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Identification of *Plasmodium falciparum* and host factors associated with cerebral malaria. A prospective, case-control study (NeuroCM)

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Abstract

Introduction: In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred worldwide, in 91 countries. Most cases and deaths were in the African region (respectively 88% and 90%), including Benin, located in West Africa. In Benin, malaria causes 26.8% of disease report in the general population and 20.9% in children under five years old.

The goal of the NeuroCM project is to identify the causative and remedial factors of neuroinflammation in the context of cerebral malaria. There are currently very few systematic data from West Africa on the etiologies and management of non-traumatic coma in small children, and NeuroCM will help to fill this gap. We postulate that an accurate understanding of molecular and cellular mechanisms involved in neuroinflammation may help to define efficient strategies to prevent and manage cerebral malaria.

Methods and analysis: This is a prospective, case-control study comparing cerebral malaria to uncomplicated malaria and non-malarial coma. This study takes place in Benin, precisely in Cotonou for the hospital's recruitment. Uncomplicated malaria recruitment proceeds in Sô-Ava district. We aim to include 300 children between 24 and 71 months divided in three different clinical groups during 12 months (from December 2017 to November 2018). Study data, including clinical, biological and research results will be collected and managed using CS online-Ennov clinical.

Ethics and dissemination: 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 *Comité consultatif de déontologie et d'éthique* of Institut de Recherche pour le Développement (IRD; 10/24/2017)

Strengths and limitations of this study

- This case-control study aims to identify the causative and remedial factors of neuroinflammation in the context of cerebral malaria
- This study will inform on the etiologies and management of non-traumatic coma in small children
- The final products of NeuroCM are expected to feed the pipeline of new therapeutic (immune intervention) and preventive (vaccine) strategies that will improve cerebral malaria outcome
- This study does not have the power to investigate all etiologies of fever in Benin. Contrary to the malaria groups, there is no information on the frequency of non-malaria coma admissions, and no certainty on the number of children who will included in the non-*Plasmodium* group.
- According to the low number of patients, conclusions will further need to be confirmed in larger studies

Introduction

Malaria is triggered by an apicomplexan parasite, *Plasmodium spp*. Six *Plasmodium* species can infect humans, with *Plasmodium falciparum* (*P. falciparum*) being the most frequent in Sub-Saharian Africa (99% of estimated cases in 2016). *P. falciparum* is the agent of severe malaria and responsible for most malarial deaths.

In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred worldwide, in 91 countries[1]. Despite a recent decrease in malaria mortality due to extensive malaria control through insecticide impregnated bednets and increased use of artemisinin derivatives, 275,000 children still die every year from malaria. Most cases and deaths were in African region (respectively 88% and 90%). Severe malaria occurs mostly in non-immune patients and in Sub-Saharian Africa, 90% of severe malaria affect young children[2]. In endemic states, malaria is one of the three major causes of hospitalization in children under five years old.

Benin is in West Africa, along the Guinea gulf. In 2018, 11.5 million people live in Benin, most of them in South-Benin. Malaria transmission occurs seasonally, during rainy seasons (from May to August and October). According to the Beninese health department in 2016, malaria is responsible for 26.8% of disease reports in consultation and hospitalization in the general population and for 20.9% in children under five years old[3]. It is also the first morbidity cause in the general population with a prevalence of 39.7% in 2013, followed by respiratory infections in 12.4% cases and gastro-intestinal disease for 6.4%[4].

According to the World Health Organization (WHO), severe *falciparum* malaria is defined by the association between *P. falciparum* asexual parasitaemia and the presence of one or more of the clinical or laboratory features (with no other confirmed cause for their symptoms) presented in table 1. Cerebral malaria is defined by the presence of asexual form of *P. falciparum* associated with Blantyre score ≤ 2 Table 2). Cerebral malaria is a coma which persists for > 1h after a seizure

irrespective of anticonvulsant medications. But clinical criteria for cerebral malaria diagnosis are currently debated. Some study highlighted that *P. falciparum* parasitaemia can be observed in comatose children with a non-malarial central nervous system disease requiring another treatment than antimalarials[5]. Diagnostic of cerebral malaria could therefore be overestimated. A recent study in Malawi found that 25% of cerebral malaria cases were misdiagnosed and that many children may have had a viral meningoencephalitis concomitant to a malarial infection[5]. Implementation of fundoscopic examination (in order to look for malaria retinopathy signs)[6] and microbiological investigations (blood culture, cerebrospinal fluid (CSF) culture, CSF multiplex PCR) could limit the overestimation of cerebral malaria diagnosis, but fundoscopic examination requires trained physicians and microbiological investigations are expensive. Clinical research needs to focus on new clinical or diagnostic tools designed to help physicians in order to better diagnose cerebral malaria.

NeuroCM study aims to collect data (such as blood and cerebrospinal fluid culture, fundoscopic examination) on coma's etiologies in Beninese young children.

Without treatment, cerebral malaria is invariably fatal. Even with parenteral artemisinin use, severe malaria death rate is 20%[7]. In case of severe or cerebral malaria, patients should be hospitalized in an intensive care unit. Conscience status, blood pressure, heart and respiratory rates, diuresis and oxygen saturation have to be monitored every six hours[8]. Parenteral artemisinin instead of quinine is recommended at a posology of 2.4 mg/kg by injection (3 mg/kg in children under 20 kg of body weight) at diagnosis, 12 and 24 hours later and then daily until patients can take oral drugs[7]. Benzodiazepine should be used to treat convulsions (diazepam 0.3 mg/kg; midazolam or lorazepam). It seems accepted that cerebral malaria surviving patients generally don't present any neurological sequelae and fully recover their neurological capacity. However, immediate neurological after-effect is described in 6.7 to 11.6% of cases[79] and a recent meta-analysis found a

relation between cerebral malaria and neurologic disease[10]. The NeuroCM study will collect data on children's clinical recovery at discharge and 1 month later.

Control means for malaria are less and less effective due to multiple parasite and vector mechanisms of resistance. First, *P. falciparum* drug resistance is a growing concern. Resistance to chloroquine, one of the main anti-malarial drugs, appeared during the sixties in South-East Asia and then spread to Africa[1112]. Artemisinin-combined therapy became the treatment of choice for malaria to reduce the risk of parasites developing resistance[13]. But artemisinin-resistance appeared in South-East Asia in 2008[14] and was confirmed by others studies[15]. It has not, hitherto, spread to Africa but this is a real concern for the WHO[16]. On the other hand, mosquitos become more and more resistant to insecticides, making antivectorial prevention more and more difficult[17]. For those different reasons, research for new therapies is important and needs to be developed.

Pathophysiology of cerebral malaria is complex and multifactorial, based on both parasite and host immune factors. It is currently believed that cerebral malaria is caused by dedicated parasite variants that specifically localize in brain through interaction between parasite proteins expressed on the surface of the infected erythrocytes (iE) and brain endothelium. This sequestration occurs with erythrocytes infected with late stage of *P. falciparum* (trophozoïtes and schizonts). Binding of iE to endothelial vascular cells is mediated by Variant Surface Antigens (VSA). *Plasmodium* virulence is linked to its ability to express VSA[18]. VSA includes three different multigenic families: *var*, *rifin* and *stevor*. More specifically, *var* genes coding for PfEMP1 proteins are highly polymorphic and present in sixty copies in *P. falciparum* genome. Those PfEMP1 are expressed on iE surface and are responsible for endothelial receptors binding, such as Chondroitin Sulfate A (CSA) and Endothelial Protein C Receptor (EPCR) or InterCellular Adhesion Molecule (ICAM) respectively involved in placental malaria[19] and severe malaria[20]. PfEMP1 family has been clearly associated with binding of iE to the microvascular endothelium of every organ and tissue[21]. We

now better understand iE's binding on placenta[22] and vaccine development to prevent gestational malaria seems an achievable goal. By contrast, research is still needed to understand which type of proteins specifically binds to cerebral endothelial receptor. In a previous study conducted in Benin, we identified several proteins associated with cerebral malaria[23].

The finding of a PfEMP1 specifically related to cerebral malaria could pave the way to the development of a vaccine targeting this specific protein. Studying the transcriptomic and proteomic profiles of plasmodial strains involved in cerebral malaria compared to strains involved in uncomplicated malaria is a first step to better understand related mechanisms to cerebral endothelium binding.

The host immune aspect of the pathophysiology of cerebral malaria are the consequences of microvascular sequestration of iEs and rosettes (non-infected red blood cells) in brain. Such sequestration leads to blood flow obstruction, ischemia/hypoxia and local inflammation resulting in neuroinflammation[24-28]. The way this cascade of events is linked and the reasons why they result in death or inflammation resolution remains to be elucidated. Acute cerebral ischemia is known to drive microglia activation and influx of myeloid immune cells to the brain. Resident microglia and infiltrating monocytes/neutrophils have a critical role in initiating, sustaining (M1 polarization) and resolving (M2 polarization) post-ischemic inflammation[29-31]. Another important immune aspect of neuroinflammation during cerebral malaria is redox equilibrium. The production reactive oxygen species both by parasites (haemoglobin digestion) and monocytes/macrophages are thought to cause damages to neurons[32]. This process leads to BBB permeability and neurodegeneration[33,34]. To counterbalance the excess of oxidants, oxidant scavengers and antioxidant enzymes may be produced. In the NeuroCM study, we intend to better understand mechanisms of neuroinflammation and its resolution in a context of cerebral malaria, by comparing data collected in children presenting with cerebral malaria, in children hospitalised for non-malaria non-traumatic

coma, and in children with uncomplicated malaria. We will focus our studies on markers of immune cell migration and polarization (towards inflammatory or resolutive phenotypes), and of pro- or anti-oxidant response, through urine and blood samples analysis at inclusion, 3 and 21 to 28 days post-inclusion.

Study objectives

The main objective is to identify the causative and remedial factors of neuroinflammation in the context of cerebral malaria. There are currently very few systematic data from West Africa on the etiologies and management of non-traumatic coma in small children, and NeuroCM will bring new information on these aspects. We postulate that an accurate understanding of molecular and cellular mechanisms involved in neuroinflammation may help to define efficient strategies to prevent and manage cerebral malaria.

There are three distinct objectives in this study.

I. To identify parasitological factors associated with P. falciparum cerebral malaria or uncomplicated malaria

We expect to identify and validate *P. falciparum* virulence factors associated with cerebral malaria by comparison with uncomplicated malaria. Once proteins of interest will be found, functional studies will help to better understand their role in cerebral malaria.

II. To identify immune host factors associated with fatal of favourable outcome of cerebral malaria

We expect to better understand which mechanisms trigger neuroinflammation and its resolution during cerebral malaria by comparing three groups of children: presenting with cerebral malaria, hospitalised for non-malaria non-traumatic coma, and presenting with uncomplicated malaria. We aim to identify therapeutic molecular targets involved in neuroinflammation resolution.

III. To describe coma's etiology in Sub-Saharian Africa

We expect to improve knowledge in non-traumatic coma's etiologies in Sub-Saharian Africa in order to improve young children's coma management and inform health public policies on the role played by infections that could be prevented by vaccination.

Methods and analysis

Design

This is a prospective, case-control study comparing cerebral malaria to uncomplicated malaria and non-*Plasmodium* coma. Patients will be recruited in South Benin, in two different hospitals for coma and in a dispensary for uncomplicated malaria. Conversely, uncomplicated malaria is rarely detected in hospitals. This study is conducted by one Beninese research team (CERPAGE, Centre d'Etude et de Recherche sur le Paludisme Associé à la Grossesse et à l'Enfance) and three French research teams (UMR D216 MERIT in Paris carrying the project, UMR D152 PHARMADEV in Toulouse, UMR S1094 NET in Limoges).

Study environment

This study takes place in Benin, precisely in Cotonou and Calavi for the hospital's recruitment. Uncomplicated malaria recruitment takes place in Sô-Ava district. Cotonou is the largest city and economic centre of Benin, with an estimated population of 679,012 habitants in 2013.

The two recruitment hospitals are the CHU-MEL (CHU-Mère et Enfant de la Lagune) and Hôpital de zone de Calavi. Routine laboratory analysis, except bacteriology, are performed on site for children included with coma and at IRCB (Institut de Recherche Clinique du Bénin) for children with uncomplicated malaria. Bacteriological analyses are performed in the microbiology laboratory of CNHU (Centre National Hospitalier Universitaire). Research analyses are done in the CERPAGE laboratory.

Participants

We aim to include 3 different clinical groups of 100 children between 24 and 71 months during 12 months (from December 2017 to November 2018). This duration has been determined according to previous studies in Benin[35].

In the **first group**, a diagnosis of cerebral malaria will be defined as follows: positive *P. falciparum* parasitaemia with a Blantyre score ≤ 2 with exclusion of patients presenting: positive bacteraemia, meningitidis proved or suspected (leucocytes in cerebrospinal fluid > 1000 per microliter and/or Gram positive in LCS and/or LCS bacterial culture positive and/or PCR positive for any bacteria or virus).

In the **second group**, a diagnosis of non-malarial non-traumatic coma will be defined as follows: Blantyre score ≤ 2 and no *Plasmodium* infection as detected by thick blood smear.

In the **third group**, uncomplicated *falciparum* malaria will be defined as follows: 1) fever at inclusion or within 24 hours before, 2) no clinical or biological sign of severe malaria (table 1), no danger signs and no other obvious cause of fever and 3) parasitaemia between 1,000 to 500,000 parasites per microliter.

Inclusion and exclusion criteria

For all children, the first inclusion criterion is parental acceptance that their child participate in the study after information has been given (see section "Ethics and safety considerations"). Inclusion criteria for coma (cerebral malaria and non-*Plasmodium* coma) are: age between 24 to 71 months, Blantyre score ≤ 2, negative HIV Rapid Diagnostic Test (RDT). Non-inclusion criteria are: pre-existent neurologic disease and traumatic or toxic coma.

Inclusion criteria for uncomplicated *falciparum* malaria are: age between 24 to 71 months, fever > 38°C at inclusion or within 24 hours before and no clinical severity/danger sign, positive malaria RDT, negative HIV RDT.

Exclusion criteria for coma are: thick and thin blood smear not realized at day 0 (D0) and/or biological blood test no realized at D0 and/or research blood test not realized at D0.

Exclusion criteria for uncomplicated *falciparum* malaria are: thick and thin blood smear not realized at day 0 (D0) and/or biological blood test no realized at D0 and/or research blood test not realized at D0 and/or laboratory indices for severe malaria and/or thick and thin blood smear negative for *P. falciparum* and/or parasite density under 1000 parasite per microliter or higher than 500,000 parasites per microliter.

Recruitment process

Step 1: Enrolment/screening

The first step is patients' screening to confirm study eligibility and provide participants with information about the study. A questionnaire assessing eligibility will inform on home addresses, sociodemographic data (number of children in the family, ethnical group…), clinical history, use of mosquito net and vaccination status. Informed consent is then obtained from the parents or caregivers.

The following tests are performed to screen for malaria and to rule out HIV infections: a RDT detecting HRP2 for *P. falciparum* detection and Determine HIV-1/2 set, Alere for HIV detection.

Step 2: Clinical examination and biological sample/analysis

A clinical examination is performed by a study physician for children hospitalised with coma, and by a study nurse for uncomplicated malaria. In the coma group, a fundoscopic assessment is performed (Eyepax 1.0 Dioptrix) and pictures are captured and downloaded on an online database. The clinical data entry is performed on an online case report form.

In order to allocate children to their respective groups, biological analyses according to severe malaria are needed. For coma's inclusion: thick and thin blood smear analysis, complete blood count (CBC) (Sysmex KX-21N, Sysmex XT-1800i and ABS Micro ES 60), biochemistry analysis (Na^+, K^+, Cl^-, Ca^{++}, HCO_3^-, albumin, urea, creatinine, glucose, lactate) with Piccolo Sysmex and ALAT plus bilirubine (Biolabo Kenza Max biochemistry and Mindray BS-200) are performed on site. Blood culture, Gram staining and bacterial culture for cerebrospinal fluid are realized in a university hospital reference laboratory. Biomérieux Biofire™ FilmArray™ Meningitis/Encephalitis Panel multiplex PCR (looking for *E. coli*, *H. influenzae*, *L. monocytogenes*, *N. meningitidis*, *S. agalactiae*, *S. pneumoniae,* cytomegalovirus, enterovirus, Epstein-Barr virus, herpes simplex virus (HSV) 1, HSV2, human herpesvirus 6, paraechovirus, varicella zona virus and *Cryptococcus neoformans* and *C. gattii)* will be further performed in France. The required following samples are needed: one EDTA tube (2 mL), one heparin tube (2 mL), one cerebrospinal sample (1 mL), one blood sample for blood culture (5 mL) for routine analyses, two additional EDTA tubes (6 mL) and 50 mL of urine for research analyses.

For uncomplicated malaria inclusions: severe malaria was ruled out according to results from blood cell count (Sysmex XS500i), biochemistry analysis (bilirubine, glucose, creatinine) on Selectra pro automate (Elitech group) and thick blood smear. The following samples are needed: one EDTA

tube (2 mL), one heparin tube (2 mL) and one fluorinated tube (2mL) for routine analyses, two additional EDTA tubes (6 mL) and 50 ml of urine for research analyses.

Step 3: Research analyses

A part of research analyses is realized in Cerpage lab. One to 1.5 mL of blood is cultured in supplemented Roswell Park Memorial Institute (RPMI) 1640 medium with Albumax (Gibco) for less than 48 hours until they reach the mature stage (from young trophozoite to schizont), then purified using magnetic-activated cell sorting (MACS; Milteny Biotec, Bergisch Gladbach, Germany) for binding and endothelial cell activation assay. The resulting mature stage are stored at -80°C for further mass spectrometry protein analysis. Two hundreds µL of whole blood samples are conserved at -20°C for DNA analysis, 200 µL are transferred in TRIzol reagent (Life technologies, France) and stored at -80°C for further RNA extraction[36], and 200 µL in liquid nitrogen for parasite cryoconservation. Plasma samples are conserved at -20°C and -80°C respectively for immune response analysis and dosage of biomarkers. Peripherical blood mononuclear cells (PBMC) are separated from red blood cells by Ficoll density gradient and stored in liquid nitrogen. Finally, urine are stored at -80°C for further analysis. See table 3 for detailed research planning.

Parasite factors analyses will be performed in several ways. We will compare CM and UM isolates with whole genome DNA sequencing (Sanger Institute, MalariaGen consortium, Illumina technology); RNA-sequencing and by quantitative MS analysis. Highly polymorphic *var* genes will be assembled and BLASTed against peptide hits from the MS approach. Nucleotide primers will be designed with DNA-sequencing data and used in RT-qPCR to validate the RNA-seq data. Associations between gene polymorphisms and modifications in RNA nature and quantity detected by RNA-seq will be investigated. Then, we will use recombinant protein and *P. falciparum* genome modification by gene disruption to study proteins' role.

Host factors study will be based on the analysis of PBMC, plasmas and urine samples of the three groups of children. PBMC analysis will focus on the phenotyping of monocytes to distinguish M1 and M2-like phenotypes. Plasmas and urine samples will allow to measure redox, pro-/anti-inflammatory and pro-resolving mediators. We will first compare data from the group of cerebral malaria to the two other groups in order to identify the biological markers best related to inflammation and neurological impairment during cerebral malaria. Second, we will analyze data obtained with the two coma groups at inclusion (Day 0), at Day 3 and Day 30 to understand the kinetics of immune events and its relation to death or favorable outcome.

Step 4: Coma follow-up

In children presenting with coma, blood sample are collected at day 3 (D3) and day 21-28 (D21-28) to collect data on malaria outcome, and for research purpose. One EDTA tube (6 mL) and 50 mL urine will be sampled. A clinical assessment is also performed at these day of follow-up.

Data management

Data, including clinical, biological and research results are collected and managed using CS online-Ennov clinical (https://ufrcb.chu-limoges.fr/crfonline/). It is a secure, web-based application designed to support data capture for research studies. Study participants are identified by a code and have their own account. The two physicians and the nurse were trained to entry the data on included children in the database. Nobody can delete a patient created in the base, except the Data manager.

Data & Safety Monitoring Board (DSMB), composed by two physicians specialized in infectious disease and one statistician, will review allocation of children to the pre-defined study groups and discuss possible deviations from the expected number of subjects in the groups.

Data analysis

In a first step, descriptive statistics will be realized by calculating mean and standard deviation (sd) for quantitative variables, and proportion for qualitative variables to determine the main characteristics of the three clinical groups.

Focusing on cerebral and uncomplicated malaria children, Maxquant software and plasmoDB[37] will be used to compare malaria protein expression between isolates of these two clinical groups. Transcriptomic data will be analyzed with Galaxy (https://usegalaxy.org/) and R software (https://www.r-project.org/)[38]. We will also use free tools from Galaxy as Cufflinks, Htseq-count and Tophat2. Data normalization will be realized with DESeq2 software, with hypothesis that there exists gene overexpressed and underexpressed. Transcript expression levels (evaluated with RT-qPCR) will be compared by Kruskal-Wallis and Wilcoxon tests.

Regarding immune response analysis, potential markers related to inflammation and neurological symptoms will be compared using variance analysis in samples from children from cerebral malaria, non-malarial coma and uncomplicated malaria groups. In a second step, data will be analyzed by regression models (linear or logistic depending on the variable analysed) and hierarchical models for repeated samples over time in blood or urine. The non-*Plasmodium* coma group will be used as a comparator to analyse specific effect of malaria in neuroinflammation development.

Patient and public involvement

From patients' experience and preference, follow-up of children admitted with coma was scheduled in order to be able to detect neurological sequelae. The diagnostic workup proposed to all children included into the study, although not affordable to all patients in routine practice, met parent's expectations on what heath facilities should provide to all patients. All patients were recruited in

health facilities were they usually seek care, and to that respect patients were involved in their recruitment process. Finally, results will not be disseminated directly to study participants but through peer-reviewed scientific journal and conference presentations.

Ethics and dissemination

Ethics and safety considerations

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).

Parents/guardians will be given an oral information by the physician or the nurse and an opportunity to ask question and refuse the protocol. Patient's confidentiality will be ensured and anonymity guaranteed by anonymous coding given at the inclusion.

Dissemination

The main benefits of NeuroCM are targeted on people living in malaria endemic countries. The study results will be disseminated through a variety of instruments to ensure that a broad range of both specialists and non-specialists are informed and can properly benefit from the findings. First, through the direct consultations with the WHO's TDR-MIM, Roll Back Malaria program to reach the wider public health audience; through scientific meetings and peer-reviewed publications in scientific or medical journals to reach the scientific/medical/public health communities; through guidelines targeting the medical and paramedical staff for optimization of severe malaria management, through booklets (e.g. first aid procedures and adapted behaviour in case of emergency) elaborated and adapted to the population of Benin.

Discussion

Cerebral malaria is the most life-threatening form of malaria with high mortality rate in young children. Mortality related to malaria is still high in children population and accurate cerebral malaria diagnosis remains challenging. Among cerebral malaria surviving children, up to 25% have long-term neuro-cognitive deficits (visual/hearing/cognitive/language impairment/ ataxia/hemiparesis/motor deficit…), and 10% show evidence of mental health disorders[39]. As cerebral malaria might be one of the more common causes of epilepsy in malaria-endemic regions, the burden of cerebral malaria neurological sequelae may be largely underestimated, but difficult to estimate because diagnosis is challenging in malaria-endemic regions. Bacterial or viral central nervous system infections may occur in children with malarial infection; this may not only originates overdiagnosis of cerebral malaria, but also may overlooks potential bacterial and viral central nervous system infections.

The expected impact of NeuroCM is of great magnitude. First, NeuroCM is an attempt to propose improvements for the diagnosis of cerebral malaria. It will provide as far as possible, for the first time in West Africa, an identification of the causes of coma in the study area. Second, thanks to DNA, RNA and protein analyses, NeuroCM will allow to identify new parasite targets for a vaccine to prevent cerebral malaria. Third, NeuroCM will provide data on the kinetics of appearance of inflammatory and pro-resolving molecular and cellular events in brain during cerebral malaria. The role of endogenous mediators in neuroinflammation resolution during cerebral malaria will be clarified, with emphasis on pro-oxidant components and lipid mediators. NeuroCM will also identify markers allowing the definition of an immunological state in the process of neuroinflammation resolution in cerebral malaria patients. Our experimental murine model will allow the formulation of new hypothesis while proof of concept will be achieved through the

correlation of our proposed targets with patient morbidity and mortality parameters. In the future, it may allow clinicians to better manage cerebral malaria, with specific pro-resolving drugs for instance.

The final products of NeuroCM are expected to feed the pipeline of new therapeutic (immune intervention) and preventive (vaccine) strategies to improve cerebral malaria outcome, as well as other diseases involving neuroinflammation.

Authors contributions

All authors have substantially contributed to the conception and design of the study. VJ and JFF drafted the manuscript. JFF, SH, PD, AA, MC, DA, NA and GB revised the manuscript. All authors approved the final version to be submitted to the journal.

Collaborators

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Competing interests

No competing interest.

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Clinical manifestations	Prognosis value	Frequency in children
Impaired consciousness	+++	+++
Respiratory distress	+++	+++
Multiple convulsions	+	+++
Prostration	+	+++
Shock	+++	+
Pulmonary oedema (radiology)	+++	+/-
Abnormal bleeding	+++	+/-
Jaundice	++	+
Laboratory indices	**Prognosis value**	**Frequency**
Severe anemia (hemoglobin < 5g/dL or hematocrit < 15%)	+	+++
Hypoglycaemia (< 40 mg/dL)	+++	+++
Acidosis (bicarbonate < 15 mM)	+++	+++
Hyperlactemia (lactates > 5 mM)	+++	+++
Renal impairment (creatinin > 3 mg/dL)	++	+
Hyperparasitemia (parasitaemia > 10%)	+/-	++

519 Table 1 – Clinical and laboratory criteria for severe malaria (from (4))

520

	Score
Best motor response	
Localises painful stimulus	2
Withdraws limb from pain	1
Non-specific or absent response	0
Verbal response	
Appropriate cry	2
Moan or inappropriate cry	1
None	0
Eye movement	
Directed	1
Not directed	0
Total	0-5

Table 2 – Blantyre score (from (4))

Task	Calendar											
	2017	2018				2019				2020		
	T4	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3
Cohort recruitment and follow-up												
Area preparation												
Inclusion												
Follow-up												
Biological samples organization												
Parasite factors												
Parasite whole genome sequencing												
Parasite RNA-Sequencing												
Mass spectrometry analysis												
Identified protein validation												
Protein's role on endothelium activation												
Host factors												
Macrophage M2 kinetics apparition in mice brain												
Endogenous mediator role in neuroinflammation												
Neuroinflammation markers identification in cerebral malaria patients												
Results exploitation												
Database validation												
Data analysis												
Dissemination												

Table 3 - Detailed research planning

BMJ Open

Identification of Plasmodium falciparum and host factors associated with cerebral malaria. Description of the protocol for a prospective, case-control study (NeuroCM)

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2018-027378.R1
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Date Submitted by the Author:	08-Jan-2019
Complete List of Authors:	Joste, Valentin; MERIT, IRD Maurice, Laurine; MERIT, IRD; PHARMADEV, IRD Bertin, Gwladys; MERIT, IRD Aubouy, Agnès; PHARMADEV, IRD Boumédiène, Farid; INSERM UMR 1094, Tropical Neuroepidemiology Houzé, Sandrine; MERIT, IRD; Hopital Bichat - Claude-Bernard, Parasitology laboratory Daniel Ajzenberg , Daniel Ajzenberg ; Inserm UMR 1094, Tropical Neuroepidemiology Argy, Nicolas; MERIT, IRD; Hopital Bichat - Claude-Bernard, Parasitology laboratory Massougbodji, Achille; Institut de Recherche Clinique du Bénin Dossou-Dagba, Ida; Calavi Hospital, Pediatric department Alao, Jules; CHU-MEL Hospital, Pediatric department Cot, Michel; MERIT, IRD Deloron, Philippe; MERIT, IRD Faucher, Jean-François; Inserm UMR 1094, Tropical Neuroepidemiology
Primary Subject Heading:	Infectious diseases
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Identification of *Plasmodium falciparum* and host factors associated with cerebral malaria. Description of the protocol for a prospective, case-control study (NeuroCM)

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Abstract

Introduction: In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred worldwide, in 91 countries. Most cases and deaths were in the African region (respectively 88% and 90%), including Benin, located in West Africa. In Benin, malaria causes 26.8% of consultation and hospitalization motif in the general population and 20.9% in children under five years old.

The goal of the NeuroCM project is to identify the causative factors of neuroinflammation in the context of cerebral malaria. There are currently very few systematic data from West Africa on the etiologies and management of non-malarial non-traumatic coma in small children, and NeuroCM will help to fill this gap. We postulate that an accurate understanding of molecular and cellular mechanisms involved in neuroinflammation may help to define efficient strategies to prevent and manage cerebral malaria.

Methods and analysis: This is a prospective, case-control study comparing cerebral malaria to uncomplicated malaria and non-malarial non traumatic coma. This study takes place in Benin, precisely in Cotonou for children with coma and in Sô-Ava district for children with uncomplicated malaria. We aim to include 300 children aged between 24 and 71 months and divided in three different clinical groups during 12 months (from December 2017 to November 2018) with a 21-28 days follow-up for coma. Study data, including clinical, biological and research results will be collected and managed using CS online-Ennov clinical.

Ethics and dissemination: 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 *Comité consultatif de déontologie et d'éthique* of Institut de Recherche pour le Développement (IRD; 10/24/2017)

Strengths and limitations of this study

- This case-control study aims to identify the causative factors of neuroinflammation in the context of cerebral malaria
- This study will inform on the etiologies and management of non-malarial non-traumatic coma in small children
- The final products of NeuroCM are expected to feed the pipeline of new therapeutic (immune intervention) and preventive (vaccine) strategies that will improve cerebral malaria outcome
- This study does not have the power to investigate all etiologies of fever in Benin. Contrary to the malaria groups, there is no information on the frequency of non-malarial non-traumatic coma admissions, and no certainty on the number of children who will included in the non-malarial non-traumatic group.
- According to the limited number of patients, conclusions will further need to be confirmed in larger studies

Introduction

Malaria is triggered by an apicomplexan parasite, *Plasmodium spp*. Six *Plasmodium* species can infect humans, with *Plasmodium falciparum* (*P. falciparum*) being the most frequent in Sub-Saharian Africa (99.7% of estimated cases in 2017). *P. falciparum* is the agent of severe malaria and responsible for most malarial deaths.

In 2017, an estimated 219 million cases and 435,000 deaths of malaria occurred worldwide, in 91 countries[1]. Despite a recent decrease in malaria mortality due to extensive malaria control through insecticide impregnated bednets and increased use of artemisinin derivatives, 275,000 children still die every year from malaria. Most cases and deaths were in African region (respectively 92% and 93%). Severe malaria occurs mostly in non-immune patients and in Sub-Saharian Africa and 90% of severe malaria affect young children[2]. In endemic states, malaria is one of the three major causes of hospitalization in children under five years old.

Benin is in West Africa, along the Guinea gulf. In 2018, 11.5 million people live in Benin, most of them in South-Benin. Malaria transmission occurs seasonally, during rainy seasons (from May to August and October). According to the Beninese health department in 2016, malaria is responsible for 26.8% of disease reports in consultation and hospitalization in the general population and for 20.9% in children under five years old[3]. It is also the first morbidity cause in the general population with a prevalence of 39.7% in 2013, followed by respiratory infections in 12.4% cases and gastro-intestinal disease for 6.4%[4].

According to the World Health Organization (WHO), severe *falciparum* malaria is defined by the association between *P. falciparum* asexual parasitaemia and the presence of one or more of the clinical or laboratory features (with no other confirmed cause for their symptoms) presented in table 1. Cerebral malaria (CM) is defined by the presence of asexual form of *P. falciparum* associated with Blantyre score ≤ 2 (Table 2). CM is a coma which persists for > 1h after a seizure irrespective of anticonvulsant medications. Clinical criteria for CM diagnosis are

currently debated, and it has been highlighted that a *P. falciparum* parasitaemia can be observed in comatose children with coma related to a non-malarial central nervous system disease[5], leading to a possible overestimation of CM cases. A recent study in Malawi found that 25% of CM cases were misdiagnosed and that many children may have had a viral meningoencephalitis concomitant to a malarial infection[5]. Implementation of fundoscopic examination (in order to look for malaria retinopathy signs)[6] and microbiological investigations (blood culture, cerebrospinal fluid (CSF) culture, CSF multiplex PCR) could limit the overestimation of CM diagnosis, but fundoscopic examination requires trained physicians and microbiological investigations are expensive. Clinical research needs to focus on new clinical or diagnostic tools designed to help physicians in order to better diagnose CM.

NeuroCM study aims to collect data (such as blood and cerebrospinal fluid culture, fundoscopic examination) on coma's etiologies in Beninese young children.

Without treatment, CM is invariably fatal. Even with parenteral artemisinin use, severe malaria death rate is 20%[7]. In case of severe or CM, patients should be hospitalized in an intensive care unit. Conscience status, blood pressure, heart and respiratory rates, diuresis and oxygen saturation have to be monitored every six hours[8]. Parenteral artemisinin instead of quinine is recommended at a posology of 2.4 mg/kg by injection (3 mg/kg in children under 20 kg of body weight) at diagnosis, 12 and 24 hours later and then daily until patients can take oral drugs[7]. Benzodiazepine should be used to treat convulsions (diazepam 0.3 mg/kg; midazolam or lorazepam). It seems accepted that CM surviving patients generally don't present any neurological sequelae and fully recover their neurological capacity. However, immediate neurological after-effect is described in 6.7 to 11.6% of cases[7,9] and a recent meta-analysis found a relation between CM and neurologic disease[10]. The NeuroCM study will collect data on children's clinical recovery at discharge and 21-28 days later.

Tools for malaria control are less and less effective. On one hand, *P. falciparum* drug resistance is a growing concern. Resistance to chloroquine, one of the main anti-malarial drugs, appeared during the sixties in South-East Asia and then spread to Africa[11,12]. Artemisinin-combined therapy became the treatment of choice for malaria with the aim to reduce the risk of parasites developing resistance[13], but resistance appeared in South-East Asia in 2008[14] and was confirmed in others studies[15]. It has not, hitherto, spread to Africa but this is a real concern for the WHO[16]. On the other hand, mosquitos become more and more resistant to insecticides, making antivectorial prevention more and more difficult[17]. Thus, research for new therapies is needed.

Pathophysiology of CM is complex and multifactorial, based on both parasite and host immune factors. It is currently believed that CM is caused by dedicated parasite variants that specifically localize in brain through interaction between parasite proteins expressed on the surface of the infected erythrocytes (iE) and brain endothelium. This sequestration occurs with erythrocytes infected with late stage of *P. falciparum* (trophozoites and schizonts). Binding of iE to endothelial vascular cells is mediated by Variant Surface Antigens (VSA). *Plasmodium* virulence is linked to its ability to express VSA[18]. VSA include three different multigenic families: *var*, *rifin* and *stevor*. More specifically, *var* genes coding for PfEMP1 proteins are highly polymorphic and present in sixty copies in *P. falciparum* genome. Those PfEMP1 are expressed on iE surface and are responsible for endothelial receptors binding, such as Chondroitin Sulfate A (CSA) and Endothelial Protein C Receptor (EPCR) or InterCellular Adhesion Molecule (ICAM) respectively involved in placental malaria[19] and severe malaria[20]. PfEMP1 family has been clearly associated with binding of iE to the microvascular endothelium of every organ and tissue[21]. We now better understand iE's binding on placenta[22] and vaccine development to prevent gestational malaria seems an achievable goal. By contrast, research is still needed to understand which type of proteins specifically binds to cerebral endothelial

receptor. In a previous study conducted in Benin, we identified several proteins associated with CM[23].

The finding of a PfEMP1 variant specifically related to CM could pave the way to the development of a vaccine targeting this specific protein. Studying the transcriptomic and proteomic profiles of plasmodial strains involved in CM compared to strains involved in uncomplicated malaria (UM) is a first step to better understand related mechanisms to cerebral endothelium binding.

The host immune aspect of the pathophysiology of CM are the consequences of microvascular sequestration of iEs and rosettes (non-infected red blood cells) in brain. Such sequestration leads to blood flow obstruction, ischemia/hypoxia and local inflammation resulting in neuroinflammation[24-28]. The way this cascade of events is linked and the reasons why they result in death or inflammation resolution remains to be elucidated. Acute cerebral ischemia is known to drive microglia activation and influx of myeloid immune cells to the brain. Resident microglia and infiltrating monocytes/neutrophils have a critical role in initiating, sustaining (M1 polarization) and resolving (M2 polarization) post-ischemic inflammation[29-31]. Another important immune aspect of neuroinflammation during CM is redox equilibrium. The production of reactive oxygen species both by parasites (haemoglobin digestion) and monocytes/macrophages are thought to cause damages to neurons[32]. This process leads to BBB permeability and neurodegeneration[33,34]. In order to counterbalance the excess of oxidants, oxidant scavengers and antioxidant enzymes may be produced. NO bioinsufficiency and subsequent vasoconstriction constitute other important aspects of CM pathophysiology[35]. Haem and superoxide anion release during infection leads to NO mobilization for detoxification, depriving vascular smooth muscle cells in NO and leading to inflammation-related vasospasm[35,36]. Although vasospasm has not been clearly associated to death risk during CM[37], NO pathway deserves a better understanding during CM pathophysiology. In the NeuroCM

study, we intend to better understand mechanisms of neuroinflammation and its resolution in a context of CM, by comparing data collected in children presenting with CM, in children hospitalized for non-malarial non-traumatic coma, and in children with UM. We will focus our studies on markers of immune cell migration and polarization (towards inflammatory or resolutive phenotypes), of pro- or anti-oxidant response, and of pro- or anti-inflammatory response through urine and blood samples analysis at inclusion, 3 and 21 to 28 days post-inclusion.

Study objectives

The main objective is to identify the causative factors of neuroinflammation in the context of CM. There are currently very few systematic data from West Africa on the etiologies and management of non-traumatic coma in small children, and NeuroCM will bring new information on these aspects. We postulate that an accurate understanding of molecular and cellular mechanisms involved in neuroinflammation may help to define efficient strategies to prevent and manage CM.

There are three distinct objectives in this study.

I. To identify parasitological factors associated with P. falciparum CM or UM

We expect to identify and validate *P. falciparum* virulence factors associated with CM by comparison with UM. Once proteins of interest will be found, functional studies will help to better understand their role in CM.

II. To identify immune host factors associated with fatal of favorable outcome of CM

We expect to better understand which mechanisms trigger neuroinflammation and its resolution during CM by comparing three groups of children: presenting with CM, hospitalized for non-malarial non-traumatic coma, and presenting with UM. We aim to identify therapeutic molecular targets involved in neuroinflammation resolution.

III. To describe coma's etiology in Sub-Saharian Africa

We expect to improve knowledge in non-malarial non-traumatic coma's etiologies in Sub-Saharian Africa in order to improve young children's coma management and inform health public policies on the role played by infections that could be prevented by vaccination.

Methods and analysis

Design

This is a prospective, case-control study comparing CM to UM and non-malarial non-traumatic coma. Patients will be recruited in South Benin, in two different hospitals for coma and in a dispensary for UM, as UM is rarely detected in hospitals where children with coma are managed. This study is conducted by one Beninese research team (CERPAGE, Centre d'Etude et de Recherche sur le Paludisme Associé à la Grossesse et à l'Enfance) and three French research teams (UMR D216 MERIT in Paris carrying the project, UMR D152 PHARMADEV in Toulouse, UMR S1094 NET in Limoges).

Study environment

This study takes place in Benin, precisely in Cotonou and Calavi for the hospital's recruitment. UM recruitment takes place in Sô-Ava district. Cotonou is the largest city and economic centre of Benin, with an estimated population of 679,012 habitants in 2013. In the study area, outpatients with UM do not seek care in the health care facilities where children with coma are

managed. A multi-center study for UM cases inclusion, using the main patient's origin from the corresponding hospital, would have been more even accurate.

The two recruitment hospitals are the CHU-MEL (CHU-Mère et Enfant de la Lagune) and Hôpital de zone de Calavi. Routine laboratory analysis, except bacteriology, are performed on site for children included with coma and at IRCB (Institut de Recherche Clinique du Bénin) for children with UM. Bacteriological analyses are performed in the microbiology laboratory of CNHU (Centre National Hospitalier Universitaire). Research analyses are done in the CERPAGE laboratory.

Participants

We aim to include 3 different clinical groups of 100 children between 24 and 71 months during 12 months (from December 2017 to November 2018). This duration has been determined according to previous studies in Benin[38].

In the **first group**, a diagnosis of CM will be defined as follows: positive *P. falciparum* parasitaemia with a Blantyre score ≤ 2 with exclusion of patients presenting: positive bacteraemia, meningitidis proved or suspected (leucocytes in cerebrospinal fluid > 1000 per microliter and/or Gram positive in LCS and/or LCS bacterial culture positive and/or PCR positive for any bacteria or virus).

In the **second group**, a diagnosis of non-malarial non-traumatic coma will be defined as follows: Blantyre score ≤ 2 and no *Plasmodium* infection as detected by thick blood smear.

In the **third group** UM will be defined as follows: 1) fever at inclusion or within 24 hours before, 2) no clinical or biological sign of severe malaria (table 1), no danger signs and no other obvious cause of fever and 3) *P. falciparum* parasitaemia between 1,000 to 500,000 parasites per microliter.

Inclusion and exclusion criteria

For all children, the first inclusion criterion is parental acceptance that their child participate in the study after information has been given (see section "Ethics and safety considerations"). Inclusion criteria for coma (CM and non-malarial non-traumatic coma) are: age between 24 to 71 months, Blantyre score ≤ 2, negative HIV Rapid Diagnostic Test (RDT). Non-inclusion criteria are: pre-existent neurologic disease and traumatic or toxic coma.

Inclusion criteria for UM are: age between 24 to 71 months, fever > 38°C at inclusion or within 24 hours before and no clinical severity/danger sign, positive malaria RDT, negative HIV RDT.

Exclusion criteria for coma are: thick and thin blood smear not realized at day 0 (D0) and/or biological blood test no realized at D0 and/or research blood test not realized at D0.

Exclusion criteria for UM are: thick and thin blood smear not realized at day 0 (D0) and/or biological blood test no realized at D0 and/or research blood test not realized at D0 and/or laboratory indices for severe malaria and/or thick and thin blood smear negative for *P. falciparum* and/or parasite density under 1000 parasite per microliter or higher than 500,000 parasites per microliter. To evidence a significant difference between CM and UM groups in the ratio of endogenous mediators associated with inflammation resolution, we estimated that a sample size of 100 subjects per group was sufficient to reach the main study target, i.e., by linear regression analysis involving a maximum of 6 predictors and an R^2 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.

Recruitment process

Step 1: Enrolment/screening

For CM and non-malarial non-traumatic coma group, every young child with neurologic symptoms is screened for eligibility. For UM group, every child presenting at the outpatient clinic with fever or fever during the previous 24 hours is screened. The first step is patients' screening to confirm study eligibility and provide participants with information about the study. A questionnaire assessing eligibility will inform on home addresses, sociodemographic data (number of children in the family, ethnical group…), clinical history, use of mosquito net and vaccination status. Informed consent is then obtained from the parents or caregivers.

The following tests are performed to screen for malaria and to rule out HIV infections: a RDT detecting HRP2 for *P. falciparum* detection and Determine HIV-1/2 set, Alere for HIV detection.

Step 2: Clinical examination and biological sample/analysis

A clinical examination is performed by a study physician for children hospitalized with coma, and by a study nurse for UM. In the coma group, a fundoscopic assessment is performed (Eyepax 1.0 Dioptrix) and pictures are captured and downloaded on an online database. The clinical data entry is performed on an online case report form.

In order to allocate children to their respective groups, biological analyses according to severe malaria are needed. For coma's inclusion: thick and thin blood smear analysis, complete blood count (CBC) (Sysmex KX-21N, Sysmex XT-1800i and ABS Micro ES 60), biochemistry analysis (Na^+, K^+, Cl^-, Ca^{++}, HCO_3^-, albumin, urea, creatinine, glucose, lactate) with Piccolo Sysmex and ALAT plus bilirubine (Biolabo Kenza Max biochemistry and Mindray BS-200) are performed on site. Blood culture, Gram staining and bacterial culture for CSF are realized in a university hospital reference laboratory. Biomérieux Biofire™ FilmArray™ Meningitis/Encephalitis Panel multiplex PCR (looking for *E. coli*, *H. influenzae*, *L. monocytogenes*, *N. meningitidis*, *S. agalactiae*, *S. pneumoniae,* cytomegalovirus, enterovirus, Epstein-Barr virus, herpes simplex virus (HSV) 1, HSV2, human herpesvirus 6, paraechovirus,

varicella zona virus and *Cryptococcus neoformans* and *C. gattii)* will be further performed in France. The required following samples are needed: one EDTA tube (2 mL) for CBC and malaria diagnostic, one heparin tube (2 mL) for biochemistry analysis, one cerebrospinal sample (1 mL), one blood sample for blood culture (5 mL) for routine analyses, two additional EDTA tubes (6 mL) and 50 mL of urine for research analyses.

For UM inclusions: severe malaria was ruled out according to results from blood cell count (Sysmex XS500i), biochemistry analysis (bilirubin, glucose, creatinine) on Selectra pro automate (Elitech group) and thick blood smear. The following samples are needed: one EDTA tube (2 mL), one heparin tube (2 mL) and one fluorinated tube (2mL) for routine analyses, two additional EDTA tubes (6 mL) and 50 ml of urine for research analyses.

Step 3: Research analyses

A part of research analyses is realized in Cerpage lab. One to 1.5 mL of blood is cultured in supplemented Roswell Park Memorial Institute (RPMI) 1640 medium with Albumax (Gibco) for less than 48 hours until parasites reach the mature stage (from young trophozoite to schizont), then purified using magnetic-activated cell sorting (MACS; Milteny Biotec, Bergisch Gladbach, Germany) for binding and endothelial cell activation assay. The resulting mature stage are stored at -80°C for further mass spectrometry protein analysis. Two hundred µL of whole blood samples are conserved at -20°C for DNA analysis, 200 µL are transferred in TRIzol reagent (Life technologies, France) and stored at -80°C for further RNA extraction[39], and 200 µL in liquid nitrogen for parasite cryoconservation. Plasma samples are conserved at -20°C and -80°C respectively for immune response analysis and dosage of biomarkers. Peripheral blood mononuclear cells (PBMC) are separated from red blood cells by Ficoll density gradient and stored in liquid nitrogen. Finally, urines are stored at -80°C for further analysis. See table 3 for detailed research planning.

Parasite factors analyses will be performed in several ways. We will compare CM and UM isolates with whole genome DNA sequencing; RNA-sequencing and by quantitative MS analysis. Highly polymorphic *var* genes will be assembled and BLASTed against peptide hits from the MS approach. Nucleotide primers will be designed with DNA-sequencing data and used in RT-qPCR to validate the RNA-seq data. Associations between gene polymorphisms and modifications in RNA nature and quantity detected by RNA-seq will be investigated. Then, we will use recombinant protein and *P. falciparum* genome modification by gene disruption to study proteins' role.

Host factors study will be based on the analysis of PBMC, plasmas and urine samples of the three groups of children. PBMC analysis will focus on the phenotyping of monocytes to distinguish M1 and M2-like phenotypes. Flow cytometry will be used to measure expression levels of CD11b and CD16 as M1 markers, and CD163 and CD206 as M2 markers. The assessment of gene expression levels of cytokines, chemokines and their receptors by RT-qPCR will complete phenotype analysis. Plasmas and urine samples will allow to measure redox (L-arginine and biopterins), pro-/anti-inflammatory (cytokines, chemokines and lipid mediators such as eicosanoids) and pro-resolving mediators (such as prostaglandins and lipoxins) by ELISA or EIA. We will first compare data from the group of CM to the two other groups in order to identify the biological markers best related to inflammation and neurological impairment during CM. Second, we will analyze data obtained with the two coma groups at inclusion (Day 0), at Day 3 and Day 30 to understand the kinetics of immune events and its relation to death or favorable outcome. Finally, we will search for severity and death risk factors within the CM groups.

Step 4: Coma follow-up

In children presenting with coma, both clinical data and blood samples are collected at day 3 (D3) and day 21-28 (D21-28) on disease outcome, and for research purpose. One EDTA tube

(6 mL) and 50 mL urine will be sampled. In order to prevent losses, parents/guardians are called a few days before D21-28 to remind them of follow-up visit. No follow-up visit is scheduled for children with UM.

Data management

Data, including clinical, biological and research results are collected and managed using CS online-Ennov clinical (https://ufrcb.chu-limoges.fr/crfonline/). It is a secure, web-based application designed to support data capture for research studies. Study participants are identified by a code and have their own account. The two physicians and the nurse were trained to entry the data on included children in the database. Nobody can delete a patient created in the base, except the Data manager.

Data & Safety Monitoring Board (DSMB), composed by two physicians specialized in infectious disease and one statistician, will review allocation of children to the pre-defined study groups and discuss possible deviations from the expected number of subjects in the groups.

Data analysis

In a first step, descriptive statistics will be realized by calculating mean and standard deviation (sd) for quantitative variables, and proportion for qualitative variables to determine the main characteristics of the three clinical groups.

Focusing on cerebral and UM children the MS/MS data will be searched against the databases (UNIPROT and PlasmoDB[40]), the proteins will be considered as positive hits with at least two peptides. The MaxQuant software will be used to compare malaria protein expression between isolates of these two clinical groups. Transcriptomic data will be analyzed with Galaxy (https://usegalaxy.org/) and R software (https://www.r-project.org/)[41]. The raw data will be trimmed with Trimmomatic tool for Phred Quality Score Qscore >20, read length >30 bases,

and ribosome sequences will be removed with tool sortMeRNA. Reads will be mapped against the *P. falciparum* 3D7 reference genome combined with *var* transcript sequences from 7 *P. falciparum* genomes. Differential expression analysis on RNAseq data will be performed using the DESeq2[42] package considering a 1 log-fold increase as significant using adjusted p value < 0.05. Data normalization will be realized with DESeq2 software, with hypothesis that there exists genes overexpressed and underexpressed and that majority of genes are not expressed in a differential way. Transcript expression levels (evaluated with RT-qPCR) will be compared by T-tests and ANOVA of transformed outcomes.

Regarding immune response analysis, potential markers related to inflammation and neurological symptoms will be compared using variance analysis in samples from children from the three groups, CM, UM and non-malarial non-traumatic group. 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. Generally speaking, the non-malarial non-traumatic coma group will be used as a comparator to analyze specific effect of malaria in neuroinflammation development. 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 this 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.

Missing data are not expected 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 will be applied, as the errors can be considered at random[43].

Patient and public involvement

From patients' experience and preference, follow-up of children admitted with coma was scheduled in order to be able to detect neurological sequelae. The diagnostic workup proposed to all children included into the study, although not affordable to all patients in routine practice, met parent's expectations on what heath facilities should provide to all patients. All patients were recruited in health facilities were they usually seek care, and to that respect patients were involved in their recruitment process. Finally, results will not be disseminated directly to study participants but through peer-reviewed scientific journal and conference presentations.

Ethics and dissemination

Ethics and safety considerations

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).

Parents/guardians will be given an oral information by the physician or the nurse and an opportunity to ask question and refuse the protocol. Patient's confidentiality will be ensured and anonymity guaranteed by anonymous coding given at the inclusion.

Dissemination

The main benefits of NeuroCM are targeted on people living in malaria endemic countries. The study results will be disseminated through a variety of instruments to ensure that a broad range of both specialists and non-specialists are informed and can properly benefit from the findings. First, through the direct consultations with the WHO's TDR-MIM, Roll Back Malaria program to reach the wider public health audience; through scientific meetings and peer-reviewed publications in scientific or medical journals to reach the scientific/medical/public health communities; through guidelines targeting the medical and paramedical staff for optimization of severe malaria management, through booklets (e.g. first aid procedures and adapted behavior in case of emergency) elaborated and adapted to the population of Benin.

Discussion

CM is the most life-threatening form of malaria with high mortality rate in young children. Mortality related to malaria is still high in children population and accurate CM diagnosis remains challenging. Among CM surviving children, up to 25% have long-term neuro-cognitive deficits (visual/hearing/cognitive/language impairment/ataxia/hemiparesis/motor deficit…), and 10% show evidence of mental health disorders[44]. As CM might be one of the more common causes of epilepsy in malaria-endemic regions, the burden of CM neurological sequelae may be largely underestimated, but difficult to estimate because diagnosis is challenging in malaria-endemic regions. Bacterial or viral central nervous system infections may occur in children with malarial infection; this may not only originate overdiagnosis of CM, but also may overlook potential bacterial and viral central nervous system infections.

The expected impact of NeuroCM is of great magnitude. First, NeuroCM is an attempt to propose improvements for the diagnosis of CM. It will provide as far as possible, for the first time in West Africa, an identification of the causes of coma in the study area. Second, thanks to DNA, RNA and protein analyses, NeuroCM will allow to identify new parasite targets for a

vaccine to prevent CM. Third, NeuroCM will provide data on the kinetics of appearance of inflammatory and pro-resolving molecular and cellular events in brain during CM. The role of endogenous mediators in neuroinflammation resolution during CM will be clarified, with emphasis on pro-oxidant components and lipid mediators. NeuroCM will also identify markers allowing the definition of an immunological state in the process of neuroinflammation resolution in CM patients. Our experimental murine model will allow the formulation of new hypothesis while proof of concept will be achieved through the correlation of our proposed targets with patient morbidity and mortality parameters. In the future, it may allow clinicians to better manage CM, with specific pro-resolving drugs for instance.

The final products of NeuroCM are expected to feed the pipeline of new therapeutic (immune intervention) and preventive (vaccine) strategies to improve CM outcome, as well as other diseases involving neuroinflammation.

Authors contributions

VJ and JFF drafted the manuscript. GB, AA, SH, DA, NA, MC, PD and JFF revised the manuscript. GB, AA, FB, SH, DA, NA, MC, PD and JFF designed the study. VJ and LM organized the study in the field. AM, IDD and JA implemented the study in the field. All members of the NeuroCM group have substantially contributed to the conception, design or organization of the study. All authors approved the final version to be submitted to the journal.

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Competing interests

No competing interest.

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Clinical manifestations	Prognosis value	Frequency in children
Impaired consciousness	+++	+++
Respiratory distress	+++	+++
Multiple convulsions	+	+++
Prostration	+	+++
Shock	+++	+
Pulmonary oedema (radiology)	+++	+/-
Abnormal bleeding	+++	+/-
Jaundice	++	+
Laboratory indices	**Prognosis value**	**Frequency**
Severe anemia (hemoglobin < 5g/dL or hematocrit < 15%)	+	+++
Hypoglycaemia (< 40 mg/dL)	+++	+++
Acidosis (bicarbonate < 15 mM)	+++	+++
Hyperlactemia (lactates > 5 mM)	+++	+++
Renal impairment (creatinin > 3 mg/dL)	++	+
Hyperparasitemia (parasitaemia > 10%)	+/-	++

579 Table 1 – Clinical and laboratory criteria for severe malaria (from (4))

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	Score
Best motor response	
Localises painful stimulus	2
Withdraws limb from pain	1
Non-specific or absent response	0
Verbal response	
Appropriate cry	2
Moan or inappropriate cry	1
None	0
Eye movement	
Directed	1
Not directed	0
Total	0-5

Table 2 – Blantyre score (from (4))

Task	Calendar											
	2017	2018				2019				2020		
	T4	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3
Cohort recruitment and follow-up												
Area preparation												
Inclusion												
Follow-up												
Biological samples organization												
Parasite factors												
Parasite whole genome sequencing												
Parasite RNA-Sequencing												
Mass spectrometry analysis												
Identified protein validation												
Protein's role on endothelium activation												
Host factors												
Macrophage M2 kinetics apparition in mice brain												
Endogenous mediator role in neuroinflammation												
Neuroinflammation markers identification in cerebral malaria patients												
Results exploitation												
Database validation												
Data analysis												
Dissemination												

584 Table 3 - Detailed research planning

BMJ Open

Identification of Plasmodium falciparum and host factors associated with cerebral malaria. Description of the protocol for a prospective, case-control study in Benin (NeuroCM)

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Identification of *Plasmodium falciparum* and host factors associated with cerebral malaria. Description of the protocol for a prospective, case-control study in Benin (NeuroCM)

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Abstract

Introduction: In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred worldwide, in 91 countries. In Benin, malaria causes 26.8% of consultation and hospitalization motif in the general population and 20.9% in children under five years old.

The goal of the NeuroCM project is to identify the causative factors of neuroinflammation in the context of cerebral malaria. There are currently very few systematic data from West Africa on the etiologies and management of non-malarial non-traumatic coma in small children, and NeuroCM will help to fill this gap. We postulate that an accurate understanding of molecular and cellular mechanisms involved in neuroinflammation may help to define efficient strategies to prevent and manage cerebral malaria.

Methods and analysis: This is a prospective, case-control study comparing cerebral malaria to uncomplicated malaria and non-malarial non traumatic coma. This study takes place in Benin, precisely in Cotonou for children with coma and in Sô-Ava district for children with uncomplicated malaria. We aim to include 300 children aged between 24 and 71 months and divided in three different clinical groups during 12 months (from December 2017 to November 2018) with a 21-28 days follow-up for coma. Study data, including clinical, biological and research results will be collected and managed using CS online-Ennov clinical.

Ethics and dissemination: 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 *Comité consultatif de déontologie et d'éthique* of Institut de Recherche pour le Développement (IRD; 10/24/2017). The study results will be disseminated through the direct consultations with the WHO's TDR-MIM and Roll Back Malaria program, through scientific meetings and peer-reviewed publications in scientific or medical journals, and through guidelines and booklets.

Strengths and limitations of this study

- This case-control study aims to identify the causative factors of neuroinflammation in the context of cerebral malaria
- This study will inform on the etiologies and management of non-malarial non-traumatic coma in small children
- The final products of NeuroCM are expected to feed the pipeline of new therapeutic (immune intervention) and preventive (vaccine) strategies that will improve cerebral malaria outcome
- This study does not have the power to investigate all etiologies of fever in Benin. Contrary to the malaria groups, there is no information on the frequency of non-malarial non-traumatic coma admissions, and no certainty on the number of children who will included in the non-malarial non-traumatic group.
- According to the limited number of patients, conclusions will further need to be confirmed in larger studies

Introduction

Malaria is triggered by an apicomplexan parasite, *Plasmodium spp*. Six *Plasmodium* species can infect humans, with *Plasmodium falciparum* (*P. falciparum*) being the most frequent in Sub-Saharian Africa (99.7% of estimated cases in 2017). *P. falciparum* is the agent of severe malaria and responsible for most malarial deaths.

In 2017, an estimated 219 million cases and 435,000 deaths of malaria occurred worldwide, in 91 countries[1]. Despite a recent decrease in malaria mortality due to extensive malaria control through insecticide impregnated bednets and increased use of artemisinin derivatives, 275,000 children still die every year from malaria. Most cases and deaths were in African region (respectively 92% and 93%). Severe malaria occurs mostly in non-immune patients and in Sub-Saharian Africa and 90% of severe malaria affect young children[2]. In endemic states, malaria is one of the three major causes of hospitalization in children under five years old.

Benin is in West Africa, along the Guinea gulf. In 2018, 11.5 million people live in Benin, most of them in South-Benin. Malaria transmission occurs seasonally, during rainy seasons (from May to August and October). According to the Beninese health department in 2016, malaria is responsible for 26.8% of disease reports in consultation and hospitalization in the general population and for 20.9% in children under five years old[3]. It is also the first morbidity cause in the general population with a prevalence of 39.7% in 2013, followed by respiratory infections in 12.4% cases and gastro-intestinal disease for 6.4%[4].

According to the World Health Organization (WHO), severe *falciparum* malaria is defined by the association between *P. falciparum* asexual parasitaemia and the presence of one or more of the clinical or laboratory features (with no other confirmed cause for their symptoms) presented in table 1. Cerebral malaria (CM) is defined by the presence of asexual form of *P. falciparum* associated with Blantyre score ≤ 2 (Table 2). CM is a coma which persists for > 1h after a seizure irrespective of anticonvulsant medications. Clinical criteria for CM diagnosis are

currently debated, and it has been highlighted that a *P. falciparum* parasitaemia can be observed in comatose children with coma related to a non-malarial central nervous system disease[5], leading to a possible overestimation of CM cases. A recent study in Malawi found that 25% of CM cases were misdiagnosed and that many children may have had a viral meningoencephalitis concomitant to a malarial infection[5]. Implementation of fundoscopic examination (in order to look for malaria retinopathy signs)[6] and microbiological investigations (blood culture, cerebrospinal fluid (CSF) culture, CSF multiplex PCR) could limit the overestimation of CM diagnosis, but fundoscopic examination requires trained physicians and microbiological investigations are expensive. Clinical research needs to focus on new clinical or diagnostic tools designed to help physicians in order to better diagnose CM.

NeuroCM study aims to collect data (such as blood and cerebrospinal fluid culture, fundoscopic examination) on coma's etiologies in Beninese young children.

Without treatment, CM is invariably fatal. Even with parenteral artemisinin use, severe malaria death rate is 20%[7]. In case of severe or CM, patients should be hospitalized in an intensive care unit. Conscience status, blood pressure, heart and respiratory rates, diuresis and oxygen saturation have to be monitored every six hours[8]. Parenteral artemisinin instead of quinine is recommended at a posology of 2.4 mg/kg by injection (3 mg/kg in children under 20 kg of body weight) at diagnosis, 12 and 24 hours later and then daily until patients can take oral drugs[7]. Benzodiazepine should be used to treat convulsions (diazepam 0.3 mg/kg; midazolam or lorazepam). It seems accepted that CM surviving patients generally don't present any neurological sequelae and fully recover their neurological capacity. However, immediate neurological after-effect is described in 6.7 to 11.6% of cases[7,9] and a recent meta-analysis found a relation between CM and neurologic disease[10]. The NeuroCM study will collect data on children's clinical recovery at discharge and 21-28 days later.

Tools for malaria control are less and less effective. On one hand, *P. falciparum* drug resistance is a growing concern. Resistance to chloroquine, one of the main anti-malarial drugs, appeared during the sixties in South-East Asia and then spread to Africa[11,12]. Artemisinin-combined therapy became the treatment of choice for malaria with the aim to reduce the risk of parasites developing resistance[13], but resistance appeared in South-East Asia in 2008[14] and was confirmed in others studies[15]. It has not, hitherto, spread to Africa but this is a real concern for the WHO[16]. On the other hand, mosquitos become more and more resistant to insecticides, making antivectorial prevention more and more difficult[17]. Thus, research for new therapies is needed.

Pathophysiology of CM is complex and multifactorial, based on both parasite and host immune factors. It is currently believed that CM is caused by dedicated parasite variants that specifically localize in brain through interaction between parasite proteins expressed on the surface of the infected erythrocytes (iE) and brain endothelium. This sequestration occurs with erythrocytes infected with late stage of *P. falciparum* (trophozoites and schizonts). Binding of iE to endothelial vascular cells is mediated by Variant Surface Antigens (VSA). *Plasmodium* virulence is linked to its ability to express VSA[18]. VSA include three different multigenic families: *var*, *rifin* and *stevor*. More specifically, *var* genes coding for PfEMP1 proteins are highly polymorphic and present in sixty copies in *P. falciparum* genome. Those PfEMP1 are expressed on iE surface and are responsible for endothelial receptors binding, such as Chondroitin Sulfate A (CSA) and Endothelial Protein C Receptor (EPCR) or InterCellular Adhesion Molecule (ICAM) respectively involved in placental malaria[19] and severe malaria[20]. PfEMP1 family has been clearly associated with binding of iE to the microvascular endothelium of every organ and tissue[21]. We now better understand iE's binding on placenta[22] and vaccine development to prevent gestational malaria seems an achievable goal. By contrast, research is still needed to understand which type of proteins specifically binds to cerebral endothelial

receptor. In a previous study conducted in Benin, we identified several proteins associated with CM[23].

The finding of a PfEMP1 variant specifically related to CM could pave the way to the development of a vaccine targeting this specific protein. Studying the transcriptomic and proteomic profiles of plasmodial strains involved in CM compared to strains involved in uncomplicated malaria (UM) is a first step to better understand related mechanisms to cerebral endothelium binding.

The host immune aspect of the pathophysiology of CM are the consequences of microvascular sequestration of iEs and rosettes (non-infected red blood cells) in brain. Such sequestration leads to blood flow obstruction, ischemia/hypoxia and local inflammation resulting in neuroinflammation[24-28]. The way this cascade of events is linked and the reasons why they result in death or inflammation resolution remains to be elucidated. Acute cerebral ischemia is known to drive microglia activation and influx of myeloid immune cells to the brain. Resident microglia and infiltrating monocytes/neutrophils have a critical role in initiating, sustaining (M1 polarization) and resolving (M2 polarization) post-ischemic inflammation[29-31]. Another important immune aspect of neuroinflammation during CM is redox equilibrium. The production of reactive oxygen species both by parasites (haemoglobin digestion) and monocytes/macrophages are thought to cause damages to neurons[32]. This process leads to BBB permeability and neurodegeneration[33,34]. In order to counterbalance the excess of oxidants, oxidant scavengers and antioxidant enzymes may be produced. NO bioinsufficiency and subsequent vasoconstriction constitute other important aspects of CM pathophysiology[35]. Haem and superoxide anion release during infection leads to NO mobilization for detoxification, depriving vascular smooth muscle cells in NO and leading to inflammation-related vasospasm[35,36]. Although vasospasm has not been clearly associated to death risk during CM[37], NO pathway deserves a better understanding during CM pathophysiology. In the NeuroCM

study, we intend to better understand mechanisms of neuroinflammation and its resolution in a context of CM, by comparing data collected in children presenting with CM, in children hospitalized for non-malarial non-traumatic coma, and in children with UM. We will focus our studies on markers of immune cell migration and polarization (towards inflammatory or resolutive phenotypes), of pro- or anti-oxidant response, and of pro- or anti-inflammatory response through urine and blood samples analysis at inclusion, 3 and 21 to 28 days post-inclusion.

Study objectives

The main objective is to identify the causative factors of neuroinflammation in the context of CM. There are currently very few systematic data from West Africa on the etiologies and management of non-traumatic coma in small children, and NeuroCM will bring new information on these aspects. We postulate that an accurate understanding of molecular and cellular mechanisms involved in neuroinflammation may help to define efficient strategies to prevent and manage CM.

There are three distinct objectives in this study.

I. To identify parasitological factors associated with P. falciparum CM or UM

We expect to identify and validate *P. falciparum* virulence factors associated with CM by comparison with UM. Once proteins of interest will be found, functional studies will help to better understand their role in CM.

II. To identify immune host factors associated with fatal of favorable outcome of CM

We expect to better understand which mechanisms trigger neuroinflammation and its resolution during CM by comparing three groups of children: presenting with CM, hospitalized for non-malarial non-traumatic coma, and presenting with UM. We aim to identify therapeutic molecular targets involved in neuroinflammation resolution.

III. To describe coma's etiology in Sub-Saharian Africa

We expect to improve knowledge in non-malarial non-traumatic coma's etiologies in Sub-Saharian Africa in order to improve young children's coma management and inform health public policies on the role played by infections that could be prevented by vaccination.

Methods and analysis

Design

This is a prospective, case-control study comparing CM to UM and non-malarial non-traumatic coma. Patients will be recruited in South Benin, in two different hospitals for coma and in a dispensary for UM, as UM is rarely detected in hospitals where children with coma are managed. This study is conducted by one Beninese research team (CERPAGE, Centre d'Etude et de Recherche sur le Paludisme Associé à la Grossesse et à l'Enfance) and three French research teams (UMR D216 MERIT in Paris carrying the project, UMR D152 PHARMADEV in Toulouse, UMR S1094 NET in Limoges).

Study environment

This study takes place in Benin, precisely in Cotonou and Calavi for the hospital's recruitment. UM recruitment takes place in Sô-Ava district. Cotonou is the largest city and economic centre of Benin, with an estimated population of 679,012 habitants in 2013.

The two recruitment hospitals are the CHU-MEL (CHU-Mère et Enfant de la Lagune) and Hôpital de zone de Calavi. Routine laboratory analysis, except bacteriology, are performed on site for children included with coma and at IRCB (Institut de Recherche Clinique du Bénin) for children with UM. Bacteriological analyses are performed in the microbiology laboratory of CNHU (Centre National Hospitalier Universitaire). Research analyses are done in the CERPAGE laboratory.

Participants

We aim to include 3 different clinical groups of 100 children between 24 and 71 months during 12 months (from December 2017 to November 2018). This duration has been determined according to previous studies in Benin[38].

In the **first group**, a diagnosis of CM will be defined as follows: positive *P. falciparum* thin blood smear with a Blantyre score ≤ 2 with exclusion of patients presenting: positive bacteraemia, meningitidis proved or suspected (leucocytes in cerebrospinal fluid > 1000 per microliter and/or Gram positive in LCS and/or LCS bacterial culture positive and/or PCR positive for any bacteria or virus).

In the **second group**, a diagnosis of non-malarial non-traumatic coma will be defined as follows: Blantyre score ≤ 2 and no *Plasmodium* infection as detected by thin blood smear.

In the **third group** UM will be defined as follows: 1) fever at inclusion or within 24 hours before, 2) no clinical or biological sign of severe malaria (table 1), no danger signs and no other obvious cause of fever and 3) *P. falciparum* parasitaemia between 1,000 to 500,000 parasites per microliter.

Inclusion and exclusion criteria

For all children, the first inclusion criterion is parental acceptance that their child participate in the study after information has been given (see section "Ethics and safety considerations"). Inclusion criteria for coma (CM and non-malarial non-traumatic coma) are: age between 24 to 71 months, Blantyre score ≤ 2, negative HIV Rapid Diagnostic Test (RDT). Non-inclusion criteria are: pre-existent neurologic disease and traumatic or toxic coma.

Inclusion criteria for UM are: age between 24 to 71 months, fever > 38°C at inclusion or within 24 hours before and no clinical severity/danger sign, positive malaria RDT, negative HIV RDT.

Exclusion criteria for coma are: thick and thin blood smear not realized at day 0 (D0) and/or biological blood test no realized at D0 and/or research blood test not realized at D0.

Exclusion criteria for UM are: thick and thin blood smear not realized at day 0 (D0) and/or biological blood test no realized at D0 and/or research blood test not realized at D0 and/or laboratory indices for severe malaria and/or thick and thin blood smear negative for *P. falciparum* and/or parasite density under 1000 parasite per microliter or higher than 500,000 parasites per microliter. To evidence a significant difference between CM and UM groups in the ratio of endogenous mediators associated with inflammation resolution, we estimated that a sample size of 100 subjects per group was sufficient to reach the main study target, i.e., by linear regression analysis involving a maximum of 6 predictors and an R^2 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.

Recruitment process

Step 1: Enrolment/screening

For CM and non-malarial non-traumatic coma group, every young child with neurologic symptoms is screened for eligibility. For UM group, every child presenting at the outpatient

clinic with fever or fever during the previous 24 hours is screened. The first step is patients' screening to confirm study eligibility and provide participants with information about the study. A questionnaire assessing eligibility will inform on home addresses, sociodemographic data (number of children in the family, ethnical group…), clinical history, use of mosquito net and vaccination status. Informed consent is then obtained from the parents or caregivers.

The following tests are performed to screen for malaria and to rule out HIV infections: a RDT detecting HRP2 for *P. falciparum* detection and Determine HIV-1/2 set, Alere for HIV detection.

Step 2: Clinical examination and biological sample/analysis

A clinical examination is performed by a study physician for children hospitalized with coma, and by a study nurse for UM. In the coma group, a fundoscopic assessment is performed (Eyepax 1.0 Dioptrix) and pictures are captured and downloaded on an online database. The clinical data entry is performed on an online case report form.

In order to allocate children to their respective groups, biological analyses according to severe malaria are needed. For coma's inclusion: thick and thin blood smear analysis, complete blood count (CBC) (Sysmex KX-21N, Sysmex XT-1800i and ABS Micro ES 60), biochemistry analysis (Na^+, K^+, Cl^-, Ca^{++}, HCO_3^-, albumin, urea, creatinine, glucose, lactate) with Piccolo Sysmex and ALAT plus bilirubine (Biolabo Kenza Max biochemistry and Mindray BS-200) are performed on site. Blood culture, Gram staining and bacterial culture for CSF are realized in a university hospital reference laboratory. Biomérieux Biofire™ FilmArray™ Meningitis/Encephalitis Panel multiplex PCR (looking for *E. coli*, *H. influenzae*, *L. monocytogenes*, *N. meningitidis*, *S. agalactiae*, *S. pneumoniae,* cytomegalovirus, enterovirus, Epstein-Barr virus, herpes simplex virus (HSV) 1, HSV2, human herpesvirus 6, paraechovirus, varicella zona virus and *Cryptococcus neoformans* and *C. gattii)* will be further performed in France. The required following samples are needed: one EDTA tube (2 mL) for CBC and

malaria diagnostic, one heparin tube (2 mL) for biochemistry analysis, one cerebrospinal sample (1 mL), one blood sample for blood culture (5 mL) for routine analyses, two additional EDTA tubes (6 mL) and 50 mL of urine for research analyses.

For UM inclusions: severe malaria was ruled out according to results from blood cell count (Sysmex XS500i), biochemistry analysis (bilirubin, glucose, creatinine) on Selectra pro automate (Elitech group) and thick and thin blood smear. The following samples are needed: one EDTA tube (2 mL), one heparin tube (2 mL) and one fluorinated tube (2mL) for routine analyses, two additional EDTA tubes (6 mL) and 50 ml of urine for research analyses.

Step 3: Research analyses

A part of research analyses is realized in Cerpage lab. One to 1.5 mL of blood is cultured in supplemented Roswell Park Memorial Institute (RPMI) 1640 medium with Albumax (Gibco) for less than 48 hours until parasites reach the mature stage (from young trophozoite to schizont), then purified using magnetic-activated cell sorting (MACS; Milteny Biotec, Bergisch Gladbach, Germany) for binding and endothelial cell activation assay. The resulting mature stage are stored at -80°C for further mass spectrometry protein analysis. Two hundred µL of whole blood samples are conserved at -20°C for DNA analysis, 200 µL are transferred in TRIzol reagent (Life technologies, France) and stored at -80°C for further RNA extraction[39], and 200 µL in liquid nitrogen for parasite cryoconservation. Plasma samples are conserved at -20°C and -80°C respectively for immune response analysis and dosage of biomarkers. Peripheral blood mononuclear cells (PBMC) are separated from red blood cells by Ficoll density gradient and stored in liquid nitrogen. Finally, urines are stored at -80°C for further analysis. See table 3 for detailed research planning.

Parasite factors analyses will be performed in several ways. We will compare CM and UM isolates with whole genome DNA sequencing; RNA-sequencing and by quantitative MS analysis. Highly polymorphic *var* genes will be assembled and BLASTed against peptide hits

from the MS approach. Nucleotide primers will be designed with DNA-sequencing data and used in RT-qPCR to validate the RNA-seq data. Associations between gene polymorphisms and modifications in RNA nature and quantity detected by RNA-seq will be investigated. Then, we will use recombinant protein and *P. falciparum* genome modification by gene disruption to study proteins' role.

Host factors study will be based on the analysis of PBMC, plasmas and urine samples of the three groups of children. PBMC analysis will focus on the phenotyping of monocytes to distinguish M1 and M2-like phenotypes. Flow cytometry will be used to measure expression levels of CD11b and CD16 as M1 markers, and CD163 and CD206 as M2 markers. The assessment of gene expression levels of cytokines, chemokines and their receptors by RT-qPCR will complete phenotype analysis. Plasmas and urine samples will allow to measure redox (L-arginine and biopterins), pro-/anti-inflammatory (cytokines, chemokines and lipid mediators such as eicosanoids) and pro-resolving mediators (such as prostaglandins and lipoxins) by ELISA or EIA. We will first compare data from the group of CM to the two other groups in order to identify the biological markers best related to inflammation and neurological impairment during CM. Second, we will analyze data obtained with the two coma groups at inclusion (Day 0), at Day 3 and Day 30 to understand the kinetics of immune events and its relation to death or favorable outcome. Finally, we will search for severity and death risk factors within the CM groups.

Step 4: Coma follow-up

In children presenting with coma, both clinical data and blood samples are collected at day 3 (D3) and day 21-28 (D21-28) on disease outcome, and for research purpose. One EDTA tube (6 mL) and 50 mL urine will be sampled. In order to prevent losses, parents/guardians are called a few days before D21-28 to remind them of follow-up visit. No follow-up visit is scheduled for children with UM.

Data management

Data, including clinical, biological and research results are collected and managed using CS online-Ennov clinical (https://ufrcb.chu-limoges.fr/crfonline/). It is a secure, web-based application designed to support data capture for research studies. Study participants are identified by a code and have their own account. The two physicians and the nurse were trained to entry the data on included children in the database. Nobody can delete a patient created in the base, except the Data manager.

Data & Safety Monitoring Board (DSMB), composed by two physicians specialized in infectious disease and one statistician, will review allocation of children to the pre-defined study groups and discuss possible deviations from the expected number of subjects in the groups.

Data analysis

In a first step, descriptive statistics will be realized by calculating mean and standard deviation (sd) for quantitative variables, and proportion for qualitative variables to determine the main characteristics of the three clinical groups.

Focusing on cerebral and UM children the MS/MS data will be searched against the databases (UNIPROT and PlasmoDB[40]), the proteins will be considered as positive hits with at least two peptides. The MaxQuant software will be used to compare malaria protein expression between isolates of these two clinical groups. Transcriptomic data will be analyzed with Galaxy (https://usegalaxy.org/) and R software (https://www.r-project.org/)[41]. The raw data will be trimmed with Trimmomatic tool for Phred Quality Score Qscore >20, read length >30 bases, and ribosome sequences will be removed with tool sortMeRNA. Reads will be mapped against the *P. falciparum* 3D7 reference genome combined with *var* transcript sequences from 7 *P. falciparum* genomes. Differential expression analysis on RNAseq data will be performed using

the DESeq2[42] package considering a 1 log-fold increase as significant using adjusted *p* value < 0.05. Data normalization will be realized with DESeq2 software, with hypothesis that there exists genes overexpressed and underexpressed and that majority of genes are not expressed in a differential way. Transcript expression levels (evaluated with RT-qPCR) will be compared by T-tests and ANOVA of transformed outcomes.

Regarding immune response analysis, potential markers related to inflammation and neurological symptoms will be compared using variance analysis in samples from children from the three groups, CM, UM and non-malarial non-traumatic group. 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. The threshold for significance level will be 0.05, and a Bonferroni correction will be applied to take into account multiple testing. It will be further determined if a global comparison between the three groups will be made. Generally speaking, the non-malarial non-traumatic coma group will be used as a comparator to analyze specific effect of malaria in neuroinflammation development. 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 this 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.

Missing data are not expected 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 will be applied, as the errors can be considered at random[43]. No proteomic analysis for immune marker will be done.

Patient and public involvement

From patients' experience and preference, follow-up of children admitted with coma was scheduled in order to be able to detect neurological sequelae. The diagnostic workup proposed to all children included into the study, although not affordable to all patients in routine practice, met parent's expectations on what heath facilities should provide to all patients. All patients were recruited in health facilities were they usually seek care, and to that respect patients were involved in their recruitment process. Finally, results will not be disseminated directly to study participants but through peer-reviewed scientific journal and conference presentations.

Ethics and dissemination

Ethics and safety considerations

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).

Parents/guardians will be given an oral information by the physician or the nurse and an opportunity to ask question and refuse the protocol. Patient's confidentiality will be ensured and anonymity guaranteed by anonymous coding given at the inclusion.

Dissemination

The main benefits of NeuroCM are targeted on people living in malaria endemic countries. The study results will be disseminated through a variety of instruments to ensure that a broad range of both specialists and non-specialists are informed and can properly benefit from the findings. First, through the direct consultations with the WHO's TDR-MIM, Roll Back Malaria program to reach the wider public health audience; through scientific meetings and peer-reviewed publications in scientific or medical journals to reach the scientific/medical/public health communities; through guidelines targeting the medical and paramedical staff for optimization of severe malaria management, through booklets (e.g. first aid procedures and adapted behavior in case of emergency) elaborated and adapted to the population of Benin.

Discussion

CM is the most life-threatening form of malaria with high mortality rate in young children. Mortality related to malaria is still high in children population and accurate CM diagnosis remains challenging. Among CM surviving children, up to 25% have long-term neuro-cognitive deficits (visual/hearing/cognitive/language impairment/ataxia/hemiparesis/motor deficit…), and 10% show evidence of mental health disorders[44]. As CM might be one of the more common causes of epilepsy in malaria-endemic regions, the burden of CM neurological sequelae may be largely underestimated, but difficult to estimate because diagnosis is challenging in malaria-endemic regions. Bacterial or viral central nervous system infections may occur in children with malarial infection; this may not only originate overdiagnosis of CM, but also may overlook potential bacterial and viral central nervous system infections.

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's 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, 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. A multi-center study for UM cases inclusion, using the main patient's origin from the corresponding hospital, would have been more even accurate. This represents a possible limitation of our study.

The expected impact of NeuroCM is of great magnitude. First, NeuroCM is an attempt to propose improvements for the diagnosis of CM. It will provide as far as possible, for the first time in West Africa, an identification of the causes of coma in the study area. Second, thanks to DNA, RNA and protein analyses, NeuroCM will allow to identify new parasite targets for a vaccine to prevent CM. Third, NeuroCM will provide data on the kinetics of appearance of inflammatory and pro-resolving molecular and cellular events in brain during CM. The role of endogenous mediators in neuroinflammation resolution during CM will be clarified, with emphasis on pro-oxidant components and lipid mediators. NeuroCM will also identify markers allowing the definition of an immunological state in the process of neuroinflammation resolution in CM patients. Our experimental murine model will allow the formulation of new hypothesis while proof of concept will be achieved through the correlation of our proposed targets with patient morbidity and mortality parameters. In the future, it may allow clinicians to better manage CM, with specific pro-resolving drugs for instance.

The final products of NeuroCM are expected to feed the pipeline of new therapeutic (immune intervention) and preventive (vaccine) strategies to improve CM outcome, as well as other diseases involving neuroinflammation.

Authors contributions

VJ and JFF drafted the manuscript. GB, AA, SH, DA, NA, MC, PD and JFF revised the manuscript. GB, AA, FB, SH, DA, NA, MC, PD and JFF designed the study. VJ and LM organized the study in the field. AM, IDD and JA implemented the study in the field. All members of the NeuroCM group have substantially contributed to the conception, design or organization of the study. All authors approved the final version to be submitted to the journal.

Collaborators

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Competing interests

No competing interest.

Data availability statement

There are no data in this work.

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Clinical manifestations	Prognosis value	Frequency in children
Impaired consciousness	+++	+++
Respiratory distress	+++	+++
Multiple convulsions	+	+++
Prostration	+	+++
Shock	+++	+
Pulmonary oedema (radiology)	+++	+/-
Abnormal bleeding	+++	+/-
Jaundice	++	+
Laboratory indices	**Prognosis value**	**Frequency**
Severe anemia (hemoglobin < 5g/dL or hematocrit < 15%)	+	+++
Hypoglycaemia (< 40 mg/dL)	+++	+++
Acidosis (bicarbonate < 15 mM)	+++	+++
Hyperlactemia (lactates > 5 mM)	+++	+++
Renal impairment (creatinin > 3 mg/dL)	++	+
Hyperparasitemia (parasitaemia > 10%)	+/-	++

596 Table 1 – Clinical and laboratory criteria for severe malaria (from (4))

597

	Score
Best motor response	
Localises painful stimulus	2
Withdraws limb from pain	1
Non-specific or absent response	0
Verbal response	
Appropriate cry	2
Moan or inappropriate cry	1
None	0
Eye movement	
Directed	1
Not directed	0
Total	0-5

Table 2 – Blantyre score (from (4))

Task	Calendar											
	2017	2018				2019				2020		
	T4	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3
Cohort recruitment and follow-up												
Area preparation												
Inclusion												
Follow-up												
Biological samples organization												
Parasite factors												
Parasite whole genome sequencing												
Parasite RNA-Sequencing												
Mass spectrometry analysis												
Identified protein validation												
Protein's role on endothelium activation												
Host factors												
Macrophage M2 kinetics apparition in mice brain												
Endogenous mediator role in neuroinflammation												
Neuroinflammation markers identification in cerebral malaria patients												
Results exploitation												
Database validation												
Data analysis												
Dissemination												

601 Table 3 - Detailed research planning