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In vivo imaging of the buccal mucosa shows loss of the endothelial glycocalyx and perivascular hemorrhages in pediatric *Plasmodium falciparum* malaria

Running title: Incident dark field imaging and malaria

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Abstract

Severe malaria is mostly caused by *Plasmodium falciparum* resulting in considerable, systemic inflammation and pronounced endothelial activation. The endothelium forms an interface between blood and tissue and vasculopathy has previously been linked with malaria severity. We studied to what extent the endothelial glycocalyx that normally maintains endothelial function is involved in *falciparum* malaria pathogenesis by using incident dark field imaging in the buccal mucosa. This enabled calculation of the perfused boundary region, which indicates to what extent erythrocytes can permeate into the endothelial glycocalyx. The perfused boundary region was significantly increased in severe malaria patients and mirrored by an increase of soluble glycocalyx components in plasma. This is suggestive of a substantial endothelial glycocalyx loss. Patients with severe malaria had significantly higher plasma levels of sulfated glycosaminoglycans than patients with uncomplicated malaria, whereas other measured glycocalyx markers were raised to comparable extent in both groups. In severe malaria, plasma levels of the glycosaminoglycan hyaluronic acid were positively correlated with perfused boundary region in the buccal cavity. Plasma hyaluronic acid and heparan sulfate were particularly high in severe malaria patients with low Blantyre Coma Score suggesting involvement in its pathogenesis. *In vivo* imaging also detected perivascular hemorrhages and sequestering late-stage parasites. In line with this, plasma angiopoietin-1 was decreased while angiopoietin-2 was increased suggesting vascular instability. Density of hemorrhages correlated negatively with plasma levels of angiopoietin-1. Our findings indicate that, similar to experimental malaria, loss of endothelial glycocalyx is associated with vascular dysfunction in human malaria and related to severity.

Keywords: Endothelial glycocalyx, microcirculation, malaria, incident dark field imaging, image analyses, *Plasmodium falciparum*, glycocalyx shedding,

Introduction

Severe malaria (SM) is caused by *Plasmodium falciparum*; a parasite that invades and multiplies in human erythrocytes. The pathogenesis of SM involves cytoadhesion of parasitized erythrocytes leading to impaired blood flow and dysregulated coagulation and inflammation.(1) The inflammatory state causes remodeling of the endothelial surface including upregulation of immune receptors on the vasculature (2-4) and further interactions between the vasculature and malaria-infected erythrocytes and leukocytes.(5, 6) The severity of malaria depends partly on what variant surface antigens the parasites express and export to the erythrocyte surface.(7, 8) Also, endothelial responsiveness to inflammatory cytokines contributes to determining whether an infection results in SM or uncomplicated malaria (UM).(9) Plasma markers of endothelial activation correlate strongly with malaria severity and discriminate between SM and UM suggesting that the level of vasculopathy is a strong predictor for the outcome of *P. falciparum* malaria.(3, 10)

One part of the endothelium that responds to activation and inflammation is the dense matrix of carbohydrates termed the endothelial glycocalyx.(11) The endothelial glycocalyx covers the luminal surface of healthy blood vessels and plays several roles in maintaining vascular homeostasis: it shields immune receptors from unwanted binding, it is a mechano-sensor and it is involved in reducing permeability over the vasculature.(5, 12-14) The endothelial glycocalyx is shed in response to inflammatory conditions and has been studied in several diseases including diabetes(15), sepsis(16), and in viral infections. (17, 18)

In experimental, murine malaria we have previously shown an association between malaria severity and glycocalyx loss in brain vessels.(19, 20) Murine malaria models have been debated (21) and we were thus interested in addressing whether loss of endothelial glycocalyx is involved in pathogenesis of human *P. falciparum* malaria. If being so, one would expect an association between the extent of glycocalyx loss and the severity of *P. falciparum* malaria. It has recently been shown that plasma levels of the glycosaminoglycan chondroitin sulfate and the proteoglycan syndecan-1 are increased in adult, Asian malaria patients and the latter marker also in the plasma of Ugandan children. (22-24) Levels of chondroitin sulfate in urine have also been shown to increase in SM. Here, we provide detailed analysis of the glycocalyx loss associated with pediatric *P. falciparum* malaria and its association with impaired microcirculation, using state-of-the-art non-invasive imaging of the buccal mucosa.

Materials and methods

Patients and enrolment

The study was a cross-sectional study designed to assess glycocalyx loss in pediatric malaria patients. Patients were admitted to Magu district hospital, Mwanza region, Tanzania during high transmission period (April-November 2017). Sample size was determined in a small pilot study (n=15). Following the Hospital's routine admission procedures, the designated nurse/clinician screened the children for malaria and, before enrolling the child into the study, informed consent was obtained from the parent/guardian. The project was approved by the ethical committee of the National Institute for Medical Research Tanzania (NIMR/HQ/R.8c/Vol. II/715). All malaria patients enrolled were rapid diagnostic test (mRDT)-positive (CareStart Malaria, Access Bio, NJ, USA) and had peripheral parasites counted in Giemsa-stained blood

smears by a skilled microscopist. A lower cut-off of 1000 infected erythrocytes/µL blood was set to avoid including non-malaria fever (NMF) in either of the groups of malaria patients. Patients were excluded from the study if they were less than six months old, had been admitted to a hospital less than a month before the current malaria attack and if they had other complications (e.g. other infections) when admitted to the hospital with malaria. Malaria-negative individuals were enrolled from the same hospital from the Reproductive and Child Health Clinic; no neonates were enrolled in the study. Blood samples were collected, using sodium citrate as anticoagulant and initially stored at -20^{O} C, then transferred to -80^{O} C.

SM was defined as malaria with at least one of the following features (following WHO guidelines): seizures, lack of verbal and motor responses (Blantyre Coma Score (BCS) less than or equal to 2), lactate >5mM, hemoglobin (Hb) <5 g/dl (severe anemia) or hyperparasitemia (>10% parasitemia). Malaria patients without features associated with SM or SMA were termed UM. Patients with NMF were RDT negative and were admitted to the children's ward with e.g. diarrhea and urinary tract infections. Characteristics are found in Table 1 and a CONSORT diagram is supplied as supplementary figure 1. Breakdown of patients in the SM group is presented in figure 2.

Assessment of the microcirculation in vivo

Microcirculatory function was measured at the day of admission by incident darkfield (IDF) imaging (Cytocam, Braedius, The Netherlands). A handheld probe emits flashes (2 ms) of green light (525 nm) that is absorbed by hemoglobin in erythrocytes. Images are captured at a frame rate of 25 frames per second. The probe was placed on the buccal mucosa between the inferior lip and the teeth without causing local damage to the microvasculature. While recording, the quality of the movies was assessed by trained nurses and according to best practice (25) at least three movies were recorded from each patient. Recordings were not included in the study if these criteria were not met (due to e.g. shaken or compressed movies).

Analyses of IDF imaging

Cytocam software (version 1.7.12, Braedius) was used to crop and stabilize movie sequences. If less than three movies were considered adequate the IDF data from that patient were excluded. For all analyses of IDF outputs only a single value (mean/median) is reported per patient. The software automatically identifies blood vessels and can calculate total vessel density (TVD), perfused vessel density (PVD), proportion of perfused vessels (PPV) and average flow speed. We calculated the heterogeneity index as previously described.(26) IDF imaging allowed a localized quantification of hemorrhages (stagnant erythrocytes). Stagnant erythrocytes were defined as immobile cells localized around vessels with flow. The field of view (~1.8 mm^2) was divided into 16 non-overlapping rectangles and the number of rectangles with hemorrhages was counted and divided by the total number of assessed rectangles; this fraction was reported in percentage for each individual. Also, the proportion of individuals with hemorrhages was compared between the groups.

Furthermore, we developed software for studying erythrocyte movements. We developed a method by applying a pixel-wise filtering approach enabling us to calculate the width of a blood vessel and analyse

how erythrocytes move temporally. Blood vessel segments were cropped and a minimum filter and a median filter were applied. Since erythrocytes absorb light and thus result in low pixel values, the minimum filter captures all positions (pixels) in which an erythrocyte has been present through all the analysed frames and displays this in one output image. The median filter captures where erythrocytes most frequently have been present throughout the image sequence.

Edge detection (Canny) was used to determine the boundary between the blood vessel and the surrounding tissue using a standard deviation of σ=1.8.(27) We applied a simplified version of Dijkstra's algorithm using pixel intensities as a cost measure for an automated selection of continuous edges of both sides of the blood vessels.(28) This enabled us to determine the Euclidean widths along the length of the vessel. We did this after blurring images with a Gaussian filter (standard deviation σ=5) and subsequently applying the Dijkstra's algorithm to obtain a medial line of the vessel followed by applying perpendicular lines to the medial line. The perpendicular lines were interpolated and their corresponding intersections on the edges of the blood vessel were used to calculate the Euclidean distances for both the minimum- and the median-filter (Matlab, R2017a, Mathworks, MA, USA) (supplemental figure 4). This enabled in-depth analyses of multiple microvessel segments from multiple small movies from each patient. With this data we calculated the perfused boundary region (PBR) as the Euclidian distance of the minimum filter minus the Euclidian distance of median filter divided by two. The median value of all the computed PBRs (>100/patient) was reported for each subject. The investigator was blinded to patient data when analyzing the movies.

Imaging of infected and uninfected erythrocytes in vitro

To assess how late-stage parasites appear in IDF imaging, *P. falciparum* (FCR3) were cultured in human type O erythrocytes as previously described (29). We produced a simple tissue phantom in 12-well plates, using 1% gelatin in RPMI 1640 (Biological Industries, Israel). Erythrocytes at 1% hematocrit diluted in 1% gelatin (diluted in RPMI1640) were seeded and after gelation imaged from above. We recorded IDF images of uninfected erythrocytes and late stage infected erythrocytes purified by magnetic assisted column separation as described previously.(29) The images were assessed by line plots using Image J (version 1.52i)(30) and the Prewitt filter (Matlab). This filter was also applied to data obtained from the buccal cavity.

Plasma analyses

Plasma was analyzed by ELISA and multiplex-based Luminex (Magpix, R&D Systems, BioTechne, UK). The following plasma constituents were analyzed by ELISA: hyaluronic acid (HA, Echelon Biosciences, UT, USA), syndecan-4 (Quantikine, R&D Systems), angiopoietin-1 (Quantikine, R&D Systems). The following plasma constituents were analyzed by Luminex: Syndecan-1, angiopoietin-2, TNF, E-selectin, thrombomodulin, CD44, endothelin-1 (R&D Systems). Some healthy controls had endothelin-1 levels below the detection limit, which was set to the lowest value on the standard curve. All assays were performed according to manufacturer's instructions besides blocking was performed using 5% BSA for angiopoietin-1, as previously reported (31).

Also, 2 µL of plasma was blotted onto positively charged nitrocellulose (Amersham Hybond N^+, GE Healthcare, IL, USA). Standards for sulfated glycosaminoglycans (GAG) (Chondroitin sulfate A, Sigma-Aldrich, MI, USA), heparan sulfate (Sigma-Aldrich) and recombinant glypican-1 (Biolegend, CA, USA) were blotted. After drying, the membranes were blocked with skim milk (5% in TBS, Sigma-Aldrich) for 1 hour. Antibodies detecting human glypican-1 (R&D Systems) and heparan sulfate (clone 10E4, United States Biologicals, MA, USA) were diluted in blocking buffer and applied over night at 4° C. Signals were detected by rabbit anti-goat HRP (ThermoFisher, MA, USA) followed by goat anti-mouse IgM (Dylight680, Rockland Immunochemicals, Limerick, PA, USA). The signals were detected using an Odyssey Fc reader (LI-COR Biosciences, NE, USA). Ultimately, the membranes were stained with Alcian Blue as previously described (19, 20). Signal density was quantified using Image J (30). Markers were run for patients with satisfactory IDF imaging and some markers (ELISA and dot blot assays) were also run for randomly selected individuals, who had poor IDF movies (i.e. shaken, compressed). All analyses were performed in a blinded manner.

Statistical analyses

Sample size required to detect a 30% difference in glycocalyx loss was calculated from a small subset of the patients enrolled in the first month of the study. Data were initially tested for normal distribution (Shapiro Wilks test) and equal variance (Bartlett's test). If data followed these criteria parametric analyses were performed (ANOVA followed by Tukey's multiple comparison tests). For some parameters data followed these criteria after logarithmic conversion ($x' = \ln(x+1)$), otherwise non-parametric analyses were performed (Kruskal-Wallis followed by Dunn's test). Chi-square test was performed to assess whether proportion of subjects with hemorrhages was higher in SM compared with healthy subjects. Logistic regression was applied to test for differences in perivascular hemorrhage densities. Correlation tests were performed with the non-parametric Spearman's rank correlation. Follow up data were analyzed with Friedman test followed by a Dunn's test. All statistical analyses were performed using R for Windows (version 2.12.1(32)). Graphs were designed using GraphPad Prism (version 8.01, CA, USA).

Results

Patients

The children enrolled are presented in Table 1. The age, 0-10 years, differed between the four groups (p=0.02) and post hoc tests showed that UM patients were significantly older than healthy children and patients with NMF (p=0.01 and p=0.047, respectively). Gender distributions were marginally different between the four groups (p=0.047). Parasitemia and plasma glucose levels were similar in UM and SM (p=0.4 and p=0.2, respectively). SM patients had significantly lower levels of plasma Hb and higher levels of lactate compared with UM patients (p<0.01). None of the admitted patients died from the infection. 8 patients had temporarily decreased consciousness after a seizure but these did not formally meet the criteria for cerebral malaria. The distribution of criteria defining SM is summarized in table 2 .

Incident dark field (IDF) imaging shows perivascular hemorrhages and sequestration in the buccal microcirculation

IDF imaging was used to visualize erythrocyte movements in the buccal microcirculation. Healthy subjects had strictly delineated blood vessels as shown in a representative still image (figure 1A). The vascular integrity in the buccal cavity was frequently impaired in malaria patients showing stagnant erythrocytes outside blood vessels (figure 1B, C). Perivascular hemorrhages were common in SM patients but not in controls; approximately 50% of all SM patients had some degree of perivascular hemorrhaging. The proportion of subjects with perivascular hemorrhages was significantly different when comparing all groups (p=0.04), although the difference between SM and UM did not reach significance in post hoc tests (p=0.15). The density of perivascular hemorrhages was comparable between the groups (figure 1D, SM vs. healthy controls p=0.09).

IDF imaging furthermore showed late stage parasites sequestering in the microvessels (figure 1E, supplemental movie 1). *In vitro* studies allowed us to substantiate that the dark cells clearly marked by a well-defined edge present in and around microvessels are late stage parasites (supplemental figure 2). Thus, the Prewitt filter allows for unbiased detection of late stage parasites (supplemental figure 3 and supplemental movie 2).

IDF imaging shows loss of endothelial glycocalyx

Image analyses of the microvessels enabled detection of spatiotemporal movements of erythrocytes. Median diameter of blood vessels analyzed was 23.1 µm, which is in the range of post-capillary venules and was similar in all groups (p=0.8). An example of three temporally separated segments is shown (figure 2A) and from the full stack of images the corresponding median and minimum filter is calculated and shown (figure 2B). Calculating the diameter at the same localization for both filters allowed us to calculate the perfused boundary region (PBR) (figure 2C). The PBR measures how well erythrocytes penetrate the glycocalyx (33). Since there is an inverse relationship between PBR and the thickness of the glycocalyx, a high PBR indicates a thin glycocalyx. The median PBR was significantly increased in SM patients as

compared with healthy children (p<0.0001, figure 2D). The median PBR of UM and SM patients was comparable. Since an increased PBR could be associated with clinical features as seen in experimental models (34) we tested if SM patients with BCS less than 3 (n=5, median: 4.5 µm) had higher PBR than in SM patients with a higher BCS score (n=38, median: 3.9 µm) and found a non-significant trend (p=0.09).

The proportion of perfused vessels (PPV) was similar in all groups (p=0.2, supplemental figure 4A). Average flow speed differed significantly between groups (p=0.03, supplemental figure 4B). This could be explained by the anemia since SMA patients had significantly higher average speed of blood flow compared with healthy children (p=0.006) and since flow speed was negatively correlated with plasma Hb (rho=-0.2, p=0.04). Despite focal hypoperfusion, the heterogeneity index was similar in malaria patients and healthy children (p>0.9, supplemental figure 4C) but significantly increased in NMF patients (p=0.03).

Increased plasma levels of glycocalyx components in malaria patients

An increased PBR implies that the glycocalyx is perturbed and possibly shed in the circulation in malaria patients. In concordance with this, plasma HA- and sulfated GAG-levels were significantly increased in SM patients (figure 3A-B, p<0.001 with post hoc analysis showing significance for SM compared with healthy controls (p<0.01)). UM and SM as well as SM and NMF were not significantly different in terms of HA, whereas levels of sulfated GAGs were significantly higher in SM vs UM (p=0.02). Plasma levels of HS and syndecan-1 were significantly increased in SM (figure 3C-D, p<0.0001) and for HS, also a significant increase was seen in UM (p=0.003). Plasma levels of HS and syndecan-1 were comparable between UM and SM (p>0.9) and these groups had shed HS levels being comparable with NMF (p>0.4). NMF, which did not affect PBR, did not change the levels of these plasma components (p>0.2). Plasma syndecan-4 was not changed in any of the groups (p=0.7), whereas glypican-1 was significantly increased in SM and NMF (figure 3E, p=0.02 for both groups). SM and UM were comparable (p>0.9). The HA-receptor, CD44 did not vary between groups (p=0.8).

The visually detected glycocalyx loss in the buccal mucosa of SM patients showed an association with plasma levels of HA (figure 4A, rho=0.7, p<0.0001) but not with other glycocalyx components. Because there was a trend towards higher PBR in SM patients with low BCS we tested if this was reflected in any of the plasma markers of glycocalyx shedding. Both plasma HA and HS were significantly increased in SM patients with low BCS compared with SM patients with a higher BCS score (figure 4B-C, p=0.01 and p=0.005, respectively). Plasma levels of glycocalyx components were neither correlated with either parasite counts nor with the density of hemorrhages in the buccal mucosa (data not shown). It could be speculated that glycocalyx loss was secondary to anaerobic metabolism, induced by impaired microcirculation but glycocalyx markers did not correlate with plasma lactate levels (p>0.16).

Shedding of endothelial glycocalyx components correlates with markers of endothelial activation

Plasma angiopoietin-1 decreased significantly in SM (figure 5A, p<0.0001) with post hoc tests showing significance for SM but not UM and NMF. Plasma levels of angiopoietin-2 were significantly increased in SM (figure 5B, p<0.0001) but not in NMF or UM patients (p>0.4). Levels of the vasoconstrictor endothelin-1

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increased significantly in SM and UM (figure 5C, p=0.002 and p=0.02, respectively). Finally, the levels of soluble thrombomodulin, which is involved in both coagulation and inflammation, increased in SM compared with healthy controls and NMF (figure 5D, p=0.002 and p=0.04, respectively).

In SM patients, plasma angiopoietin-1 was negatively associated with HA levels (rho=-0.31, p=0.02) and with density of hemorrhages figure 5E, rho=-0.36, p=0.02) but not with angiopoietin-2 (p=0.9). All subjects with hemorrhages in the buccal cavity had plasma angiopoietin-1 levels below 20 ng/ml. Angiopoietin-2 was positively associated with syndecan-1 (rho=0.5, p=0.001) and with HS levels (rho=0.34, p=0.03). Also, thrombomodulin was positively associated with PBR (figure 5F, rho=0.38, p=0.02).

Plasma levels of the pro-inflammatory cytokine TNF and plasma E-selectin were significantly increased in SM patients (p<0.0001 vs healthy controls for both markers, supplementary figure 5A, B).

Glycocalyx components are cleared slowly from the plasma

Five SM and four UM patients returned for follow-up investigations. IDF showed that micro hemorrhages persisted to some extent as long as 28 days after admission. The gradual reduction of hemorrhages appeared faster in UM than in SM, but the low number of follow-up patients precludes any meaningful comparison between the groups. PBR, HA and syndecan-1 levels returned to those of healthy controls at day 28 post admission (figure 6A-C). In patients with UM and SM, HA stayed elevated at day 14 after admission, while being significantly reduced and comparable with that in healthy controls at day 28 (figure 6B, p=0.03). The persistent increase in shed glycocalyx components was to some extent mirrored by plasma angiopoietin-1 levels but these were temporal changes were not significant (figure 6D, p>0.07). Also, angiopoietin-2 levels seemed to drop but not significantly (figure 6E, p>0.07). These persistent signatures are mirrored by E-selectin levels that were also normalized (i.e. significantly lower than day 0) at day 28 post admission (figure 6F, p=0.03).

We hypothesized that the level of glycocalyx components in the plasma would correlate with the level of TNF over time, but this was not the case for any glycocalyx component (p>0.2).

Discussion

By *using* state-of-the-art *in vivo* imaging of the microcirculation, we were able to quantify loss of the endothelial glycocalyx and identify other malaria-induced microcirculatory changes in the buccal microvessels. Increased numbers of microhemorrhages, thinning of the glycocalyx and plasma levels of shed HS and HA were associated with disease severity. This indicates that the glycocalyx loss previously shown in experimental malaria (19, 20) also occurs in human *P. falciparum* malaria and may contribute to disease severity.

In vivo imaging using IDF allowed us to assess the microcirculation to unprecedented detail in real time. This enabled demonstration of microvascular alterations and showed *in vivo* evidence of malaria-infected erythrocytes sequestering in the microcirculation and frequent perivascular hemorrhages. The finding that the buccal microcirculation is impaired in malaria points towards a systemic intravascular accumulation of

infected erythrocytes as previously described.(35) One previous study using *in vivo* imaging in adult malaria patients from Asia showed obstructed vessels and heterogeneous flow patterns in the rectal mucosal during CM, but to a less pronounced degree in the sublingual mucosa (36) suggesting notable regional differences in the microcirculation. There are clear differences between adults and pediatric SM and microvascular damage and perivascular hemorrhages may be of particular importance in children (1).

IDF can also indicate whether the patients have loss of hemodynamic coherence as seen in e.g. sepsis.(25) We did not notice a change in PPV or heterogeneity, suggesting that obstruction of microvessels in the buccal cavity was not pronounced. Another factor leading to loss of hemodynamic coherence is hemodilution.(37) In patients with SMA we saw significantly increased RBC velocity which was negatively correlated with Hb. It has been suggested that moderate anemia plays a protective role in patients with SM, possibly due to hemodilution leading to improved blood flow (38). Our IDF findings support this hypothesis.

IDF showed that perivascular hemorrhages in the buccal mucosa were frequent in SM patients and differed in numbers between patients similarly to what is seen with retinal assessment during CM.(39) The number of perivascular hemorrhages was negatively correlated with plasma angiopoietin-1, supporting the role of angiopoietin-1 in prevention of vascular leakage.(40) Our data suggests that perivascular hemorrhages in SM are mostly seen when plasma angiopoietin-1 gets below 20 ng/ml. This drop corresponds with low angiopoietin-1 levels that have been associated with malaria severity in studies in African children (3, 4).

Our *in vitro* experiments showed late-stage infected erythrocytes appearing as dark spots with clearly defined edges. This can be explained by hemozoin absorbing light at 525 nm to a greater extent than Hb.(41) Since the *in vitro* experiment was conducted in the absence of leukocytes we cannot rule out the possibility that some of the perivascular spots that were observed in the buccal mucosa *in vivo* were hemozoin-containing leukocytes (42, 43).

A main purpose of using IDF imaging was to assess glycocalyx loss. We designed software to automatically calculate PBR in random vessel segments in an unbiased manner. PBR has previously been used to assess glycocalyx loss in e.g. obese patients (33). Our analysis showed significantly larger PBR in SM compared with healthy controls, providing direct *in vivo* evidence for malaria-induced glycocalyx loss in humans. The use of PBR as an indicator of glycocalyx shedding was further supported by the positive correlation between PBR and plasma HA. HA constitutes an outer flexible part of the glycocalyx acting as a canopy (44), which could explain why HA showed the strongest association with PBR of the glycocalyx components. The study we performed in a Nigerian cohort (supplementary figure 5) as well as other studies further support the loss of endothelial glycocalyx in *P. falciparum* malaria (22-24).

An increase in PBR during malaria implies that the infected erythrocytes can penetrate deeper into the glycocalyx getting in close contact with receptors anchored on the endothelial surface. A healthy glycocalyx leaves only nanometer-sized pores open to entry (45) thereby shielding endothelial receptors (12, 46) and *in vitro* it prevents infected erythrocytes from optimally interacting with CD36.(47) SM patients with low BCS had significantly higher plasma levels of some GAGs suggesting that glycocalyx shedding is associated with disease severity.

Glycocalyx loss has been seen for other diseases involving inflammation and endothelial activation (15, 17, 18, 33) and is, thus, not unique to malaria. However, *P. falciparum* uses endothelial receptors for cytoadhesion and changes to the glycocalyx may lead to increased exposure of e.g. CD54 as previously shown *in vitro (*47).

Patients with impaired consciousness had signs of endothelial glycocalyx loss. In sepsis it was recently shown that circulating HS components were associated with cognitive impairment since they have affinity for brain-derived neurotrophic factor (48). Whether circulating HS fragments also contribute to cognitive impairment in cerebral malaria needs further investigation.

Finally, IDF enabled us to assess PBR over time demonstrating that the restoration of the glycocalyx is a slow process lasting up 2-4 weeks after malaria. This is in line with *in vitro* studies showing a slow recovery of the glycocalyx (47, 49, 50). Furthermore, elevated levels of angiopoetin-2 and inflammatory markers (51) have been shown to last for weeks after a malaria attack hampering restoration (52).

The study had some limitations since UM patients were significantly older than healthy controls and NMF. Glycocalyx coverage may be influenced by age although this has only been shown for young vs aged adults (53). Thus, the relatively small difference in age in this study is not expected to have an impact on interpretations. Gender has not shown to result in any differences on glycocalyx coverage and the difference in gender composition in the four groups is not expected to affect findings in the study. Furthermore, an increase in sample size, in particular for NMF patients, and more complete follow up, would have been desirable. Nevertheless, our data show robust differences between patient groups and to some extent support two recently published study on glycocalyx loss in SM (23, 54). Plasma HRP2 was not measured and a relationship between total parasite biomass and glycocalyx loss as well as level of hemorrhages could not be established.

In summary, IDF imaging has confirmed a previous study on microcirculatory changes in malaria. As a novel finding, it enabled assessment of micro hemorrhages and visualization of sequestering parasites *in vivo.* Furthermore, it allowed us to demonstrate loss of the endothelial glycocalyx in the buccal mucosa of human malaria patients. These vascular changes were mirrored by increased plasma levels of multiple glycocalyx components including HA, which was positively correlated with PBR. Shedding of the glycocalyx seemed related to endothelial activation and malaria severity and the multitude of glycocalyx markers detected in plasma suggests that several proteases and glycosidases are activated during the disease. This should be investigated in further studies.

381 Authorship Contributions:

382 EL: Performed experiments, analyzed data, contributed to writing of manuscript

383 LEH: Developed software, performed experiments, analyzed data, contributed to writing of manuscript

384 TR: Developed software, performed experiments, analyzed data, contributed to writing of manuscript

385 CWW: Supervised the project, research infrastructure, contributed to writing of manuscript

386 AMF: Collected data, contributed to writing of manuscript

387 AM: Supervised the project, research infrastructure, contributed to writing of manuscript

388 VM: Supervised the project, research infrastructure, contributed to writing of manuscript

389 JL: Supervised the project, research infrastructure, contributed to writing of manuscript

390 TGT: Supervised the project, research infrastructure, contributed to writing of manuscript

391 JALK: Supervised the project, contributed to writing of manuscript

392 RP: Developed software, analyzed data, contributed to writing of manuscript

393 CH: Conceived and planned experiments, attracted funding, analyzed data, wrote the manuscript with
394 input from all co-authors

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545 Table 1. Baseline characteristics of patients from the Tanzanian cohort. Subjects were stratified into
546 category of disease. ND= not determined. Most data are presented as means and ranges; parasitemia as
547 median and ranges.

	Healthy subjects (n=31)	**Non-malaria fever (NMF) (n=7)**	**Uncomplicated malaria (UM) (n=12)**	**Severe malaria (SM) (n=69)**
Age (years)	2.6 (0.8-4.3)	2.28 (1.0-4.0)	5.5 (1.1-10.1)	4.1 (0.6-10.0)
Sex (female %)	21%	67%	62%	48%
Parasitemia (/µL blood)	0	0	55560 (1360-156560)	53560 (1000-2789320)
Hemoglobin (Hb) (g/dL)	8.5 (6.2-11.3)	6.8 (2.2-11.3)	9.3 (7.0-13.4)	7.4 (2.3-12.6)
Glucose (mmol/L)	ND	7.8 (6.9-9.2)	6.2 (3.8-8.2)	6.8 (1.4-26.6)
Lactate (mmol/L)	ND	5.5 (4.6-7.4)	4.1 (3.1-5.0)	7.4 (2.4-21.9)

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549 Table 2. Breakdown of the SM subgroup. Patients were enrolled in the SM group due to hyperparasitemia,
550 severe anemia and Blantyre coma score (BCS) of 2 or less. Some patients belonged to more than one of the
551 subgroups.

Number of SM patients with plasma lactate > 5 mmol/L	**Number of SM patients with hyperparasitemia**	**Number of SM patients with severe anemia**	**Number of SM patients with seizures**	**Number of SI Blantyre com**
56	24	14	8	

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554 Supplementary table. Overview of parameters quantitated in the paper. Values represent median values
555 and ranges in brackets. n equals the number of subjects behind the numbers.

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Figure legends

Figure 1. IDF imaging shows malaria-induced changes in the buccal microcirculation. A) A still image of a healthy volunteer showing highly delineated blood vessels. Still images from malaria-infected individuals can be with multiple hemorrhages (B). Still image of malaria-infected individual without hemorrhage is seen in supplemental figure 1A. Arrows in B denote perivascular hemorrhages. C) Close ups of representative hemorrhages; I is selected from the still shown in B, while II is a close up from a different still image. D) Density of perivascular hemorrhages as determined by IDF imaging. E) Still images from movie showing an infected erythrocyte sequestering in a capillary (supplemental movie 1). Still images are temporally separated by 40 milliseconds. Scale bars equal 100 µm in A and B, 50 µm in C and E.

Figure 2. Quantitative analyses of IDF imaging shows substantial loss of endothelial glycocalyx in the buccal cavity as measured by increased PBR. A) A temporal sequence of vessel segments. B) The corresponding minimum and median filters. C) Model of measuring the Euclidian distance. The shortest path is calculated (centered line) and perpendicular to this, the Euclidian distance is calculated after application of a minimum and a medium filter. D) The PBR was significantly increased in SM ($p<0.0001$) as well as in UM ($p=0.04$) when compared to healthy controls. All data points represent one individual. Data are summarized as median (for average speed mean is shown) and error bars show 95% confidence intervals. ***: p value <0.001, **: p value <0.01 and >0.001, *: p value <0.05 and >0.01.

Figure 3. Glycocalyx components are shed and detected at an increased level in the plasma of patients with SM. A) Plasma HA increased significantly in SM ($p<0.0005$) but not in patients with UM. B) Sulfated GAGs in plasma increased significantly in patients with SM ($p=0.01$) when compared with healthy controls and levels in SM were increased when compared with UM ($p=0.02$). C) Plasma HS increased significantly in SM ($p<0.0001$) and also in UM ($p=0.003$). Plasma levels were comparable in UM and SM. D) Compared with healthy subjects, plasma syndecan-1 increased significantly in SM ($p<0.0001$). Plasma levels were comparable in UM and SM. E) Glypican-1 increased significantly in SM ($p=0.02$) and in NMF ($p=0.02$). Plasma levels were comparable in UM and SM. All data points represent one individual. Data are summarized as median and error bars show 95% confidence intervals. ***: p value <0.001; **: p value <0.01 and >0.001, *: p value <0.05 and >0.01.

Figure 4. Some glycocalyx components in the plasma are particularly high in patients with low BCS. A) Plasma HA correlated positively with PBR ($p<0.0001$). B) Plasma HA was significantly increased in plasma from SM individuals with low BCS compared with patients without ($p=0.01$). C) Plasma HS was significantly increased in plasma from SM individuals with low BCS compared with patients without ($p=0.005$). All data points represent one individual. Data are summarized as median and error bars show 95% confidence intervals. ***: p value <0.001; **: p value <0.01 and >0.001, *: p value <0.05 and >0.01.

Figure 5. Plasma markers show marked endothelial dysfunction and associations with impaired microcirculation. A) Plasma angiopoietin-1 decreased significantly in SM ($p=0.0002$). No change was seen for UM and NMF. B) Angiopoietin-2 levels increased significantly in SM ($p<0.0001$). C) Plasma endothelin-1 was increased, in SM ($p=0.002$) and in UM ($p=0.02$). D) Plasma thrombomodulin was significantly increased in SM ($p=0.002$). Plasma levels for all four markers were comparable in UM and SM. E). Plasma

angiopoietin-1 correlated negatively with the frequency of hemorrhages detected in fields of views analyzed (rho=-0.36, p=0.02). F) Plasma thrombomodulin was positively correlated with PBR (rho=0.38, p=0.02). All data points represent one individual. Data are summarized as median and error bars show 95% confidence intervals. ***: p value <0.001; **: p value <0.01 and >0.001, *: p value <0.05 and >0.01.

Figure 6. Glycocalyx components are increased in plasma for several weeks after admission. A) PBR persisted to be increased but decreased to levels comparable to healthy controls at day 28 post admission. The bold line shows the linear regression of the data points. B) Plasma HA decreased significantly at day 28 post admission (p=0.02), while no change compared today 0 was noted at day 14 post admission (p=0.4). Also, in UM, a time dependent decrease was seen (p=0.03, day 0 vs day 28). C) Plasma syndecan-1 was significantly reduced at day 28 in SM patients (p=0.03). D) Plasma angiopoietin-1 levels did not change significantly during 28 days post admission. E) Plasma levels of angiopoietin-2 decreased but not significantly compared with levels at admission. F) Plasma E-selectin levels were reduced back to normal levels at day 28 post admission (p=0.03). All data points represent one individual. Data are summarized as median and error bars show 95% confidence intervals. ***: p value <0.001; **: p value <0.01 and >0.001, *: p value <0.05 and >0.01.

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