Author’s response to reviews

Title: Microvascular obstruction and endothelial activation are independently associated with the clinical manifestations of severe falciparum malaria in adults.

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Author’s response to reviews: see over
26 April 2015

To The Editor,

Thank you for giving us the opportunity to reply to the reviewers’ comments on our manuscript “Microvascular obstruction and endothelial activation contribute independently to the pathophysiology of severe falciparum malaria in adults” (MS: 2007987475163109).

We thank the reviewers for the time that they have taken to evaluate the manuscript and to offer such thoughtful and constructive comments. We feel that our revised manuscript – which has been modified to address the reviewers’ concerns – has improved as a result. Please find below our responses to their comments. For clarity the reviewers’ comments are presented in grey italics, while our responses are in normal black text in Calibri font. When we present our modified text we use bold, black Times New Roman text, underlining any modifications to the text. The title has also been changed to reflect the reviewers’ comments. This has been updated at biomedcentral.com

We have also endeavoured to highlight the novel findings in the paper by rewriting the discussion and emphasising the fact that this is the first time that microvascular obstruction in vivo has been shown to correlate with subsequent death. It is also the first time that microvascular obstruction has been linked to multi-organ dysfunction and acute kidney injury in life. We emphasise in the introduction that this is the first time that microvascular obstruction and endothelial dysfunction have been assessed concurrently in patients. Our findings potentially unify two schools of thought – that have sometime disagreed vehemently - about the pathophysiology of the disease.

If you have any additional queries, please do not hesitate to contact me.

Yours sincerely

Josh Hanson
On behalf of all the authors
Reviewer 1: Malcolm Edward Molyneux

Reviewer’s report:

Major comments for attention

1. The paper carries a declamatory title (“Microvascular obstruction and endothelial activation contribute independently to the pathophysiology...”). The title should be less emphatic, because as written it does not accurately reflect the findings reported: the word ‘contribute’ implies a causal role, where only associations or correlations have been shown; and independence is deduced from statistical relationships between microvascular obstruction and circulating plasma concentrations of Ang-2 and lactate, all of which, as surrogates of the mechanisms they are representing, are imprecise, especially when based on a single point in time. (In relation to microvascular obstruction, the authors mention on p.12 ‘the marked heterogeneity that is seen in the distribution of sequestration in different organs of the body’). Unlike the title, the text (p.11) is appropriately tentative: ‘this finding is consistent with the hypothesis that the two processes make a separate contribution to disease severity’. The same considerations apply to the last sentence of the abstract. The first sentence of the Discussion also makes greater claims than are warranted by the data from this study – the paper reports associations, not a ‘role’, in pathogenesis, and in the absence of control groups (non-malarial severe disease; non-severe malaria), the data do not demonstrate that the findings are peculiar to either malaria or its severe forms.

Response: Professor Molyneux is absolutely correct in his assertion that we have only demonstrated an association between the clinical manifestations of severe malaria and our measures of endothelial activation and microvascular obstruction. While our findings are consistent with the dominant prevailing theory of pathogenesis, they fall short of proving causation. We have therefore modified the title of the manuscript to the less declamatory:

“Microvascular obstruction and endothelial activation are independently associated with the clinical manifestations of severe falciparum malaria in adults.”

The last sentence of the abstract has also been modified in response to Professor Molyneux’s comments:

“Microvascular obstruction and systemic endothelial activation are independently associated with plasma lactate, the strongest predictor of death in adults with falciparum malaria. This supports the hypothesis that the two processes make an independent contribution to the pathogenesis and clinical manifestations of the disease.”

We feel that we have been appropriately circumspect in the first sentence of the Discussion where we emphasise that these data only provide further support for the fundamental role of microvascular obstruction in the pathogenesis of disease. We have toned the declarative language down a little however in response to Professor Molyneux’s comments by adding the phrase “for the belief”:

“This detailed clinical study provides further support for the belief that microvascular obstruction has a fundamental role in the pathogenesis of severe falciparum malaria.”
The data that we present builds on over a century of clinico-pathological correlation and are consistent with the hypothesis that microvascular obstruction – the pathological signature of falciparum malaria – plays a causal role in the pathogenesis of the unique clinical presentation of the disease.

There is no mention in this paper of the timing of the recordings of rectal microvascular blood flow in relation to the start of either supportive therapy (oxygen, fluids, blood transfusion, anticonvulsant drugs etc) or specific antiparasitic drugs. These measures might affect capillary perfusion. Nine patients were treated with quinine, a drug known to have effects on circulation – the timing of the OPS-recordings may be particularly important in these patients. An exclusion criterion was: having ‘received parenteral antimalarial treatment for >24h before enrolment’; this suggests that some patients may have been receiving such treatment for up to 24h before the OPS recordings – how many patients, and which parenteral drugs?

Response: Professor Molyneux is correct to note that we have not included the timing of OPS measurement in the methods; we have therefore amended the text:

“Video recordings of the microcirculation in the rectal mucosa were collected at the time of study enrolment, with an OPS device (either Cytosc an® from Cytometrics or Microscan® from Microvision Medical), which was applied gently to prevent pressure artefact.”

We have changed the wording in our discussion to be more specific about the OPS findings in severe malaria which is quite different to that seen in bacterial sepsis:

“In contrast, in severe falciparum malaria the striking finding is one of numerous persistently obstructed capillaries, an appearance which exactly replicates the histopathological findings at post-mortem examination.”

Professor Molyneux suggests that “oxygen, fluids, blood transfusion, anticonvulsant drugs etc” might be responsible for the OPS changes that we have seen in this study, however all these supportive therapies are routinely administered to patients with bacterial sepsis and the OPS findings in that population are quite different to those that are seen in falciparum malaria. Rather than seeing a persistently obstructed microcirculation in patients with bacterial sepsis, instead “In patients with bacterial sepsis there is reduced capillary density and intermittent or absent perfusion in vessels of all sizes, changes which are reversible with the application of acetylcholine”

Furthermore the microvascular obstruction seen in patients with severe malaria in vivo exactly replicate the findings seen in post-mortem cases at a time when oxygen, fluids and blood transfusion no longer have any influence.

Whilst quinine does have some cardiovascular effects it would not be expected to induce the findings that we have seen in this study. At a microvascular level quinine has an alpha-blocking effect which, if anything, would result in vasodilation and increased blood flow rather than microvascular obstruction (Bateman D & Dyson EH (1986) Quinine toxicity. Adv Drug React Ac Pois Rev 5: 215-233).

Examination of the data does not suggest a role of the anti-malarial therapy on the OPS appearances that we have seen. There were 32 (22.5%) patients who received parenteral anti-malarial therapy prior to study enrolment, 25 received quinine and 7 received artesunate. Patients who had received
parenteral therapy prior to enrolment had a median (IQR) 9.1% (2.1-25.7%) of capillaries blocked compared with 13.3% (3.3-26.6%) (p=0.73) in patients who had not received prior therapy. There was similarly no difference between the patients receiving quinine (10.0% (2.5-45.7%)) and those receiving artesunate (6.6% (1.7-14.9%)) (p=0.34).

(II) It would be helpful to know whether rectal capillaries in the malaria patients would sometimes open and close variably (as described in sepsis) – or when a vessel was ‘blocked’, was it continuously obstructed? The latter situation would particularly support the idea that the impaired flow is due to mechanical obstruction – eg by sequestered parasites – (although sequestered parasites may roll on, causing intermittent obstruction). It may be useful, to readers not familiar with this field, to point out that histopathology does not provide information about vessel blood flow, but only about what vessels contain – ‘blockage’ is a deduction, not an observation. A particular value of this study is that non-flow of blood is directly observed, and during life.

Response: As we mentioned in the previous response we have adjusted the wording of the discussion to address this point more specifically.

“In contrast, in severe falciparum malaria there are no changes in larger vessels; instead the striking finding is one of numerous persistently obstructed capillaries.”

We have also adjusted the text of the discussion to emphasise Professor Molyneux’s well-made point that one of the strengths of the study is the fact that the microvasculature can be observed during life with OPS imaging.

In response to Professor Molyneux’s comment that “It may be useful, to readers not familiar with this field, to point out that histopathology does not provide information about vessel blood flow” we do, to be fair, note that these histopathological changes are noted at post-mortem examination, which should alert the reader to the fact that blood flow is not expected!

(III) In the first group of patients, the video recordings of rectal vessels were read by 2 readers, and in the second group by 3. In the third (the largest) group the recordings were analyzed by only one reader. Is there a reason for this? How consistent were the various readers in their interpretations of the first two groups of patients? – were they so consistent that 2nd/3rd readings were considered unnecessary thereafter? On p8 para 2, and p 10 para 1, controlling for ‘inter-rater variability’ is mentioned: how was this possible in the largest group for whom there was only one reader?

Response: When we use the term inter-rater variability we are referring to the fact that the OPS videos were analysed on 3 separate occasions (2006, 2009 & 2013) with 2 readers contributing to the values from 2006. We outline when and why different examiners analysed the OPS recordings in the methods:

“Assessment was performed on three occasions: two reviewers (JH & PC) separately analyzed the recordings from the first group blinded to patient details and outcome in 2006. In this group of patients a mean of 50 (95% confidence interval 37-62) capillaries were assessed. In 2009 the recordings from second group were analysed by the clinician (JH) who...
had managed the patients a year previously (although patients were identified only by a patient code and the results were validated independently by blinded reviewers (RJM and HK) [15]). In 2013 the third group was analyzed by a single reviewer (JH) blinded to patient details and outcome.”

The variation in the number of readers on each of the 3 occasions is more an accident of history than anything else. In 2006 the video recordings were read by JH & PC who were blinded to patient details and outcome. Two readers were used on this occasion as neither had performed the analysis before and it was therefore anticipated that the measuring would be more laborious as they learned the technique. The measurements of the two readers were not compared.

In 2009 the video recordings were read by JH alone. On this occasion there was no “learning curve” to consider, but as he had managed the patients the year before there was the potential for him not to be completely blinded to patient details and outcome (although each of the 28 patients in this study had an individual study code which would have made recollection of individual patient characteristics effectively impossible). Accordingly the OPS films were also assessed separately by RJM and HWFK (who had no knowledge of the patient details or coding) to ensure a lack of bias using a semi-quantitative approach as outlined in the methods in reference 15. Two readers were used to check the values in the interests of time. There was a good correlation between JH’s values and the values of RJM & HWFK (r =0.48, p=0.03).

In 2013 the video recordings were read by JH alone who had not managed the patients and was blinded to their details and outcome. As these readings were completely blinded there was no reason for the readings to be verified. Only JH read these OPS films because by this stage he was able to perform the analysis efficiently. Also during this period JH was completing a PhD also, had no clinical commitments and so had more time to perform the somewhat laborious analysis process!

More succinctly: In 2006 the values were recorded by JH & PC (both blinded). In 2009 the values were recorded by JH alone (semi-blinded) (checked by RJM & HWFK to exclude bias). In 2013 the values were recorded by JH (blinded).

The values recorded on the three separate occasions are similar:
2006 (measurements by JH & PC): median (IQR) % of obstructed capillaries: 8.3 (0-14.9)
2009 (measurements by JH alone): median (IQR) % of obstructed capillaries: 14.1 (1.7-32.0)
2013 (measurements by JH alone): median (IQR) % of obstructed capillaries: 13.3 (5.8-33.2)

However we adjust for the potential unobserved heterogeneity that reading the OPS films on 3 separate occasions may introduce – particularly the fact that PC read some of the OPS films in 2006 - by using a logistic regression model that was stratified by year and reader (random effects model) as we note in the statistics section.

(iv) More detail on the characteristics of the patients’ severe malaria syndromes would help us to understand the studied cases better. How many of the 52 patients with a single criterion of severity had each defining syndrome? And among the 90 with multi-organ dysfunction, how many had which combinations of organs affected? Was there any explanation for the remarkable disparity of case-fatality rates in the different study years (8.3%-60%)?
Response: In the case of the patients satisfying with multi-organ involvement the combinations of the various criteria are numerous and the resulting table presenting this data is somewhat unwieldy. The characteristics of the patients are presented in the manuscript in table 1 and we believe these data offer a reasonable “snapshot” of the patients. We’re not sure that the mix of the different syndromes would assist reader understanding, however if the editorial staff feel that it would be helpful to present the data in the form that Professor Molyneux suggests we are happy to do so. These data are presented here:

Of the 52 with a single criterion:

<table>
<thead>
<tr>
<th>Severity criterion</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coma</td>
<td>27</td>
</tr>
<tr>
<td>Acidosis</td>
<td>11</td>
</tr>
<tr>
<td>ARF</td>
<td>8</td>
</tr>
<tr>
<td>Seizures without coma</td>
<td>2</td>
</tr>
<tr>
<td>APO</td>
<td>1</td>
</tr>
<tr>
<td>Bleeding</td>
<td>1</td>
</tr>
<tr>
<td>Anaemia</td>
<td>1</td>
</tr>
<tr>
<td>Hyperparasitaemia alone</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>52</strong></td>
</tr>
</tbody>
</table>

(APO: acute pulmonary oedema, ARF: acute renal failure)

In the 90 patients with multi-organ involvement, 69 had acidosis, 64 had coma, 28 had jaundice, 27 had Acute Kidney Injury, 10 had anaemia, 7 had shock, 5 had acute pulmonary oedema, 4 had bleeding and 3 had seizures without coma.

<table>
<thead>
<tr>
<th>Severity criteria</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coma/seizures + Acidosis</td>
<td>32</td>
</tr>
<tr>
<td>Acidosis + ARF</td>
<td>9</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis + Jaundice</td>
<td>7</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis + ARF + Hypoglycaemia + Jaundice</td>
<td>3</td>
</tr>
<tr>
<td>Acidosis + Anaemia + ARF + Jaundice</td>
<td>3</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis + ARF</td>
<td>3</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis</td>
<td>3</td>
</tr>
<tr>
<td>Acidosis + Jaundice</td>
<td>3</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis + Hypoglycaemia</td>
<td>2</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis + Shock</td>
<td>2</td>
</tr>
<tr>
<td>Coma/seizures + Shock</td>
<td>2</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis + Anaemia + Hypoglycaemia + Jaundice</td>
<td>1</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis + Anaemia + ARF + Jaundice</td>
<td>1</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis + Jaundice + Bleeding</td>
<td>1</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis + Hypoglycaemia + Jaundice</td>
<td>1</td>
</tr>
<tr>
<td>Coma/seizures + Acidosis + Shock + Jaundice</td>
<td>1</td>
</tr>
</tbody>
</table>
It is true that there was a notable variation in mortality by study year. This is largely explained by variation in disease severity year by year – as we note in the results - but also by the fact that there was variable availability of renal replacement therapy, mechanical ventilation year by year (as we point out in the methods).

However we have now stated this more explicitly by adding:

“Death also varied by study year (range 8.3% - 60%, p=0.02, degrees of freedom=8). The highest case fatality rates occurred in the first two study years (60% and 52.3% respectively) when few patients had access to RRT and mechanical ventilation. In the ensuing years, when this support was more accessible, the median (IQR) case fatality rate was 27.9% (12.8-36.1). However, using a stepwise model, admission plasma lactate was identified as the strongest predictor of outcome (adjusted odds ratio: 1.39, 95% CI 1.15 – 1.68). When controlled for disease severity (plasma lactate on admission), the association between death and multi-organ involvement and death and study year was no longer significant (p=0.07 and p=0.06, respectively).”

Minor points for attention.

(i) ‘Malaria transmission is seasonal at both sites.’ Still it would be helpful to know if there are any data from these sites as to the prevalence and densities of asymptomatic or ‘incidental’ parasitaemia in the population of the same age range and time of year as the patients. This could strengthen the evidence for P falciparum being the causative agent of the illnesses in the studied patients – all severe malaria syndromes can be mimicked by non-malarial diseases such as sepsis, encephalitides, etc.
Response: Unfortunately we do not have parasitaemia data in asymptomatic people in this population. In locations with similar transmission it has indeed been noted that the proportion of asymptomatic parasite carriers is much larger than previously assumed, despite the low endemicity and resulting low population immunity against the parasite. However, the level of parasitemia observed in these asymptomatic individuals was only discovered with the introduction of an ultrasensitive qPCR technique that had a lower limit of detection of 20 parasites per millilitre. In this study the median (IQR) parasite count was 67133 (20253-274499) per microlitre. Therefore, we think that it is unlikely that the high parasitaemia in these patients with (a strict and prospectively defined) diagnosis of severe malaria is coincidental. This hypothesis is also supported by the very low proportion of positive blood cultures reported in Asian adult patients with severe falciparum malaria; a situation is quite different to the relatively high rate of concomitant bacteraemia in paediatric severe malaria in moderate to high endemic settings in Africa.

(ii) page 5 line 6: ‘...coma and seizures and acidosis and hyperlactatemia were classified as a single criterion;...’. This may be confusing to readers, especially if their software fails to show italics. Better may be: ‘...coma and seizures were classified as a single criterion, as were acidosis and hyperlactatemia;...’

Response: We agree. The text has been adjusted accordingly.

(iii) In p8 para 3 and the next paragraph, for some of the comparatives listed the comparator is not stated and is not obvious: for example ‘Admission plasma Ang-2 concentrations were significantly lower in patients with coma on admission’ – does this mean ‘than in controls’ or ‘than in patients without coma’? Please note that there are several other instances like this that need clarification.

Response: The text has been adjusted to highlight the comparator.

“Admission plasma Ang-2 concentrations were significantly lower in the patients with coma on admission than in the non-comatose patients (p=0.002).”

(iv) p10 last sentence before Discussion: ‘Plasma Ang-2 had a stronger correlation with plasma lactate than [***] parasite biomass...’. It would clarify your meaning to add, at [***], either ‘with’ or ‘did’, to indicate which correlations you are referring to.

Response: this is a well-made point. We have adjusted the text accordingly by adding “did” as Professor Molyneux recommends.

(v) p11 para 2 ‘...numerous obstructed capillaries, an appearance which exactly replicates the histopathological findings from post-mortem cases’ – most of the quoted references and other studies show at least as much sequestration in small venules as in capillaries. It would be interesting if the authors could mention whether venular flow is ever impaired when capillary flow appears normal, in the rectal vasculature.

Response: The technique that we used to examine blood flow in the rectal mucosa dictated that we only systematically assessed capillaries. The vessels in the rectal mucosa form a hexagonal pattern of capillaries that surround the crypts at the centre. When assessing the patient we only recorded video images when we saw this hexagonal appearance. This way we were able to ensure that we were performing a standardized assessment that could be reliably replicated over several years and by different study staff. Larger vessels do cross the visualised field in the OPS recordings - in a somewhat random manner - and no obstruction is seen within them. However as venules lack an
anatomical landmark to confirm their identity it is difficult to systematically record and quantify blood flow within them using the OPS technique we present in this paper. We therefore cannot offer a definitive comment on venular flow, but agree that it would be an interesting point for future investigation. We have adjusted the text of our discussion to address the fact that “larger vessels” (which include venules) were not formally assessed in this study, by deleting the phrase “there are no changes in larger vessels”.

(vi) p 14 last para: ‘The inverse association between parasite biomass is hypothesized to result...’
There are obviously some words missing after ‘biomass’
Response: We thank Professor Molyneux for noting this typographical oversight! We have added the missing words:
“The inverse association between parasite biomass and VEGF is hypothesized to result from the absorption and accumulation of host VEGF within the parasitophorous vacuole by mature P. falciparum parasites, sequestered within the microvasculature”

(vii) p14 last sentence. Do you mean that deficiency of VEGF could provide another mechanism?
Response: Yes. We have stated this more explicitly in revised text:
“VEGF stimulates the release of NO and upregulates the expression of nitric oxide synthase, thus the decreased VEGF concentrations provide another mechanism for decreased NO bioavailability.”

(viii) In all the figures it would be helpful to have the number of tests (n=) above or below each column of data pictured.
Response: This is a question of style as is the suggestion from reviewer 3 that the OPS graphs and Ang-2 graphs should be combined into 2 panels. We would be happy to present the graphs in any way that the Editorial staff feel improve their readability.

(ix) In 43 patients drawn from the previous study (ref 14), a mean of 50 vessels had been assessed per field for the original publication, but the criteria for the present study were that 20 should be assessed per field – which denominator was used for those patients in this report?
Response: Professor Molyneux is right to point out that the methods section here is somewhat confusing. All patients in the study had video recording at three sites in the rectal mucosa. In the first group a mean of 50 vessels in total were assessed. In the second and third groups the protocol dictated that each of the three sites would have 20 capillaries’ flow measured and so each of the patients in these latter two groups had 60 capillaries analysed. We have re-written this section it to hopefully make it clearer.

Minor point for discretionary consideration
(i) P11 line 4: ‘...relatively underpowered to assess its relative contribution...’ Relative to what in each case?
Response: Professor Molyneux is right to point out that our use of language here is a little loose. We have rewritten this sentence accordingly:
“This larger series was able to demonstrate an association between the degree of microvascular obstruction and death in univariate analysis although it was still underpowered to confirm the association in multivariable analysis.”
Reviewer's report:

The paper by Hanson et al describes the relationship of a range of clinical parameters, including microvascular obstruction and endothelial activation, with severe malaria in adults. The study is well-designed and has been described clearly and accurately. Their main findings are that this study supports previous work (with smaller clinical groups) showing independent associations between vessel blockage and endothelial inflammation with disease, but no link between these observations. The discussion provides a good balance with the rigour that an extensive and detailed study such as this provides but indicating potential weaknesses in the arguments.

I have only a few comments (which should be seen as discretionary revisions) to add to the paper:

1. There are a couple of issues in claiming that microvascular obstruction and endothelial activation are independent variables; (i) people may read the title of the paper as meaning that these two phenotypes are not linked at all. I do not believe that the authors meant to imply this and indeed they say that endothelial activation could contribute to vessel blockage, for example, through upregulation of PRBC adhesion receptors; (ii) the measurements made for microvascular blockage and endothelial activation are different in character, with the former being a localized phenomenon (i.e. what is actually happening in the vessel itself) and the latter being a measurement of a systemic condition rather than localized endothelial activation. Measuring plasma biomarkers of activation is understandable as although it is possible to investigate endothelial cell activation, this is far from easy and requires significant intervention to obtain samples (rather than just taking a blood sample). I do not think that this detracts from the overall message of the paper but it should be flagged to remove any confusion.

Response: We agree with Professor Craig's comments and he is right to say that we do not feel that the two phenotypes are not linked at all. As he points out we spend some time describing how the two processes might influence one another in the discussion.

Fundamentally the lack of a statistical association between the OPS measurements and the Ang-2 values means that it is not valid - at least in our dataset - to say that they are linked but Professor Craig is right to say that our approach in assessing such an interaction is imperfect.

We have therefore modified the text in our discussion accordingly to more explicitly state the limitations of our analysis, incorporating some of Professor Craig’s suggestions:

“It is clear to see how this might evolve into a vicious cycle, but in this series plasma Ang-2 concentrations did not correlate strongly with the visualized obstruction. This suggests that there is either a temporal dissociation between Ang-2 release and microvascular sequestration, that elevated Ang-2 concentrations are not the result of a simple, direct interaction between pRBCs and the endothelium, or that our statistical analysis is limited by the fact that we are comparing a local phenomenon (directly visualized microvascular appearances) with a systemic one (circulating plasma Ang-2 levels).”

2. The authors chose to record “severe malaria” as a single variable but this covers a fairly broad spectrum of pathology, which may not have a single aetiology. The balance between having enough numbers to drive ‘significance’ of a study against the need to reduce noise and favour
‘specificity’ is a difficult one and, as above, I do not disagree with the approach taken by the authors. I would only note, for the unwary, that severe malaria is not the same as cerebral malaria. This is done in detail in the methods section where the clinical definitions are well-described but readers may skip this section.

**Response:** Professor Craig’s point is well made. Too often in the literature the terms severe malaria and cerebral malaria are used interchangeably. In clinical practice this can result in physicians underappreciating the significance of other manifestations of the disease, particularly the metabolic acidosis and renal impairment that are independent predictors of outcome. However, as Professor Craig acknowledges, we do take some time to define “severe malaria” in the methods and the figures also emphasise the association between microvascular obstruction and Ang-2 concentrations and lactic acidosis, acute kidney injury and multi-organ dysfunction. To be fair, it is difficult to write for the reader who skips sections!

3. Microvascular obstruction in the rectal vessels may not reflect the situation in another organ, such as the brain. Of note are two major differences in the microvascular endothelia of the gut and brain, with the latter lacking Weibel-Palade Bodies (which may affect Ang-2 release) and CD36 expression. In some studies PRBC adhesion to CD36 has been linked to uncomplicated malaria and it is possible that gut provides a ‘reservoir’ for cytoadherence that is less pathogenic than in other organs (although this is just speculation). The finding that microvascular obstruction, presumably due to sequestration, in the rectal mucosa is associated with severe malaria suggests that these endothelial compartments share at least some properties in terms of pathology.

**Response:** We agree with Professor Craig on this point and make a similar observation in the discussion

“This presumably reflects the marked heterogeneity that is seen in the distribution of sequestration in different organs of the body [4], different parts of the brain [30, 31] and even in different vessels from the same area of the brain [31].”

However an extended discussion of the different endothelial receptors in different organs is probably beyond the scope of this article.

4. Is an increase in white cell count (Table 1) associated with death in severe malaria commonly seen? A comment on this finding would be useful, even if only to say that the preliminary significant difference was lost on further analysis.

**Response:** This is true. However we have presented this data (incorporated into a larger data set) - and discussed it in some depth - in our manuscript, “The clinical implications of thrombocytopenia in adults with severe falciparum malaria; a retrospective analysis”, which was published in Biomed Central Medicine on April 24th.

5. I am not a statistician so it is with some trepidation that I comment on this area. The multivariable regression analysis does not find a significant (p < 0.05) association between microvascular obstruction and Ang-2 concentration (a marker of endothelial activation) but with a p-value = 0.06 this does fall into a ‘zone’ that some statisticians define as “equivocal” or “borderline significance” (usually when p < 0.1 but > 0.05). So, along similar lines from my
comment above, I wonder whether the message from the paper that these two properties are not associated is portrayed a bit too strongly.

Response: We agree with Professor Craig that this can be a grey area. It is likely that the sophisticated readership of Biomed Central Medicine is likely to appreciate that this lack of strictly defined statistical significance is likely to be due at least partly to a type II error (the result of the small sample size). In a larger dataset the correlation may have become statistically significant, although the spearman’s rho for the correlation is only 0.17 suggesting that even if a correlation were present in a larger dataset it would be a relatively weak one.

We do “hedge our bets” a little by writing:

“There was no statistically significant association between microvascular obstruction and plasma Ang-2 concentrations (r_s=0.17, p=0.057)”

However we agree that we may have portrayed the lack of association a little too strongly. Accordingly we have reworded our discussion:

“Plasma Ang-2 concentrations did not correlate strongly with the visualized obstruction which suggests that there is either a temporal dissociation between Ang-2 release and microvascular sequestration, that…”

We also applied the strict 0.05 cut-off to the logistic regression where outcome was the dependent variable and as a result we were similarly unable to link both microvascular obstruction and endothelial activation to outcome:

“When combined in a logistic regression model with random effects for study year and inter-rater variability, neither microvascular obstruction nor ang-2 concentration were significantly associated with outcome (p=0.06 for both, n=126).”

So we have lived and died by the same sword!

We note the statistical review did not raise any objections to our use of this terminology.

Reviewer 3: Xianghua Luo

Reviewer’s report:

This review only focuses on the statistical methods and interpretation of statistical results in the paper.

Major Compulsory Revisions: 1. There are different stepwise variable selection procedures such as backward elimination, forward selection, and combination of backward elimination and forward selection available in statistical software. It is not clear which specific procedure was used in this paper. It is not clear what cut-off p value value(s) was used in the stepwise procedure. It is also not clear what specific variables were candidate variables in the stepwise regression, which need to be presented clearly in the Results section. Actually, even though the stepwise approach was mentioned
in the Statistics section, it does not seem that the stepwise regression result was presented anywhere in the Results section.

Response: The model used a backward stepwise estimation (and the results were confirmed using a forward stepwise approach), with variables at P < 0.05 retained in the final model. All variables from Table 1 that were significant were included in the initial model.

The wording in the methods has now been modified to clarify which procedure and cut-off p-value was used and Table 1 is referenced to inform the reader which variables were considered for inclusion:

“The strongest predictor of outcome was identified using backward stepwise estimation in a logistic regression model with random effects for study year. All risk factors that were significant in the univariate analysis at p < 0.05 were included in the initial model (Table 1). Only variables that were significant at p < 0.05 were retained in the final model.”

The result from this analysis was specified in the middle of the first paragraph of the results: “The strongest predictor of outcome in the study was plasma lactate on admission (adjusted odds ratio: 1.39, 95% CI 1.15 – 1.68).”

This has now been changed to help the reader understand that this was from the stepwise regression:

“Using a stepwise model, admission plasma lactate was identified as the strongest predictor of outcome (adjusted odds ratio: 1.39, 95% CI 1.15 – 1.68).”

We have also changed the title of Table 1 to “Baseline characteristics of the patients and association with outcome” to highlight the fact that we have used these data to determine the factors most linked to outcome.

2. Page 8, lines 13-14: It says that “The relationship [between percentage of obstructed capillaries and death] remained significant when controlled for inter-rater variability and study year.” Please provide the adjusted OR ratio for death and p value.

Response: These data have been presented as requested:

“The relationship remained significant when controlled for inter-rater variability and study year (AOR 1.03 (95% CI 1.00 to 1.05), p = 0.027).”

3. In both the Abstract and the Discussion section, the authors claimed that microvascular obstruction and systemic endothelial activation have potentially “synergistic” effects. However, the analysis result did not support this conclusion because the interaction term of the two factors was not found significant in the multivariable model for death (lines 4-6 in page 10).

Response: Associate Professor Luo is absolutely correct. We have removed the word synergistic from the text entirely.
Minor Essential Revisions:

1. The authors use “outcome” and “death” exchangeably in the manuscript (e.g., line 10 in page 7, line 1 in page 8, and line 4 in page 10, to name just a few). This can be misleading because there are other outcomes, e.g. disease severity, also presented in the paper.

**Response:** Associate Professor Luo is correct to note that the word “outcome” has a precise meaning in the field of statistics. However we were at pains to only use it in relation to death in the paper. None of the other variables (such as disease severity) are ever described as outcome. The other two reviewers – who have less of a statistical background - do not express concern about our use of the word in the manuscript, however we have endeavoured to replace “outcome” with “death” as frequently as the readability of the text allows.

2. Can a table be provided to summarize the difference (or no difference) between malaria cases and healthy controls?

**Response:** Unfortunately only measures of endothelial activation (Ang-2, ADMA, L-arginine and VEGF) were collected in the controls, no other measures (clinical or demographic) so there would be no data to present in such a table beyond those that are presented in the text. The requested table would therefore add little to the paper.

3. Figures 2 – 6 can be rearranged into 2 multi-panel figures with one devoted to % of obstructed capillaries only and one to Ang-2.

**Response:** Associate Professor Luo is right to suggest that the figures could be rearranged into 2 multi-panel figures however, as with Professor Molyneux’s suggestion about the graph layouts, this is largely a question of style. We agree that it would be good to have all the graphs on a single panel, but when we try to prepare the graphs in the manner in which Associate Professor Luo suggests the x-axis is quite cramped and difficult to read. We would be happy to present the graphs in any format that the Editorial staff feel would make them easiest to understand and in keeping with the layout of the Journal.