PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Identification of Plasmodium falciparum and host factors associated with cerebral malaria. Description of the protocol for a prospective, case-control study in Benin (NeuroCM)
AUTHORS	Joste, Valentin; Maurice, Laurine; Bertin, Gwladys; Aubouy, Agnès; Boumédiène, Farid; Houzé, Sandrine; Daniel Ajzenberg, Daniel Ajzenberg; Argy, Nicolas; Massougbodji, Achille; Dossou-Dagba, Ida; Alao, Jules; Cot, Michel; Deloron, Philippe; Faucher, Jean-François

VERSION 1 – REVIEW

REVIEWER	Dr. Michael Eisenhut Luton&Dunstable University Hospital NHS Foundation Trust, United Kingdom
REVIEW RETURNED	07-Nov-2018

GENERAL COMMENTS	The authors need to explain what they mean by “disease report” in sentence 2 of the abstract. In page 5 of 25 line 55 the authors state they want to identify “remedial factors”. This cannot be achieved with this study design as it would require a case control study comparing patients with cerebral malaria who survive and who die and in large numbers Page 7 of 25, line 96: Please rephrase the sentence starting “Diagnostic of (…) to “Cerebral malaria could therefore be overdiagnosed” Page 8 of 25: The paragraphs on drug and vector resistance are redundant. Page 9 of 25: The authors have not included a discussion of confirmed hypothesis that inflammation induced cerebral vasospasm is a key event in the pathophysiology of cerebral malaria (See Eisenhut M. The evidence of vasospasm in the pathogenesis of cerebral malaria. Malaria Journal 2015;14:405) The authors need to include a comparison of survivors and non-survivors of cerebral malaria and include and discuss the role of nitric oxide metabolites and free haemoglobin and RBC microparticles as a nitric oxide scavenger which needs to be analysed as nitric oxide depletion is a key event due to it causing predisposition to cerebral vasospasm. The study authors need to investigate the role of haemoxygenase-1 (HO-1) in cerebral malaria and cerebral malaria outcome. This is done by measurement of ferritin, bilirubin and iron in the CSF of patients who had a lumbar puncture because those substances are the end product of haem metabolism by HO-1.

REVIEWER	Clarissa Valim Michigan State University

REVIEW RETURNED	05-Dec-2018

GENERAL COMMENTS	The study by Valentin et al. proposes and very comprehensive approach to address a very important topic. I stated below some of my recommendations to strengthen the manuscript. MAJOR REVISIONS 1 – Could the authors include in the abstract the length of follow-up time of the three groups the the time of visits? 2 – The authors may want to clarify which children will be screened, i.e., they clearly stated that children will undergo consent and if they consent, the inclusion criteria will be assessed. However, it is unclear what will be the criteria used to decide the children in which they will seek (or sought) for consent. 3 – One of the inclusion criteria for the three groups is based on RDT and exclusion criteria for all three groups is missing a malaria blood smear result. Positivity to microscopy is included in the definition of the non-malarial coma and uncomplicated malaria. However, it was not clearly stated in lines 223-225 that positivity to blood smear (and not only to RDT) will be used in the definition of cerebral malaria. The authors may want to clearly state that. 4 – In the assessment of host factors, the authors state that they will evaluate: phenotype of monocytes, redox, pro- and anti-inflammatory and pro-resolving mediators. Assessment of inflammatory mediators is a central part of their work but I could not find any description of how those inflammatory mediators will be measured or examples of the inflammatory mediators that will be measured. 5 - In lines 351-352, it is stated that children with coma will be followed to detect neurological sequelae. For how long? Could that be clarified? In line 118 it is stated that follow-up will happen for 1 month after discharge. However, when describing the research analysis, the authors mention that subjects will be followed at day 0, 3 and 30 or 21-28, depending on the analysis. Could the authors clarify all follow-up visits that will be done for all purposes in each group in the Research Analysis section? That description should include the group with uncomplicated malaria. What will be the follow-up of this group? They will not be admitted so an end of follow-up one month after discharge cannot be defined. Also, it would be important to read how the 1 month follow-up visit will (or in fact was) done, e.g. whether patients were contacted at home or they came back to the hospital… Which measures will (or were) put in place to prevent losses to follow-up after discharge? 6 - My understanding is that some of the aims are: a) Assess whether neurological sequelae occurred and if it was attributed to cerebral malaria b) Evaluate markers related to “inflammation and neurological impairment during cerebral malaria” (line 312) With regards to (a), what will be the comparison groups involved in this analysis, i.e., how will the authors determine that the sequelae were associated with malarial coma? With regards to (b), do the authors expect to have a sample size large enough for this analysis? Could they clarify how they will attain this aim? 7 – The study outcomes (features to be compared across groups) could be further detailed. For instance, there are no details about inflammatory markers or outcomes associated with phenotype of monocytes. 8 – In the Data Analysis section:

	8.1- All packages that will be used to analyze genomics and transcriptomics data are listed but not the methods that will be used. Moreover, we could not find any description of the normalization procedure that will be used but only the package that will be used to conduct normalization of expresssion data. 8.2 - – Something should be said about how missing data will be handled in the Data Analysis section. 8.3 – In lines 340 and 341, it is stated that RT-qPCR data will be analyzed through Wilcoxon and Kruskal-Wallis. The authors may want to compare the variability of transcripts between groups. Those tests are not appropriate and yield biased results when the variance across comparison groups are very different. T-tests and ANOVA of transformed outcomes may be more appropriate. 8.4 – Lines 342-348 describes how immune markers will be analyzed. Since we do not know the type of markers the authors are referring to, we cannot comment on the appropriateness of the choice of analytical methods. For instance, when those makers are predictors they may be highly correlated and a simple regression analysis would not be appropriate. 8.5 - Generally more details need to be given about the proposed analysis. For instance: a) for which outcomes they are planning to use linear or logistic regression specifically; b) will they use random intercept and slope (those are hierarchical models)? for which factors they would consider including a random slope? how will they decide whether a random slope should be included?; c) when using ANOVA, which time point will be compared across the 3 groups; d) criteria for variable selection (or defining that a marker is associated with the outcome of interest). All these details can be minimally presented in a few sentences 8.6 – It is critical to have stated the threshold for significance level and method for adjustment for multiple testing. 8.7 – Will proteomic screening for markers be done? If so, how will this data be analyzed? 9 – It would be important to see in the description of the limitations in the discussion the potential for selection bias and confounding resulting from lack of comparability across the 3 comparison groups of interest. For validity of inferences, the three groups need to be comparable with respect to every factor that could lead to the events of interest (malarial coma, non-malarial coma, and uncomplicated malaria) other than the study exposures. If patients come from different areas, their infecting parasites may be different just because they are exposed to different parasites although those parasites are not associated with the event of the group. Patients with cerebral malaria will be identified in hospitals that are likely to be referral centers from patients coming from different geographic areas. Patients with uncomplicated malaria, apparently, will be identified in a delimited geographic area. Therefore, their parasites are expected to be different therefore associated with cerebral vs. uncomplicated malaria solely because of geographical area. Would that be correct? If so, that should be included in the discussion of study limitations. MINOR REVISIONS 1 - In lines 223-227, the authors describe how cerebral malaria will be defined. It was unclear to us: a) the reason they are doing a fundoscopic examination but not using rethinopathy in their definition; b) whether they are excluding other possible causes of

	bacterial disease, e.g., would patients with history of clinical pneumonia or evidence of skin infection be excluded? 2 - In line 122 the reference after “Africa” seems mistyped. 3 – A list of samples that will be obtained is provided in lines 282-284 but not the purpose of each of those samples. 4 – We could not find evidence that the study was registered. We only found the IRB approval number. I do not believe that is a requirement for an observational study but wanted to justify why I marked no one of the questions in the standardized review form.

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1
Reviewer Name: Dr. Michael Eisenhut

Institution and Country: Luton&Dunstable University Hospital NHS Foundation Trust, United Kingdom

Please state any competing interests or state ‘None declared’: None declared

Please leave your comments for the authors below

The authors need to explain what they mean by “disease report” in sentence 2 of the abstract.

Disease report refers to consultation and hospitalization motif, as mentioned line 34.

In page 5 of 25 line 55 the authors state they want to identify “remedial factors”. This cannot be achieved with this study design as it would require a case control study comparing patients with cerebral malaria who survive and who die and in large numbers

We changed our sentence line 56.

Page 7 of 25, line 96: Please rephrase the sentence starting “Diagnostic of (…) to “Cerebral malaria could therefore be overdiagnosed”

We rephrased it lines 96 and 97.

Page 8 of 25: The paragraphs on drug and vector resistance are redundant.

We modified this paragraph lines 119 to 127.

Page 9 of 25: The authors have not included a discussion of confirmed hypothesis that inflammation induced cerebral vasospasm is a key event in the pathophysiology of cerebral malaria (See Eisenhut M. The evidence of vasospasm in the pathogenesis of cerebral malaria. Malaria Journal 2015;14:405)
The authors need to include a comparison of survivors and non-survivors of cerebral malaria and include and discuss the role of nitric oxide metabolites and free haemoglobin and RBC microparticles as a nitric oxide scavenger which needs to be analysed as nitric oxide depletion is a key event due to it causing predisposition to cerebral vasospasm.
The study authors need to investigate the role of haemoxygenase-1 (HO-1) in cerebral malaria and cerebral malaria outcome. This is done by measurement of ferritin, bilirubin and iron in the CSF of patients who had a lumbar puncture because those substances are the end product of haem metabolism by HO-1.

We agree with Reviewer 1 that vasospasm and NO metabolites are key factors in the pathogenesis of cerebral malaria. We added this aspect in the introduction, see lines 163-166 in the revised article. We also emphasized the idea of pro- and anti-inflammatory response at the end of the introduction (see line 173).
However, measuring accurately vasospasm relies on the use of specific equipment as transcranial Doppler and angiography which requires users' training. Such equipment is not available in the two hospital centers we work with in Benin. In addition, CSF samples of patients who will have a lumbar puncture is planned for bacterial culture and multiplex PCR looking for 16 possible coinfections. As you know, CSF sampling in young children is not easy and volumes will be low. That's why we haven't planned any further analyses on these CSF samples. However, we plan to measure plasmatic or urine arginine levels. We will also investigate in monocytes the mRNA expression levels of NOS2 and arginase 1, as well as HO-1, a key enzyme for detoxifying haem and for inhibiting ROS production. Levels will be compared between groups, and within the CM group between survivors and non-survivors. Such comparison was added in the text (see lines 332 and 376-378 in the revised paper).

Reviewer: 2
Reviewer Name: Clarissa Valim

Institution and Country: Michigan State University

Please state any competing interests or state 'None declared': None declared

Please leave your comments for the authors below
The study by Valentin et al. proposes and very comprehensive approach to address a very important topic. I stated below some of my recommendations to strengthen the manuscript.

MAJOR REVISIONS
1 – Could the authors include in the abstract the length of follow-up time of the three groups the the time of visits?

This information on duration of follow-up is now included in the abstract, lines 46-47.

2 – The authors may want to clarify which children will be screened, i.e., they clearly stated that children will undergo consent and if they consent, the inclusion criteria will be assessed. However, it is unclear what will be the criteria used to decide the children in which they will seek (or sought) for consent.

Thanks for that comment. We clarified which children will be screened lines 267 to 269.

3 – One of the inclusion criteria for the three groups is based on RDT and exclusion criteria for all three groups is missing a malaria blood smear result. Positivity to microscopy is included in the definition of the non-malarial coma and uncomplicated malaria. However, it was not clearly stated in lines 223-225 that positivity to blood smear (and not only to RDT) will be used in the definition of cerebral malaria. The authors may want to clearly state that.

All HIV negative children with coma were included in a first step since some of the characteristics (CSF results, blood cultures) which enabled us to classify them severe malaria or non-malarial coma were not available at admission. For children with uncomplicated malaria, screening for malaria was based on malaria RDT, not on blood smear results.

4 – In the assessment of host factors, the authors state that they will evaluate: phenotype of monocytes, redox, pro- and anti-inflammatory and pro-resolving mediators. Assessment of inflammatory mediators is a central part of their work but I could not find any description of how those inflammatory mediators will be measured or examples of the inflammatory mediators that will be measured.

As our goal was to describe mainly the protocol for patients' inclusion, we didn't detail much the research parts. However, to clarify our study, we specified in the method that monocyte phenotyping will be studied by flow cytometry (M1 and M2 markers studied will be respectively CD11b, CD16, and CD163, CD206) and RT-qPCR (gene expression levels of cytokines, chemokines and their receptors), and that mediators studied will be L-arginine and biopterin levels for redox balance, cytokines, chemokines and eicosanoids for pro- and anti-inflammatory response, and prostaglandins and lipoxins for pro-resolving mediators. See lines 329-337.

5 - In lines 351-352, it is stated that children with coma will be followed to detect neurological sequelae. For how long? Could that be clarified?

Children with coma will be followed until D21-28 post diagnostis. It is clarified line 339-340.

In line 118 it is stated that follow-up will happen for 1 month after discharge.

We modified the sentence line 118.

However, when describing the research analysis, the authors mention that subjects will be followed at day 0, 3 and 30 or 21-28, depending on the analysis. Could the authors clarify all follow-up visits that will be done for all purposes in each group in the Research Analysis section? That description should include the group with uncomplicated malaria. What will be the follow-up of this group? They will not be admitted so an end of follow-up one month after discharge cannot be defined. Also, it would be important to read how the 1 month follow-up visit will (or in fact was) done, e.g. whether patients were contacted at home or they came back to the hospital… Which measures will (or were) put in place to prevent losses to follow-up after discharge?

We add some precisions line 339 to 343. In fact, no follow-up was performed for uncomplicated falciparum malaria group. In order to prevent losses, parents/guardians are called a few days before the follow-up scheduled date to remind them the of follow-up visit.

6 - My understanding is that some of the aims are:
a) Assess whether neurological sequelae occurred and if it was attributed to cerebral malaria
b) Evaluate markers related to "inflammation and neurological impairment during cerebral malaria" (line 312)

With regards to (a), what will be the comparison groups involved in this analysis, i.e., how will the authors determine that the sequelae were associated with malarial coma?

The follow-up data are an attempt to describe in children with coma neurological disorders still observed 3 to 4 weeks after admission, assuming that the coma episode has played a role in these disorders. The study was not designed to show evidence that what was observed 3 to 4 weeks after admission is a direct consequence of the recent coma episode.

With regards to (b), do the authors expect to have a sample size large enough for this analysis? Could they clarify how they will attain this aim?

The reviewer's understanding is quite right. Of note, in the first phase of the statistical analysis, there will be more than a single assessment of neurological sequelae after comatose children's discharge. A complete study of the general characteristics and clinical factors associated with a favorable outcome (i.e. survival without sequelae) vs death will be undertaken, such as the duration between the first symptoms and the admission to the hospital, the administration of a treatment (antimalarials or antibiotics) before hospitalization, etc…
Regarding aim (b), we estimated that a sample size of 100 subjects per group was sufficient to reach the main study target, i.e. to evidence a significant difference between CM and UM groups in the ratio of endogenous mediators associated with inflammation resolution (LTB4/LXA4), by linear regression analysis involving a maximum of 6 predictors and an R2 value of 0.400, ensuring an 80% power and a 5% probability of type I error. This sample size also complies with the requirements of the RT-qPCR analysis used to validate the discrimination of CM and UM samples obtained by SARTools, and finally with the overall funding request of the project. It may also be noted that, independently from the statistical analysis requirements which are met here, the recruitment of more than 100 subjects in the CM group would require another season transmission, i.e. a supplementary year, which is inconsistent with the time limits of this project.

7 – The study outcomes (features to be compared across groups) could be further detailed. For instance, there are no details about inflammatory markers or outcomes associated with phenotype of monocytes.

Again, details have been added in the method to explain the markers of interest we will study.

8 – In the Data Analysis section:
8.1- All packages that will be used to analyze genomics and transcriptomics data are listed but not the methods that will be used. Moreover, we could not find any description of the normalization procedure that will be used but only the package that will be used to conduct normalization of expression data.

We made some precisions lines 364 to 373.

8.2 - – Something should be said about how missing data will be handled in the Data Analysis section.

From the preliminary data we have already collected, we do not expect missing data to affect more than 10% of the records for the main factors that will be analyzed. Should they be over 5%, an imputation method such as the MICE method that has previously been used by our team (see Van Buuren S, Boshuizen H, Knook D, 1999. Multiple imputation of missing blood pressure covariates in survival analysis. Statistics Medicine 18: 681–699) will be applied, as the errors can be considered at random.

8.3 – In lines 340 and 341, it is stated that RT-qPCR data will be analyzed through Wilcoxon and Kruskal-Wallis. The authors may want to compare the variability of transcripts between groups. Those tests are not appropriate and yield biased results when the variance across comparison groups are very different. T-tests and ANOVA of transformed outcomes may be more appropriate.

We are agreed with that remark. We modified our manuscript lines 372 and 373.

8.4 – Lines 342-348 describes how immune markers will be analyzed. Since we do not know the type of markers the authors are referring to, we cannot comment on the appropriateness of the choice of analytical methods.
For instance, when those makers are predictors they may be highly correlated and a simple regression analysis would not be appropriate.

We hope that the details now provided in the method do clarify the proposed method of analysis.

8.5 - Generally more details need to be given about the proposed analysis. For instance: a) for which outcomes they are planning to use linear or logistic regression specifically; b) will they use random intercept and slope (those are hierarchical models)? for which factors they would consider including a random slope? how will they decide whether a random slope should be included?; c) when using ANOVA, which time point will be compared across the 3 groups; d) criteria for variable selection (or defining that a marker is associated with the outcome of interest). All these details can be minimally presented in a few sentences

Regarding points 8.4 and 8.5, we reconsidered the whole statistical analysis plan in the light of the variables that would be collected and of the questions that could be answered through the project design. For each question, univariate then multivariate analyses will be performed in order to determine the potential risk factors and adjustment variables to be kept in the final models. Finally, as the processes involved in the changes of inflammatory markers from D0 (admission), D3 (initiation of recovery or death), and D21-D30 (complete or partial recovery in the survivors) are complex and concern a variable number of subjects, it was decided not to apply a hierarchical model on the three time points, but to consider separately D0 and D3 on one hand, and D3 and D21 (survivors only) on the other hand.
The first step will be a comparison of the three groups, CM, UM and NMC, for a selection of inflammation markers. Those will be markers of the inflammatory response (such as TNFα or IL-10), or mores specific markers of oxidative stress, of NOsynthase activity, of monocyte/T lymphocyte migration, or cell signaling paths.
The groups will be compared two by two with a linear regression, with a special attention to CM/UM comparison. Adjustment variables such as age, sex, ethnical group, time to hospital transfer, body temperature, and co-morbidities will be taken into account in the model. It will be further determined if a global comparison between the three groups will be made.
The second major question to be answered to is, within the CM group, whether the changes of the inflammation markers between D0 (admission) and D3 are predictive of the outcome (survival/death). A logistic model (univariate then multivariate) will be used for the analysis. The same adjustment variables will be used as in the comparison between groups. The dependent variable will be the outcome survival/death.
The last model (also a logistic regression) will study the changes in inflammation markers between D3 and D21 in the survivors in order to determine if they are predictive of a favorable evolution. The dependent variable will be the outcome, here the discharge from the hospital without apparent sequelae.

8.6 – It is critical to have stated the threshold for significance level and method for adjustment for multiple testing.

Several inflammation markers will be tested. The threshold for significance level will be 0.05, and a Bonferroni correction will be applied to take into account multiple testing.

8.7 – Will proteomic screening for markers be done? If so, how will this data be analyzed?

No proteomic analysis for immune marker will be done.

9 – It would be important to see in the description of the limitations in the discussion the potential for selection bias and confounding resulting from lack of comparability across the 3 comparison groups of interest.

For validity of inferences, the three groups need to be comparable with respect to every factor that could lead to the events of interest (malarial coma, non-malarial coma, and uncomplicated malaria) other than the study exposures. If patients come from different areas, their infecting parasites may be different just because they are exposed to different parasites although those parasites are not associated with the event of the group. Patients with cerebral malaria will be identified in hospitals that are likely to be referral centers from patients coming from different geographic areas. Patients with uncomplicated malaria, apparently, will be identified in a delimited geographic area. Therefore, their parasites are expected to be different therefore associated with cerebral vs. uncomplicated malaria solely because of geographical area. Would that be correct? If so, that should be included in the discussion of study limitations.

Patients were included in different areas reflecting the health care system in Benin. UM patients could not be included in hospital centers such as the CHU-MEL (Cotonou) hospital, and Calavi Hospital, because outpatients with UM rarely seek care in these centers. In 2014, a pilot study aimed to include UM patients in the Cotonou CHU-MEL, and highlighted the absence of UM cases in hospitals. However, patients from the So-Ava areas are referred to the main hospital centers when patients present severe malaria (or any severe illness that cannot be monitored and managed in dispensary. In 2016, we aimed to include patients suffering from cerebral malaria in the So-Ava area (Centre hospitalier Saint Joseph), and realized that first, patients were directly sent to the main hospitals, and second, that it would not be ethical to include severe malaria cases in these health structures due to the facility itself. We included a sentence in the discussion regarding this problematic lines 216 to 219.

MINOR REVISIONS
1 - In lines 223-227, the authors describe how cerebral malaria will be defined. It was unclear to us: a) the reason they are doing a fundoscopic examination but not using rethinopathy in their definition; b) whether they are excluding other possible causes of bacterial disease, e.g., would patients with history of clinical pneumonia or evidence of skin infection be excluded?

We followed the WHO recommendations for cerebral malaria diagnosis. A normal fundoscopic examination cannot rule out CM, alternatively fundoscopic abnormalities encountered in CM are not specific of CM. The study deals with infectious/inflammatory disorders affecting the central nervous system: this is why infections not likely to produce a coma are not an exclusion criterion.

2 - In line 122 the reference after “Africa” seems mistyped.

We correct it line 121.

3 – A list of samples that will be obtained is provided in lines 282-284 but not the purpose of each of those samples.

We add precisions lines 293 and 294.

4 – We could not find evidence that the study was registered. We only found the IRB approval number. I do not believe that is a requirement for an observational study but wanted to justify why I marked no one of the questions in the standardized review form.

This work was supported by the French Agence Nationale de la Recherche, ANR-17-CEI 7-0001-01. French Agence Nationale de la Recherche is listed by the Juliet project (http://v2.sherpa.ac.uk/id/funder/30). Ethics approval for the NeuroCM study has been obtained from Comité National d’Ethique pour la Recherche en santé of Benin (n°67/MS/DC/SGM/DRFMT/CNERS/SA; 10/17/2017). NeuroCM study has also been approved by the

Comité consultatif de déontologie et d'éthique of Institut de Recherche pour le Développement (IRD; 10/24/2017).

VERSION 2 – REVIEW

REVIEWER	Dr. Michael Eisenhut Luton&Dunstable University Hospital NHS Foundation Trust, Luton, United Kingdom
REVIEW RETURNED	11-Jan-2019

GENERAL COMMENTS	All comments have been addressed adequately.