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Circulating endothelial cells in severe *Plasmodium falciparum* infection

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ABSTRACT

Plasmodium falciparum infection is associated with diffuse vascular dys-regulation. Levels of blood circulating endothelial cells (CECs; $CD146^+CD45^-$) are a marker of vascular injury. This study aimed to measure blood CECs by flow cytometery in patients with acute malaria infection before and after treatment and to evaluate the prognostic value of that measurement for that disease. The subjects were allocated into: Group I: uncomplicated malaria (UM, $n = 32$), Group II: severe malaria (SM, $n = 12$), Group III: the treated UM (TUM, $n = 32$), Group IV: the treated SM (TSM, $n = 12$) and Group V: healthy controls (HC, $n = 25$). Before treatment, SM patients showed the highest mean number of CECs (30,658.3 ± 2658.2/5 × 10^6 peripheral blood mononuclear cells (PBMCs)), followed by UM patients (19,481.56 ± 866.83/5x10^6PBMCs) and both groups were significantly higher than HC (2034 ± 300.59/5x10^6PBMCs, $P < .001$). The level of CECs decreased significantly in both infected groups after treatment; in TUM it became 5602.18 ± 509.72/5 × 10^6PBMCs and in TSM it reached 8457.5 ± 452.4/5 × 10^6 PBMCs (both values $P < .001$ in comparison with SM). By receiver operating characteristic curve analysis, the best cut-off count for CECs which enables prediction of the occurrence of severe malaria infection was 27,150/5 × 10^6 PBMCs or more, with 100% sensitivity, 100% specificity, and 100% accuracy. CECs had a significant positive correlation with parasitemia index and serum creatinine and a significant negative correlation with hemoglobin concentration in patients with acute malaria. In conclusion, the level of CECs could be used as a biomarker denoting endothelium damage during acute *P. falciparum* infection, and it correlated with infection severity and predicted its prognosis.

1. Introduction

Malaria is the most important parasitic disease worldwide, present in 97 countries. The parasite affects around 219 million people, of which over 90% are found in Africa. It causes about 400,000 deaths every year and there are nearly 3.3 billion people who are at risk of infection [1,2]. Although malaria is endemic in many tropical states, many cases of malaria have been reported in malaria non-endemic areas because of opportunities for international travel [3]. Among malaria species, *P. falciparum* is the main cause of severe infection and death. In most cases, infected patients may present with UM infection with mild symptoms such as fever, chills and headaches. However, a few cases may have severe malaria with one or more serious symptoms such as cerebral malaria, pulmonary edema, acute renal failure or hepatic dysfunction [4].

The pathological effects of *P. falciparum* infection are due to trapping of the parasitized red blood cells (PRBCs) in the microvasculature as a result of adherence of PRBCs to endothelial cells (ECs) [5]. Cyto-adherence is a consequence of binding of *P. falciparum*-derived surface proteins such as *P. falciparum* erythrocyte membrane protein 1 to specific host ligands such as endothelial cell adhesion molecule-1 [6] and endothelial protein C receptor [7]. Additionally, pro-inflammatory cytokines, especially interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) increase during the infection leading to more surface expression of the molecules on ECs [8]. Therefore, diffuse activation and inflammation of the vascular endothelium may occur [9,10]. Cyto-adherence could also be an escape mechanism exploited by the parasite to avoid its removal by macrophages found in spleen [11]. Blood vessel endothelium plays many vital roles, such as maintaining vascular tone and permeability. It helps preserve the balance between pro-inflammatory and anti-inflammatory mediators locally and it acts as a barrier for the micro-vascular system

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[12]. Any vascular dys-regulation that occurs during malaria infection may, however, be asymptomatic which in turn may cause long-term cardiovascular morbidity [13].

Circulating endothelial cells (CECs) are mature cells that are shed from the endothelium and circulate in blood, but they still have proliferative capacity. These cells are usually identified by expressing certain surface endothelial cell markers, for example CD146, CD31 or von Willebrand factor, but they are negative for CD45 (a blood cells marker) [14]. Imbalances between pro-angiogenic and anti-angiogenic factors, pro-inflammatory cytokines, proteases, breakdown of intercellular adhesions, mechanical vascular injury, and the impact of some medications are examples of mechanisms which can cause the detachment of CECs from the vascular endothelium [15].

In healthy individuals, the level of CECs is low in blood [16]. However, their level increases significantly in medical disorders that are associated with vascular defects, like myocardial infarction [17], heart failure [18], Type 1 diabetes [19], sepsis [20] and cancers [21]. An increase in the number of CECs in blood has been reported to be a novel biomarker for the occurrence of endothelial damage and can be used to evaluate the extent of the lesion [14]. Flow cytometry is becoming the most generally used method for detecting CECs, since it is a rapid, quantitative technique and can be used for simultaneous determination of different specific markers [22].

The aim of this study was to measure CECs in blood of *P. falciparum*-infected patients with different clinical presentations before and after treatment and to evaluate its prognostic value during severe malarial infection.

2. Materials and methods

2.1. Study population and enrollment

This study was a case-controlled and conducted during the whole of 2017 and 2018. The cases were recruited from Abbasia Fever Hospital, Cairo, Egypt. Any suspected *P. falciparum*-infected patient admitted to the hospital was subjected to complete history taking, clinical examination, abdominal ultrasound, blood film microscopy and other routine laboratory investigations. The diagnosis of positive *P. falciparum* malaria infection was based on a positive blood film. Each participant signed an informed consent form before his/her enrollment. The control group consisted of 25 healthy subjects (Egyptians and other nationalities from Africa) matched for the same age, gender and nationality. This work was approved by the Menoufia Faculty of Medicine Ethics Committee for the Conduct of Scientific Research.

Subjects having any medical disorder that could be associated with vascular damage, such as sickle cell trait/disease, diabetes, cardiovascular disease or cancer, were excluded from this study. Also, pregnant women and patients suffering from diseases other than malaria were not included herein.

2.2. Patient groups

Two groups of acute malaria patients were compared to a healthy group before and after treatment. The subjects were allocated into the followings groups: Group I: Uncomplicated malaria (UM), Group II: Severe malaria (SM), Group III: patients of UM group after treatment (TUM), Group IV: patients of SM group after treatment (TSM) and Group V: Healthy controls (HC).

Malaria patients were classified into UM and SM according to WHO [4] criteria, where the UM malaria patients were positive for *P. falciparum* blood film with fever ($\geq$ 37.5 °C) and presence of mild symptoms, while SM patients had one or more WHO criteria of severe malaria. Both UM and SM patients received treatment following WHO guidelines [4].

According to the local protocol of malaria treatment, the treated patients were discharged from the hospital when the blood film was negative for parasitemia and their clinical examination and laboratory investigations were normal. In the present study the treated UM patients left the hospital after 5–7 days, but the treated severe ones left after 10–15 days, according to their individual condition. The blood samples were collected from them for CECs count on the day of leaving the hospital.

2.3. Parasitemia index

From each subject, drops of blood were taken to prepare thin and thick blood films stained with Giemsa. The thin film confirmed infection in all the studied patients. The parasitaemia index was calculated by counting the number of the parasite asexual stages per 200 white blood cells (WBCs) in a thick blood film, but if fewer than nine parasites were detected, another 300 leucocytes were counted [4].

2.4. Blood collection and laboratory investigations

From each participant, 15 ml blood was withdrawn through a freshly placed cannula because venipuncture accompanied with trauma may increase the number of CECs in the sample. The blood sample was divided into 10 ml for counting CECs and 5 ml for measuring other laboratory parameters. WBCs, Hb and platelets were analyzed in an Advia 2120 automated blood counter (Siemens Healthcare Diagnostics, USA). Serum creatinine levels were measured by Chemistry Analyzer AU480 (Beckman Coulter Inc., USA).

2.5. Flow cytometric detection of CECs

Ten ml of blood was mixed with an equal volume of phosphate buffer solution (PBS), pH 7.4, layered carefully over Ficoll reagent, 1.077 g/ml (Sigma-Aldrich, USA), then centrifuged at 800*g* for 20 min (min). The buffy coat was recovered, loaded again with 10 ml PBS, and centrifuged at 3000*g* for 10 min. After that, the resulting cell pellet was re-suspended in 1 ml PBS and the count of peripheral mononuclear cells (PBMCs) adjusted to 1×10^6/ml. From the resulting PBMCs suspension, 100 µl of PBMCs was incubated with 10 µl of each of the mouse anti-human fluorescein isothiocyanate-conjugated CD146 antibody (MiltenyiBiotec, Germany) and mouse anti-human phycoerythrin-conjugated CD45 antibody (MiltenyiBiotec) for 30 min at room temperature in the dark. An isotype control antibody was added to distinguish between the signal of the specific antibody from that of the non-specific background one. A FACSCalibur flow cytometer (BD Biosciences, USA) was used to analyze the assay. Stained samples were analyzed with internal complexity (SSC) versus a size (FSC) dot plot was developed, after which a region containing a PBMC population was gated. For each sample we used an unstained auto-control tube containing 100 µl PBMCs and 300 µl PBS without any conjugated monoclonal antibodies. CECs were identified as $CD146^+$ and $CD45^-$. For each sample, 100,000 PBMCs were evaluated by FCS to obtain the number of CECs present in 5×10^6 PBMCs.

2.6. Statistical analysis

Data were analyzed by SPSS version 23 (SPSS Inc. Released 2015. IBM SPSS statistics for windows, version 23.0, Armnok, NY: IBM Corp.). Analysis of the variance (ANOVA) was used to test the difference of normally distributed means among > 2 groups with the Tukey test as a post Hoc test while the Kruskal Wallis test was used for means that were not normally distributed, with Tamhane's test as a post hoc test. The paired-*t*-test and Wilcoxon test were used for comparing paired data before and after treatment for normal and not normal distributed data, respectively. The Spearman method was used to demonstrate correlations between CECs and other laboratory investigations. A receiver operator characteristic (ROC) with respective points of maximal accuracy for sensitivity and specificity was generated to determine CECs

performance. The Wilcoxon test was used to compare means of related readings of a variable in the same group. A two sided P value of $< .05$ was considered statistically significant.

3. Results

3.1. Baseline subject characteristics

The current study included 44 *P. falciparum* infected patients. Their age ranged from 22 to 40 years with a mean of 25 ± 2 years. Males represented 68.2% (30/44). Of the malaria infected patients, 10 were Egyptian travellers who had recently visited different African countries. The other 34 patients were Africans who were visiting Egypt for study or work; they originated from North Sudan, South Sudan, Nigeria, Guinea, Gambia and Malawi. The recruited healthy controls ($n = 25$) were matched with the infected patients with no significant differences regarding their age (range = 21–38; mean = 24 ± 2 years), gender distribution (16 males and 9 females) or nationality (10 Egyptians and 15 Africans).

3.2. Laboratory characteristics (Table 1)

There was a statistically significant ($P < .001$) difference of the parasite densities between SM (2.7 ± 1.64%) and UM (1.2 ± 0.52%) patients.

Hb level was significantly lower in patients with SM (7.85 ± 2 g/dl) in comparison with either UM (10.54 ± 1.45 g/dl; $P = .002$) or the HC group (12.98 ± 1 g/dl; $P < .001$). Also, the Hb level of UM was significantly ($P < .001$) lower than in the HC group. After anti-malarial treatment there was an improvement in Hb level in TUM (11.22 ± 1.11 g/dl) and TSM (10.69 ± 0.76 g/dl) groups with a non-significant ($P = .22$) difference between both groups. However, it was still significantly ($P < .001$) lower than that of HC group.

The platelet count was significantly lower in patients with SM (54.9 ± 33.79 × 10^3/μl) compared with both those of UM (85.19 ± 39 × 10^3/μl; $P = .041$) and HC (301.7 ± 78 × 10^3/μl; $P < .001$) patients. Also the UM group showed a significantly ($P < .001$) lower platelet count compared to HC. After receiving the treatment the platelet count improved to 277.5 ± 100.4 × 10^3/μl in TUM and 236.7 ± 74.7 × 10^3/μl in TSM, levels which did not differ significantly from each other ($P = .113$) or from the HC group.

The WBCs count was significantly lower in SM patients (4.1 ± 0.96 × 10^3/μl) compared with both that of UM (5 ± 1.1 × 10^3/μl; $P = .060$) and HC (7 ± 1.91 × 10^3/μl; $P < .001$) groups. Also, UM patients had a significantly lower WBCs count compared to HC ($P < .001$). The number of WBCs in TUM (5.3 ± 1.1 × 10^3/μl; $P < .001$) and TSM (5.3 ± 0.95 × 10^3/μl; $P = .003$) were significantly lower than in HC, but still in the normal range.

Regarding serum creatinine, it was significantly higher in SM patients (3.09 ± 1.4 mg/dl) compared with either those with UM (1.18 ± 0.4 mg/dl; $P = .003$) or HC (0.83 ± 0.2 mg/dl; $P < .001$). It was significantly higher in UM than in HC ($P < .001$). The creatinine levels decreased in TUM and TSM to 1.04 ± 0.24 and 1.32 ± 0.39 g/dl, respectively, but these levels were still significantly higher than HC ($P = .014$ and $P = .007$, respectively). There was a non-significant difference between both treated groups ($P = .127$).

3.3. Flow cytometry CECs numbers

Representative flow cytometry analysis of CECs is shown in Fig. 1. The highest mean number of CECs among all the investigated groups was found in SM patients (30,658.33 ± 2658.24/5 × 10^6 PBMCs) and it was significantly higher than in both UM (19,481.56 ± 866.83/5 × 10^6 PBMCs, $P < .001$) and HC (2034 ± 300.59/5 × 10^6 PBMCs, $P < .001$) groups. After receiving anti-malarial treatment the mean number of CECs decreased significantly in both TUM (5602.18 ± 509.72/5 × 10^6 PBMCs, $P < .001$) and TSM (8457.5 ± 452.41/5 × 10^6 PBMCs, $P < .001$) patients in comparison with the pre-treatment levels, but both treated groups still had a significantly higher mean number of CECs than HC ($P < .001$). Also, TSM had higher mean number of CECs than TUM, a difference that was highly significant: $P < .001$ (Fig. 2).

3.4. Determining the predictive cut-off value of CECs for severe malaria

Through ROC curve analysis, CECs numbers could be used to clearly distinguish uncomplicated malaria cases (the UM group) from cases with severe malarial infection (the SM group). A cut-off value of 27,150 CECs per 5 × 10^6 PBMCs predicted severe malaria with maximum (100%) sensitivity, specificity and accuracy (Fig. 3).

3.5. Correlation between CECs and laboratory investigations

A significant negative correlation was found between a CEC counts of malaria patients (44 patients) and Hb concentrations ($r = -0.344$, $P = .022$), whereas platelet counts ($r = 0.255$, $P = .075$) and WBCs ($r = -0.257$, $P = .096$) correlated negatively and non-significantly with the CECs count. The mean CECs counts of the patients had a significant positive correlations with both parasitemia index ($r = 0.445$, $P = .002$) and serum creatinine ($r = 0.506$, $P = .001$) (Table 2 and Fig. 4).

Table 1
Laboratory investigations of malaria-infected patients

Variable	Before treatment		HC ($n = 32$)	P value	Post Hoc	After treatment		P value	Post Hoc
	UM ($n = 32$)	SM ($n = 12$)				TUM ($n = 32$)	TSM ($n = 12$)		
Parasitic index	1.24 ± 0.52	2.70 ± 1.64	–	< .001					
HB	10.54 ± 1.45	7.85 ± 2.00	12.98 ± 1.04	< .001	$P1 = 0.002$ $P2 < 0.001$ $P3 < 0.001$	11.22 ± 1.11	10.69 ± 0.76	< .001	$P4 = 0.220$ $P5 < 0.001$ $P6 < 0.001$
Platelets (x10^3/μl)	85.19 ± 39	54.9 ± 33.79	301.7 ± 78	< .001	$P1 = 0.041$ $P2 < 0.001$ $P3 < 0.001$	277.5 ± 100.4	236.7 ± 74.7	.113	–
WBCs (x10^3/μl)	5 ± 1.1	4.1 ± 0.96	7 ± 1.91	< .001	$P1 = 0.060$ $P2 < 0.001$ $P3 < 0.001$	5.3 ± 1.1	5.3 ± 0.95	.001	$P4 = 1.00$ $P5 < 0.001$ $P6 = 0.003$
Creatinine	1.18 ± 0.41	3.09 ± 1.41	0.83 ± 0.26	< .001	$P1 = 0.003$ $P2 < 0.001$ $P3 < 0.001$	1.04 ± 0.24	1.32 ± 0.39	< .001	$P4 = 0.127$ $P5 = 0.014$ $P6 = 0.007$

$P1$: UM Vs SM, $P2$: UM Vs HC, $P3$: SM Vs HC, $P4$: TUM Vs TSM, $P5$: TUM Vs HC, $P6$: TSM Vs HC. UM = uncomplicated malaria, SM = severe malaria, HC = healthy controls, TUM = treated uncomplicated malaria, TSM = treated severe malaria.

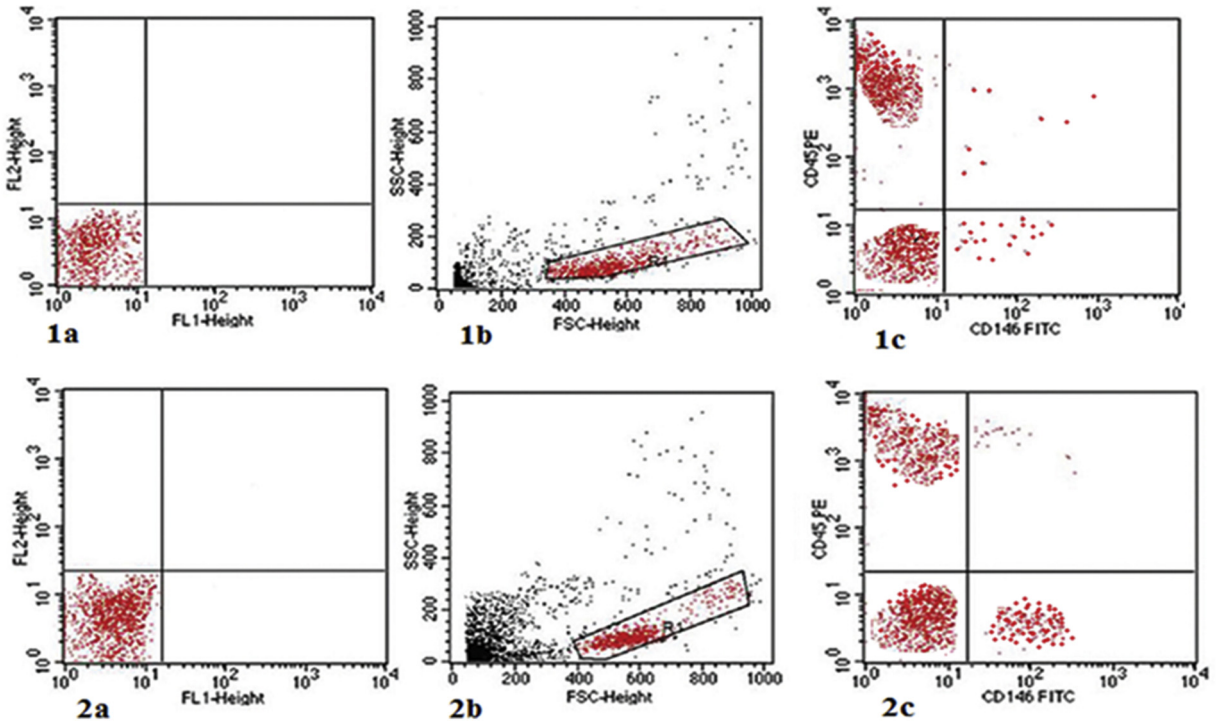

Fig. 1. Representative flow cytometry analysis of circulating endothelial cells (CECs). 1. Healthy controls: (1a) unstained auto-control sample, (1b) gating on peripheral mononuclear cells (PBMCs) (R1), and (1c) CECs ($CD146^+CD45^-$). 2. Patients with severe malaria: (2a) unstained auto-control sample, (2b) gating on PBMCs (R1), and (2c) mature CECs with increased number of CECs compared to the healthy controls.

4. Discussion

In the current study, CECs were detected by flow-cytometric analysis where they were $CD45^-$ and $CD146^+$. CD146 is the most widely used surface marker for the identification of CECs [14]. Flow cytometry is a fast and easy technique, and can be used to detect several cell markers at the same time [22].

To our knowledge, this report is the first one which measured the level of CECs in malaria-infected patients. It was found that the mean CECs count was significantly greater in both UM and SM patient groups than in the healthy controls, and patients with SM had a higher number of CECs than UM patients. This indicated an occurrence of broad endothelial injury during the course of acute *P. falciparum* infection, which increased in severity in accordance with the disease's severity.

Our results correlated well with other studies which revealed that malaria infection is associated with endothelial activation and

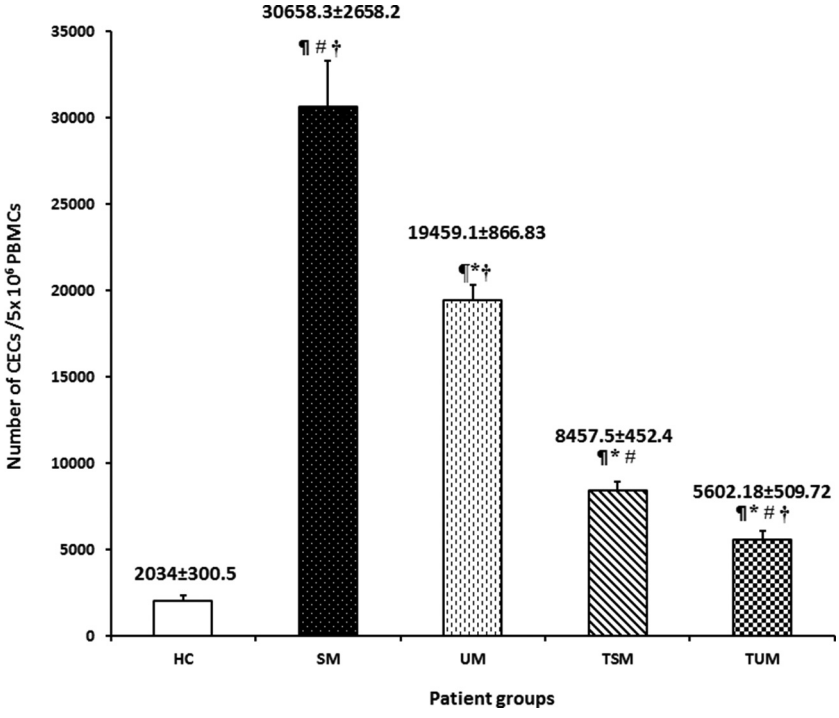

Fig. 2. Count of CECs. Results are presented as mean ± SD for each group. ¶ indicates a statistically significant difference from HC group ($P < .001$). * indicates a statistically significant difference from SM group ($P < .001$); # indicates statistically significant difference from UM group ($P < .001$) and † indicates a statistically significant difference from TSM group ($P < .001$) using one-way ANOVA followed by a post-hoc test.

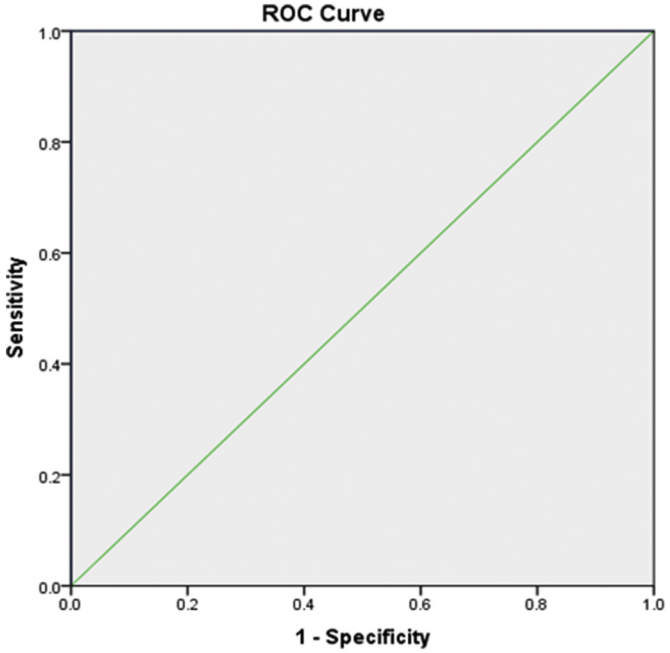

Fig. 3. ROC curve analysis for calculation of cut-off value of CECs for prediction of severe malaria.

Table 2
Correlation between the mean counts of CECs and estimated laboratory parameters.

Laboratory data	CECs number	
	r	*P*-value
Parasitic index	0.445	.002
Hb	−0.344	.022
Platelets	−0.255	.075
WBCs	−0.257	.096
Creatinine	0.506	.001

inflammation. For example, a study done by Combes et al. [10] found that the mean number of circulating endothelial micro-particles increased significantly in patients with SM, but not in UM malaria, in comparison with healthy controls. A significant rise in von Willebrand factor (VWF), a marker of chronic ECs activation, VWF propeptide, a marker of acute ECs activation, and activated VWF were detected in malaria patients suffering from cerebral and non-cerebral malaria [23,24]. Also, a study done by Moxon et al. [13] revealed that Malawian children with UM or cerebral malaria showed higher plasma levels of C-reactive protein, angiopoetin 2 and soluble intracellular adhesion molecule 1 than controls, confirming an occurrence of endothelial inflammation. Moreover, different isolates of *P. falciparum* induced various degrees of apoptosis in human endothelial cells [25].

The mechanisms which mediate endothelial dysfunction during *P. falciparum* infection have not been completely clarified; however, this could be multifactorial in origin [26]. TNF-α, released during malaria infection initiates local and broad stimulatory effects on vascular ECs [9], and other cytokines and *P. falciparum* antigens can alter the ECs phenotype [13,27–28]. A malaria-induced endothelial inflammation may be partly responsible for detachment of CECs observed here. Added to that, the merozoites and digestive vacuole antigens incorporated by ECs may lead to disruption of endothelial tight-junctions followed by detachment of CECs [29]. Detection of increasing CECs, herein, referred to that malaria infection caused endothelial injury.

We observed that the mean number of CECs decreased significantly in both treated groups in comparison with the corresponding infected group, but both still had a significantly higher cell count than healthy controls. It seems that clearance of the malaria infection helped reduce the endothelial dys-regulation including vascular damage. This is in agreement with Gyan et al. [30] who suggested that repair of endothelial damage occurs in treated *P. falciparum* patients. However, treated patients should be followed for a longer time than was the case here in order to monitor whether the level of CECs reduces to base-line levels indicating complete healing of the vascular injury. Unfortunately a longer follow-up was not possible here.

In this study the partial endothelial repair, indicated by significant lowering of the CECs levels in the treated patients, could be partly explained by recruitment of circulating endothelial progenitor cells (CEPCs) from bone marrow to the injured endothelium. This suggestion is in accordance with Gyan et al. [30] who found that CEPCs decreased more significantly in cerebral malaria than in UM or healthy participants. Furthermore, CEPCs increased significantly in survivor than non-survivor children suffering from sepsis [20]. CEPCs make a well-documented contribution to re-endothelialization [31]. Several growth factors, such as vascular endothelial growth factor, stroma-derived factor 1 and erythropoietin promote CEPCs mobilization and proliferation from bone marrow to the damaged endothelium [32,33].

On the other hand, our results were in contrast to those of Moxon et al. [13] who observed persistent endothelial activation and inflammation, even after elimination of PRBCs from blood, as indicated by increased plasma levels of soluble intracellular adhesion molecule 1, angiopoetin 2, and C-reactive protein at 1 month follow-up visits. The difference regarding timing of resolution of endothelial dys-regulation between our results and that found by Moxon and his colleagues could be related to several factors. First, the nature of the patient cohorts: Moxon et al. [13] studied children living in endemic areas who were exposed frequently to malaria infection with a liability of persistent endothelial inflammation, while our patients were adults of mixed nationalities. Second, the respective markers that were examined: CECs are a marker of endothelium damage which starts to recover before complete relief of inflammation of the endothelium after malaria treatment, while the markers used by Moxon et al. [13] were of inflammation and activation. Third, the number of CEPCs usually returns to pre-injury levels within 2–3 days [34], and this could explain the reduced number of CECs within a relatively short time after treatment of the infection.

In addition to confirming the occurrence of acute malaria-induced vascular damage, and a contribution to diagnosis of malaria infection, a CECs count can help in predicting which patients are suffering a severe form of malaria form, as demonstrated by the ROC analysis in this study giving 100% sensitivity, 100% specificity and 100% accuracy. The present study showed a significant negative correlation between CECs counts and Hb concentration, though both the parasitemia index and creatinine level had a significant positive correlation with CEC count. These correlations show the potential importance of detecting CECs for evaluating the prognosis of malaria.

It is worth mentioning that the endothelial activation or damage associated with repeated malaria infection may have a long-term negative cardiovascular effect in hyper-endemic countries, so this impact needs further evaluation and even intervention [13,30].

The elevated level of CECs during acute malaria infection may have a role with other immune cells in combating the parasite infection. It is well known that ECs can function as antigen-presenting cells presenting antigens (APCs) in the context of major histocompatibility complex (MHC) class I and class II to CD4+ and CD8+ T cells, thus initiating their activation [26,35]. In vitro studies revealed that ECs can cross-present *P. berghei* antigens in the context of MHC class I to CD8+ T cells [36,37]. Furthermore, it is documented that CECs can work as APCs, where they express TLR4, HLA-DR and CD91, so they can present endogenous pathogen-associated molecules, such as HSPs and chaperoned peptides to T cells [38,39]. The same scenario could reveal a role for CECs in controlling malaria infection, an interesting possibility that

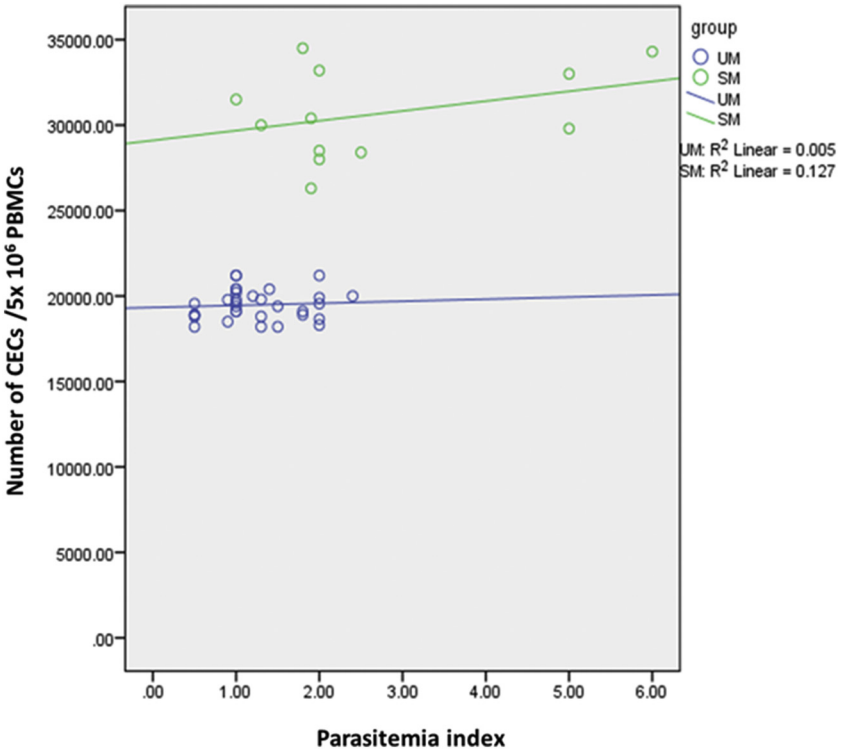

Fig. 4. Scatter plot of CECs count and parasitemia index in UM and SM patient groups.

may warrant further investigation.

5. Conclusion

CEC counts in blood could be a useful biomarker to monitor endothelial damage during acute *P. falciparum* infection and its partial recovery after treatment, being elevated in severe and uncomplicated malaria and decreased after treatment. Also, CECs could be used to predict the prognosis of disease in malaria patients. This study has revealed a new aspect of endothelium pathology during malaria infection and further assessment of CECs levels in larger patient samples with different forms of severe malaria in multiple centers is to be recommended.

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