean ± SD, y	10 ± 2.241	11.45 ± 2.695	10.24 ± 2.737	.14[b]
Parasitemia, median (IQR), /µL	NA	NA	935.5 (288.8–1973)	NA
Hemoglobin level, median (IQR), g/dL	11.75 (10.8–12.6)	11.25 (10.7–12.48)	11.05 (10.5–12.63)	.41[c]

Abbreviations: IQR, interquartile range; NA, not applicable.

[a]Chi-square test.

[b]Analysis of variance.

[c] Kruskal-Wallis test.

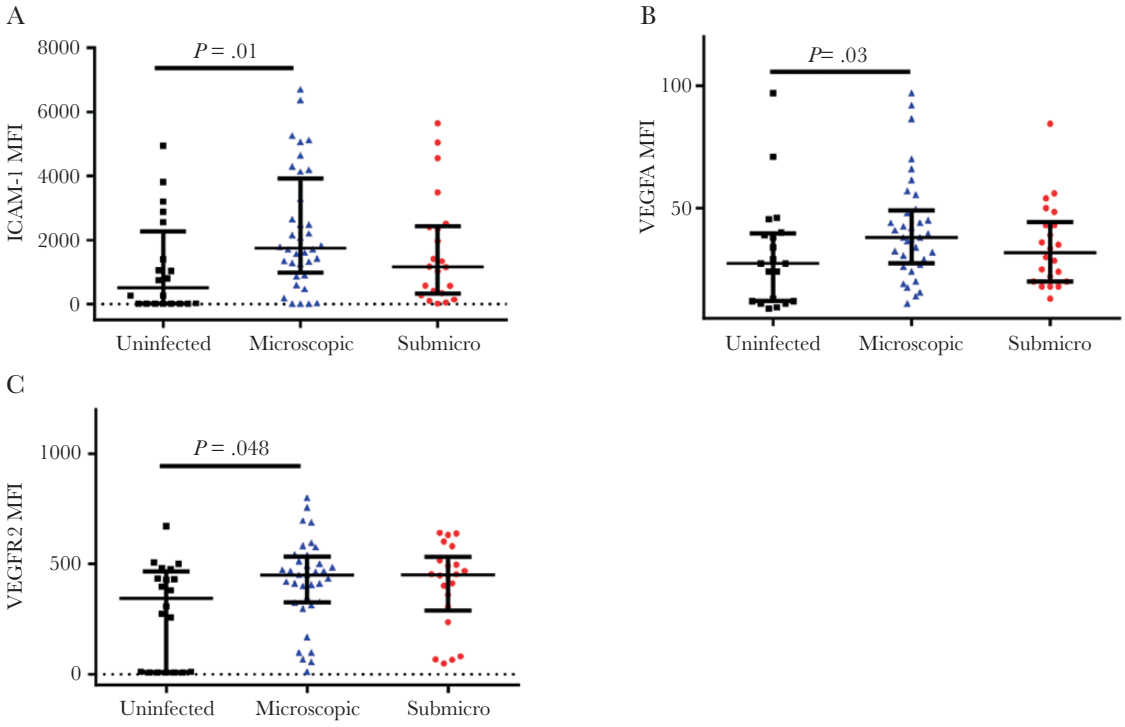

Figure 1. Levels of ICAM-1 and angiogenic growth factors VEGF-A and VEGFR2 in microscopic asymptomatic malaria. The levels of (A) ICAM-1, (B) VEGF-A, and (C) VEGFR2 were determined in children with microscopic asymptomatic malaria, submicroscopic asymptomatic malaria, and uninfected controls. Dot plots show the distribution of the data in the various groups. Medians are indicated by horizontal lines, with vertical lines indicating the confidence interval. Analyte levels were compared by the Kruskal-Wallis followed by a Dunn's test when necessary. Abbreviations: ICAM-1, intracellular adhesion molecule–1; VEGF-A, vascular endothelial growth factor–A; VEGFR2, vascular endothelial growth factor receptor 2.

Increased Levels of VEGF-A Are Negatively Associated With Age in Microscopic Asymptomatic Malaria

To determine whether there was any association between any of the angiogenic molecules with parasitemia or age in children with microscopic and submicroscopic asymptomatic malaria, a generalized additive model was used. First, it was observed that VEGF-A did not show any significant association with levels of parasitemia ($P = .67$). However, VEGF-A levels were negatively associated with age ($P = .015$) in children with microscopic asymptomatic parasitemia but not in children with submicroscopic parasitemia (Figure 2, Table 3). Therefore, we stratified the ages for the microscopic asymptomatic parasitemia group to determine if VEGF-A levels may vary across different age ranges. Even though the difference was not significant, there was a trend of decreasing VEGF-A levels with increasing age ($P = .33$) (Figure 3). However, for VEGFR2, no significant association was found with parasitemia (Table 2) or age (Table 3).

Plasma Levels of ICAM-1 Can Discriminate Microscopic Asymptomatic Infection From Uninfected Controls

To assess the potential of the 3 molecules to differentiate individuals with asymptomatic microscopic malaria from uninfected controls, a receiver operating characteristic analysis was performed to determine the area under the curve (AUC). An AUC value of 1.00 is an accurate marker for differentiation,

Table 2. A Generalized Additive Model Reporting on the Parametric Coefficient and *P* Values for the Effect of Parasitemia on Levels of ICAM-1, VEGF-A, and VEGFR2

	Estimate	Standard Error	T-Value	*P* Value
Parametric coefficients				
ICAM-1	0.3524	0.3147	1.120	.2712
VEGF-A	12.7617	29.2645	0.436	.6657
VEGFR2	–5.5143	2.8546	–1.932	.0623

Abbreviations: ICAM-1, intracellular adhesion molecule–1; VEGF-A, vascular endothelial growth factor–A; VEGFR2, vascular endothelial growth factor receptor 2.

Table 3. A Generalized Additive Model Reporting on the Parametric Coefficient and Estimated Degrees of Freedom, Reference Degrees of Freedom, F-Statistics, and *P* Values for the Effect of Age on ICAM-1, VEGF-A, and VEGFR2

	Estimate	Standard Error	T-Value	*P* Value
Parametric coefficients				
ICAM-1	0.00028	0.00029	0.961	.345
VEGF-A	–0.06813	0.026	–2.606	.0147
Nonparametric (smooth terms)	Edf	Ref.df	F-Value	*P* Value
VEGFR2	4.194	5.162	0.82	.558

Abbreviations: ICAM-1, intracellular adhesion molecule–1; Ref.df, reference degrees of freedom; VEGF-A, vascular endothelial growth factor–A; VEGFR2, vascular endothelial growth factor receptor 2.

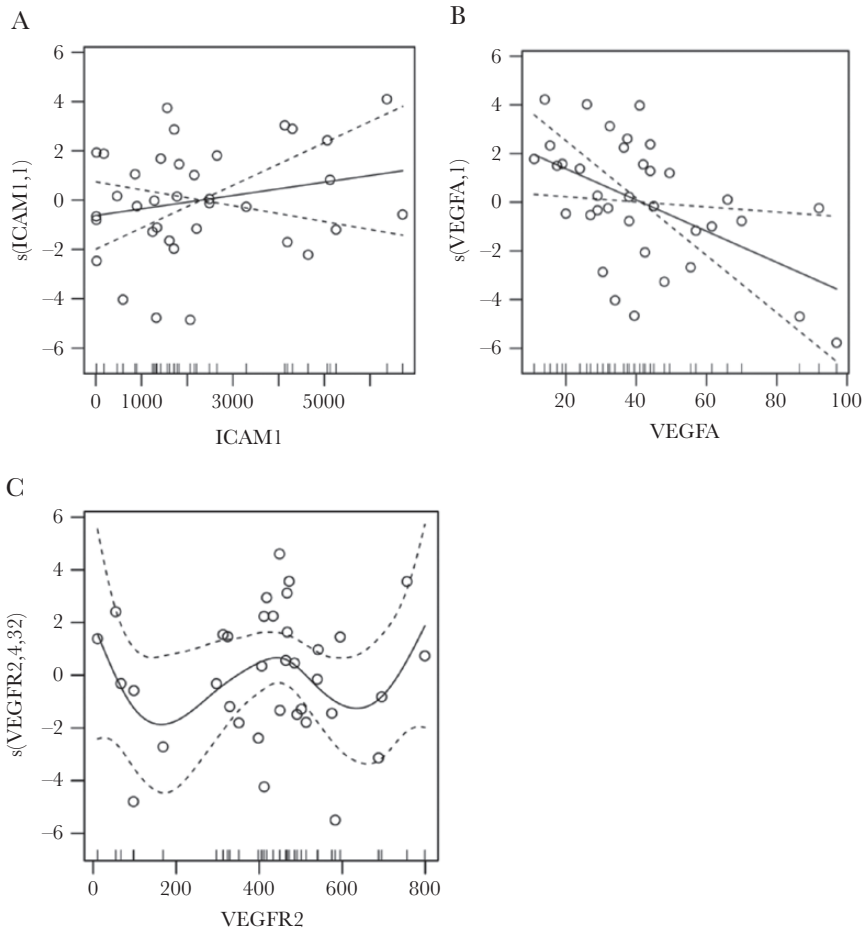

Figure 2. A plot showing the association between endothelial molecules and age. The partial effects of each covariate (ICAM-1, VEGF-A, and VEGFR2) are plotted as smoothed fits. The broken lines represent 2 standard errors, above and below the estimate of the nonlinear effect (smooth) being plotted. The short vertical lines on the x-axes of each of the plots denote the values of the observations made. Abbreviations: ICAM-1, intracellular adhesion molecule–1; VEGF-A, vascular endothelial growth factor–A; VEGFR2, vascular endothelial growth factor receptor 2.

whereas a value of 0.5 indicates the inability to differentiate between groups [42]. Comparing the molecules, ICAM-1 was a better predictive marker and showed potential for discriminating between the groups, with an AUC of 0.72 (95% CI, 0.587–0.834), sensitivity of 0.75, and specificity of 0.70 (Figure 4), compared with VEGF-A and VEGFR2, with an AUC of 0.67 and 0.69, respectively. Both VEGF-A and VEGFR2 had similar sensitivity and specificity of 0.97 and 0.35, respectively.

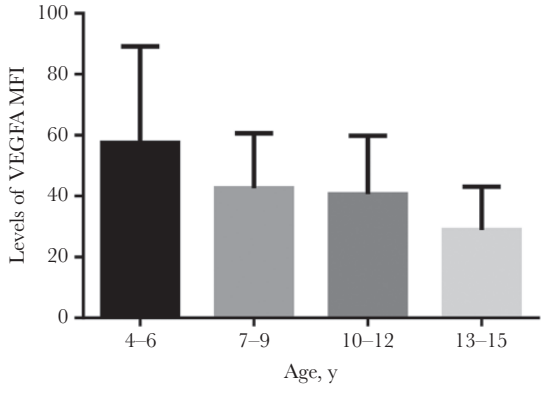

Figure 3. The bar chart shows the levels of VEGF-A across different age groups—4–6 years (n = 5), 7–9 years (n = 8), 10–12 years (n = 17), and 13–15 years (n = 6)—in children with microscopic asymptomatic malaria. Abbreviation: VEGF-A, vascular endothelial growth factor–A.

DISCUSSION

There is a strong evidence of endothelial activation in symptomatic malaria including uncomplicated and severe malaria [37, 38, 43], while similar data in asymptomatically infected persons are limited. In this study, we determined whether plasma levels of the angiogenic marker VEGF-A and its receptor VEGFR2 as well as the endothelial activation marker ICAM-1 are increased in asymptomatic children with either microscopic parasitemia or submicroscopic parasitemia compared with uninfected age-matched controls. The results from this study showed that ICAM-1, VEGF-A, and VEGFR2 are upregulated in children with microscopic malaria compared with uninfected children.

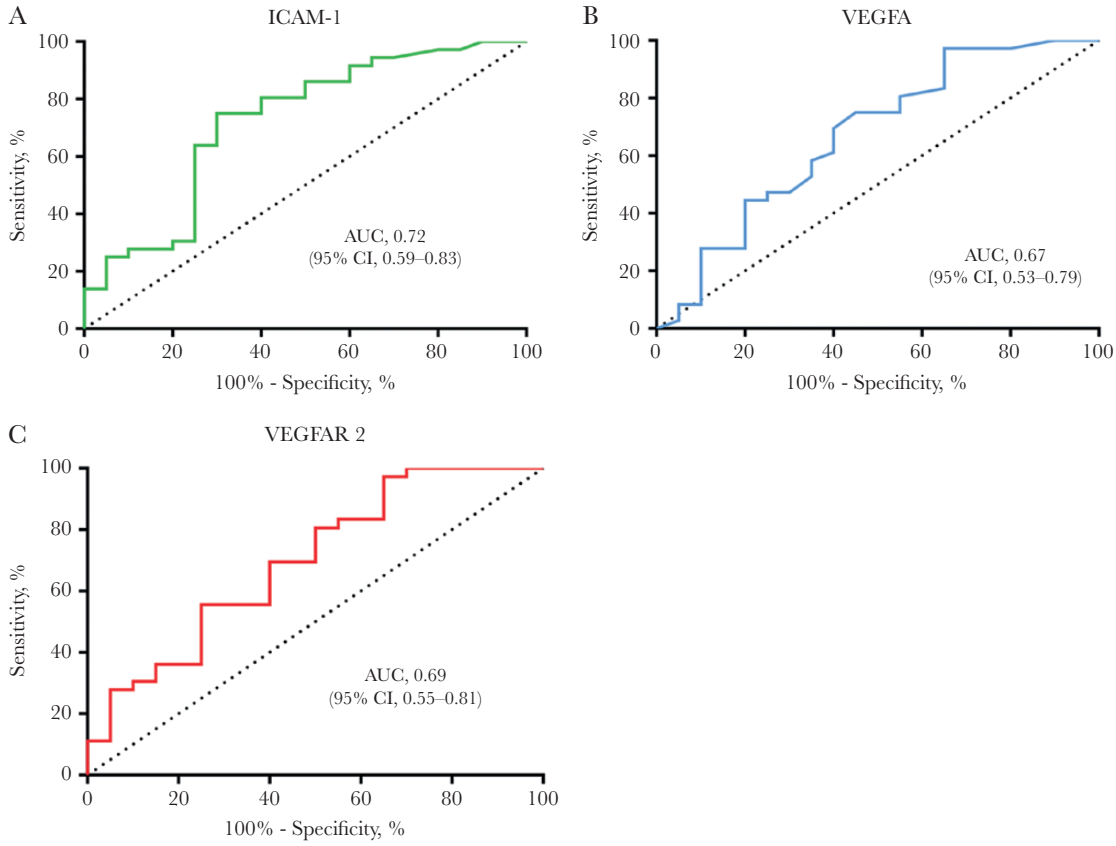

Figure 4. Receiver operating characteristic curves for ICAM-1, VEGF-A, and VEGFR2 in discriminating between children with microscopic asymptomatic malaria and uninfected controls. The sensitivity is found on the y-axis, while the x-axis demonstrates the specificity. Abbreviations: ICAM-1, intracellular adhesion molecule–1; VEGF-A, vascular endothelial growth factor–A; VEGFR2, vascular endothelial growth factor receptor 2.

ICAM-1 showed good potential in discriminating between children with microscopic asymptomatic parasitemia and uninfected controls. The study shows that asymptomatic malaria in children characterized by peripheral parasitemia is associated with endothelial activation. The results generally indicate that these molecules may have significance in disease pathogenesis.

In this study, we observed that significant differences only occurred when comparisons were made between uninfected controls and children with microscopic parasitemia, even though there was no significant association between the levels of these molecules and parasite levels. This is in agreement with the findings by Park et al. [9] and Mast et al. [8], who observed increased levels of vWf, VCAM-1, sICAM-1, and C-reactive protein, respectively, in children with microscopic asymptomatic malaria. This may indicate that endothelial cells may be one of the early host responders to parasite presence in infected persons, especially when parasitemia reaches detectable levels by microscopy as compared with persons with submicroscopic infections. However, a previous study observed increased levels of endothelial activation molecules in a naïve population that developed submicroscopic infection during a controlled human malaria infection [44]. The contrast between this and our study may indicate that some level of tolerance to the parasite due to repeated exposure to *Plasmodium* parasites exists in naturally infected persons, such that at the subpatent level, there is no significant upregulation of endothelial molecules.

Vascular endothelial growth factor promotes vascular permeability, proliferation, survival, and inflammation of endothelial cells. Levels of VEGF have been shown to increase in inflammatory diseases, which may occur as a result of endothelial dysfunction [45, 46]. However, increasing age has been associated with a decline in endothelial repair activity. This has been reported to result in a decline in the production of pro-angiogenic cytokines and growth factors [47–49]. Even though the inverse association between VEGF-A and age was a bit moderate, it could denote that age is a contributing factor to the levels of VEGF-A and may impact angiogenesis, endothelial cell activation, and repair of damaged endothelial cells. Probably, it could also imply that repeated exposure to the parasite will result in tolerance, leading to a decrease in inflammation or a balanced pro- and anti-inflammatory response [50, 51]. For instance, in a previous study, levels of VEGF-A were comparable between pregnant women with asymptomatic malaria and healthy controls [52], implying that for persons in

endemic areas, continuous exposure to malaria will lead to the establishment of immunological tolerance as one ages. This may indicate that tolerance to malaria will likely be associated with decreased levels and activity of VEGF-A as well as the development of other complications that may be associated with increased levels of this growth factor.

Increasing levels of endothelial activation molecules have been associated with adverse outcomes in diseases like diabetes, resulting in endothelial dysfunction and organ damage [43, 53, 54]. The recent malaria–high-blood pressure hypothesis, which seeks to determine the effect of chronic endothelial activation in malaria on the development of cardiovascular diseases, may suggest that endothelial cell activation in asymptomatic infection is an early indicator of some potential health complications [55, 56]. Besides, other studies have associated the presence of microscopic asymptomatic parasitemia in children with poor cognitive function and educational outcomes, as well as susceptibility to invasive bacterial infections [57, 58]. This indicates that children with asymptomatic malaria infection do not only serve as silent reservoirs for malaria transmission but may also suffer some adverse consequences even if the infection does not progress to clinical disease. It will be interesting if this can be investigated in longitudinal studies in larger sample cohorts. Moreover, other endothelial cell activation molecules such as angiopoietins (Ang)-2, which decreases the integrity of the endothelial barrier, have been reported to be similar in children with mild and asymptomatic malaria [59]. Therefore, further studies on the role of both (Ang)-1 and Ang-2 in children with microscopic asymptomatic malaria will help determine if (Ang)-1 and Ang-2 have any associations with the development of cardiovascular disease.

Lastly, it was observed that plasma levels of ICAM-1 compared with VEGF-A and VEGFR2 were a moderate biomarker that could potentially help in discriminating children with microscopic asymptomatic malaria from uninfected controls. Also, the moderate specificity and sensitivity of ICAM-1 compared with VEGF-A and VEGFR2 make ICAM-1 a much preferred biomarker in determining disease progression and pathogenesis.

In summary, this study provides evidence that there are increased levels of the endothelial activation marker ICAM-1 and the pro-angiogenic molecules VEGF-A and VEGFR2 in children with microscopic asymptomatic parasitemia. This suggests that even during asymptomatic malaria infections and before clinical symptoms develop, endothelial activation and angiogenic events can occur. Therefore, the upregulation of these molecules may be associated with damage caused by the parasite, probably through sequestration to the host endothelium when the threshold between submicroscopic and microscopic parasitemia is crossed. As microscopic asymptomatic malaria may already predispose to possible endothelial damage and adverse health consequences, malaria control strategies should aim at identifying and eliminating these asymptomatic infections.

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Author contributions. A.F. and K.A.K. conceived the idea and designed the experiments. K.A.K. and A.F. supervised the work. J.A., D.A., and A.S.A. performed the experiments in the study and were assisted by S.E.A. and N.A.E. A.F., D.O., L.E.A., and K.A.K. wrote the manuscript. All authors reviewed and approved the final manuscript. All authors have read and agreed to the content of this manuscript and its publication upon acceptance.

Availability of data and materials. The data sets generated and analyzed for the current study are available from the corresponding author(s) on reasonable request.

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