Elevated levels of endothelial molecules ICAM-1, VEGF-A and VEGFR2 in microscopic asymptomatic malaria

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Abstract

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

Methods
Blood samples were obtained from community children for malaria diagnosis using microscopy and PCR. A multiplex immunoassay was used to determine the levels of Intracellular adhesion molecule (ICAM)-1, VEGF-A, and VEGFR2 in the plasma of children with microscopic or submicroscopic asymptomatic parasitaemia and compared with levels in uninfected controls.

Results
Levels of ICAM-1, VEGF-A and VEGFR2 were significantly increased in children with microscopic asymptomatic parasitaemia 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 (AUC =0.72), showed a moderate potential in discriminating between children with microscopic malaria from uninfected controls when compared to VEGF-A (AUC =0.67) and VEGFR2 (AUC =0.69).
Conclusion

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: malaria, asymptomatic, microscopic, submicroscopic, ICAM-1, VEGF-A, VEGFR2.
Summary: This article highlights the significance of endothelial molecules during microscopic and sub-microscopic asymptomatic malaria. Using a multiplex immunoassay, the study shows that levels of ICAM-1, VEGF-A and VEGFR2 are upregulated in children with microscopic asymptomatic malaria compared to non-infected children.
Introduction

Even though malaria cases continue to decline globally due to concerted control efforts, malaria burden remains high with about 409,000 deaths in 2019 and most of these deaths occurred in the WHO African region [1]. Infection with Plasmodium spp such as P. falciparum and P. vivax are associated with most of the 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 serve as silent reservoirs that sustain malaria transmission [4, 5]. Whether these asymptomatic infections may be responsible for the development of any other health complications remain unclear [6, 7].

Notwithstanding, previous studies have identified that children with microscopic asymptomatic parasitaemia 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 parasitaemia compared to 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]. These 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 pre-existing ones. Inflammation 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 the VEGF receptor VEGFR2 to maintain endothelial cell homoeostasis [17-19]. It is essential for endothelial cell proliferation, 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 have 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 host endothelium and 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, 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 a dysregulated endothelial activity [8, 9]. Therefore, we hypothesized that asymptomatic malaria results in
dysregulated endothelial function that 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 as well as determine if any of the molecules could help differentiate the study populations.

Materials and Methods

Patient consent statement
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 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 aparasitaemic 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 a one-time point in December. The malaria parasite prevalence in Obom estimated by microscopy during the raining season is 65% and dry season is 28%[39]. Children who qualified for this study were those ≤ 15 years of age. Asymptomatic cases were defined by the presence of parasitaemia and having an axillary temperature of <37.5 °C and not taken any anti-malarial medication within the last two weeks before sampling. Parasitaemia 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 had not been on any anti-malarial medication within the last two 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 the genomic DNA (gDNA) extraction and plasma preparation. Plasma was stored in Eppendorf tubes at -80 °C till they were used for experiments.

Determination of parasitaemia by microscopy and polymerase chain reaction (PCR)
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/ul of blood. Blood films were read by two 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, 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 with a limit of detection of 1-0.1 parasites/ul [40]. The amplified products were visualized on a 2% agarose gel stained with 0.5 µg/ml ethidium bromide. After electrophoresis, gels were viewed under ultraviolet light and images captured using 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 whereas 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, 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 duplicates on each plate to ensure uniformity. Likewise, for each individual, plasma sample was run in duplicates. Plates were read using the LUMINEX®200™ system, running on the Xponent 3.1 software. 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 one-way ANOVA followed by a post hoc test.
Correlation between endothelial molecules was determined using Spearman’s rank correlation. Additionally, a generalized additive model which provides a very robust tool to assess the association between predictors and dependent variables was used. This GAM-based analysis was used to: (i) determine the effect of age on the levels of the endothelial activation molecule (ICAM-1), angiogenic molecules (VEGF-A and VEGFR2); (ii) the effect of parasitaemia 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-values < 0.05.

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 parasitaemia and 36 with microscopic parasitaemia. There was no significant difference in the proportions of males to females (p = 0.93). Likewise, age and haemoglobin levels did not differ between children with either microscopic or submicroscopic parasitaemia 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 parasitaemia compared to uninfected controls (p = 0.01) but were not significantly different when compared to children with submicroscopic parasitaemia (p = 0.53). Also, ICAM-1 levels were not significantly different between children with submicroscopic parasites and uninfected controls (p = 0.48 Fig. 1A). Additionally, in a generalized additive model ICAM-1
did not have any significant association with parasitaemia (p = 0.27, Table 2) or age (p = 0.35, 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 parasitaemia compared to uninfected controls (p=0.03, p=0.048). However, levels between children with submicroscopic infections and uninfected controls were comparable for both VEGF-A and VEGFR2 (p>0.05 Fig. 1B and C). Additionally, levels of VEGF-A and VEGFR2 were positively correlated (r = 0.43, p = 0.009, Spearman rank correlation).

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 parasitaemia 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 parasitaemia (p = 0.67). However, VEGF-A levels were negatively associated with age (p = 0.015) in children with microscopic asymptomatic parasitaemia but not in children with submicroscopic parasitaemia (Fig. 2; Table 3). Therefore, we stratified the ages for the microscopic asymptomatic parasitaemia 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 = 0.33; Fig. 3). However, for VEGFR2, no significant association was found with parasitaemia (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 three 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 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 a 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 (Fig. 4) compared to 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.

Discussion

There is a strong evidence of endothelial activation in symptomatic malaria including uncomplicated and severe malaria [37, 38, 43] while similar data in asymptotically infected persons is 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 parasitaemia or submicroscopic parasitaemia 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 to uninfected children. ICAM-1 showed a good potential
in discriminating between children with microscopic asymptomatic parasitaemia from 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 probable 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 parasitaemia 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 parasitaemia reaches detectable levels by microscopy as compared to persons with submicroscopic infections. However, a previous study observed increased levels of endothelial activation molecules in naïve population who 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 sub-patent 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 that 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]. With the recent malaria-high blood pressure hypothesis that seeks to determine the effect of chronic endothelial activation in malaria to the development of cardiovascular diseases, it may suggest that endothelial cell activation in asymptomatic infection may be an early indicator of some potential health complications [55, 56]. Besides, other studies have associated the presence of microscopic asymptomatic parasitaemia 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, the study of 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 they may have any associations with the development of cardiovascular disease.

Lastly, it was observed that plasma levels of ICAM-1 compared to VEGF-A and VEGFR2 was 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 to VEGF-A and VEGFR2 makes ICAM-1 a much-preferred biomarker in determining disease progression and pathogenesis.

In summary, the study provides evidence that there are increased levels of endothelial activation marker ICAM-1 and pro-angiogenic molecules VEGF-A and VEGFR2 in children with microscopic asymptomatic parasitaemia. This suggests that even during asymptomatic malaria infections and before clinical symptoms may 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 host endothelium when the threshold between sub-microscopic and microscopic parasitaemia is crossed. Since 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.
Abbreviations

Ang-1: Angiopoeitin -1
AUC: Area Under the Curve
CI: Confidence Interval
DNA: Deoxynucleic acid
EDTA: Ethylenediamine tetraacetic acid
ICAM-1: Intracellular Adhesion Molecule-1
MFI: Median Fluorescence Intensity
PCR: Polymerase Chain Reaction
ROC: Receiver Operating Characteristic
VCAM-1: Vascular Growth Endothelial Growth Factor-1
VEGF-A: Vascular Endothelial Growth Factor A
VEGFR2: Vascular Endothelial Growth Factor Receptor 2
vWf: soluble von Willebrand factor
WHO: World Health Organization
Consent for publication

All authors have read and agreed to the content of this manuscript and its publication upon acceptance.

Availability of data and materials

The datasets generated and analyzed for the current study are available from the corresponding author(s) on reasonable request.

Authors’ contributions

AF and KAK conceived the idea and designed the experiments. KAK and AF supervised the work. JA, DA, ASA, performed the experiments in the study and were assisted by SEA and NAE. AF, DO, LEA and KAK wrote the manuscript. All authors reviewed and approved the final manuscript.

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Potential conflicts of interest

The authors have no potential conflict of interest.
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### Table 1: Clinical characteristics of the study participants

| Characteristics          | Controls | Sub-microscopic | Microscopic | P-value |
|--------------------------|----------|-----------------|-------------|---------|
| Sample size (n)          | 20       | 22              | 36          |         |
| Sex (n)                  |          |                 |             |         |
| Male (%)                 | 8 (40%)  | 10 (45.5%)      | 16 (44.4%)  | 0.93a   |
| Age (±SD) years          | 10±2.241 | 11.45±2.695     | 10.24±2.737 | 0.14b   |
| Parasitaemia (IQR), /µl | NA       | NA              | 935.5(288.8-1973) | NA     |
| Haemoglobin, level (IQR), g/dl | 11.75(10.8-12.6) | 11.25(10.7-12.48) | 11.05(10.5-12.63) | 0.41c   |

SD = Standard deviation; IQR = interquartile range; NA not applicable; a Chi square test; b ANOVA; c Kruskal–Wallis test
Table 2: A generalized additive model reporting on the parametric coefficient and p-values for the effect of parasitaemia on levels of ICAM-1, VEGF-A and VEGFR2.

|                     | Estimate | Standard error | t-value | p-value |
|---------------------|----------|----------------|---------|---------|
| ICAM-1              | 0.3524   | 0.3147         | 1.120   | 0.2712  |
| VEGF-A              | 12.7617  | 29.2645        | 0.436   | 0.6657  |
| VEGFR2              | -5.5143  | 2.8546         | -1.932  | 0.0623  |
Table 3: A generalized additive model reporting on the parametric coefficient and Estimated degrees of Freedom (Edf), Reference Degrees of Freedom (Ref.df), 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   | 0.345   |
| VEGF-A               | -0.06813 | 0.026          | -2.606  | 0.0147  |
| **Non parametric (smooth terms)** |          |                |         |         |
| VEGFR2               | 4.194    | 5.162          | 0.82    | 0.558   |
Figure legends

**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 as 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.

**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 denotes the values of the observations made.

**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.

**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 whilst the x-axis has the Specificity (100-specificity).
Figure 1

A

B

C

p = 0.01

p = 0.03

p = 0.048
Figure 4

A

ICAM-1

AUC: 0.72
(95% CI: 0.59-0.83)

B

VEGFA

AUC: 0.67
(95% CI: 0.53-0.79)

C

VEGFR2

AUC: 0.69
(95% CI: 0.55-0.81)