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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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1 Identification of *Plasmodium falciparum* and host factors associated with cerebral malaria. A
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5 prospective, case-control study (NeuroCM)
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14 30 **Abstract**

15 31 **Introduction:** In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred
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17 32 worldwide, in 91 countries. Most cases and deaths were in the African region (respectively 88%
18
19 33 and 90%), including Benin, located in West Africa. In Benin, malaria causes 26.8% of disease
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21 34 report in the general population and 20.9% in children under five years old.
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24 35 The goal of the NeuroCM project is to identify the causative and remedial factors of
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26 36 neuroinflammation in the context of cerebral malaria. There are currently very few systematic data
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28 37 from West Africa on the etiologies and management of non-traumatic coma in small children, and
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30 38 NeuroCM will help to fill this gap. We postulate that an accurate understanding of molecular and
31
32 39 cellular mechanisms involved in neuroinflammation may help to define efficient strategies to
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34 40 prevent and manage cerebral malaria.
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37 41 **Methods and analysis:** This is a prospective, case-control study comparing cerebral malaria to
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39 42 uncomplicated malaria and non-malarial coma. This study takes place in Benin, precisely in
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41 43 Cotonou for the hospital's recruitment. Uncomplicated malaria recruitment proceeds in Sô-Ava
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43 44 district. We aim to include 300 children between 24 and 71 months divided in three different
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45 45 clinical groups during 12 months (from December 2017 to November 2018). Study data, including
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47 46 clinical, biological and research results will be collected and managed using CS online-Ennov
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49 47 clinical.
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3 48 **Ethics and dissemination:** Ethics approval for the NeuroCM study has been obtained from *Comité*
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5 49 *National d’Ethique pour la Recherche en santé* of Benin
6
7 50 (n°67/MS/DC/SGM/DRFMT/CNERS/SA; 10/17/2017). NeuroCM study has also been approved
8
9 51 by *Comité consultatif de déontologie et d’éthique* of Institut de Recherche pour le Développement
10
11 52 (IRD; 10/24/2017)
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17 54 **Strengths and limitations of this study**

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19 55 ➤ This case-control study aims to identify the causative and remedial factors of
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21 56 neuroinflammation in the context of cerebral malaria
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23 57 ➤ This study will inform on the etiologies and management of non-traumatic coma in small
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25 58 children
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27 59 ➤ The final products of NeuroCM are expected to feed the pipeline of new therapeutic
28
29 60 (immune intervention) and preventive (vaccine) strategies that will improve cerebral
30
31 61 malaria outcome
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33 62 ➤ This study does not have the power to investigate all etiologies of fever in Benin. Contrary
34
35 63 to the malaria groups, there is no information on the frequency of non-malaria coma
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37 64 admissions, and no certainty on the number of children who will included in the non-
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39 65 *Plasmodium* group.
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41 66 ➤ According to the low number of patients, conclusions will further need to be confirmed in
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43 67 larger studies
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69 Introduction

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

74 In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred worldwide, in 91
75 countries¹. Despite a recent decrease in malaria mortality due to extensive malaria control through
76 insecticide impregnated bednets and increased use of artemisinin derivatives, 275,000 children still
77 die every year from malaria. Most cases and deaths were in African region (respectively 88% and
78 90%). Severe malaria occurs mostly in non-immune patients and in Sub-Saharan Africa, 90% of
79 severe malaria affect young children². In endemic states, malaria is one of the three major causes
80 of hospitalization in children under five years old.

81 Benin is in West Africa, along the Guinea gulf. In 2018, 11.5 million people live in Benin, most of
82 them in South-Benin. Malaria transmission occurs seasonally, during rainy seasons (from May to
83 August and October). According to the Beninese health department in 2016, malaria is responsible
84 for 26.8% of disease reports in consultation and hospitalization in the general population and for
85 20.9% in children under five years old³. It is also the first morbidity cause in the general population
86 with a prevalence of 39.7% in 2013, followed by respiratory infections in 12.4% cases and gastro-
87 intestinal disease for 6.4%⁴.

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

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3 93 irrespective of anticonvulsant medications. But clinical criteria for cerebral malaria diagnosis are
4
5 94 currently debated. Some study highlighted that *P. falciparum* parasitaemia can be observed in
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8 95 comatose children with a non-malarial central nervous system disease requiring another treatment
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10 96 than antimalarials⁵. Diagnostic of cerebral malaria could therefore be overestimated. A recent study
11
12 97 in Malawi found that 25% of cerebral malaria cases were misdiagnosed and that many children
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14 98 may have had a viral meningoencephalitis concomitant to a malarial infection⁵. Implementation of
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17 99 fundoscopic examination (in order to look for malaria retinopathy signs)⁶ and microbiological
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19 100 investigations (blood culture, cerebrospinal fluid (CSF) culture, CSF multiplex PCR) could limit
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21 101 the overestimation of cerebral malaria diagnosis, but fundoscopic examination requires trained
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24 102 physicians and microbiological investigations are expensive. Clinical research needs to focus on
25
26 103 new clinical or diagnostic tools designed to help physicians in order to better diagnose cerebral
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28 104 malaria.

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31 105 NeuroCM study aims to collect data (such as blood and cerebrospinal fluid culture, fundoscopic
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33 106 examination) on coma's etiologies in Beninese young children.

35 107 Without treatment, cerebral malaria is invariably fatal. Even with parenteral artemisinin use, severe
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37 108 malaria death rate is 20%⁷. In case of severe or cerebral malaria, patients should be hospitalized in
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40 109 an intensive care unit. Conscience status, blood pressure, heart and respiratory rates, diuresis and
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42 110 oxygen saturation have to be monitored every six hours⁸. Parenteral artemisinin instead of quinine
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44 111 is recommended at a posology of 2.4 mg/kg by injection (3 mg/kg in children under 20 kg of body
45
46 112 weight) at diagnosis, 12 and 24 hours later and then daily until patients can take oral drugs⁷.
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49 113 Benzodiazepine should be used to treat convulsions (diazepam 0.3 mg/kg; midazolam or
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51 114 lorazepam). It seems accepted that cerebral malaria surviving patients generally don't present any
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54 115 neurological sequelae and fully recover their neurological capacity. However, immediate
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56 116 neurological after-effect is described in 6.7 to 11.6% of cases⁷⁹ and a recent meta-analysis found a

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3 117 relation between cerebral malaria and neurologic disease¹⁰. The NeuroCM study will collect data
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5 118 on children's clinical recovery at discharge and 1 month later.

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8 119 Control means for malaria are less and less effective due to multiple parasite and vector
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10 120 mechanisms of resistance. First, *P. falciparum* drug resistance is a growing concern. Resistance to
11
12 121 chloroquine, one of the main anti-malarial drugs, appeared during the sixties in South-East Asia
13
14 122 and then spread to Africa¹¹¹². Artemisinin-combined therapy became the treatment of choice for
15
16 123 malaria to reduce the risk of parasites developing resistance¹³. But artemisinin-resistance appeared
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18 124 in South-East Asia in 2008¹⁴ and was confirmed by others studies¹⁵. It has not, hitherto, spread to
19
20 125 Africa but this is a real concern for the WHO¹⁶. On the other hand, mosquitos become more and
21
22 126 more resistant to insecticides, making antivectional prevention more and more difficult¹⁷. For those
23
24 127 different reasons, research for new therapies is important and needs to be developed.

25
26 128 Pathophysiology of cerebral malaria is complex and multifactorial, based on both parasite and host
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28 129 immune factors. It is currently believed that cerebral malaria is caused by dedicated parasite
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30 130 variants that specifically localize in brain through interaction between parasite proteins expressed
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32 131 on the surface of the infected erythrocytes (iE) and brain endothelium. This sequestration occurs
33
34 132 with erythrocytes infected with late stage of *P. falciparum* (trophozoites and schizonts). Binding
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36 133 of iE to endothelial vascular cells is mediated by Variant Surface Antigens (VSA). *Plasmodium*
37
38 134 virulence is linked to its ability to express VSA¹⁸. VSA includes three different multigenic families:
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40 135 *var*, *rifin* and *stevor*. More specifically, *var* genes coding for PfEMP1 proteins are highly
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42 136 polymorphic and present in sixty copies in *P. falciparum* genome. Those PfEMP1 are expressed
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44 137 on iE surface and are responsible for endothelial receptors binding, such as Chondroitin Sulfate A
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46 138 (CSA) and Endothelial Protein C Receptor (EPCR) or InterCellular Adhesion Molecule (ICAM)
47
48 139 respectively involved in placental malaria¹⁹ and severe malaria²⁰. PfEMP1 family has been clearly
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50 140 associated with binding of iE to the microvascular endothelium of every organ and tissue²¹. We
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3 141 now better understand iE's binding on placenta²² and vaccine development to prevent gestational
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5 142 malaria seems an achievable goal. By contrast, research is still needed to understand which type of
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7 143 proteins specifically binds to cerebral endothelial receptor. In a previous study conducted in Benin,
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10 144 we identified several proteins associated with cerebral malaria²³.

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12 145 The finding of a PfEMP1 specifically related to cerebral malaria could pave the way to the
13
14 146 development of a vaccine targeting this specific protein. Studying the transcriptomic and proteomic
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17 147 profiles of plasmodial strains involved in cerebral malaria compared to strains involved in
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19 148 uncomplicated malaria is a first step to better understand related mechanisms to cerebral
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22 149 endothelium binding.

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24 150 The host immune aspect of the pathophysiology of cerebral malaria are the consequences of
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26 151 microvascular sequestration of iEs and rosettes (non-infected red blood cells) in brain. Such
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28 152 sequestration leads to blood flow obstruction, ischemia/hypoxia and local inflammation resulting
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31 153 in neuroinflammation²⁴⁻²⁸. The way this cascade of events is linked and the reasons why they result
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33 154 in death or inflammation resolution remains to be elucidated. Acute cerebral ischemia is known to
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35 155 drive microglia activation and influx of myeloid immune cells to the brain. Resident microglia and
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37 156 infiltrating monocytes/neutrophils have a critical role in initiating, sustaining (M1 polarization) and
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39 157 resolving (M2 polarization) post-ischemic inflammation²⁹⁻³¹. Another important immune aspect of
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42 158 neuroinflammation during cerebral malaria is redox equilibrium. The production reactive oxygen
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44 159 species both by parasites (haemoglobin digestion) and monocytes/macrophages are thought to
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46 160 cause damages to neurons³². This process leads to BBB permeability and neurodegeneration^{33,34}.
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49 161 To counterbalance the excess of oxidants, oxidant scavengers and antioxidant enzymes may be
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51 162 produced. In the NeuroCM study, we intend to better understand mechanisms of
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54 163 neuroinflammation and its resolution in a context of cerebral malaria, by comparing data collected
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56 164 in children presenting with cerebral malaria, in children hospitalised for non-malaria non-traumatic

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3 165 coma, and in children with uncomplicated malaria. We will focus our studies on markers of immune
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5 166 cell migration and polarization (towards inflammatory or resolutive phenotypes), and of pro- or
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8 167 anti-oxidant response, through urine and blood samples analysis at inclusion, 3 and 21 to 28 days
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10 168 post-inclusion.

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14 170 **Study objectives**

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17 171 The main objective is to identify the causative and remedial factors of neuroinflammation in the
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19 172 context of cerebral malaria. There are currently very few systematic data from West Africa on the
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21 173 etiologies and management of non-traumatic coma in small children, and NeuroCM will bring new
22
23 174 information on these aspects. We postulate that an accurate understanding of molecular and cellular
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25 175 mechanisms involved in neuroinflammation may help to define efficient strategies to prevent and
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27 176 manage cerebral malaria.

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33 178 There are three distinct objectives in this study.

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37 180 *I. To identify parasitological factors associated with P. falciparum cerebral malaria or*
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40 181 *uncomplicated malaria*

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42 182 We expect to identify and validate *P. falciparum* virulence factors associated with cerebral malaria
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44 183 by comparison with uncomplicated malaria. Once proteins of interest will be found, functional
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46 184 studies will help to better understand their role in cerebral malaria.

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51 186 *II. To identify immune host factors associated with fatal of favourable outcome of*
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54 187 *cerebral malaria*

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3 188 We expect to better understand which mechanisms trigger neuroinflammation and its resolution
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5 189 during cerebral malaria by comparing three groups of children: presenting with cerebral malaria,
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7 190 hospitalised for non-malaria non-traumatic coma, and presenting with uncomplicated malaria. We
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9 191 aim to identify therapeutic molecular targets involved in neuroinflammation resolution.
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13 14 193 *III. To describe coma's etiology in Sub-Saharan Africa*

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17 194 We expect to improve knowledge in non-traumatic coma's etiologies in Sub-Saharan Africa in
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19 195 order to improve young children's coma management and inform health public policies on the role
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21 196 played by infections that could be prevented by vaccination.
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25 26 198 **Methods and analysis**

27 28 199 Design

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31 200 This is a prospective, case-control study comparing cerebral malaria to uncomplicated malaria and
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33 201 non-*Plasmodium* coma. Patients will be recruited in South Benin, in two different hospitals for
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35 202 coma and in a dispensary for uncomplicated malaria. Conversely, uncomplicated malaria is rarely
36
37 203 detected in hospitals. This study is conducted by one Beninese research team (CERPAGE, Centre
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39 204 d'Etude et de Recherche sur le Paludisme Associé à la Grossesse et à l'Enfance) and three French
40
41 205 research teams (UMR D216 MERIT in Paris carrying the project, UMR D152 PHARMADEV in
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43 206 Toulouse, UMR S1094 NET in Limoges).
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48 49 208 Study environment

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51 209 This study takes place in Benin, precisely in Cotonou and Calavi for the hospital's recruitment.
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53 210 Uncomplicated malaria recruitment takes place in Sô-Ava district. Cotonou is the largest city and
54
55 211 economic centre of Benin, with an estimated population of 679,012 habitants in 2013.
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3 212 The two recruitment hospitals are the CHU-MEL (CHU-Mère et Enfant de la Lagune) and Hôpital
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5 213 de zone de Calavi. Routine laboratory analysis, except bacteriology, are performed on site for
6
7 214 children included with coma and at IRCB (Institut de Recherche Clinique du Bénin) for children
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9 215 with uncomplicated malaria. Bacteriological analyses are performed in the microbiology laboratory
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11 216 of CNHU (Centre National Hospitalier Universitaire). Research analyses are done in the
12
13 217 CERPAGE laboratory.
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19 219 Participants

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21 220 We aim to include 3 different clinical groups of 100 children between 24 and 71 months during 12
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23 221 months (from December 2017 to November 2018). This duration has been determined according
24
25 222 to previous studies in Benin³⁵.

26
27 223 In the **first group**, a diagnosis of cerebral malaria will be defined as follows: positive *P. falciparum*
28
29 224 parasitaemia with a Blantyre score ≤ 2 with exclusion of patients presenting: positive bacteraemia,
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31 225 meningitidis proved or suspected (leucocytes in cerebrospinal fluid > 1000 per microliter and/or
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33 226 Gram positive in LCS and/or LCS bacterial culture positive and/or PCR positive for any bacteria
34
35 227 or virus).

36
37 228 In the **second group**, a diagnosis of non-malarial non-traumatic coma will be defined as follows:
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39 229 Blantyre score ≤ 2 and no *Plasmodium* infection as detected by thick blood smear.

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41 230 In the **third group**, uncomplicated *falciparum* malaria will be defined as follows: 1) fever at
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43 231 inclusion or within 24 hours before, 2) no clinical or biological sign of severe malaria (table 1), no
44
45 232 danger signs and no other obvious cause of fever and 3) parasitaemia between 1,000 to 500,000
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47 233 parasites per microliter.
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53 234 Inclusion and exclusion criteria

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

241 Inclusion criteria for uncomplicated *falciparum* malaria are: age between 24 to 71 months, fever $>$
242 38°C at inclusion or within 24 hours before and no clinical severity/danger sign, positive malaria
243 RDT, negative HIV RDT.

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

246 Exclusion criteria for uncomplicated *falciparum* malaria are: thick and thin blood smear not
247 realized at day 0 (D0) and/or biological blood test no realized at D0 and/or research blood test not
248 realized at D0 and/or laboratory indices for severe malaria and/or thick and thin blood smear
249 negative for *P. falciparum* and/or parasite density under 1000 parasite per microliter or higher than
250 500,000 parasites per microliter.

251

252 Recruitment process

253 *Step 1: Enrolment/screening*

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

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

261 *Step 2: Clinical examination and biological sample/analysis*

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

265 The clinical data entry is performed on an online case report form.

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

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

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

285 *Step 3: Research analyses*

286 A part of research analyses is realized in Cerpage lab. One to 1.5 mL of blood is cultured in
287 supplemented Roswell Park Memorial Institute (RPMI) 1640 medium with Albumax (Gibco) for
288 less than 48 hours until they reach the mature stage (from young trophozoite to schizont), then
289 purified using magnetic-activated cell sorting (MACS; Milteny Biotec, Bergisch Gladbach,
290 Germany) for binding and endothelial cell activation assay. The resulting mature stage are stored
291 at -80°C for further mass spectrometry protein analysis. Two hundreds μ L of whole blood samples
292 are conserved at -20°C for DNA analysis, 200 μ L are transferred in TRIzol reagent (Life
293 technologies, France) and stored at -80°C for further RNA extraction³⁶, and 200 μ L in liquid
294 nitrogen for parasite cryoconservation. Plasma samples are conserved at -20°C and -80°C
295 respectively for immune response analysis and dosage of biomarkers. Peripheral blood
296 mononuclear cells (PBMC) are separated from red blood cells by Ficoll density gradient and stored
297 in liquid nitrogen. Finally, urine are stored at -80°C for further analysis. See table 3 for detailed
298 research planning.

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

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3 307 Host factors study will be based on the analysis of PBMC, plasmas and urine samples of the three
4
5 308 groups of children. PBMC analysis will focus on the phenotyping of monocytes to distinguish M1
6
7 309 and M2-like phenotypes. Plasmas and urine samples will allow to measure redox, pro-/anti-
8
9 310 inflammatory and pro-resolving mediators. We will first compare data from the group of cerebral
10
11 311 malaria to the two other groups in order to identify the biological markers best related to
12
13 312 inflammation and neurological impairment during cerebral malaria. Second, we will analyze data
14
15 313 obtained with the two coma groups at inclusion (Day 0), at Day 3 and Day 30 to understand the
16
17 314 kinetics of immune events and its relation to death or favorable outcome.
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22 315 *Step 4: Coma follow-up*

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24 316 In children presenting with coma, blood sample are collected at day 3 (D3) and day 21-28 (D21-
25
26 317 28) to collect data on malaria outcome, and for research purpose. One EDTA tube (6 mL) and 50
27
28 318 mL urine will be sampled. A clinical assessment is also performed at these day of follow-up.
29
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31

32 319

33 320 Data management

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35 321 Data, including clinical, biological and research results are collected and managed using CS online-
36
37 322 Ennov clinical (<https://ufrcb.chu-limoges.fr/crfonline/>). It is a secure, web-based application
38
39 323 designed to support data capture for research studies. Study participants are identified by a code
40
41 324 and have their own account. The two physicians and the nurse were trained to entry the data on
42
43 325 included children in the database. Nobody can delete a patient created in the base, except the Data
44
45 326 manager.
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48
49 327 Data & Safety Monitoring Board (DSMB), composed by two physicians specialized in infectious
50
51 328 disease and one statistician, will review allocation of children to the pre-defined study groups and
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53 329 discuss possible deviations from the expected number of subjects in the groups.
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331 Data analysis

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

335 Focusing on cerebral and uncomplicated malaria children, Maxquant software and plasmoDB³⁷
336 will be used to compare malaria protein expression between isolates of these two clinical groups.
337 Transcriptomic data will be analyzed with Galaxy (<https://usegalaxy.org/>) and R software
338 (<https://www.r-project.org/>)³⁸. We will also use free tools from Galaxy as Cufflinks, Htseq-count
339 and Tophat2. Data normalization will be realized with DESeq2 software, with hypothesis that there
340 exists gene overexpressed and underexpressed. Transcript expression levels (evaluated with RT-
341 qPCR) will be compared by Kruskal-Wallis and Wilcoxon tests.

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

349

350 Patient and public involvement

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

1
2
3 355 health facilities were they usually seek care, and to that respect patients were involved in their
4
5 356 recruitment process. Finally, results will not be disseminated directly to study participants but
6
7 357 through peer-reviewed scientific journal and conference presentations.
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10 358

11 359 **Ethics and dissemination**

12 360 Ethics and safety considerations

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17 361 Ethics approval for the NeuroCM study has been obtained from *Comité National d’Ethique pour*
18
19 362 *la Recherche en santé* of Benin (n°67/MS/DC/SGM/DRFMT/CNERS/SA; 10/17/2017). NeuroCM
20
21 363 study has also been approved by the *Comité consultatif de déontologie et d’éthique* of Institut de
22
23 364 Recherche pour le Développement (IRD; 10/24/2017).

24
25
26 365 Parents/guardians will be given an oral information by the physician or the nurse and an opportunity
27
28 366 to ask question and refuse the protocol. Patient’s confidentiality will be ensured and anonymity
29
30 367 guaranteed by anonymous coding given at the inclusion.
31
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33 368

34 369 Dissemination

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36
37 370 The main benefits of NeuroCM are targeted on people living in malaria endemic countries. The
38
39 371 study results will be disseminated through a variety of instruments to ensure that a broad range of
40
41 372 both specialists and non-specialists are informed and can properly benefit from the findings. First,
42
43 373 through the direct consultations with the WHO’s TDR-MIM, Roll Back Malaria program to reach
44
45 374 the wider public health audience; through scientific meetings and peer-reviewed publications in
46
47 375 scientific or medical journals to reach the scientific/medical/public health communities; through
48
49 376 guidelines targeting the medical and paramedical staff for optimization of severe malaria
50
51 377 management, through booklets (e.g. first aid procedures and adapted behaviour in case of
52
53 378 emergency) elaborated and adapted to the population of Benin.
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3 379
45 380 **Discussion**

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8 381 Cerebral malaria is the most life-threatening form of malaria with high mortality rate in young
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10 382 children. Mortality related to malaria is still high in children population and accurate cerebral
11
12 383 malaria diagnosis remains challenging. Among cerebral malaria surviving children, up to 25% have
13
14 384 long-term neuro-cognitive deficits (visual/hearing/cognitive/language impairment/
15
16 385 ataxia/hemiparesis/motor deficit...), and 10% show evidence of mental health disorders³⁹. As
17
18 386 cerebral malaria might be one of the more common causes of epilepsy in malaria-endemic regions,
19
20 387 the burden of cerebral malaria neurological sequelae may be largely underestimated, but difficult
21
22 388 to estimate because diagnosis is challenging in malaria-endemic regions. Bacterial or viral central
23
24 389 nervous system infections may occur in children with malarial infection; this may not only
25
26 390 originates overdiagnosis of cerebral malaria, but also may overlooks potential bacterial and viral
27
28 391 central nervous system infections.

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32
33 392 The expected impact of NeuroCM is of great magnitude. First, NeuroCM is an attempt to propose
34
35 393 improvements for the diagnosis of cerebral malaria. It will provide as far as possible, for the first
36
37 394 time in West Africa, an identification of the causes of coma in the study area. Second, thanks to
38
39 395 DNA, RNA and protein analyses, NeuroCM will allow to identify new parasite targets for a vaccine
40
41 396 to prevent cerebral malaria. Third, NeuroCM will provide data on the kinetics of appearance of
42
43 397 inflammatory and pro-resolving molecular and cellular events in brain during cerebral malaria. The
44
45 398 role of endogenous mediators in neuroinflammation resolution during cerebral malaria will be
46
47 399 clarified, with emphasis on pro-oxidant components and lipid mediators. NeuroCM will also
48
49 400 identify markers allowing the definition of an immunological state in the process of
50
51 401 neuroinflammation resolution in cerebral malaria patients. Our experimental murine model will
52
53 402 allow the formulation of new hypothesis while proof of concept will be achieved through the
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3 403 correlation of our proposed targets with patient morbidity and mortality parameters. In the future,
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5 404 it may allow clinicians to better manage cerebral malaria, with specific pro-resolving drugs for
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7
8 405 instance.

9
10 406 The final products of NeuroCM are expected to feed the pipeline of new therapeutic (immune
11
12 407 intervention) and preventive (vaccine) strategies to improve cerebral malaria outcome, as well as
13
14 408 other diseases involving neuroinflammation.

15 16 17 409 18 19 410 **Authors contributions**

20
21 411 All authors have substantially contributed to the conception and design of the study. VJ and JFF
22
23 412 drafted the manuscript. JFF, SH, PD, AA, MC, DA, NA and GB revised the manuscript. All
24
25 413 authors approved the final version to be submitted to the journal.

26 27 414 28 29 30 415 **Collaborators**

31
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33
34 417 Agn es Coste, Jean-Eudes Degbelo, Latifou Dramane, Sayeh Jafari-Guemouri, Claire Kamaliddin,
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36 418 Elis e Kinkpe, Ana is Labrunie, Y el e Ladipo, Thomas Lathiere, Audrey Mowendabeka, Jade Papin,
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38 419 Bernard Pippy, Pierre-Marie Preux, Marie Raymondeau, Jade Royo, Darius Sossou, Brigitte
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40 420 Techer, Bertin Vianou.

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50 51 425 52 53 54 426 **Competing interests**

1
2
3 427 No competing interest.
4
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6 428

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8 429 **Word Count**
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10 430 3,964 words
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12 431
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15 432 **References**
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46 518

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

521

	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

522 Table 2 – Blantyre score (from (4))

523

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												■

524 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:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2018-027378.R1
Article Type:	Protocol
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
Secondary Subject Heading:	Paediatrics, Neurology
Keywords:	Paediatric neurology < NEUROLOGY, Paediatric infectious disease & immunisation < PAEDIATRICS, TROPICAL MEDICINE, PARASITOLOGY

SCHOLARONE™
Manuscripts

1 Identification of *Plasmodium falciparum* and host factors associated with cerebral malaria.

2 Description of the protocol for a prospective, case-control study (NeuroCM)

3
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15 31 **Abstract**

16
17 32 **Introduction:** In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred
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19 33 worldwide, in 91 countries. Most cases and deaths were in the African region (respectively 88%
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21 34 and 90%), including Benin, located in West Africa. In Benin, malaria causes 26.8% of
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23 35 consultation and hospitalization motif in the general population and 20.9% in children under
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25 36 five years old.
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28 37 The goal of the NeuroCM project is to identify the causative factors of neuroinflammation in
29
30 38 the context of cerebral malaria. There are currently very few systematic data from West Africa
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32 39 on the etiologies and management of non-malarial non-traumatic coma in small children, and
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34 40 NeuroCM will help to fill this gap. We postulate that an accurate understanding of molecular
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36 41 and cellular mechanisms involved in neuroinflammation may help to define efficient strategies
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38 42 to prevent and manage cerebral malaria.
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42 43 **Methods and analysis:** This is a prospective, case-control study comparing cerebral malaria to
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44 44 uncomplicated malaria and non-malarial non traumatic coma. This study takes place in Benin,
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46 45 precisely in Cotonou for children with coma and in Sô-Ava district for children with
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48 46 uncomplicated malaria. We aim to include 300 children aged between 24 and 71 months and
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50 47 divided in three different clinical groups during 12 months (from December 2017 to November
51
52 48 2018) with a 21-28 days follow-up for coma. Study data, including clinical, biological and
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54 49 research results will be collected and managed using CS online-Ennov clinical.
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3 50 **Ethics and dissemination:** Ethics approval for the NeuroCM study has been obtained from
4
5 51 *Comité National d’Ethique pour la Recherche en santé* of Benin
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7 52 (n°67/MS/DC/SGM/DRFMT/CNERS/SA; 10/17/2017). NeuroCM study has also been
8
9 53 approved by *Comité consultatif de déontologie et d’éthique* of Institut de Recherche pour le
10
11 54 Développement (IRD; 10/24/2017)
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17 56 **Strengths and limitations of this study**

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19 57 ➤ This case-control study aims to identify the causative factors of neuroinflammation in
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21 58 the context of cerebral malaria
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23 59 ➤ This study will inform on the etiologies and management of non-malarial non-traumatic
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25 60 coma in small children
26
27 61 ➤ The final products of NeuroCM are expected to feed the pipeline of new therapeutic
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29 62 (immune intervention) and preventive (vaccine) strategies that will improve cerebral
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31 63 malaria outcome
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33 64 ➤ This study does not have the power to investigate all etiologies of fever in Benin.
34
35 65 Contrary to the malaria groups, there is no information on the frequency of non-malarial
36
37 66 non-traumatic coma admissions, and no certainty on the number of children who will
38
39 67 included in the non-malarial non-traumatic group.
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41 68 ➤ According to the limited number of patients, conclusions will further need to be
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43 69 confirmed in larger studies
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71 Introduction

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

76 In 2017, an estimated 219 million cases and 435,000 deaths of malaria occurred worldwide, in
77 91 countries¹. Despite a recent decrease in malaria mortality due to extensive malaria control
78 through insecticide impregnated bednets and increased use of artemisinin derivatives, 275,000
79 children still die every year from malaria. Most cases and deaths were in African region
80 (respectively 92% and 93%). Severe malaria occurs mostly in non-immune patients and in Sub-
81 Saharian Africa and 90% of severe malaria affect young children². In endemic states, malaria
82 is one of the three major causes of hospitalization in children under five years old.

83 Benin is in West Africa, along the Guinea gulf. In 2018, 11.5 million people live in Benin, most
84 of them in South-Benin. Malaria transmission occurs seasonally, during rainy seasons (from
85 May to August and October). According to the Beninese health department in 2016, malaria is
86 responsible for 26.8% of disease reports in consultation and hospitalization in the general
87 population and for 20.9% in children under five years old³. It is also the first morbidity cause
88 in the general population with a prevalence of 39.7% in 2013, followed by respiratory infections
89 in 12.4% cases and gastro-intestinal disease for 6.4%⁴.

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

1
2
3 96 currently debated, and it has been highlighted that a *P. falciparum* parasitaemia can be observed
4
5 97 in comatose children with coma related to a non-malarial central nervous system disease⁵,
6
7 98 leading to a possible overestimation of CM cases. A recent study in Malawi found that 25% of
8
9 99 CM cases were misdiagnosed and that many children may have had a viral meningoencephalitis
10
11
12 100 concomitant to a malarial infection⁵. Implementation of fundoscopic examination (in order to
13
14 101 look for malaria retinopathy signs)⁶ and microbiological investigations (blood culture,
15
16 102 cerebrospinal fluid (CSF) culture, CSF multiplex PCR) could limit the overestimation of CM
17
18 103 diagnosis, but fundoscopic examination requires trained physicians and microbiological
19
20 104 investigations are expensive. Clinical research needs to focus on new clinical or diagnostic tools
21
22 105 designed to help physicians in order to better diagnose CM.
23
24 106 NeuroCM study aims to collect data (such as blood and cerebrospinal fluid culture, fundoscopic
25
26 107 examination) on coma's etiologies in Beninese young children.
27
28 108 Without treatment, CM is invariably fatal. Even with parenteral artemisinin use, severe malaria
29
30 109 death rate is 20%⁷. In case of severe or CM, patients should be hospitalized in an intensive care
31
32 110 unit. Conscience status, blood pressure, heart and respiratory rates, diuresis and oxygen
33
34 111 saturation have to be monitored every six hours⁸. Parenteral artemisinin instead of quinine is
35
36 112 recommended at a posology of 2.4 mg/kg by injection (3 mg/kg in children under 20 kg of body
37
38 113 weight) at diagnosis, 12 and 24 hours later and then daily until patients can take oral drugs⁷.
39
40 114 Benzodiazepine should be used to treat convulsions (diazepam 0.3 mg/kg; midazolam or
41
42 115 lorazepam). It seems accepted that CM surviving patients generally don't present any
43
44 116 neurological sequelae and fully recover their neurological capacity. However, immediate
45
46 117 neurological after-effect is described in 6.7 to 11.6% of cases^{7,9} and a recent meta-analysis
47
48 118 found a relation between CM and neurologic disease¹⁰. The NeuroCM study will collect data
49
50 119 on children's clinical recovery at discharge and 21-28 days later.
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3 120 Tools for malaria control are less and less effective. On one hand, *P. falciparum* drug resistance
4
5 121 is a growing concern. Resistance to chloroquine, one of the main anti-malarial drugs, appeared
6
7 122 during the sixties in South-East Asia and then spread to Africa^{11,12}. Artemisinin-combined
8
9 123 therapy became the treatment of choice for malaria with the aim to reduce the risk of parasites
10
11 124 developing resistance¹³, but resistance appeared in South-East Asia in 2008¹⁴ and was
12
13 125 confirmed in others studies¹⁵. It has not, hitherto, spread to Africa but this is a real concern for
14
15 126 the WHO¹⁶. On the other hand, mosquitos become more and more resistant to insecticides,
16
17 127 making antivectorial prevention more and more difficult¹⁷. Thus, research for new therapies is
18
19 128 needed.

20
21
22
23 129 Pathophysiology of CM is complex and multifactorial, based on both parasite and host immune
24
25 130 factors. It is currently believed that CM is caused by dedicated parasite variants that specifically
26
27 131 localize in brain through interaction between parasite proteins expressed on the surface of the
28
29 132 infected erythrocytes (iE) and brain endothelium. This sequestration occurs with erythrocytes
30
31 133 infected with late stage of *P. falciparum* (trophozoites and schizonts). Binding of iE to
32
33 134 endothelial vascular cells is mediated by Variant Surface Antigens (VSA). *Plasmodium*
34
35 135 virulence is linked to its ability to express VSA¹⁸. VSA include three different multigenic
36
37 136 families: *var*, *rifin* and *stevor*. More specifically, *var* genes coding for PfEMP1 proteins are
38
39 137 highly polymorphic and present in sixty copies in *P. falciparum* genome. Those PfEMP1 are
40
41 138 expressed on iE surface and are responsible for endothelial receptors binding, such as
42
43 139 Chondroitin Sulfate A (CSA) and Endothelial Protein C Receptor (EPCR) or InterCellular
44
45 140 Adhesion Molecule (ICAM) respectively involved in placental malaria¹⁹ and severe malaria²⁰.
46
47 141 PfEMP1 family has been clearly associated with binding of iE to the microvascular endothelium
48
49 142 of every organ and tissue²¹. We now better understand iE's binding on placenta²² and vaccine
50
51 143 development to prevent gestational malaria seems an achievable goal. By contrast, research is
52
53 144 still needed to understand which type of proteins specifically binds to cerebral endothelial
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3 145 receptor. In a previous study conducted in Benin, we identified several proteins associated with
4
5 146 CM²³.

7
8 147 The finding of a PfEMP1 variant specifically related to CM could pave the way to the
9
10 148 development of a vaccine targeting this specific protein. Studying the transcriptomic and
11
12 149 proteomic profiles of plasmodial strains involved in CM compared to strains involved in
13
14 150 uncomplicated malaria (UM) is a first step to better understand related mechanisms to cerebral
15
16 151 endothelium binding.

18
19 152 The host immune aspect of the pathophysiology of CM are the consequences of microvascular
20
21 153 sequestration of iEs and rosettes (non-infected red blood cells) in brain. Such sequestration
22
23 154 leads to blood flow obstruction, ischemia/hypoxia and local inflammation resulting in
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25 155 neuroinflammation²⁴⁻²⁸. The way this cascade of events is linked and the reasons why they result
26
27 156 in death or inflammation resolution remains to be elucidated. Acute cerebral ischemia is known
28
29 157 to drive microglia activation and influx of myeloid immune cells to the brain. Resident
30
31 158 microglia and infiltrating monocytes/neutrophils have a critical role in initiating, sustaining
32
33 159 (M1 polarization) and resolving (M2 polarization) post-ischemic inflammation²⁹⁻³¹. Another
34
35 160 important immune aspect of neuroinflammation during CM is redox equilibrium. The
36
37 161 production of reactive oxygen species both by parasites (haemoglobin digestion) and
38
39 162 monocytes/macrophages are thought to cause damages to neurons³². This process leads to BBB
40
41 163 permeability and neurodegeneration^{33,34}. In order to counterbalance the excess of oxidants,
42
43 164 oxidant scavengers and antioxidant enzymes may be produced. NO bioinsufficiency and
44
45 165 subsequent vasoconstriction constitute other important aspects of CM pathophysiology³⁵. Haem
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47 166 and superoxide anion release during infection leads to NO mobilization for detoxification,
48
49 167 depriving vascular smooth muscle cells in NO and leading to inflammation-related
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51 168 vasospasm^{35,36}. Although vasospasm has not been clearly associated to death risk during CM³⁷,
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53 169 NO pathway deserves a better understanding during CM pathophysiology. In the NeuroCM
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3 170 study, we intend to better understand mechanisms of neuroinflammation and its resolution in a
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5 171 context of CM, by comparing data collected in children presenting with CM, in children
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7 172 hospitalized for non-malarial non-traumatic coma, and in children with UM. We will focus our
8
9 173 studies on markers of immune cell migration and polarization (towards inflammatory or
10
11 174 resolutive phenotypes), of pro- or anti-oxidant response, and of pro- or anti-inflammatory
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13 175 response through urine and blood samples analysis at inclusion, 3 and 21 to 28 days post-
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15 176 inclusion.
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178 **Study objectives**

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23 179 The main objective is to identify the causative factors of neuroinflammation in the context of
24
25 180 CM. There are currently very few systematic data from West Africa on the etiologies and
26
27 181 management of non-traumatic coma in small children, and NeuroCM will bring new
28
29 182 information on these aspects. We postulate that an accurate understanding of molecular and
30
31 183 cellular mechanisms involved in neuroinflammation may help to define efficient strategies to
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33 184 prevent and manage CM.
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40 186 There are three distinct objectives in this study.
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44 188 *I. To identify parasitological factors associated with P. falciparum CM or UM*

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46 189 We expect to identify and validate *P. falciparum* virulence factors associated with CM by
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48 190 comparison with UM. Once proteins of interest will be found, functional studies will help to
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50 191 better understand their role in CM.
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55 193 *II. To identify immune host factors associated with fatal or favorable outcome of CM*
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3 194 We expect to better understand which mechanisms trigger neuroinflammation and its resolution
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5 195 during CM by comparing three groups of children: presenting with CM, hospitalized for non-
6
7 196 malarial non-traumatic coma, and presenting with UM. We aim to identify therapeutic
8
9 197 molecular targets involved in neuroinflammation resolution.
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14 199 *III. To describe coma's etiology in Sub-Saharan Africa*

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16
17 200 We expect to improve knowledge in non-malarial non-traumatic coma's etiologies in Sub-
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19 201 Saharian Africa in order to improve young children's coma management and inform health
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21 202 public policies on the role played by infections that could be prevented by vaccination.
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25
26 204 **Methods and analysis**

27
28 205 Design

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30 206 This is a prospective, case-control study comparing CM to UM and non-malarial non-traumatic
31
32 207 coma. Patients will be recruited in South Benin, in two different hospitals for coma and in a
33
34 208 dispensary for UM, as UM is rarely detected in hospitals where children with coma are
35
36 209 managed. This study is conducted by one Beninese research team (CERPAGE, Centre d'Etude
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38 210 et de Recherche sur le Paludisme Associé à la Grossesse et à l'Enfance) and three French
39
40 211 research teams (UMR D216 MERIT in Paris carrying the project, UMR D152 PHARMADEV
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42 212 in Toulouse, UMR S1094 NET in Limoges).
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49 214 Study environment

50
51 215 This study takes place in Benin, precisely in Cotonou and Calavi for the hospital's recruitment.
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53 216 UM recruitment takes place in Sô-Ava district. Cotonou is the largest city and economic centre
54
55 217 of Benin, with an estimated population of 679,012 habitants in 2013. In the study area,
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57 218 outpatients with UM do not seek care in the health care facilities where children with coma are
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3 219 managed. A multi-center study for UM cases inclusion, using the main patient's origin from
4
5 220 the corresponding hospital, would have been more even accurate.

6
7 221 The two recruitment hospitals are the CHU-MEL (CHU-Mère et Enfant de la Lagune) and
8
9 222 Hôpital de zone de Calavi. Routine laboratory analysis, except bacteriology, are performed on
10
11 223 site for children included with coma and at IRCB (Institut de Recherche Clinique du Bénin) for
12
13 224 children with UM. Bacteriological analyses are performed in the microbiology laboratory of
14
15 225 CNHU (Centre National Hospitalier Universitaire). Research analyses are done in the
16
17 226 CERPAGE laboratory.

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23 24 228 Participants

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26 229 We aim to include 3 different clinical groups of 100 children between 24 and 71 months during
27
28 230 12 months (from December 2017 to November 2018). This duration has been determined
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30 231 according to previous studies in Benin³⁸.

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32
33 232 In the **first group**, a diagnosis of CM will be defined as follows: positive *P. falciparum*
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35 233 parasitaemia with a Blantyre score ≤ 2 with exclusion of patients presenting: positive
36
37 234 bacteraemia, meningitidis proved or suspected (leucocytes in cerebrospinal fluid > 1000 per
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39 235 microliter and/or Gram positive in LCS and/or LCS bacterial culture positive and/or PCR
40
41 236 positive for any bacteria or virus).

42
43
44 237 In the **second group**, a diagnosis of non-malarial non-traumatic coma will be defined as
45
46 238 follows: Blantyre score ≤ 2 and no *Plasmodium* infection as detected by thick blood smear.

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48
49 239 In the **third group** UM will be defined as follows: 1) fever at inclusion or within 24 hours
50
51 240 before, 2) no clinical or biological sign of severe malaria (table 1), no danger signs and no other
52
53 241 obvious cause of fever and 3) *P. falciparum* parasitaemia between 1,000 to 500,000 parasites
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55 242 per microliter.

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244 Inclusion and exclusion criteria

245 For all children, the first inclusion criterion is parental acceptance that their child participate in
246 the study after information has been given (see section “Ethics and safety considerations”).

247 Inclusion criteria for coma (CM and non-malarial non-traumatic coma) are: age between 24 to
248 71 months, Blantyre score ≤ 2 , negative HIV Rapid Diagnostic Test (RDT). Non-inclusion
249 criteria are: pre-existent neurologic disease and traumatic or toxic coma.

250 Inclusion criteria for UM are: age between 24 to 71 months, fever $> 38^{\circ}\text{C}$ at inclusion or within
251 24 hours before and no clinical severity/danger sign, positive malaria RDT, negative HIV RDT.

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

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

256 laboratory indices for severe malaria and/or thick and thin blood smear negative for *P.*
257 *falciparum* and/or parasite density under 1000 parasite per microliter or higher than 500,000

258 parasites per microliter. To evidence a significant difference between CM and UM groups in
259 the ratio of endogenous mediators associated with inflammation resolution, we estimated that

260 a sample size of 100 subjects per group was sufficient to reach the main study target, i.e., by
261 linear regression analysis involving a maximum of 6 predictors and an R^2 value of 0.400,

262 ensuring an 80% power and a 5% probability of type I error. This sample size also complies
263 with the requirements of the RT-qPCR analysis used to validate the discrimination of CM and

264 UM samples obtained by SARTools, and finally with the overall funding request of the project.

265

266 Recruitment process

267 *Step 1: Enrolment/screening*

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3 268 For CM and non-malarial non-traumatic coma group, every young child with neurologic
4
5 269 symptoms is screened for eligibility. For UM group, every child presenting at the outpatient
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7 270 clinic with fever or fever during the previous 24 hours is screened. The first step is patients'
8
9 271 screening to confirm study eligibility and provide participants with information about the study.
10
11 272 A questionnaire assessing eligibility will inform on home addresses, sociodemographic data
12
13 273 (number of children in the family, ethnical group...), clinical history, use of mosquito net and
14
15 274 vaccination status. Informed consent is then obtained from the parents or caregivers.
16
17 275 The following tests are performed to screen for malaria and to rule out HIV infections: a RDT
18
19 276 detecting HRP2 for *P. falciparum* detection and Determine HIV-1/2 set, Alere for HIV
20
21 277 detection.

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26 278 *Step 2: Clinical examination and biological sample/analysis*

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28 279 A clinical examination is performed by a study physician for children hospitalized with coma,
29
30 280 and by a study nurse for UM. In the coma group, a fundoscopic assessment is performed
31
32 281 (Eyepax 1.0 Dioptrix) and pictures are captured and downloaded on an online database. The
33
34 282 clinical data entry is performed on an online case report form.

35
36 283 In order to allocate children to their respective groups, biological analyses according to severe
37
38 284 malaria are needed. For coma's inclusion: thick and thin blood smear analysis, complete blood
39
40 285 count (CBC) (Sysmex KX-21N, Sysmex XT-1800i and ABS Micro ES 60), biochemistry
41
42 286 analysis (Na^+ , K^+ , Cl^- , Ca^{++} , HCO_3^- , albumin, urea, creatinine, glucose, lactate) with Piccolo
43
44 287 Sysmex and ALAT plus bilirubine (Biolabo Kenza Max biochemistry and Mindray BS-200)
45
46 288 are performed on site. Blood culture, Gram staining and bacterial culture for CSF are realized
47
48 289 in a university hospital reference laboratory. Biomérieux Biofire™ FilmArray™
49
50 290 Meningitis/Encephalitis Panel multiplex PCR (looking for *E. coli*, *H. influenzae*, *L.*
51
52 291 *monocytogenes*, *N. meningitidis*, *S. agalactiae*, *S. pneumoniae*, cytomegalovirus, enterovirus,
53
54 292 Epstein-Barr virus, herpes simplex virus (HSV) 1, HSV2, human herpesvirus 6, paraechovirus,
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3 293 varicella zona virus and *Cryptococcus neoformans* and *C. gattii*) will be further performed in
4
5 294 France. The required following samples are needed: one EDTA tube (2 mL) for CBC and
6
7
8 295 malaria diagnostic, one heparin tube (2 mL) for biochemistry analysis, one cerebrospinal
9
10 296 sample (1 mL), one blood sample for blood culture (5 mL) for routine analyses, two additional
11
12 297 EDTA tubes (6 mL) and 50 mL of urine for research analyses.

14 298 For UM inclusions: severe malaria was ruled out according to results from blood cell count
15
16
17 299 (Sysmex XS500i), biochemistry analysis (bilirubin, glucose, creatinine) on Selectra pro
18
19 300 automate (Elitech group) and thick blood smear. The following samples are needed: one EDTA
20
21 301 tube (2 mL), one heparin tube (2 mL) and one fluorinated tube (2mL) for routine analyses, two
22
23 302 additional EDTA tubes (6 mL) and 50 ml of urine for research analyses.

26 303 *Step 3: Research analyses*

28 304 A part of research analyses is realized in Cerpage lab. One to 1.5 mL of blood is cultured in
29
30 305 supplemented Roswell Park Memorial Institute (RPMI) 1640 medium with Albumax (Gibco)
31
32 306 for less than 48 hours until parasites reach the mature stage (from young trophozoite to
33
34 307 schizont), then purified using magnetic-activated cell sorting (MACS; Milteny Biotec, Bergisch
35
36 308 Gladbach, Germany) for binding and endothelial cell activation assay. The resulting mature
37
38 309 stage are stored at -80°C for further mass spectrometry protein analysis. Two hundred µL of
39
40 310 whole blood samples are conserved at -20°C for DNA analysis, 200 µL are transferred in
41
42 311 TRIZol reagent (Life technologies, France) and stored at -80°C for further RNA extraction³⁹,
43
44 312 and 200 µL in liquid nitrogen for parasite cryoconservation. Plasma samples are conserved at -
45
46 313 20°C and -80°C respectively for immune response analysis and dosage of biomarkers.
47
48 314 Peripheral blood mononuclear cells (PBMC) are separated from red blood cells by Ficoll
49
50 315 density gradient and stored in liquid nitrogen. Finally, urines are stored at -80°C for further
51
52 316 analysis. See table 3 for detailed research planning.
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2
3 317 Parasite factors analyses will be performed in several ways. We will compare CM and UM
4
5 318 isolates with whole genome DNA sequencing; RNA-sequencing and by quantitative MS
6
7 319 analysis. Highly polymorphic *var* genes will be assembled and BLASTed against peptide hits
8
9 320 from the MS approach. Nucleotide primers will be designed with DNA-sequencing data and
10
11 321 used in RT-qPCR to validate the RNA-seq data. Associations between gene polymorphisms
12
13 322 and modifications in RNA nature and quantity detected by RNA-seq will be investigated. Then,
14
15 323 we will use recombinant protein and *P. falciparum* genome modification by gene disruption to
16
17 324 study proteins' role.

18
19 325 Host factors study will be based on the analysis of PBMC, plasmas and urine samples of the
20
21 326 three groups of children. PBMC analysis will focus on the phenotyping of monocytes to
22
23 327 distinguish M1 and M2-like phenotypes. Flow cytometry will be used to measure expression
24
25 328 levels of CD11b and CD16 as M1 markers, and CD163 and CD206 as M2 markers. The
26
27 329 assessment of gene expression levels of cytokines, chemokines and their receptors by RT-qPCR
28
29 330 will complete phenotype analysis. Plasmas and urine samples will allow to measure redox (L-
30
31 331 arginine and biopterins), pro-/anti-inflammatory (cytokines, chemokines and lipid mediators
32
33 332 such as eicosanoids) and pro-resolving mediators (such as prostaglandins and lipoxins) by
34
35 333 ELISA or EIA. We will first compare data from the group of CM to the two other groups in
36
37 334 order to identify the biological markers best related to inflammation and neurological
38
39 335 impairment during CM. Second, we will analyze data obtained with the two coma groups at
40
41 336 inclusion (Day 0), at Day 3 and Day 30 to understand the kinetics of immune events and its
42
43 337 relation to death or favorable outcome. Finally, we will search for severity and death risk factors
44
45 338 within the CM groups.

339 *Step 4: Coma follow-up*

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

1
2
3 342 (6 mL) and 50 mL urine will be sampled. In order to prevent losses, parents/guardians are called
4
5 343 a few days before D21-28 to remind them of follow-up visit. No follow-up visit is scheduled
6
7 344 for children with UM.
8
9

10 345

11 12 346 Data management

13
14 347 Data, including clinical, biological and research results are collected and managed using CS
15
16 348 online-Ennov clinical (<https://ufrcb.chu-limoges.fr/crffonline/>). It is a secure, web-based
17
18 349 application designed to support data capture for research studies. Study participants are
19
20 350 identified by a code and have their own account. The two physicians and the nurse were trained
21
22 351 to entry the data on included children in the database. Nobody can delete a patient created in
23
24 352 the base, except the Data manager.

25
26 353 Data & Safety Monitoring Board (DSMB), composed by two physicians specialized in
27
28 354 infectious disease and one statistician, will review allocation of children to the pre-defined study
29
30 355 groups and discuss possible deviations from the expected number of subjects in the groups.
31
32

33 356

34 35 36 37 357 Data analysis

38
39 358 In a first step, descriptive statistics will be realized by calculating mean and standard deviation
40
41 359 (sd) for quantitative variables, and proportion for qualitative variables to determine the main
42
43 360 characteristics of the three clinical groups.

44
45 361 Focusing on cerebral and UM children the MS/MS data will be searched against the databases
46
47 362 (UNIPROT and PlasmoDB⁴⁰), the proteins will be considered as positive hits with at least two
48
49 363 peptides. The MaxQuant software will be used to compare malaria protein expression between
50
51 364 isolates of these two clinical groups. Transcriptomic data will be analyzed with Galaxy
52
53 365 (<https://usegalaxy.org/>) and R software (<https://www.r-project.org/>)⁴¹. The raw data will be
54
55 366 trimmed with Trimmomatic tool for Phred Quality Score Qscore >20, read length >30 bases,
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3 367 and ribosome sequences will be removed with tool sortMeRNA. Reads will be mapped against
4
5 368 the *P. falciparum* 3D7 reference genome combined with *var* transcript sequences from 7 *P.*
6
7 369 *falciparum* genomes. Differential expression analysis on RNAseq data will be performed using
8
9 370 the DESeq2⁴² package considering a 1 log-fold increase as significant using adjusted *p* value <
10
11 371 0.05. Data normalization will be realized with DESeq2 software, with hypothesis that there
12
13 372 exists genes overexpressed and underexpressed and that majority of genes are not expressed in
14
15 373 a differential way. Transcript expression levels (evaluated with RT-qPCR) will be compared by
16
17 374 T-tests and ANOVA of transformed outcomes.

18
19 375 Regarding immune response analysis, potential markers related to inflammation and
20
21 376 neurological symptoms will be compared using variance analysis in samples from children from
22
23 377 the three groups, CM, UM and non-malarial non-traumatic group. The groups will be compared
24
25 378 two by two with a linear regression, with a special attention to CM/UM comparison. Adjustment
26
27 379 variables such as age, sex, ethnical group, time to hospital transfer, body temperature, and co-
28
29 380 morbidities will be taken into account in the model. It will be further determined if a global
30
31 381 comparison between the three groups will be made. Generally speaking, the non-malarial non-
32
33 382 traumatic coma group will be used as a comparator to analyze specific effect of malaria in
34
35 383 neuroinflammation development. The second major question to be answered to is, within the
36
37 384 CM group, whether the changes of the inflammation markers between D0 (admission) and D3
38
39 385 are predictive of the outcome (survival/death). A logistic model (univariate then multivariate)
40
41 386 will be used for this analysis. The same adjustment variables will be used as in the comparison
42
43 387 between groups. The dependent variable will be the outcome survival/death.

44
45 388 The last model (also a logistic regression) will study the changes in inflammation markers
46
47 389 between D3 and D21 in the survivors in order to determine if they are predictive of a favorable
48
49 390 evolution. The dependent variable will be the outcome, here the discharge from the hospital
50
51 391 without apparent sequelae.

392 Missing data are not expected to affect more than 10% of the records for the main factors that
393 will be analyzed. Should they be over 5%, an imputation method such as the MICE method will
394 be applied, as the errors can be considered at random⁴³.

395

396 Patient and public involvement

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

404

405 **Ethics and dissemination**

406 Ethics and safety considerations

407 Ethics approval for the NeuroCM study has been obtained from *Comité National d'Ethique*
408 *pour la Recherche en santé* of Benin (n°67/MS/DC/SGM/DRFMT/CNERS/SA; 10/17/2017).
409 NeuroCM study has also been approved by the *Comité consultatif de déontologie et d'éthique*
410 of Institut de Recherche pour le Développement (IRD; 10/24/2017).

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

414

415 Dissemination

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2
3 416 The main benefits of NeuroCM are targeted on people living in malaria endemic countries. The
4
5 417 study results will be disseminated through a variety of instruments to ensure that a broad range
6
7 418 of both specialists and non-specialists are informed and can properly benefit from the findings.
8
9
10 419 First, through the direct consultations with the WHO's TDR-MIM, Roll Back Malaria program
11
12 420 to reach the wider public health audience; through scientific meetings and peer-reviewed
13
14 421 publications in scientific or medical journals to reach the scientific/medical/public health
15
16 422 communities; through guidelines targeting the medical and paramedical staff for optimization
17
18 423 of severe malaria management, through booklets (e.g. first aid procedures and adapted behavior
19
20 424 in case of emergency) elaborated and adapted to the population of Benin.
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26 426 **Discussion**

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28 427 CM is the most life-threatening form of malaria with high mortality rate in young children.
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30 428 Mortality related to malaria is still high in children population and accurate CM diagnosis
31
32 429 remains challenging. Among CM surviving children, up to 25% have long-term neuro-cognitive
33
34 430 deficits (visual/hearing/cognitive/language impairment/ataxia/hemiparesis/motor deficit...),
35
36 431 and 10% show evidence of mental health disorders⁴⁴. As CM might be one of the more common
37
38 432 causes of epilepsy in malaria-endemic regions, the burden of CM neurological sequelae may be
39
40 433 largely underestimated, but difficult to estimate because diagnosis is challenging in malaria-
41
42 434 endemic regions. Bacterial or viral central nervous system infections may occur in children with
43
44 435 malarial infection; this may not only originate overdiagnosis of CM, but also may overlook
45
46 436 potential bacterial and viral central nervous system infections.

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51 437 The expected impact of NeuroCM is of great magnitude. First, NeuroCM is an attempt to
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53 438 propose improvements for the diagnosis of CM. It will provide as far as possible, for the first
54
55 439 time in West Africa, an identification of the causes of coma in the study area. Second, thanks
56
57 440 to DNA, RNA and protein analyses, NeuroCM will allow to identify new parasite targets for a
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3 441 vaccine to prevent CM. Third, NeuroCM will provide data on the kinetics of appearance of
4
5 442 inflammatory and pro-resolving molecular and cellular events in brain during CM. The role of
6
7 443 endogenous mediators in neuroinflammation resolution during CM will be clarified, with
8
9 444 emphasis on pro-oxidant components and lipid mediators. NeuroCM will also identify markers
10
11 445 allowing the definition of an immunological state in the process of neuroinflammation
12
13 446 resolution in CM patients. Our experimental murine model will allow the formulation of new
14
15 447 hypothesis while proof of concept will be achieved through the correlation of our proposed
16
17 448 targets with patient morbidity and mortality parameters. In the future, it may allow clinicians to
18
19 449 better manage CM, with specific pro-resolving drugs for instance.
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21
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23
24 450 The final products of NeuroCM are expected to feed the pipeline of new therapeutic (immune
25
26 451 intervention) and preventive (vaccine) strategies to improve CM outcome, as well as other
27
28 452 diseases involving neuroinflammation.
29
30

31 453

32 454 **Authors contributions**

33
34
35 455 VJ and JFF drafted the manuscript. GB, AA, SH, DA, NA, MC, PD and JFF revised the
36
37 456 manuscript. GB, AA, FB, SH, DA, NA, MC, PD and JFF designed the study. VJ and LM
38
39 457 organized the study in the field. AM, IDD and JA implemented the study in the field. All
40
41 458 members of the NeuroCM group have substantially contributed to the conception, design or
42
43 459 organization of the study. All authors approved the final version to be submitted to the journal.
44
45
46

47 460

48 461 **Collaborators**

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53 463 Coste, Jean-Eudes Degbelo, Latifou Dramane, Sayeh Jafari-Guemouri, Claire Kamaliddin,
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55 464 Elis ee Kinkpe, Ana ıs Labrunie, Y el e Ladipo, Thomas Lathiere, Audrey Mowendabeka, Jade
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3 465 Papin, Bernard Pippy, Pierre-Marie Preux, Marie Raymondeau, Jade Royo, Darius Sossou,
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5 466 Brigitte Techer, Bertin Vianou.
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9
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18
19 472 **Competing interests**

20
21 473 No competing interest.
22
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24 474

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26 475 **Word Count**

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28 476 4,536 words
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33 478 **References**

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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))

580

581

	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

582 Table 2 – Blantyre score (from (4))

583

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)

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2018-027378.R2
Article Type:	Protocol
Date Submitted by the Author:	08-Apr-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
Secondary Subject Heading:	Paediatrics, Neurology
Keywords:	Paediatric neurology < NEUROLOGY, Paediatric infectious disease & immunisation < PAEDIATRICS, TROPICAL MEDICINE, PARASITOLOGY

SCHOLARONE™
Manuscripts

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3 1 Identification of *Plasmodium falciparum* and host factors associated with cerebral
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5 2 malaria. Description of the protocol for a prospective, case-control study in Benin (NeuroCM)
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55 23 Mowendabeka³, Jade Papin¹, Bernard Pipy², Pierre-Marie Preux³, Marie Raymondeau³, Jade
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13
14 31 **Abstract**

15
16 32 **Introduction:** In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred
17
18 33 worldwide, in 91 countries. In Benin, malaria causes 26.8% of consultation and hospitalization
19
20 34 motif in the general population and 20.9% in children under five years old.

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22 35 The goal of the NeuroCM project is to identify the causative factors of neuroinflammation in
23
24 36 the context of cerebral malaria. There are currently very few systematic data from West Africa
25
26 37 on the etiologies and management of non-malarial non-traumatic coma in small children, and
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28 38 NeuroCM will help to fill this gap. We postulate that an accurate understanding of molecular
29
30 39 and cellular mechanisms involved in neuroinflammation may help to define efficient strategies
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32 40 to prevent and manage cerebral malaria.
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37 41 **Methods and analysis:** This is a prospective, case-control study comparing cerebral malaria to
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39 42 uncomplicated malaria and non-malarial non traumatic coma. This study takes place in Benin,
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41 43 precisely in Cotonou for children with coma and in Sô-Ava district for children with
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43 44 uncomplicated malaria. We aim to include 300 children aged between 24 and 71 months and
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45 46 divided in three different clinical groups during 12 months (from December 2017 to November
46
47 47 2018) with a 21-28 days follow-up for coma. Study data, including clinical, biological and
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49 48 research results will be collected and managed using CS online-Ennov clinical.
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54 49 **Ethics and dissemination:** Ethics approval for the NeuroCM study has been obtained from
55
56 49 *Comité National d’Ethique pour la Recherche en santé* of Benin
57
58 50 (n°67/MS/DC/SGM/DRFMT/CNERS/SA; 10/17/2017). NeuroCM study has also been
59
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3 51 approved by *Comité consultatif de déontologie et d'éthique* of Institut de Recherche pour le
4
5 52 Développement (IRD; 10/24/2017). The study results will be disseminated through the direct
6
7 53 consultations with the WHO's TDR-MIM and Roll Back Malaria program, through scientific
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9 54 meetings and peer-reviewed publications in scientific or medical journals, and through
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11 55 guidelines and booklets.
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57 **Strengths and limitations of this study**

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19 58 ➤ This case-control study aims to identify the causative factors of neuroinflammation in
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21 59 the context of cerebral malaria
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23 60 ➤ This study will inform on the etiologies and management of non-malarial non-traumatic
24
25 61 coma in small children
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27 62 ➤ The final products of NeuroCM are expected to feed the pipeline of new therapeutic
28
29 63 (immune intervention) and preventive (vaccine) strategies that will improve cerebral
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31 64 malaria outcome
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33 65 ➤ This study does not have the power to investigate all etiologies of fever in Benin.
34
35 66 Contrary to the malaria groups, there is no information on the frequency of non-malarial
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37 67 non-traumatic coma admissions, and no certainty on the number of children who will
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39 68 included in the non-malarial non-traumatic group.
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41 69 ➤ According to the limited number of patients, conclusions will further need to be
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43 70 confirmed in larger studies
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72 Introduction

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

77 In 2017, an estimated 219 million cases and 435,000 deaths of malaria occurred worldwide, in
78 91 countries¹. Despite a recent decrease in malaria mortality due to extensive malaria control
79 through insecticide impregnated bednets and increased use of artemisinin derivatives, 275,000
80 children still die every year from malaria. Most cases and deaths were in African region
81 (respectively 92% and 93%). Severe malaria occurs mostly in non-immune patients and in Sub-
82 Saharian Africa and 90% of severe malaria affect young children². In endemic states, malaria
83 is one of the three major causes of hospitalization in children under five years old.

84 Benin is in West Africa, along the Guinea gulf. In 2018, 11.5 million people live in Benin, most
85 of them in South-Benin. Malaria transmission occurs seasonally, during rainy seasons (from
86 May to August and October). According to the Beninese health department in 2016, malaria is
87 responsible for 26.8% of disease reports in consultation and hospitalization in the general
88 population and for 20.9% in children under five years old³. It is also the first morbidity cause
89 in the general population with a prevalence of 39.7% in 2013, followed by respiratory infections
90 in 12.4% cases and gastro-intestinal disease for 6.4%⁴.

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

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2
3 97 currently debated, and it has been highlighted that a *P. falciparum* parasitaemia can be observed
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5 98 in comatose children with coma related to a non-malarial central nervous system disease⁵,
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7
8 99 leading to a possible overestimation of CM cases. A recent study in Malawi found that 25% of
9
10 100 CM cases were misdiagnosed and that many children may have had a viral meningoencephalitis
11
12 101 concomitant to a malarial infection⁵. Implementation of fundoscopic examination (in order to
13
14 102 look for malaria retinopathy signs)⁶ and microbiological investigations (blood culture,
15
16 103 cerebrospinal fluid (CSF) culture, CSF multiplex PCR) could limit the overestimation of CM
17
18 104 diagnosis, but fundoscopic examination requires trained physicians and microbiological
19
20 105 investigations are expensive. Clinical research needs to focus on new clinical or diagnostic tools
21
22 106 designed to help physicians in order to better diagnose CM.
23
24 107 NeuroCM study aims to collect data (such as blood and cerebrospinal fluid culture, fundoscopic
25
26 108 examination) on coma's etiologies in Beninese young children.
27
28
29 109 Without treatment, CM is invariably fatal. Even with parenteral artemisinin use, severe malaria
30
31 110 death rate is 20%⁷. In case of severe or CM, patients should be hospitalized in an intensive care
32
33 111 unit. Conscience status, blood pressure, heart and respiratory rates, diuresis and oxygen
34
35 112 saturation have to be monitored every six hours⁸. Parenteral artemisinin instead of quinine is
36
37 113 recommended at a posology of 2.4 mg/kg by injection (3 mg/kg in children under 20 kg of body
38
39 114 weight) at diagnosis, 12 and 24 hours later and then daily until patients can take oral drugs⁷.
40
41 115 Benzodiazepine should be used to treat convulsions (diazepam 0.3 mg/kg; midazolam or
42
43 116 lorazepam). It seems accepted that CM surviving patients generally don't present any
44
45 117 neurological sequelae and fully recover their neurological capacity. However, immediate
46
47 118 neurological after-effect is described in 6.7 to 11.6% of cases^{7,9} and a recent meta-analysis
48
49 119 found a relation between CM and neurologic disease¹⁰. The NeuroCM study will collect data
50
51 120 on children's clinical recovery at discharge and 21-28 days later.
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3 121 Tools for malaria control are less and less effective. On one hand, *P. falciparum* drug resistance
4
5 122 is a growing concern. Resistance to chloroquine, one of the main anti-malarial drugs, appeared
6
7 123 during the sixties in South-East Asia and then spread to Africa^{11,12}. Artemisinin-combined
8
9 124 therapy became the treatment of choice for malaria with the aim to reduce the risk of parasites
10
11 125 developing resistance¹³, but resistance appeared in South-East Asia in 2008¹⁴ and was
12
13 126 confirmed in others studies¹⁵. It has not, hitherto, spread to Africa but this is a real concern for
14
15 127 the WHO¹⁶. On the other hand, mosquitos become more and more resistant to insecticides,
16
17 128 making antivectorial prevention more and more difficult¹⁷. Thus, research for new therapies is
18
19 129 needed.

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22
23 130 Pathophysiology of CM is complex and multifactorial, based on both parasite and host immune
24
25 131 factors. It is currently believed that CM is caused by dedicated parasite variants that specifically
26
27 132 localize in brain through interaction between parasite proteins expressed on the surface of the
28
29 133 infected erythrocytes (iE) and brain endothelium. This sequestration occurs with erythrocytes
30
31 134 infected with late stage of *P. falciparum* (trophozoites and schizonts). Binding of iE to
32
33 135 endothelial vascular cells is mediated by Variant Surface Antigens (VSA). *Plasmodium*
34
35 136 virulence is linked to its ability to express VSA¹⁸. VSA include three different multigenic
36
37 137 families: *var*, *rifin* and *stevor*. More specifically, *var* genes coding for PfEMP1 proteins are
38
39 138 highly polymorphic and present in sixty copies in *P. falciparum* genome. Those PfEMP1 are
40
41 139 expressed on iE surface and are responsible for endothelial receptors binding, such as
42
43 140 Chondroitin Sulfate A (CSA) and Endothelial Protein C Receptor (EPCR) or InterCellular
44
45 141 Adhesion Molecule (ICAM) respectively involved in placental malaria¹⁹ and severe malaria²⁰.
46
47 142 PfEMP1 family has been clearly associated with binding of iE to the microvascular endothelium
48
49 143 of every organ and tissue²¹. We now better understand iE's binding on placenta²² and vaccine
50
51 144 development to prevent gestational malaria seems an achievable goal. By contrast, research is
52
53 145 still needed to understand which type of proteins specifically binds to cerebral endothelial
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3 146 receptor. In a previous study conducted in Benin, we identified several proteins associated with
4
5 147 CM²³.

6
7
8 148 The finding of a PfEMP1 variant specifically related to CM could pave the way to the
9
10 149 development of a vaccine targeting this specific protein. Studying the transcriptomic and
11
12 150 proteomic profiles of plasmodial strains involved in CM compared to strains involved in
13
14 151 uncomplicated malaria (UM) is a first step to better understand related mechanisms to cerebral
15
16 152 endothelium binding.

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18
19 153 The host immune aspect of the pathophysiology of CM are the consequences of microvascular
20
21 154 sequestration of iEs and rosettes (non-infected red blood cells) in brain. Such sequestration
22
23 155 leads to blood flow obstruction, ischemia/hypoxia and local inflammation resulting in
24
25 156 neuroinflammation²⁴⁻²⁸. The way this cascade of events is linked and the reasons why they result
26
27 157 in death or inflammation resolution remains to be elucidated. Acute cerebral ischemia is known
28
29 158 to drive microglia activation and influx of myeloid immune cells to the brain. Resident
30
31 159 microglia and infiltrating monocytes/neutrophils have a critical role in initiating, sustaining
32
33 160 (M1 polarization) and resolving (M2 polarization) post-ischemic inflammation²⁹⁻³¹. Another
34
35 161 important immune aspect of neuroinflammation during CM is redox equilibrium. The
36
37 162 production of reactive oxygen species both by parasites (haemoglobin digestion) and
38
39 163 monocytes/macrophages are thought to cause damages to neurons³². This process leads to BBB
40
41 164 permeability and neurodegeneration^{33,34}. In order to counterbalance the excess of oxidants,
42
43 165 oxidant scavengers and antioxidant enzymes may be produced. NO bioinsufficiency and
44
45 166 subsequent vasoconstriction constitute other important aspects of CM pathophysiology³⁵. Haem
46
47 167 and superoxide anion release during infection leads to NO mobilization for detoxification,
48
49 168 depriving vascular smooth muscle cells in NO and leading to inflammation-related
50
51 169 vasospasm^{35,36}. Although vasospasm has not been clearly associated to death risk during CM³⁷,
52
53 170 NO pathway deserves a better understanding during CM pathophysiology. In the NeuroCM
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3 171 study, we intend to better understand mechanisms of neuroinflammation and its resolution in a
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5 172 context of CM, by comparing data collected in children presenting with CM, in children
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7 173 hospitalized for non-malarial non-traumatic coma, and in children with UM. We will focus our
8
9 174 studies on markers of immune cell migration and polarization (towards inflammatory or
10
11 175 resolutive phenotypes), of pro- or anti-oxidant response, and of pro- or anti-inflammatory
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13 176 response through urine and blood samples analysis at inclusion, 3 and 21 to 28 days post-
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15 177 inclusion.
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23 179 **Study objectives**

24 180 The main objective is to identify the causative factors of neuroinflammation in the context of
25
26 181 CM. There are currently very few systematic data from West Africa on the etiologies and
27
28 182 management of non-traumatic coma in small children, and NeuroCM will bring new
29
30 183 information on these aspects. We postulate that an accurate understanding of molecular and
31
32 184 cellular mechanisms involved in neuroinflammation may help to define efficient strategies to
33
34 185 prevent and manage CM.
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40 187 There are three distinct objectives in this study.
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44 189 *I. To identify parasitological factors associated with P. falciparum CM or UM*

46 190 We expect to identify and validate *P. falciparum* virulence factors associated with CM by
47
48 191 comparison with UM. Once proteins of interest will be found, functional studies will help to
49
50 192 better understand their role in CM.
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56 194 *II. To identify immune host factors associated with fatal or favorable outcome of CM*
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3 195 We expect to better understand which mechanisms trigger neuroinflammation and its resolution
4
5 196 during CM by comparing three groups of children: presenting with CM, hospitalized for non-
6
7 197 malarial non-traumatic coma, and presenting with UM. We aim to identify therapeutic
8
9 198 molecular targets involved in neuroinflammation resolution.
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14 200 *III. To describe coma's etiology in Sub-Saharan Africa*

16 201 We expect to improve knowledge in non-malarial non-traumatic coma's etiologies in Sub-
17
18 202 Saharian Africa in order to improve young children's coma management and inform health
19
20 203 public policies on the role played by infections that could be prevented by vaccination.
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204

25 205 **Methods and analysis**

26 206 Design

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29 207 This is a prospective, case-control study comparing CM to UM and non-malarial non-traumatic
30
31 208 coma. Patients will be recruited in South Benin, in two different hospitals for coma and in a
32
33 209 dispensary for UM, as UM is rarely detected in hospitals where children with coma are
34
35 210 managed. This study is conducted by one Beninese research team (CERPAGE, Centre d'Etude
36
37 211 et de Recherche sur le Paludisme Associé à la Grossesse et à l'Enfance) and three French
38
39 212 research teams (UMR D216 MERIT in Paris carrying the project, UMR D152 PHARMADEV
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41 213 in Toulouse, UMR S1094 NET in Limoges).
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47 215 Study environment

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50 216 This study takes place in Benin, precisely in Cotonou and Calavi for the hospital's recruitment.
51
52 217 UM recruitment takes place in Sô-Ava district. Cotonou is the largest city and economic centre
53
54 218 of Benin, with an estimated population of 679,012 habitants in 2013.
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3 219 The two recruitment hospitals are the CHU-MEL (CHU-Mère et Enfant de la Lagune) and
4
5 220 Hôpital de zone de Calavi. Routine laboratory analysis, except bacteriology, are performed on
6
7 221 site for children included with coma and at IRCB (Institut de Recherche Clinique du Bénin) for
8
9 222 children with UM. Bacteriological analyses are performed in the microbiology laboratory of
10
11 223 CNHU (Centre National Hospitalier Universitaire). Research analyses are done in the
12
13 224 CERPAGE laboratory.
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19 226 Participants

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21 227 We aim to include 3 different clinical groups of 100 children between 24 and 71 months during
22
23 228 12 months (from December 2017 to November 2018). This duration has been determined
24
25 229 according to previous studies in Benin³⁸.

26
27 230 In the **first group**, a diagnosis of CM will be defined as follows: positive *P. falciparum* thin
28
29 231 blood smear with a Blantyre score ≤ 2 with exclusion of patients presenting: positive
30
31 232 bacteraemia, meningitis proved or suspected (leucocytes in cerebrospinal fluid > 1000 per
32
33 233 microliter and/or Gram positive in LCS and/or LCS bacterial culture positive and/or PCR
34
35 234 positive for any bacteria or virus).

36
37 235 In the **second group**, a diagnosis of non-malarial non-traumatic coma will be defined as
38
39 236 follows: Blantyre score ≤ 2 and no *Plasmodium* infection as detected by thin blood smear.

40
41 237 In the **third group** UM will be defined as follows: 1) fever at inclusion or within 24 hours
42
43 238 before, 2) no clinical or biological sign of severe malaria (table 1), no danger signs and no other
44
45 239 obvious cause of fever and 3) *P. falciparum* parasitaemia between 1,000 to 500,000 parasites
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47 240 per microliter.
48
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53 241

54 242 Inclusion and exclusion criteria

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3 243 For all children, the first inclusion criterion is parental acceptance that their child participate in
4
5 244 the study after information has been given (see section “Ethics and safety considerations”).
6
7 245 Inclusion criteria for coma (CM and non-malarial non-traumatic coma) are: age between 24 to
8
9 246 71 months, Blantyre score ≤ 2 , negative HIV Rapid Diagnostic Test (RDT). Non-inclusion
10
11 247 criteria are: pre-existent neurologic disease and traumatic or toxic coma.
12
13 248 Inclusion criteria for UM are: age between 24 to 71 months, fever $> 38^{\circ}\text{C}$ at inclusion or within
14
15 249 24 hours before and no clinical severity/danger sign, positive malaria RDT, negative HIV RDT.
16
17 250 Exclusion criteria for coma are: thick and thin blood smear not realized at day 0 (D0) and/or
18
19 251 biological blood test no realized at D0 and/or research blood test not realized at D0.
20
21 252 Exclusion criteria for UM are: thick and thin blood smear not realized at day 0 (D0) and/or
22
23 253 biological blood test no realized at D0 and/or research blood test not realized at D0 and/or
24
25 254 laboratory indices for severe malaria and/or thick and thin blood smear negative for *P.*
26
27 255 *falciparum* and/or parasite density under 1000 parasite per microliter or higher than 500,000
28
29 256 parasites per microliter. To evidence a significant difference between CM and UM groups in
30
31 257 the ratio of endogenous mediators associated with inflammation resolution, we estimated that
32
33 258 a sample size of 100 subjects per group was sufficient to reach the main study target, i.e., by
34
35 259 linear regression analysis involving a maximum of 6 predictors and an R^2 value of 0.400,
36
37 260 ensuring an 80% power and a 5% probability of type I error. This sample size also complies
38
39 261 with the requirements of the RT-qPCR analysis used to validate the discrimination of CM and
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41 262 UM samples obtained by SARTools, and finally with the overall funding request of the project.
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264 Recruitment process

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Step 1: Enrolment/screening

266 For CM and non-malarial non-traumatic coma group, every young child with neurologic
267 symptoms is screened for eligibility. For UM group, every child presenting at the outpatient
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1
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3 268 clinic with fever or fever during the previous 24 hours is screened. The first step is patients'
4
5 269 screening to confirm study eligibility and provide participants with information about the study.
6
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8 270 A questionnaire assessing eligibility will inform on home addresses, sociodemographic data
9
10 271 (number of children in the family, ethnical group...), clinical history, use of mosquito net and
11
12 272 vaccination status. Informed consent is then obtained from the parents or caregivers.
13
14 273 The following tests are performed to screen for malaria and to rule out HIV infections: a RDT
15
16 274 detecting HRP2 for *P. falciparum* detection and Determine HIV-1/2 set, Alere for HIV
17
18
19 275 detection.

20
21 276 *Step 2: Clinical examination and biological sample/analysis*

22
23 277 A clinical examination is performed by a study physician for children hospitalized with coma,
24
25 278 and by a study nurse for UM. In the coma group, a fundoscopic assessment is performed
26
27 279 (Eyepax 1.0 Dioptrix) and pictures are captured and downloaded on an online database. The
28
29 280 clinical data entry is performed on an online case report form.

30
31 281 In order to allocate children to their respective groups, biological analyses according to severe
32
33 282 malaria are needed. For coma's inclusion: thick and thin blood smear analysis, complete blood
34
35 283 count (CBC) (Sysmex KX-21N, Sysmex XT-1800i and ABS Micro ES 60), biochemistry
36
37 284 analysis (Na⁺, K⁺, Cl⁻, Ca⁺⁺, HCO₃⁻, albumin, urea, creatinine, glucose, lactate) with Piccolo
38
39 285 Sysmex and ALAT plus bilirubine (Biolabo Kenza Max biochemistry and Mindray BS-200)
40
41 286 are performed on site. Blood culture, Gram staining and bacterial culture for CSF are realized
42
43 287 in a university hospital reference laboratory. Biomérieux Biofire™ FilmArray™
44
45 288 Meningitis/Encephalitis Panel multiplex PCR (looking for *E. coli*, *H. influenzae*, *L.*
46
47 289 *monocytogenes*, *N. meningitidis*, *S. agalactiae*, *S. pneumoniae*, cytomegalovirus, enterovirus,
48
49 290 Epstein-Barr virus, herpes simplex virus (HSV) 1, HSV2, human herpesvirus 6, parvovirus,
50
51 291 varicella zona virus and *Cryptococcus neoformans* and *C. gattii*) will be further performed in
52
53 292 France. The required following samples are needed: one EDTA tube (2 mL) for CBC and
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293 malaria diagnostic, one heparin tube (2 mL) for biochemistry analysis, one cerebrospinal
294 sample (1 mL), one blood sample for blood culture (5 mL) for routine analyses, two additional
295 EDTA tubes (6 mL) and 50 mL of urine for research analyses.

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

301 *Step 3: Research analyses*

302 A part of research analyses is realized in Cerpage lab. One to 1.5 mL of blood is cultured in
303 supplemented Roswell Park Memorial Institute (RPMI) 1640 medium with Albumax (Gibco)
304 for less than 48 hours until parasites reach the mature stage (from young trophozoite to
305 schizont), then purified using magnetic-activated cell sorting (MACS; Milteny Biotec, Bergisch
306 Gladbach, Germany) for binding and endothelial cell activation assay. The resulting mature
307 stage are stored at -80°C for further mass spectrometry protein analysis. Two hundred µL of
308 whole blood samples are conserved at -20°C for DNA analysis, 200 µL are transferred in
309 TRIzol reagent (Life technologies, France) and stored at -80°C for further RNA extraction³⁹,
310 and 200 µL in liquid nitrogen for parasite cryoconservation. Plasma samples are conserved at -
311 20°C and -80°C respectively for immune response analysis and dosage of biomarkers.
312 Peripheral blood mononuclear cells (PBMC) are separated from red blood cells by Ficoll
313 density gradient and stored in liquid nitrogen. Finally, urines are stored at -80°C for further
314 analysis. See table 3 for detailed research planning.

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

1
2
3 318 from the MS approach. Nucleotide primers will be designed with DNA-sequencing data and
4
5 319 used in RT-qPCR to validate the RNA-seq data. Associations between gene polymorphisms
6
7 320 and modifications in RNA nature and quantity detected by RNA-seq will be investigated. Then,
8
9 321 we will use recombinant protein and *P. falciparum* genome modification by gene disruption to
10
11 322 study proteins' role.

12
13
14 323 Host factors study will be based on the analysis of PBMC, plasmas and urine samples of the
15
16 324 three groups of children. PBMC analysis will focus on the phenotyping of monocytes to
17
18 325 distinguish M1 and M2-like phenotypes. Flow cytometry will be used to measure expression
19
20 326 levels of CD11b and CD16 as M1 markers, and CD163 and CD206 as M2 markers. The
21
22 327 assessment of gene expression levels of cytokines, chemokines and their receptors by RT-qPCR
23
24 328 will complete phenotype analysis. Plasmas and urine samples will allow to measure redox (L-
25
26 329 arginine and biopterins), pro-/anti-inflammatory (cytokines, chemokines and lipid mediators
27
28 330 such as eicosanoids) and pro-resolving mediators (such as prostaglandins and lipoxins) by
29
30 331 ELISA or EIA. We will first compare data from the group of CM to the two other groups in
31
32 332 order to identify the biological markers best related to inflammation and neurological
33
34 333 impairment during CM. Second, we will analyze data obtained with the two coma groups at
35
36 334 inclusion (Day 0), at Day 3 and Day 30 to understand the kinetics of immune events and its
37
38 335 relation to death or favorable outcome. Finally, we will search for severity and death risk factors
39
40 336 within the CM groups.

41 42 43 44 45 46 337 *Step 4: Coma follow-up*

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48 338 In children presenting with coma, both clinical data and blood samples are collected at day 3
49
50 339 (D3) and day 21-28 (D21-28) on disease outcome, and for research purpose. One EDTA tube
51
52 340 (6 mL) and 50 mL urine will be sampled. In order to prevent losses, parents/guardians are called
53
54 341 a few days before D21-28 to remind them of follow-up visit. No follow-up visit is scheduled
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56 342 for children with UM.
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344 Data management

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

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

354

355 Data analysis

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

359 Focusing on cerebral and UM children the MS/MS data will be searched against the databases
360 (UNIPROT and PlasmoDB⁴⁰), the proteins will be considered as positive hits with at least two
361 peptides. The MaxQuant software will be used to compare malaria protein expression between
362 isolates of these two clinical groups. Transcriptomic data will be analyzed with Galaxy
363 (<https://usegalaxy.org/>) and R software (<https://www.r-project.org/>)⁴¹. The raw data will be
364 trimmed with Trimmomatic tool for Phred Quality Score Qscore >20, read length >30 bases,
365 and ribosome sequences will be removed with tool sortMeRNA. Reads will be mapped against
366 the *P. falciparum* 3D7 reference genome combined with *var* transcript sequences from 7 *P.*
367 *falciparum* genomes. Differential expression analysis on RNAseq data will be performed using

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2
3 368 the DESeq2⁴² package considering a 1 log-fold increase as significant using adjusted p value <
4
5 369 0.05. Data normalization will be realized with DESeq2 software, with hypothesis that there
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7
8 370 exists genes overexpressed and underexpressed and that majority of genes are not expressed in
9
10 371 a differential way. Transcript expression levels (evaluated with RT-qPCR) will be compared by
11
12 372 T-tests and ANOVA of transformed outcomes.

13
14 373 Regarding immune response analysis, potential markers related to inflammation and
15
16
17 374 neurological symptoms will be compared using variance analysis in samples from children from
18
19 375 the three groups, CM, UM and non-malarial non-traumatic group. The groups will be compared
20
21 376 two by two with a linear regression, with a special attention to CM/UM comparison. Adjustment
22
23 377 variables such as age, sex, ethnical group, time to hospital transfer, body temperature, and co-
24
25 378 morbidities will be taken into account in the model. The threshold for significance level will be
26
27 379 0.05, and a Bonferroni correction will be applied to take into account multiple testing. It will
28
29 380 be further determined if a global comparison between the three groups will be made. Generally
30
31 381 speaking, the non-malarial non-traumatic coma group will be used as a comparator to analyze
32
33 382 specific effect of malaria in neuroinflammation development. The second major question to be
34
35 383 answered to is, within the CM group, whether the changes of the inflammation markers between
36
37 384 D0 (admission) and D3 are predictive of the outcome (survival/death). A logistic model
38
39 385 (univariate then multivariate) will be used for this analysis. The same adjustment variables will
40
41 386 be used as in the comparison between groups. The dependent variable will be the outcome
42
43 387 survival/death.

44
45 388 The last model (also a logistic regression) will study the changes in inflammation markers
46
47 389 between D3 and D21 in the survivors in order to determine if they are predictive of a favorable
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49 390 evolution. The dependent variable will be the outcome, here the discharge from the hospital
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51 391 without apparent sequelae.
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3 392 Missing data are not expected to affect more than 10% of the records for the main factors that
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5 393 will be analyzed. Should they be over 5%, an imputation method such as the MICE method will
6
7 394 be applied, as the errors can be considered at random⁴³. No proteomic analysis for immune
8
9 395 marker will be done.
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16 17 398 Patient and public involvement

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19 399 From patients' experience and preference, follow-up of children admitted with coma was
20
21 400 scheduled in order to be able to detect neurological sequelae. The diagnostic workup proposed
22
23 401 to all children included into the study, although not affordable to all patients in routine practice,
24
25 402 met parent's expectations on what health facilities should provide to all patients. All patients
26
27 403 were recruited in health facilities where they usually seek care, and to that respect patients were
28
29 404 involved in their recruitment process. Finally, results will not be disseminated directly to study
30
31 405 participants but through peer-reviewed scientific journal and conference presentations.
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35 406

37 407 **Ethics and dissemination**

39 408 Ethics and safety considerations

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41
42 409 Ethics approval for the NeuroCM study has been obtained from *Comité National d'Ethique*
43
44 410 *pour la Recherche en santé* of Benin (n°67/MS/DC/SGM/DRFMT/CNERS/SA; 10/17/2017).
45
46 411 NeuroCM study has also been approved by the *Comité consultatif de déontologie et d'éthique*
47
48 412 of Institut de Recherche pour le Développement (IRD; 10/24/2017).
49
50 413 Parents/guardians will be given an oral information by the physician or the nurse and an
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52 414 opportunity to ask question and refuse the protocol. Patient's confidentiality will be ensured
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54 415 and anonymity guaranteed by anonymous coding given at the inclusion.
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3 417 Dissemination
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5 418 The main benefits of NeuroCM are targeted on people living in malaria endemic countries. The
6
7 419 study results will be disseminated through a variety of instruments to ensure that a broad range
8
9 420 of both specialists and non-specialists are informed and can properly benefit from the findings.
10
11 421 First, through the direct consultations with the WHO's TDR-MIM, Roll Back Malaria program
12
13 422 to reach the wider public health audience; through scientific meetings and peer-reviewed
14
15 423 publications in scientific or medical journals to reach the scientific/medical/public health
16
17 424 communities; through guidelines targeting the medical and paramedical staff for optimization
18
19 425 of severe malaria management, through booklets (e.g. first aid procedures and adapted behavior
20
21 426 in case of emergency) elaborated and adapted to the population of Benin.
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28 428 **Discussion**
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30 429 CM is the most life-threatening form of malaria with high mortality rate in young children.
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32 430 Mortality related to malaria is still high in children population and accurate CM diagnosis
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34 431 remains challenging. Among CM surviving children, up to 25% have long-term neuro-cognitive
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36 432 deficits (visual/hearing/cognitive/language impairment/ataxia/hemiparesis/motor deficit...),
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38 433 and 10% show evidence of mental health disorders⁴⁴. As CM might be one of the more common
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40 434 causes of epilepsy in malaria-endemic regions, the burden of CM neurological sequelae may be
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42 435 largely underestimated, but difficult to estimate because diagnosis is challenging in malaria-
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44 436 endemic regions. Bacterial or viral central nervous system infections may occur in children with
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46 437 malarial infection; this may not only originate overdiagnosis of CM, but also may overlook
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48 438 potential bacterial and viral central nervous system infections.
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51 439 Patients were included in different areas reflecting the health care system in Benin. UM patients
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53 440 could not be included in hospital centers such as the CHU-MEL (Cotonou) hospital, and
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55 441 Calavi's hospital, because outpatients with UM rarely seek care in these centers. In 2014, a pilot
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3 442 study aimed to include UM patients in the Cotonou CHU-MEL, and highlighted the absence of
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5 443 UM cases in hospitals. However, patients from the So-Ava areas are referred to the main
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7 444 hospital centers when patients present severe malaria (or any severe illness that cannot be
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9 445 monitored and managed in dispensary). In 2016, we aimed to include patients suffering from
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11 446 cerebral malaria in the So-Ava, and realized that first, patients were directly sent to the main
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13 447 hospitals, and second, that it would not be ethical to include severe malaria cases in these health
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15 448 structures due to the facility itself. A multi-center study for UM cases inclusion, using the main
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17 449 patient's origin from the corresponding hospital, would have been more even accurate. This
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19 450 represents a possible limitation of our study.

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23 451 The expected impact of NeuroCM is of great magnitude. First, NeuroCM is an attempt to
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25 452 propose improvements for the diagnosis of CM. It will provide as far as possible, for the first
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27 453 time in West Africa, an identification of the causes of coma in the study area. Second, thanks
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29 454 to DNA, RNA and protein analyses, NeuroCM will allow to identify new parasite targets for a
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31 455 vaccine to prevent CM. Third, NeuroCM will provide data on the kinetics of appearance of
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33 456 inflammatory and pro-resolving molecular and cellular events in brain during CM. The role of
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35 457 endogenous mediators in neuroinflammation resolution during CM will be clarified, with
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37 458 emphasis on pro-oxidant components and lipid mediators. NeuroCM will also identify markers
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39 459 allowing the definition of an immunological state in the process of neuroinflammation
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41 460 resolution in CM patients. Our experimental murine model will allow the formulation of new
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43 461 hypothesis while proof of concept will be achieved through the correlation of our proposed
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45 462 targets with patient morbidity and mortality parameters. In the future, it may allow clinicians to
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47 463 better manage CM, with specific pro-resolving drugs for instance.

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51 464 The final products of NeuroCM are expected to feed the pipeline of new therapeutic (immune
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53 465 intervention) and preventive (vaccine) strategies to improve CM outcome, as well as other
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55 466 diseases involving neuroinflammation.
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5 **468 Authors contributions**

6
7 469 VJ and JFF drafted the manuscript. GB, AA, SH, DA, NA, MC, PD and JFF revised the
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9
10 470 manuscript. GB, AA, FB, SH, DA, NA, MC, PD and JFF designed the study. VJ and LM
11
12 471 organized the study in the field. AM, IDD and JA implemented the study in the field. All
13
14 472 members of the NeuroCM group have substantially contributed to the conception, design or
15
16 473 organization of the study. All authors approved the final version to be submitted to the journal.
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35 48136
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47 **486 Competing interests**

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49 487 No competing interest.
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55 **489 Data availability statement**

56 490 There are no data in this work.
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5 493 4,696 words
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10 495 **References**
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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

598

	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

599 Table 2 – Blantyre score (from (4))

600

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