Markers of vascular endothelial cell damage and *P. falciparum* malaria: association between levels of both sE-selectin and thrombomodulin, and cytokines, hemoglobin and clinical presentation

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ABSTRACT. We investigated associations between markers of damage of vascular endothelial cells (MDVECs) and plasma cytokine levels, hemoglobin level and temperature in individuals with acute uncomplicated malaria, as well as healthy controls, using enzyme linked immunosorbent assay (ELISA) for the presence of soluble endothelial cell adhesion molecule-1 (sE-selectin), circulating granule membrane protein-140 (sP-selectin), circulating thrombomodulin (TM), circulating von Willebrand factor (VWf), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). Significant differences were observed between *falciparum* malaria patients and the healthy people in term of levels of both sE-selectin and TM. The serum levels of sP-selectin and VWf were comparable between the two groups. The levels of both sE-Selectin and TM correlated positively with temperature, levels of IFN-γ and levels of TNF-α; and negatively with hemoglobin levels. Trends of positive correlations were observed between level of sP-selectin or VWf and temperature. Furthermore, sE-selectin levels correlated with vomiting. These data suggest that sE-selectin and TM might be useful markers of endothelium activation in *in vivo* studies. Moreover, our results highlight the use of both sE-selctin and TM as markers of anemia.

Keywords: adhesion molecules, *Plasmodium falciparum*, TNF-α, IFN-γ, Gabon

P. falciparum malaria is the most devastating parasitic disease in the world, and it is estimated that around 500 million clinical cases occur yearly, with about 1-2 million deaths [1]. In holo-endemic areas of *P. falciparum* malaria, the presentation of the disease is heterogeneous, ranging from asymptomatic to severe malaria. Geographical, biological, clinical, immunological and genetic factors are responsible for these differences [2-7].

P. falciparum infection is characterized by sequestration of parasitized red blood cells in the microvessels [8, 9]. This sequestration causes vascular endothelial cell activation, which is considered to be a common feature of malaria and it also plays an important role in the pathogenesis of the disease [10-12]. It has been shown that sequestration is mediated, in part, by endothelial leukocyte adhesion molecule 1 (ELAM-1 or E-selectin) [13], intracellular adhesion molecule 1 (ICAM 1) [14], CD36 [15], platelet/endothelial cell adhesion molecule (PECAM) [16], P-selectin [17] and thrombospondin [18].

Damage to the surface membrane of endothelial cells (EC) is a feature of endothelial activation. This is induced by several factors including drugs, mechanical forces, immune cell proliferation and accumulation, nitric oxide, enzymes and cytokines (including tumour necrosis factor [TNF] and interferon [IFN]) [19]. Endothelium damage can be evaluated by quantification of soluble or circulating molecules (including von Willebrand factor [VWf], thrombomodulin [TM], sP-and sE-selectins), microparticles or circulating endothelial cells [20-22]. Von Willebrand factor is a multimeric protein, which is a component of platelet α-granules, found in the Weibel-Palade bodies in the endothelial cells [23], and is involved in blood coagulation [24]. TM on the other hand, is a surface protein of endothelial cells, which acts as a thrombin receptor and serves as an anticoagulant factor [25]. An increase in VWf is considered a marker of endothelial damage, and the soluble form of TM is released after endothelial cell injury [26, 27].

P- and E-selectins are adhesion molecules of the selectin family that bind to passing immune cells. They allow the fast moving leukocytes in the blood to bind to the cells lining the blood vessels and slow down the mechanism

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known as leukocytes rolling [28]. P-selectin is a membrane molecule found in the α-granules of platelets or in the endothelial Weibel-Palade bodies while E-selectin is observed only on activated endothelium [29-31]. Both play an important role in inflammatory disorders and a variety of other pathophysiological processes. They have been reported to increase in the circulation or at lesioned sites in several diseases [32, 33].

Pro-inflammatory cytokines, such as TNF-α, interleukin-1 (IL-1) and IFN-γ, are responsible for endothelial cell activation during *P. falciparum* infection [34] and have been associated with malaria [35-37]. A few studies have reported positive correlations between markers of vascular endothelium damage and the presentation of malaria, or certain biological factors [38-45]. However, the association between malaria-induced endothelial damage and clinical or immunological factors have highlighted some contradictory results [46, 47].

Pro-inflammatory cytokines are, in general, inducers of the expression of endothelial molecules [19]. It is known that TNF-α decreases the expression of TM on the endothelium [48, 49] and, in contrast, a positive correlation is found between serum TM levels and malaria [50]. Thus, malaria is considered to be a disease with increased levels of inflammatory cytokines and reduced number of peripheral blood lymphocytes [51, 52]. It is also known that TNF-α, but not IFN-γ, activates E-selectin expression [53, 54]. Also, the combination of both cytokines does not produce any effect on the increase of *in vitro* E-selectin expression [55]. However, in 2007, Weiser and colleagues reported that IFN-γ is crucial for synergistic induction of E-selectin and P-selectin mRNA in mouse brain endothelial cells [56]. We therefore conducted a cross-sectional study to assess the association between levels of markers of vascular endothelium damage and the immunological, biological and clinical presentation of *falciparum* malaria.

DONORS AND METHODS

Study area, patients and plasma samples

The study was conducted in Lambaréné and Fougamou, two areas of the Republic of Gabon. The two communities are located at about 100 km apart and malaria transmission is holo-endemic, with about 50 infected mosquito bites per person per year [57].

From October 2007 to January 2008, individuals aged eight months to 40 years attending the Albert Schweitzer hospital and the Centre de Santé de Fougamou, were enrolled in the study. A patient was enrolled in the study if he or she had uncomplicated *falciparum* malaria (axillary temperature ≥ 37.5°C or history of fever in the previous 48 hours, in the presence of *P. falciparum* ranging from 2 000-200 000 parasites/μL for children and at any lower level of parasite density up to 200 000 parasites/μL for adults). Informed consent was obtained from each patient, parent or parental guide. A questionnaire comprising history of medication, myalgia, vomiting and headache was also completed at the time of enrolment. The study was approved by the Regional Ethics Committee of Lambaréné. All patients were treated according to the standard protocol of the Albert Schweitzer Hospital or the Centre de Santé de Fougamou.

Before treatment, one ml of venous blood was collected into three different tubes (EDTA, heparinized and citrated), from consenting patients. EDTA blood served for haematology while heparinized and citrated blood was used for immunological assessments. About 15 minutes after blood collection, plasma was isolated after blood centrifugation at 3,500 RPM for 10 minutes in a Rotanta 460 RV4 centrifuge (Hettich). The plasma was stored at -20°C until used.

A group of healthy people living in Lambaréné and Fougamou was asked for their consent to participate in the study to serve as healthy controls. They also completed the same questionnaire described above, and their blood was also treated in the same manner as patients' blood. The presence of symptoms suggestive of malaria, the presence of co-infections and prior drug intake constituted exclusion criteria, and samples from such patients and control individuals were not analysed.

Laboratory procedures

Parasitemia estimation

The *P. falciparum* asexual blood stage was assessed using thick blood smears stained with Giemsa and examined by two microscopists following standard quality-controlled procedures. Parasite load was expressed as the number of asexual forms of *P. falciparum*/μL of blood [58].

Measurement of haemoglobin levels

Haemoglobin levels were determined with a HORIBA ABX Diagnostics Pentra 60 automated analyzer (Axon lab GmbH).

Cytokine quantification

TNF-α and IFN-γ were quantified in individual plasma samples using a commercial human TNF-α ELISA Kit (catalogue number: 950 090 15B, Diaclone, Germany) and a commercial human IFN-γ ELISA Kit (catalogue number: 95 000 15B, Diaclone, Germany) respectively, according to the manufacturer's instructions. Following the assay, absorbance was read at 450 nm, on a plate reader (Multiskan Ex, ThermoElectron Cop, Vantaa, Finland).

Quantification of markers of endothelial damage

Measurement of plasma concentration of VWf by sandwich ELISA: 96-well plates (Costar) were coated in carbonate-bicarbonate buffer pH 7.4 for at least 16 hours, and with the standard von Willebrand (S59219, NIBSC) and mouse anti-human VWf monoclonal antibody (catalogue number: Ab13417, abcam) in columns 1 and 2 and from columns 3 to 12, respectively. The plates were washed and blocked with PBS/2% milk. After two hours of incubation, the plates were patted dry and filled with 100μl of either PBS or diluted plasma (1/40). The standards and samples were run in duplicate; and for each plate a blank (PBS) and a positive control (von Willebrarand peptide, ab47296-100) were loaded. The plates were incubated for 1.5 hours at room temperature. Following washing, 100 μL of the detection antibody (polyclonal rabbit anti-human Von Willebrand Factor/HRP, P022602, Dako), diluted to 1/8 000 in blocking buffer, was added. Following a wash-

ing step, and within two hours, the reaction was revealed, with100µLofready-to-useTMB-one(3,3′,5,5′-tetramethylbenzidine) (KEM-EN-TEC Diagnostic, Tasstrup, Denmark). The reaction was stopped with 2M H_2S0_4 and the absorbance read at 450 nm on a plate reader.

Measurement of the plasma concentration of TM: human thrombomodulin ELISA Kit (catalogue number: 837, American Diagnostica) was used according to the manufacturer's instructions. Following the assay, the absorbance was read at 450 nm on a plate reader.

Estimation of level of sP- and sE-selectin: levels of sP- and sE-selectin were estimated using commercial, monoclonal antibody-based ELISA kits. Human soluble P-selectin (sP-selectin) ELISA kit (catalogue number: BBE6, R&D Systems) and human soluble E-selectin (sE-selectin) ELISA kit (catalogue number: BBE2B, R&D system) were used for sP- and sE-selectin quantification according to the manufacturer's instructions, respectively. The absorbances were read at 450 nm on a reader plate.

STATISTICAL ANALYSIS

Statistical analyses were performed with StatView Version 5.0.1. Spearman's test was used for correlation between markers of damage of vascular endothelial cells (MDVECs) and both clinical and immunological factors. Kruskal-Wallis test was used for between-groups comparison of all variables, and for correlation between MDVECs and biological factors. For all analyses, $p < 0.05$ was considered to be significant.

RESULTS

Characteristics of the study population

A total of 81 people, of both sexes, participated in the study. They included: 62 (76.54%) people with uncomplicated *P. falciparum* malaria and 19 (23.46%) healthy people without malaria parasites. A summary of clinical, biological and immunological parameters of the study population is given in *table 1*. Patients with uncomplicated malaria were younger and had lower haemoglobin levels compared to the healthy individuals. Temperature was higher in people with uncomplicated malaria compared to healthy controls.

Mean plasma levels of both soluble sE-selectin and TM factor differed significantly between people with uncomplicated malaria and healthy controls ($p < 0.001$ for the two markers) *(table 1)*.

The mean plasma levels of IFN-γ differed significantly ($p = 0.04$) between people with uncomplicated malaria and healthy people. However, there was no difference between the two groups in terms of TNF-α levels.

Relationship between levels of markers of vascular endothelial cell damage and clinical malaria parameters

It was observed that the levels of the four MDVECs (sE-electin, sP-selectin, VWf and TM) increased with temperature. A significant association was observed only for sE-seletin, VWf and TM ($p < 0.007$, $p = 0.01$ and $p = 0.04$, respectively) *(figure 1)*.

Within uncomplicated malaria patients, we grouped people with uncomplicated malaria into those who vomited, having myalgia and headache into one group; and those without vomit, myalgia and headache, in the other group. Our comparisons showed that only levels of sE-selectin were significantly different between people who vomited (54.29%) and those who did not (45.71%) ($p = 0.04$) *(figure 2)*. Any other comparisons between headache, myalgia and the four MDECs yielded no significant differences (data not shown).

Relationship between levels of markers of vascular endothelial cell damage and biological malaria parameters

We observed a negative association between levels of sE-selectin, sP-selectin and TM with haemoglobin levels. The negative correlation was significant for levels of both sE-selectin and TM ($p = 0.002$ and $p = 0.008$, respectively) *(figure 3)*. Meanwhile, there was a positive association between levels of VWf and haemoglobin levels (data not shown).

Table 1
Base line characteristics of the study population

Variables	Uncomplicated malaria	Healthy malaria negative	P-value
Number of subjects (%)	62 (76.5)	19 (23.5)	
Age* (months) [P_{25}-P_{75}]	108 [42.5-216]	324.5 [276.5-333.5]	< 0.001
Temperature °C (SD)	38.3 (1.2)	36.7 (0.5)	< 0.001
Parasites/µL* [P_{25}-P_{75}]	3, 856 [2, 286-59, 147]		
Hemoglobin			
Level* (g/dL) [P_{25}-P_{75}]	10.3 [8.6-11.4	13.6 [12.8-14.5]	< 0.001
Sex ratio F/M	35/27	10/9	
sP-Selectin* (ng/mL) [P_{25}-P_{75}]	49.9 [42.9-57.6]	52.1 [45.7-61.3]	0.3
sE-Selectin* (ng/mL) [P_{25}-P_{75}]	4 [2-6.1]	1.5 [1-1.5]	< 0.001
VWf* (µg/mL)[P_{25}-P_{75}]	2016.3 [1610.5-2754.7]	1405.8 [765.1-2788.6]	0.1
TM* (ng/m) [P_{25}-P_{75}]	1.3 [1.2-1.9]	0.8 [0.7-1.2]	< 0.001
TNFα* [P_{25}-P_{75}]	bdl# [bdl-15.2]	bdl# [bdl - bdl]	0.09
IFNγ* [P_{25}-P_{75}]	bdl# [bdl -10.1]	bdl# [bdl - bdl]	0.04

P_{25} and P_{75} : first and third quartile; bdl#: below detection limit.
* Median.

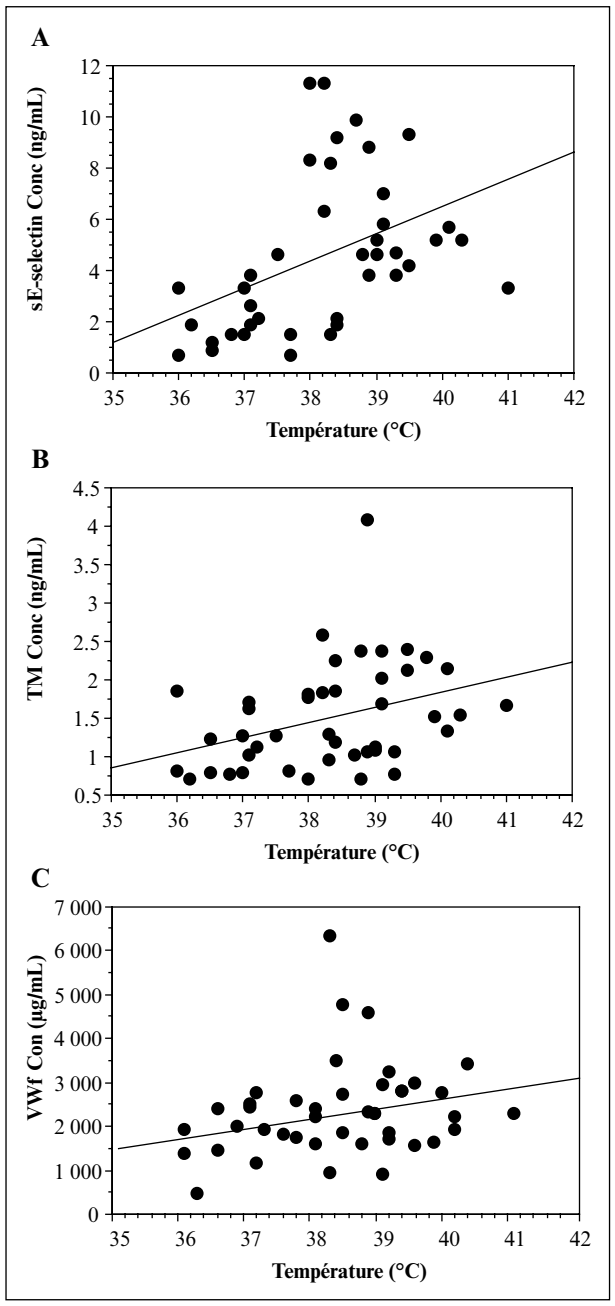

Figure 1
Correlation between temperature and levels of (**A**) sE-selectin, (**B**) TM and (**C**) VWf among people with uncomplicated *falciparum* malaria.

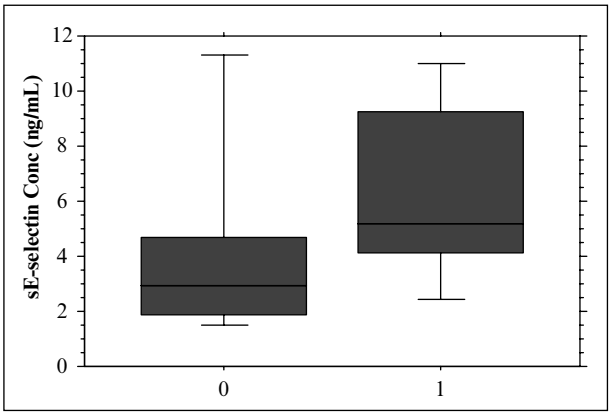

Figure 2
Comparison of levels of sE-selectin between *falciparum* malaria patients who vomited (1) and those who did not vomit (0).

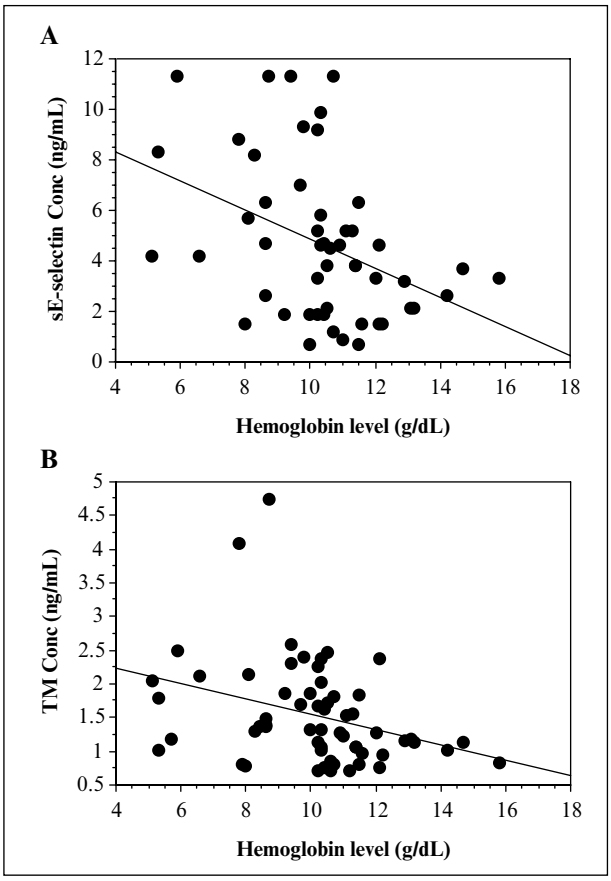

Figure 3
Correlation between hemoglobin levels and levels of (**A**) sE-selectin and (**B**) TM among people with uncomplicated *falciparum* malaria.

Relationship between levels of markers of vascular endothelial cell damage and immunological malaria parameters

Increasing levels of TNF-α were associated with increasing levels of sE-selectin, TM and VWf. Also, a positive association was observed between levels of TNF-α, with levels of both sE-selectin and TM ($p < 0.001$ for both) *(figure 4)*, but there was no significant correlation with levels of VWf; the level of sP-selectin decreased with increasing levels of TNF-α (data not shown).

The level of IFN-γ was associated with levels of the four parameters. A significant association was observed only between levels of INF-γ and levels of both sE-Selectin and TM ($p = 0.007$ for both) *(figure 5)*. However, significant correlation was not observed between the levels of IFN-γ and levels of either sP-selectin or VWf.

DISCUSSION

Plasmodium falciparum malaria remains a major public health hazard in sub-Saharan African children. While the factors that determine the variations in clinical, biological and immunological outcomes of *falciparum* malaria have not been completely defined, both host and parasite fac-

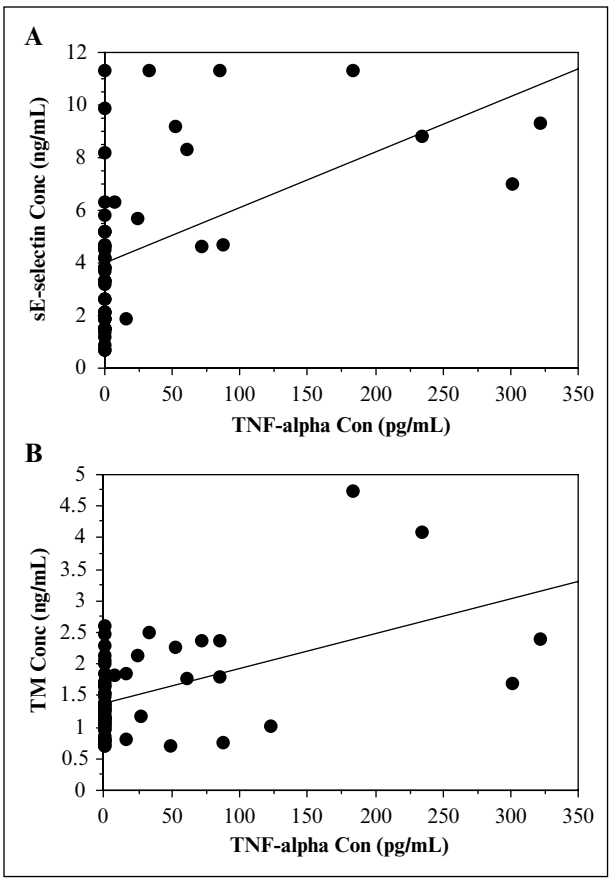

Figure 4
Correlation between levels of TNF-α and levels of (**A**) sE-selectin and (**B**) TM among people with uncomplicated *falciparum* malaria.

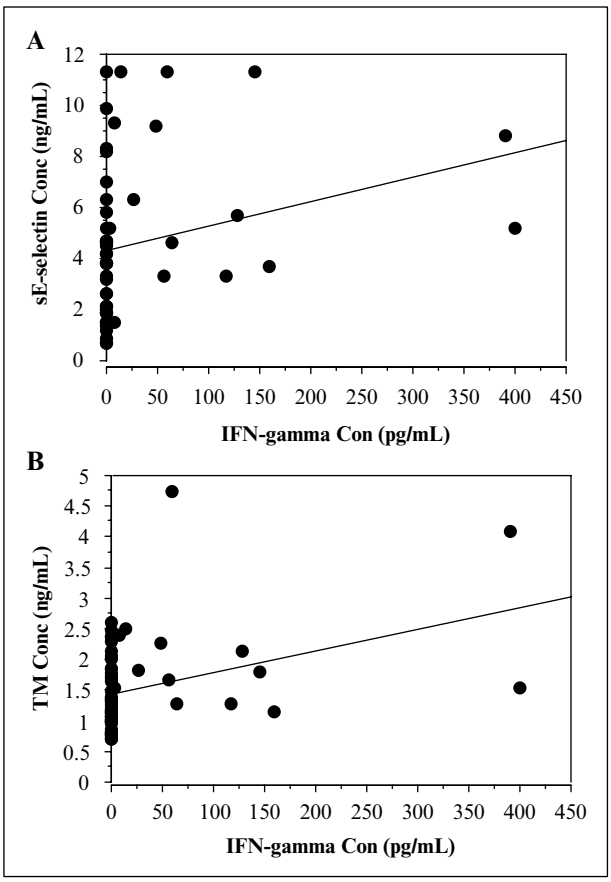

Figure 5
Correlation between levels of IFN-γ and levels of (**A**) sE-selectin and (**B**) TM among people with uncomplicated *falciparum* malaria.

tors, and the complex molecular interactions between them have been implicated. We have quantified some markers of vascular endothelial cell damage (MDVECs) in people living in a holo-endemic area for *falciparum* malaria, in order to assess for their possible correlation with clinical, biological and immunological factors of the disease. Among the MDVECs we evaluated in our study areas, two (sE-selectin and TM) showed significant correlations with clinical, biological and immunological parameters of *falciparum* malaria.

E-selctin is a constitutive molecule of the vascular endothelium and can be found in cytokine-induced endothelial activation. Its first function is to allow the rolling of moving leukocytes on the endothelium [28]. However, this molecule can also serve as a molecule for adhesion of *P. falciparum* to the endothelium, leading to blood vessel clumping [10, 13]. sE-selectin is the soluble form of E-selectin found in the circulation after endothelial cell activation. Elevated levels of sE-selectin have been associated with clinical presentations of some diseases, including uncomplicated *falciparum* malaria [38, 41], and this is well corroborated in our study. On the other hand, some other studies did not report this association [59]. The discrepancy may be the result of different study designs and also the polymorphism carriage reported in the E-selectin gene [60, 61].

sE-selectin has been related to parasitaemia in patients with *falciparum* malaria [41]. The same was observed in our study (data not shown). We found a significant difference between levels of sE-selectin in our patients compared to healthy people, and because the level of sE-selectin correlated negatively with haemoglobin levels, as observed by Tchinda *et al*. [46], we argue that sE-selectin might be considered to be a marker of anaemia in *falciparum* malaria. Indeed, anaemia may develop after an accelerated breakdown and/or clearance of pRBCs producing free haemoglobin or haeme which may be converted into methaemoglobin. It has been shown that methaemoglobin is a potent activator of endothelial cells by stimulating E-selectin expression [62].

The significant correlation observed between levels of sE-selectin and TNF-α [46] and the speculation that elevation of sE-Selectin levels is caused by release from TNF-α-stimulated endothelial cells observed in this study, is in line with previous findings. Although we found a significant correlation between levels of sE-selectin and INF-γ, it is known that INF-γ does not induce E-selectin release [54]. However, it has been shown that, *in vitro*, IFN-γ may synergize with TNF-α in the release of E-selectin [56]. Thus, we conclude that the correlation between levels of sE-selectin and IFN-γ observed in our study reflects the synergic action of both TNF-α and IFN-γ *in vivo*.

Vomiting is the forceful expulsion of the contents of the stomach. It is a reflex action, which involves the coordinated actions of the muscles of the throat, oesophagus, diaphragm, abdominal wall, and stomach. Of all the analyzed MDVECs, only sE-selectin had a positive correlation with vomiting. sE-selectin might therefore be considered as having an emetic action which might be responsible for inducing the vomiting sometimes seen

during *falciparum* malaria infection. Soluble E-selectin might act directly on the vomiting centre, localized in the medulla of the nervous system [63], or on the peripheral sensory structures of one/or more of the above mentioned organs to stimulate the nervous system through efferent nerves. Further studies are needed to confirm this finding.

Thrombomodulin, a vascular endothelial cell receptor for thrombin, is widely distributed on the surface of the endothelia of arteries, veins, capillaries, and lymphatics in all human organs and tissues except for the brain [64, 65]. It has also been described that TM is present on leukocytes [66, 67]. We noticed a significant difference, in our study population, in terms of TM levels. Moreover, levels of TM correlated significantly with temperature and parasite density (data not shown); and a negative correlation was found with haemoglobin. Our results support the use of TM as a MDVEC in *falciparum* malaria as reported in a previous study [39].

In vivo, endothelial expression of TM regulates the balance between pro- and anticoagulant events by inhibiting the myriad effects of thrombin and promoting the generation of the activated protein C (aPC) [68]. In the circulation, thrombin is required to transform fibrinogen in fibrin, and is also able to activate platelets. Since one of the functions of thrombomodulin is to fix thrombin, and because the down-regulation of TM expression is associated with a loss in the capacity of the EC to catalyze conversion of protein C (PC) to aPC [69], it may be possible that circulating TM is not able to fix thrombin. This observation may lead to two consequences. The first consequence is microvessel thrombosis, by increasing levels of fibrin in the plasma. Although rare, thrombosis is an event which occurs during *falciparum* malaria [70, 71]. Its rarity may be the result of the direct examination of microvessels made only post-mortem. Therefore, high levels of TM may be a sign of a predisposition to severe malaria. In addition, thrombosis may be responsible for mechanical haemolytic anaemia because RBCs may be destroyed due to hydrodynamic turbulence when they are forced over gross obstruction [72]. The second consequence is the activation of platelets, which may result in their aggregation [73], and their accumulation on the microvessels. It has been shown that accumulation of platelet induces, alone or in the presence of *P. falciparum* erythrocytes, EC apoptosis through production of transforming growth factor beta-1 (TGF-β1) on TNF-activated EC [74]. Our results therefore allow the suggestion that TM may have an indirect effect on EC apoptosis during *falciparum* malaria. It appears important to elucidate the phenotype of soluble TM, which could be implicated in the thrombosis and/or the aggregation of platelets.

The significant association between levels of TM and TNF-α observed in our study is in line with the previous finding by Hemmer *et al*. [40]. However, this correlation is debatable as TNF-α is not able to trigger TM release, as shown by *in vitro* experiments [48, 67, 75] and also reduces the level of TM release-proteins [75]. In this study, such a correlation could not be reported due to the limitations of the study design. It might be important to investigate further this correlation. Similarly, as with TNF-α, we made a positive observation between levels of TM and IFN-γ. Such a correlation in *falciparum* malaria has not yet been observed elsewhere. However, IFN-γ has the same effect on EC as TNF-α, although less; but its effect is increased when in association with TNF-α [48].

Levels of VWf and sP-selectin did not show any association with clinical, biological or immunological factors of *falciparum* malaria. Levels of VWf have been correlated with clinical *falciparum* malaria in Ghana [12], contradicting our finding. However, the same study reported no correlation between levels of sP-selectin and clinical malaria, a result which is in line with ours. Since we used citrated tubes as was done in the study by Hollestelle *et al.* [12], we have no explanation for these discrepant findings. This could not be related to the difference in the age groups because we reported no correlation between VWf level and age (data not shown).

In summary, we found positive associations between levels of both sE-selectin and TM, and clinical and immunological parameters; however, a negative association with haemoglobin levels in patients with *falciparum* malaria was observed. Based on our results and those by others, sE-selectin and circulating TM might therefore be useful markers of endothelial damage during *falciparum* malaria infection in *in vivo* studies. Moreover, our results highlight the use of both sE-selctin and TM as markers of anaemia. It will be important to continue investigating the role of sE-selectin in the induction of vomiting, as, with our study design, it was difficult to conclude that sE-selectin has an emetic action in *falciparum* malaria.

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