

BMJ Open

BMJ Open is committed to open peer review. As part of this commitment we make the peer review history of every article we publish publicly available.

When an article is published we post the peer reviewers' comments and the authors' responses online. We also post the versions of the paper that were used during peer review. These are the versions that the peer review comments apply to.

The versions of the paper that follow are the versions that were submitted during the peer review process. They are not the versions of record or the final published versions. They should not be cited or distributed as the published version of this manuscript.

BMJ Open is an open access journal and the full, final, typeset and author-corrected version of record of the manuscript is available on our site with no access controls, subscription charges or pay-per-view fees (<http://bmjopen.bmj.com>).

If you have any questions on BMJ Open's open peer review process please email info.bmjopen@bmj.com

BMJ Open

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2018-027378
Article Type:	Protocol
Date Submitted by the Author:	25-Oct-2018
Complete List of Authors:	<p>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 Affolabi, Dissou; Calavi Hospital, Pediatric department Authier, Hélène; PHARMADEV, IRD Ayedadjou, Linda; Hopital Bichat - Claude-Bernard Biokou, Bibiane; CHU-MEL hospital, Pediatric department Coste, Agnès; PHARMADEV, IRD Degbelo, Jean-Eudes; Institut de Recherche Clinique du Bénin Dramane, Latifou; Hopital Bichat - Claude-Bernard, Parasitology laboratory Jafari-Guemouri, Sayeh; MERIT, IRD Kamaliddin, Claire; MERIT, IRD Kinkpe, Elisée; Hopital Bichat - Claude-Bernard Labrunie, Anais; Inserm UMR 1094, Tropical Neuroepidemiology Ladipo, Yélé; CHU-MEL hospital, Pediatric department Lathiere, Thomas; Inserm UMR 1094, Tropical Neuroepidemiology Mowendabeka, Audrey; Inserm UMR 1094, Tropical Neuroepidemiology Papin, Jade; MERIT, IRD Pipy, Bernard; Inserm UMR 1094, Tropical Neuroepidemiology Preux, Pierre-Marie; UMR 1094, Tropical Neuroepidemiology Raymondeau, Marie; UMR 1094, Tropical Neuroepidemiology Royo, Jade; PHARMADEV, IRD Sossou, Darius; Hopital Bichat - Claude-Bernard, Parasitology laboratory Techer, Brigitte; MERIT, IRD</p>

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

	Vianou, Bertin; Hopital Bichat - Claude-Bernard, Parasitology laboratory
Keywords:	Paediatric neurology < NEUROLOGY, Paediatric infectious disease & immunisation < PAEDIATRICS, TROPICAL MEDICINE, PARASITOLOGY



1
2
3 1 Identification of *Plasmodium falciparum* and host factors associated with cerebral malaria. A
4
5 2 prospective, case-control study (NeuroCM)
6
7 3

8
9
10 4 Valentin Joste¹, Laurine Maurice^{1,2}, GI Bertin¹, Agnès Aubouy², Farid Boumédiène³, Sandrine
11
12 5 Houzé^{1,4,9}, Daniel Ajzenberg³, Nicolas Argy^{1,4,9}, Achille Massougbodji⁵, Ida Dossou-Dagba⁶, Jules
13
14 6 Alao⁷, Michel Cot¹, Philippe Deloron¹, Jean François Faucher^{3,8} and the NeuroCM group.
15
16
17 7

18
19 8 ¹. MERIT, IRD, Université Paris 5, Sorbonne Paris Cité, Paris, 75006, France
20

21 9 ². PHARMADEV, IRD, Université Paul Sabatier Toulouse III, Toulouse, France
22

23
24 10 ³. NET, INSERM, Université de Limoges, Limoges, France
25

26 11 ⁴. Laboratoire de Parasitologie-Mycologie, AP-HP, Hôpital Bichat, Paris
27

28 12 ⁵. Institut de Recherche Clinique du Bénin (IRCB), Calavi, Benin
29

30 13 ⁶. Pediatric Department, Calavi Hospital, Calavi, Benin
31
32

33 14 ⁷. Pediatric Department, Mother and Child University and Hospital Center (CHUMEL), Cotonou,
34
35 15 Benin.
36

37 16 ⁸. Department of Infectious Diseases, Limoges University Hospital, Limoges, France
38

39 17 ⁹. National French Malaria Reference Center, Bichat-Claude Bernard hospital, Paris
40
41
42 18

43
44 19 **NeuroCM group**: Dissou Affolabi⁶, Hélène Authier², Linda Ayedadjou⁴, Bibiane Biokou⁷, Agnès
45
46 20 Coste², Jean-Eudes Degbelo⁵, Latifou Dramane⁴, Sayeh Jafari-Guemouri¹, Claire Kamaliddin¹,
47
48 21 Elisée Kinkpe⁴, Anaïs Labrunie³, Yélé Ladipo⁷, Thomas Lathiere³, Audrey Mowendabeka³, Jade
49
50 22 Papin¹, Bernard Pipy², Pierre-Marie Preux³, Marie Raymondeau³, Jade Royo², Darius Sossou⁴,
51
52 23 Brigitte Techer¹, Bertin Vianou⁴.
53
54
55
56 24

1
2
3 25 **Corresponding author:**
4

5 26 Joste Valentin, MERIT, IRD, Université Paris 5, Sorbonne Paris Cité, Paris, 75006, France

7 27 Phone: +33617435543

9 28 Email: valentinjoste@gmail.com
10
11
12 29

13
14 30 **Abstract**

15
16 31 **Introduction:** In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred
17
18 32 worldwide, in 91 countries. Most cases and deaths were in the African region (respectively 88%
19
20 33 and 90%), including Benin, located in West Africa. In Benin, malaria causes 26.8% of disease
21
22 34 report in the general population and 20.9% in children under five years old.

23
24 35 The goal of the NeuroCM project is to identify the causative and remedial factors of
25
26 36 neuroinflammation in the context of cerebral malaria. There are currently very few systematic data
27
28 37 from West Africa on the etiologies and management of non-traumatic coma in small children, and
29
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
33
34 40 prevent and manage cerebral malaria.

35
36 41 **Methods and analysis:** This is a prospective, case-control study comparing cerebral malaria to
37
38 42 uncomplicated malaria and non-malarial coma. This study takes place in Benin, precisely in
39
40 43 Cotonou for the hospital's recruitment. Uncomplicated malaria recruitment proceeds in Sô-Ava
41
42 44 district. We aim to include 300 children between 24 and 71 months divided in three different
43
44 45 clinical groups during 12 months (from December 2017 to November 2018). Study data, including
45
46 46 clinical, biological and research results will be collected and managed using CS online-Ennov
47
48 47 clinical.
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 48 **Ethics and dissemination:** Ethics approval for the NeuroCM study has been obtained from *Comité*
4
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)
12
13
14
15
16

17 54 **Strengths and limitations of this study**

- 18
19 55 ➤ This case-control study aims to identify the causative and remedial factors of
20
21 56 neuroinflammation in the context of cerebral malaria
22
23 57 ➤ This study will inform on the etiologies and management of non-traumatic coma in small
24
25 58 children
26
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
32
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
36
37 64 admissions, and no certainty on the number of children who will included in the non-
38
39 65 *Plasmodium* group.
40
41 66 ➤ According to the low number of patients, conclusions will further need to be confirmed in
42
43 67 larger studies
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
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
6
7 95 comatose children with a non-malarial central nervous system disease requiring another treatment
8
9 96 than antimalarials⁵. Diagnostic of cerebral malaria could therefore be overestimated. A recent study
10
11 97 in Malawi found that 25% of cerebral malaria cases were misdiagnosed and that many children
12
13 98 may have had a viral meningoencephalitis concomitant to a malarial infection⁵. Implementation of
14
15 99 fundoscopic examination (in order to look for malaria retinopathy signs)⁶ and microbiological
16
17 100 investigations (blood culture, cerebrospinal fluid (CSF) culture, CSF multiplex PCR) could limit
18
19 101 the overestimation of cerebral malaria diagnosis, but fundoscopic examination requires trained
20
21 102 physicians and microbiological investigations are expensive. Clinical research needs to focus on
22
23 103 new clinical or diagnostic tools designed to help physicians in order to better diagnose cerebral
24
25 104 malaria.

26
27 105 NeuroCM study aims to collect data (such as blood and cerebrospinal fluid culture, fundoscopic
28
29 106 examination) on coma's etiologies in Beninese young children.

30
31 107 Without treatment, cerebral malaria is invariably fatal. Even with parenteral artemisinin use, severe
32
33 108 malaria death rate is 20%⁷. In case of severe or cerebral malaria, patients should be hospitalized in
34
35 109 an intensive care unit. Conscience status, blood pressure, heart and respiratory rates, diuresis and
36
37 110 oxygen saturation have to be monitored every six hours⁸. Parenteral artemisinin instead of quinine
38
39 111 is recommended at a posology of 2.4 mg/kg by injection (3 mg/kg in children under 20 kg of body
40
41 112 weight) at diagnosis, 12 and 24 hours later and then daily until patients can take oral drugs⁷.
42
43 113 Benzodiazepine should be used to treat convulsions (diazepam 0.3 mg/kg; midazolam or
44
45 114 lorazepam). It seems accepted that cerebral malaria surviving patients generally don't present any
46
47 115 neurological sequelae and fully recover their neurological capacity. However, immediate
48
49 116 neurological after-effect is described in 6.7 to 11.6% of cases⁷⁹ and a recent meta-analysis found a

1
2
3 117 relation between cerebral malaria and neurologic disease¹⁰. The NeuroCM study will collect data
4
5 118 on children's clinical recovery at discharge and 1 month later.

6
7 119 Control means for malaria are less and less effective due to multiple parasite and vector
8
9 120 mechanisms of resistance. First, *P. falciparum* drug resistance is a growing concern. Resistance to
10
11 121 chloroquine, one of the main anti-malarial drugs, appeared during the sixties in South-East Asia
12
13 122 and then spread to Africa¹¹¹². Artemisinin-combined therapy became the treatment of choice for
14
15 123 malaria to reduce the risk of parasites developing resistance¹³. But artemisinin-resistance appeared
16
17 124 in South-East Asia in 2008¹⁴ and was confirmed by others studies¹⁵. It has not, hitherto, spread to
18
19 125 Africa but this is a real concern for the WHO¹⁶. On the other hand, mosquitos become more and
20
21 126 more resistant to insecticides, making antivectorial prevention more and more difficult¹⁷. For those
22
23 127 different reasons, research for new therapies is important and needs to be developed.

24
25
26 128 Pathophysiology of cerebral malaria is complex and multifactorial, based on both parasite and host
27
28 129 immune factors. It is currently believed that cerebral malaria is caused by dedicated parasite
29
30 130 variants that specifically localize in brain through interaction between parasite proteins expressed
31
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
35
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:
39
40 135 *var*, *rifin* and *stevor*. More specifically, *var* genes coding for PfEMP1 proteins are highly
41
42 136 polymorphic and present in sixty copies in *P. falciparum* genome. Those PfEMP1 are expressed
43
44 137 on iE surface and are responsible for endothelial receptors binding, such as Chondroitin Sulfate A
45
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
49
50 140 associated with binding of iE to the microvascular endothelium of every organ and tissue²¹. We

1
2
3 141 now better understand iE's binding on placenta²² and vaccine development to prevent gestational
4
5 142 malaria seems an achievable goal. By contrast, research is still needed to understand which type of
6
7 143 proteins specifically binds to cerebral endothelial receptor. In a previous study conducted in Benin,
8
9 144 we identified several proteins associated with cerebral malaria²³.

11
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
15
16 147 profiles of plasmodial strains involved in cerebral malaria compared to strains involved in
17
18 148 uncomplicated malaria is a first step to better understand related mechanisms to cerebral
19
20 149 endothelium binding.

21
22
23
24 150 The host immune aspect of the pathophysiology of cerebral malaria are the consequences of
25
26 151 microvascular sequestration of iEs and rosettes (non-infected red blood cells) in brain. Such
27
28 152 sequestration leads to blood flow obstruction, ischemia/hypoxia and local inflammation resulting
29
30 153 in neuroinflammation²⁴⁻²⁸. The way this cascade of events is linked and the reasons why they result
31
32 154 in death or inflammation resolution remains to be elucidated. Acute cerebral ischemia is known to
33
34 155 drive microglia activation and influx of myeloid immune cells to the brain. Resident microglia and
35
36 156 infiltrating monocytes/neutrophils have a critical role in initiating, sustaining (M1 polarization) and
37
38 157 resolving (M2 polarization) post-ischemic inflammation²⁹⁻³¹. Another important immune aspect of
39
40 158 neuroinflammation during cerebral malaria is redox equilibrium. The production reactive oxygen
41
42 159 species both by parasites (haemoglobin digestion) and monocytes/macrophages are thought to
43
44 160 cause damages to neurons³². This process leads to BBB permeability and neurodegeneration^{33,34}.
45
46
47
48
49 161 To counterbalance the excess of oxidants, oxidant scavengers and antioxidant enzymes may be
50
51 162 produced. In the NeuroCM study, we intend to better understand mechanisms of
52
53 163 neuroinflammation and its resolution in a context of cerebral malaria, by comparing data collected
54
55 164 in children presenting with cerebral malaria, in children hospitalised for non-malaria non-traumatic

1
2
3 165 coma, and in children with uncomplicated malaria. We will focus our studies on markers of immune
4
5 166 cell migration and polarization (towards inflammatory or resolutive phenotypes), and of pro- or
6
7 167 anti-oxidant response, through urine and blood samples analysis at inclusion, 3 and 21 to 28 days
8
9 168 post-inclusion.
10
11
12
13

14 169
15 170 **Study objectives**
16
17 171 The main objective is to identify the causative and remedial factors of neuroinflammation in the
18
19 172 context of cerebral malaria. There are currently very few systematic data from West Africa on the
20
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
24
25 175 mechanisms involved in neuroinflammation may help to define efficient strategies to prevent and
26
27 176 manage cerebral malaria.
28
29
30

31 177
32
33 178 There are three distinct objectives in this study.
34
35

36 179
37 180 *I. To identify parasitological factors associated with P. falciparum cerebral malaria or*
38
39
40 181 *uncomplicated malaria*
41

42 182 We expect to identify and validate *P. falciparum* virulence factors associated with cerebral malaria
43
44 183 by comparison with uncomplicated malaria. Once proteins of interest will be found, functional
45
46 184 studies will help to better understand their role in cerebral malaria.
47
48

49 185
50
51 186 *II. To identify immune host factors associated with fatal of favourable outcome of*
52
53 187 *cerebral malaria*
54
55
56
57
58
59

1
2
3 188 We expect to better understand which mechanisms trigger neuroinflammation and its resolution
4
5 189 during cerebral malaria by comparing three groups of children: presenting with cerebral malaria,
6
7 190 hospitalised for non-malaria non-traumatic coma, and presenting with uncomplicated malaria. We
8
9 191 aim to identify therapeutic molecular targets involved in neuroinflammation resolution.
10
11
12
13

14 193 *III. To describe coma's etiology in Sub-Saharan Africa*

15
16
17 194 We expect to improve knowledge in non-traumatic coma's etiologies in Sub-Saharan Africa in
18
19 195 order to improve young children's coma management and inform health public policies on the role
20
21 196 played by infections that could be prevented by vaccination.
22
23
24
25

26 198 **Methods and analysis**

27
28 199 Design

29
30
31 200 This is a prospective, case-control study comparing cerebral malaria to uncomplicated malaria and
32
33 201 non-*Plasmodium* coma. Patients will be recruited in South Benin, in two different hospitals for
34
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
38
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
42
43 206 Toulouse, UMR S1094 NET in Limoges).
44
45
46
47
48

49 208 Study environment

50
51 209 This study takes place in Benin, precisely in Cotonou and Calavi for the hospital's recruitment.
52
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.
56
57
58
59
60

1
2
3 212 The two recruitment hospitals are the CHU-MEL (CHU-Mère et Enfant de la Lagune) and Hôpital
4
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
8
9 215 with uncomplicated malaria. Bacteriological analyses are performed in the microbiology laboratory
10
11 216 of CNHU (Centre National Hospitalier Universitaire). Research analyses are done in the
12
13
14 217 CERPAGE laboratory.
15
16

17 218

18

19 219 Participants

20
21 220 We aim to include 3 different clinical groups of 100 children between 24 and 71 months during 12
22
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,
30
31 225 meningitidis proved or suspected (leucocytes in cerebrospinal fluid > 1000 per microliter and/or
32
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:
38
39 229 Blantyre score ≤ 2 and no *Plasmodium* infection as detected by thick blood smear.

40
41 230 In the **third group**, uncomplicated *falciparum* malaria will be defined as follows: 1) fever at
42
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
46
47 233 parasites per microliter.
48
49
50
51
52

53 234

54

55

56 235 Inclusion and exclusion criteria

1
2
3 236 For all children, the first inclusion criterion is parental acceptance that their child participate in the
4
5 237 study after information has been given (see section “Ethics and safety considerations”). Inclusion
6
7 238 criteria for coma (cerebral malaria and non-*Plasmodium* coma) are: age between 24 to 71 months,
8
9 239 Blantyre score ≤ 2 , negative HIV Rapid Diagnostic Test (RDT). Non-inclusion criteria are: pre-
10
11 240 existent neurologic disease and traumatic or toxic coma.

12
13
14 241 Inclusion criteria for uncomplicated *falciparum* malaria are: age between 24 to 71 months, fever $>$
15
16 242 38°C at inclusion or within 24 hours before and no clinical severity/danger sign, positive malaria
17
18 243 RDT, negative HIV RDT.

19
20
21 244 Exclusion criteria for coma are: thick and thin blood smear not realized at day 0 (D0) and/or
22
23 245 biological blood test no realized at D0 and/or research blood test not realized at D0.

24
25
26 246 Exclusion criteria for uncomplicated *falciparum* malaria are: thick and thin blood smear not
27
28 247 realized at day 0 (D0) and/or biological blood test no realized at D0 and/or research blood test not
29
30 248 realized at D0 and/or laboratory indices for severe malaria and/or thick and thin blood smear
31
32 249 negative for *P. falciparum* and/or parasite density under 1000 parasite per microliter or higher than
33
34 250 500,000 parasites per microliter.

35
36
37 251

38 39 40 252 Recruitment process

41 42 253 *Step 1: Enrolment/screening*

43
44 254 The first step is patients’ screening to confirm study eligibility and provide participants with
45
46 255 information about the study. A questionnaire assessing eligibility will inform on home addresses,
47
48 256 sociodemographic data (number of children in the family, ethnical group...), clinical history, use
49
50 257 of mosquito net and vaccination status. Informed consent is then obtained from the parents or
51
52 258 caregivers.

1
2
3 259 The following tests are performed to screen for malaria and to rule out HIV infections: a RDT
4
5 260 detecting HRP2 for *P. falciparum* detection and Determine HIV-1/2 set, Alere for HIV detection.

7
8 261 *Step 2: Clinical examination and biological sample/analysis*

9
10 262 A clinical examination is performed by a study physician for children hospitalised with coma, and
11
12 263 by a study nurse for uncomplicated malaria. In the coma group, a fundoscopic assessment is
13
14 264 performed (Eyepax 1.0 Dioptrix) and pictures are captured and downloaded on an online database.

15
16
17 265 The clinical data entry is performed on an online case report form.

18
19 266 In order to allocate children to their respective groups, biological analyses according to severe
20
21 267 malaria are needed. For coma's inclusion: thick and thin blood smear analysis, complete blood
22
23 268 count (CBC) (Sysmex KX-21N, Sysmex XT-1800i and ABS Micro ES 60), biochemistry analysis
24
25 269 (Na^+ , K^+ , Cl^- , Ca^{++} , HCO_3^- , albumin, urea, creatinine, glucose, lactate) with Piccolo Sysmex and
26
27 270 ALAT plus bilirubine (Biolabo Kenza Max biochemistry and Mindray BS-200) are performed on
28
29 271 site. Blood culture, Gram staining and bacterial culture for cerebrospinal fluid are realized in a
30
31 272 university hospital reference laboratory. Biomérieux Biofire™ FilmArray™
32
33 273 Meningitis/Encephalitis Panel multiplex PCR (looking for *E. coli*, *H. influenzae*, *L.*
34
35 274 *monocytogenes*, *N. meningitidis*, *S. agalactiae*, *S. pneumoniae*, cytomegalovirus, enterovirus,
36
37 275 Epstein-Barr virus, herpes simplex virus (HSV) 1, HSV2, human herpesvirus 6, paraechovirus,
38
39 276 varicella zona virus and *Cryptococcus neoformans* and *C. gattii*) will be further performed in
40
41 277 France. The required following samples are needed: one EDTA tube (2 mL), one heparin tube (2
42
43 278 mL), one cerebrospinal sample (1 mL), one blood sample for blood culture (5 mL) for routine
44
45 279 analyses, two additional EDTA tubes (6 mL) and 50 mL of urine for research analyses.

46
47 280 For uncomplicated malaria inclusions: severe malaria was ruled out according to results from blood
48
49 281 cell count (Sysmex XS500i), biochemistry analysis (bilirubine, glucose, creatinine) on Selectra pro
50
51 282 automate (Elitech group) and thick blood smear. The following samples are needed: one EDTA

1
2
3 283 tube (2 mL), one heparin tube (2 mL) and one fluorinated tube (2mL) for routine analyses, two
4
5 284 additional EDTA tubes (6 mL) and 50 ml of urine for research analyses.
6

7
8 285 *Step 3: Research analyses*
9

10 286 A part of research analyses is realized in Cerpage lab. One to 1.5 mL of blood is cultured in
11
12 287 supplemented Roswell Park Memorial Institute (RPMI) 1640 medium with Albumax (Gibco) for
13
14 288 less than 48 hours until they reach the mature stage (from young trophozoite to schizont), then
15
16 289 purified using magnetic-activated cell sorting (MACS; Milteny Biotec, Bergisch Gladbach,
17
18 290 Germany) for binding and endothelial cell activation assay. The resulting mature stage are stored
19
20 291 at -80°C for further mass spectrometry protein analysis. Two hundreds µL of whole blood samples
21
22 292 are conserved at -20°C for DNA analysis, 200 µL are transferred in TRIzol reagent (Life
23
24 293 technologies, France) and stored at -80°C for further RNA extraction³⁶, and 200 µL in liquid
25
26 293 nitrogen for parasite cryoconservation. Plasma samples are conserved at -20°C and -80°C
27
28 294 respectively for immune response analysis and dosage of biomarkers. Peripheral blood
29
30 295 mononuclear cells (PBMC) are separated from red blood cells by Ficoll density gradient and stored
31
32 296 in liquid nitrogen. Finally, urine are stored at -80°C for further analysis. See table 3 for detailed
33
34 297 research planning.
35
36 298
37
38
39

40 299 Parasite factors analyses will be performed in several ways. We will compare CM and UM isolates
41
42 300 with whole genome DNA sequencing (Sanger Institute, MalariaGen consortium, Illumina
43
44 301 technology); RNA-sequencing and by quantitative MS analysis. Highly polymorphic *var* genes
45
46 302 will be assembled and BLASTed against peptide hits from the MS approach. Nucleotide primers
47
48 303 will be designed with DNA-sequencing data and used in RT-qPCR to validate the RNA-seq data.
49
50 304 Associations between gene polymorphisms and modifications in RNA nature and quantity detected
51
52 305 by RNA-seq will be investigated. Then, we will use recombinant protein and *P. falciparum* genome
53
54 306 modification by gene disruption to study proteins' role.
55
56
57
58
59
60

1
2
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.
18
19
20
21

22 315 *Step 4: Coma follow-up*

23
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
30
31
32

33 320 Data management

34
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.
46
47
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
52
53 329 discuss possible deviations from the expected number of subjects in the groups.
54
55
56
57
58
59
60

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.
8
9

10 358

11 359 **Ethics and dissemination**

12 360 Ethics and safety considerations

13
14
15
16
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
32

33 368

34 369 Dissemination

35
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.
54
55
56
57
58
59
60

1
2
3 3794
5 **380 Discussion**

6
7
8 381 Cerebral malaria is the most life-threatening form of malaria with high mortality rate in young
9
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.

29
30
31
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
54
55
56
57
58
59

1
2
3 403 correlation of our proposed targets with patient morbidity and mortality parameters. In the future,
4
5 404 it may allow clinicians to better manage cerebral malaria, with specific pro-resolving drugs for
6
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
15 408 other diseases involving neuroinflammation.

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
26 413 authors approved the final version to be submitted to the journal.

27 28 414 29 30 31 415 **Collaborators**

32
33 416 NeuroCM study group: Dissou Affolabi, H el ene Authier, Linda Ayedadjou, Bibiane Biokou,
34
35 417 Agn es Coste, Jean-Eudes Degbelo, Latifou Dramane, Sayeh Jafari-Guemouri, Claire Kamaliddin,
36
37
38 418 Elis e Kinkpe, Ana is Labrunie, Y el e Ladipo, Thomas Lathiere, Audrey Mowendabeka, Jade Papin,
39
40 419 Bernard Pippy, Pierre-Marie Preux, Marie Raymondeau, Jade Royo, Darius Sossou, Brigitte
41
42 420 Techer, Bertin Vianou.

43 44 421 45 46 47 422 **Funding**

48
49 423 This work was supported by the French Agence Nationale de la Recherche, under contract ANR-
50
51 424 17-CEI 7-0001-01.

52 53 425 54 55 56 426 **Competing interests**

1
2
3 427 No competing interest.
4

5 428
6

7
8 429 **Word Count**
9

10 430 3,964 words
11

12 431
13

14
15 432 **References**
16

- 17 433 1. World Health Organization. World malaria report 2017 Available at:
18 434 <http://www.who.int/malaria/publications/world-malaria-report-2017/en/>
19
- 20 435 2. Black RE, Cousens S, Johnson HL, *et al.* Global, regional, and national causes of child
21 436 mortality in 2008: a systematic analysis. *Lancet* 2010;375:1969–87.
- 23 437 3. Beninese health department. Annuaire des statistiques sanitaires 2016. Available at:
24 438 http://www2.sante.gouv.bj/IMG/pdf/annuaire_stat_pas_2016.pdf
26
- 27 439 4. World Health Organization. Stratégie de coopération de l’OMS avec le Bénin: 2016-2019.
28 440 Available at: <http://apps.who.int/iris/handle/10665/246191>
29
- 30 441 5. Mallewa M, Valley P, Faragher B, *et al.* Viral CNS infections in children from a malaria-
31 442 endemic area of Malawi: a prospective cohort study. *Lancet Glob Health* 2013;1:e153-160.
- 33 443 6. Beare NAV, Taylor TE, Harding SP, *et al.* Malarial retinopathy: a newly established diagnostic
34 444 sign in severe malaria. *Am J Trop Med Hyg* 2006 Nov;75:790–7.
- 36 445 7. Dondorp AM, Fanello CI, Hendriksen ICE, *et al.* Artesunate versus quinine in the treatment of
38 446 severe *falciparum* malaria in African children (AQUAMAT): an open-label, randomised trial.
39 447 *Lancet* 2010;376:1647–57.
- 41 448 ➤ 8. World Health Organization. La prise en charge du paludisme grave – guide pratique.
42 449 Troisième édition. Available at:
43 450 <http://www.who.int/malaria/publications/atoz/9789241548526/fr/>
44
- 45 451 9. Severe malaria. *Trop Med Int Health TM IH* 2014;19 Suppl 1:7–131.
- 47 452 10. Christensen SS, Eslick GD. Cerebral malaria as a risk factor for the development of epilepsy
49 453 and other long-term neurological conditions: a meta-analysis. *Trans R Soc Trop Med Hyg*
50 454 2015;109:233–8.
- 52 455 11. Wernsdorfer WH. The development and spread of drug-resistant malaria. *Parasitol Today*
53 456 1991;7:297–303.
54
55
56
57
58
59

- 1
2
3 457 12. Mengesha T, Makonnen E. Comparative efficacy and safety of chloroquine and alternative
4 458 antimalarial drugs: a meta-analysis from six African countries. *East Afr Med J* 1999;76:314–
5 459 9.
- 7 460 13. World Health Organization. WHO calls for an immediate halt to provision of single-drug
8 461 artemisinin malaria pills. Available at:
9 462 <http://www.who.int/mediacentre/news/releases/2006/pr02/en/>
- 11 463 14. Noedl H, Se Y, Schaecher K, *et al.* Evidence of artemisinin-resistant malaria in western
12 464 Cambodia. *N Engl J Med* 2008;359:2619–20.
- 15 465 15. Dondorp AM, Nosten F, Yi P, *et al.* Artemisinin resistance in *Plasmodium falciparum* malaria.
16 466 *N Engl J Med* 2009;361:455–67.
- 18 467 16. World Health Organization. Status report on artemisinin resistance and ACT efficacy. Available
19 468 at: <http://www.who.int/malaria/publications/atoz/artemisinin-resistance-august2018/en/>
- 21 469 17. World Health Organization. Global report on insecticide resistance in malaria vectors: 2010–
22 470 2016. Available at: <http://www.who.int/malaria/publications/atoz/9789241514057/en/>
- 24 471 18. Kraemer SM, Smith JD. A family affair: *var* genes, PfEMP1 binding, and malaria disease. *Curr*
25 472 *Opin Microbiol* 2006;9:374–80.
- 28 473 19. Tuikue Ndam NG, Salanti A, Bertin G *et al.* High level of *var2csa* transcription by *Plasmodium*
29 474 *falciparum* isolated from the placenta. *J Infect Dis* 2005;192:331–5.
- 31 475 20. Moussiliou A, Alao MJ, Denoed-Ndam L, *et al.* High plasma levels of soluble endothelial
32 476 protein C receptor are associated with increased mortality among children with cerebral
33 477 malaria in Benin. *J Infect Dis* 2015;211:1484–8.
- 35 478 21. Miller LH, Baruch DI, Marsh K *et al.* The pathogenic basis of malaria. *Nature* 2002;415:673–9.
- 38 479 22. Tuikue Ndam N, Deloron P. Towards a vaccine against pregnancy-associated malaria. *Parasite*
39 480 2008 Sep;15:515–21.
- 41 481 23. Bertin GI, Sabbagh A, Argy N, *et al.* Proteomic analysis of *Plasmodium falciparum* parasites
42 482 from patients with cerebral and uncomplicated malaria. *Sci Rep* 2016;6:26773.
- 44 483 24. White NJ, Turner GDH, Day NPJ, *et al.* Lethal malaria: Marchiafava and Bignami were right.
45 484 *J Infect Dis* 2013;208:192–8.
- 48 485 25. Berendt AR, Tumer GD, Newbold CI. Cerebral malaria: the sequestration hypothesis.
49 486 *Parasitol Today* 1994 Oct;10:412–4.
- 51 487 26. Clark IA, Cowden WB, Rockett KA. The pathogenesis of human cerebral malaria. *Parasitol*
52 488 *Today* 1994;10:417–8.
- 54 489 27. Beare NAV, Harding SP, Taylor TE, *et al.* Perfusion abnormalities in children with cerebral
55 490 malaria and malarial retinopathy. *J Infect Dis* 2009;199:263–71.

- 1
2
3 491 28. Dorovini-Zis K, Schmidt K, Huynh H, *et al.* The neuropathology of fatal cerebral malaria in
4 492 malawian children. *Am J Pathol* 2011;178:2146–58.
- 6 493 29. McDonough A, Weinstein JR. Neuroimmune Response in Ischemic Preconditioning.
7 494 *Neurother J Am Soc Exp Neurother* 2016;13:748–61.
- 9 495 30. Kim E, Cho S. Microglia and Monocyte-Derived Macrophages in Stroke. *Neurother J Am Soc*
10 496 *Exp Neurother* 2016;13:702–18.
- 13 497 31. Xia C-Y, Zhang S, Gao Y, *et al.* Selective modulation of microglia polarization to M2
14 498 phenotype for stroke treatment. *Int Immunopharmacol* 2015;25:377–82.
- 16 499 32. Kumar A, Barrett JP, Alvarez-Croda D-M, *et al.* NOX2 drives M1-like microglial/macrophage
17 500 activation and neurodegeneration following experimental traumatic brain injury. *Brain Behav*
18 501 *Immun* 2016;58:291–309.
- 21 502 33. Pino P, Taoufiq Z, Nitcheu J, *et al.* Blood-brain barrier breakdown during cerebral malaria:
22 503 suicide or murder? *Thromb Haemost* 2005;94:336–40.
- 24 504 34. Postma NS, Mommers EC, Eling WM, *et al.* Oxidative stress in malaria; implications for
25 505 prevention and therapy. *Pharm World Sci* 1996;18:121–9.
- 27 506 35. Bertin GI, Lavstsen T, Guillonneau F, *et al.* Expression of the domain cassette 8 *Plasmodium*
28 507 *falciparum* erythrocyte membrane protein 1 is associated with cerebral malaria in Benin. *PloS*
29 508 *One* 2013;8:e68368.
- 32 509 36. Ponts N, Chung D-WD, Le Roch KG. Strand-specific RNA-seq applied to malaria samples.
33 510 *Methods Mol Biol* 2012;883:59–73.
- 35 511 37. Bertin GI, Sabbagh A, Guillonneau F, *et al.* Differential protein expression profiles between
36 512 *Plasmodium falciparum* parasites isolated from subjects presenting with pregnancy-
37 513 associated malaria and uncomplicated malaria in Benin. *J Infect Dis* 2013;208:1987–97.
- 39 514 38. Otto TD, Wilinski D, Assefa S, *et al.* New insights into the blood-stage transcriptome of
40 515 *Plasmodium falciparum* using RNA-Seq. *Mol Microbiol* 2010;76:12–24.
- 43 516 39. Idro R, Kakooza-Mwesige A, Asea B, *et al.* Cerebral malaria is associated with long-term
44 517 mental health disorders: a cross sectional survey of a long-term cohort. *Malar J* 2016;15:184.

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

4 Valentin Joste¹, Laurine Maurice^{1,2}, GI Bertin¹, Agnès Aubouy², Farid Boumédiène³, Sandrine
5 Houzé^{1,4,9}, Daniel Ajzenberg³, Nicolas Argy^{1,4,9}, Achille Massougbodji⁵, Ida Dossou-Dagba⁶,
6 Jules Alao⁷, Michel Cot¹, Philippe Deloron¹ on behalf of the NeuroCM group, Jean François
7 Faucher^{3,8}

8

9 ¹. MERIT, IRD, Université Paris 5, Sorbonne Paris Cité, Paris, 75006, France

10 ². PHARMADEV, IRD, Université Paul Sabatier Toulouse III, Toulouse, France

11 ³. NET, INSERM, Université de Limoges, Limoges, France

12 ⁴. Laboratoire de Parasitologie-Mycologie, AP-HP, Hôpital Bichat, Paris

13 ⁵. Institut de Recherche Clinique du Bénin (IRCB), Calavi, Benin

14 ⁶. Pediatric Department, Calavi Hospital, Calavi, Benin

15 ⁷. Pediatric Department, Mother and Child University and Hospital Center (CHUMEL),
16 Cotonou, Benin.

17 ⁸. Department of Infectious Diseases, Limoges University Hospital, Limoges, France

18 ⁹. National French Malaria Reference Center, Bichat-Claude Bernard hospital, Paris

19

20 **NeuroCM group:** Dissou Affolabi⁶, Hélène Authier², Linda Ayedadjou⁴, Bibiane Biokou⁷,
21 Agnès Coste², Jean-Eudes Degbelo⁵, Latifou Dramane⁴, Sayeh Jafari-Guemouri¹, Claire
22 Kamaliddin¹, Elisée Kinkpe⁴, Anaïs Labrunie³, Yélé Ladipo⁷, Thomas Lathiere³, Audrey
23 Mowendabeka³, Jade Papin¹, Bernard Pipy², Pierre-Marie Preux³, Marie Raymondeau³, Jade
24 Royo², Darius Sossou⁴, Brigitte Techer¹, Bertin Vianou⁴.

25

1
2
3 26 **Corresponding author:**
4

5 27 Joste Valentin, MERIT, IRD, Université Paris 5, Sorbonne Paris Cité, Paris, 75006, France
6

7 28 Phone: +33617435543
8

9 29 Email: valentinjoste@gmail.com
10
11
12
13
14

15 31 **Abstract**

16 32 **Introduction:** In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred
17
18 33 worldwide, in 91 countries. Most cases and deaths were in the African region (respectively 88%
19
20 34 and 90%), including Benin, located in West Africa. In Benin, malaria causes 26.8% of
21
22 35 consultation and hospitalization motif in the general population and 20.9% in children under
23
24 36 five years old.
25
26
27

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
31
32 39 on the etiologies and management of non-malarial non-traumatic coma in small children, and
33
34 40 NeuroCM will help to fill this gap. We postulate that an accurate understanding of molecular
35
36 41 and cellular mechanisms involved in neuroinflammation may help to define efficient strategies
37
38 42 to prevent and manage cerebral malaria.
39
40
41

42 43 **Methods and analysis:** This is a prospective, case-control study comparing cerebral malaria to
43
44 44 uncomplicated malaria and non-malarial non traumatic coma. This study takes place in Benin,
45
46 45 precisely in Cotonou for children with coma and in Sô-Ava district for children with
47
48 46 uncomplicated malaria. We aim to include 300 children aged between 24 and 71 months and
49
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
53
54 49 research results will be collected and managed using CS online-Ennov clinical.
55
56
57
58
59
60

1
2
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
6
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)
12
13
14
15
16

17 56 **Strengths and limitations of this study**

- 18
19 57 ➤ This case-control study aims to identify the causative factors of neuroinflammation in
20
21 58 the context of cerebral malaria
22
23 59 ➤ This study will inform on the etiologies and management of non-malarial non-traumatic
24
25 60 coma in small children
26
27 61 ➤ The final products of NeuroCM are expected to feed the pipeline of new therapeutic
28
29 62 (immune intervention) and preventive (vaccine) strategies that will improve cerebral
30
31 63 malaria outcome
32
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.
40
41 68 ➤ According to the limited number of patients, conclusions will further need to be
42
43 69 confirmed in larger studies
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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.
51
52
53
54
55
56
57
58
59
60

1
2
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 antivectional 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
54
55
56
57
58
59
60

1
2
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
24
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
46
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
50
51 168 vasospasm^{35,36}. Although vasospasm has not been clearly associated to death risk during CM³⁷,
52
53 169 NO pathway deserves a better understanding during CM pathophysiology. In the NeuroCM
54
55
56
57
58
59
60

1
2
3 170 study, we intend to better understand mechanisms of neuroinflammation and its resolution in a
4
5 171 context of CM, by comparing data collected in children presenting with CM, in children
6
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
12
13 175 response through urine and blood samples analysis at inclusion, 3 and 21 to 28 days post-
14
15 176 inclusion.
16
17
18
19
20

177

178 **Study objectives**

21
22
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
32
33 184 prevent and manage CM.
34
35
36
37
38
39

185

40 186 There are three distinct objectives in this study.
41
42
43

187

44 188 *I. To identify parasitological factors associated with P. falciparum CM or UM*

45
46 189 We expect to identify and validate *P. falciparum* virulence factors associated with CM by
47
48 190 comparison with UM. Once proteins of interest will be found, functional studies will help to
49
50 191 better understand their role in CM.
51
52
53

192

54
55 193 *II. To identify immune host factors associated with fatal or favorable outcome of CM*
56
57
58
59
60

1
2
3 194 We expect to better understand which mechanisms trigger neuroinflammation and its resolution
4
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.
10
11
12 198

13 14 199 *III. To describe coma's etiology in Sub-Saharan Africa*

15
16
17 200 We expect to improve knowledge in non-malarial non-traumatic coma's etiologies in Sub-
18
19 201 Saharian Africa in order to improve young children's coma management and inform health
20
21 202 public policies on the role played by infections that could be prevented by vaccination.
22
23

24 203

25 26 204 **Methods and analysis**

27 28 205 Design

29
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
37
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
41
42 212 in Toulouse, UMR S1094 NET in Limoges).
43
44
45
46

47 213

48 49 214 Study environment

50
51 215 This study takes place in Benin, precisely in Cotonou and Calavi for the hospital's recruitment.
52
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,
56
57 218 outpatients with UM do not seek care in the health care facilities where children with coma are
58
59
60

1
2
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.

18
19
20
21
22 227

23 24 228 Participants

25
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
29
30 231 according to previous studies in Benin³⁸.

31
32
33 232 In the **first group**, a diagnosis of CM will be defined as follows: positive *P. falciparum*
34
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
38
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.

47
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
54
55 242 per microliter.

56
57
58 243
59
60

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*

1
2
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
6
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.

22
23
24
25
26 278 *Step 2: Clinical examination and biological sample/analysis*

27
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,
55
56
57
58
59
60

1
2
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
16 299 (Sysmex XS500i), biochemistry analysis (bilirubin, glucose, creatinine) on Selectra pro
17
18 300 automate (Elitech group) and thick blood smear. The following samples are needed: one EDTA
19
20 301 tube (2 mL), one heparin tube (2 mL) and one fluorinated tube (2mL) for routine analyses, two
21
22 302 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.

1
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/crfonline/>). 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
27

28 353 Data & Safety Monitoring Board (DSMB), composed by two physicians specialized in
29
30 354 infectious disease and one statistician, will review allocation of children to the pre-defined study
31
32 355 groups and discuss possible deviations from the expected number of subjects in the groups.
33
34
35

36 356

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

46 361 Focusing on cerebral and UM children the MS/MS data will be searched against the databases
47
48 362 (UNIPROT and PlasmoDB⁴⁰), the proteins will be considered as positive hits with at least two
49
50 363 peptides. The MaxQuant software will be used to compare malaria protein expression between
51
52 364 isolates of these two clinical groups. Transcriptomic data will be analyzed with Galaxy
53
54 365 (<https://usegalaxy.org/>) and R software (<https://www.r-project.org/>)⁴¹. The raw data will be
55
56 366 trimmed with Trimmomatic tool for Phred Quality Score Qscore >20, read length >30 bases,
57
58
59
60

1
2
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

1
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
9 419 First, through the direct consultations with the WHO's TDR-MIM, Roll Back Malaria program
10
11 420 to reach the wider public health audience; through scientific meetings and peer-reviewed
12
13 421 publications in scientific or medical journals to reach the scientific/medical/public health
14
15 422 communities; through guidelines targeting the medical and paramedical staff for optimization
16
17 423 of severe malaria management, through booklets (e.g. first aid procedures and adapted behavior
18
19 424 in case of emergency) elaborated and adapted to the population of Benin.
20
21
22
23
24
25

26 426 **Discussion**

27
28 427 CM is the most life-threatening form of malaria with high mortality rate in young children.
29
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.

47
48
49
50
51 437 The expected impact of NeuroCM is of great magnitude. First, NeuroCM is an attempt to
52
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
58
59
60

1
2
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.
20
21
22

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 33 454 **Authors contributions**

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 49 461 **Collaborators**

50
51 462 NeuroCM group: Dissou Affolabi, H el ene Authier, Linda Ayedadjou, Bibiane Biokou, Agn es
52
53 463 Coste, Jean-Eudes Degbelo, Latifou Dramane, Sayeh Jafari-Guemouri, Claire Kamaliddin,
54
55 464 Elis e Kinkpe, Ana ıs Labrunie, Y el e Ladipo, Thomas Lathiere, Audrey Mowendabeka, Jade
56
57
58
59
60

1
2
3 465 Papin, Bernard Pippy, Pierre-Marie Preux, Marie Raymondeau, Jade Royo, Darius Sossou,
4
5 466 Brigitte Techer, Bertin Vianou.
6
7

8 467

9
10 468 **Funding**

11
12 469 This work was supported by the French Agence Nationale de la Recherche, under contract
13
14 470 ANR-17-CEI 7-0001-01.
15

16
17 471

18
19 472 **Competing interests**

20
21 473 No competing interest.
22
23

24 474

25
26 475 **Word Count**

27
28 476 4,536 words
29
30

31 477

32
33 478 **References**

34
35 479 1. World Health Organization. World malaria report 2018. Available at:
36
37 480 <https://www.who.int/malaria/publications/world-malaria-report-2018/en/>
38

39
40 481 2. Black RE, Cousens S, Johnson HL, *et al*. Global, regional, and national causes of child
41
42 482 mortality in 2008: a systematic analysis. *Lancet* 2010;375:1969–87.
43

44
45 483 3. Beninese health department. Annuaire des statistiques sanitaires 2016. Available at:
46
47 484 http://www2.sante.gouv.bj/IMG/pdf/annuaire_stat_pas_2016.pdf
48

49
50 485 4. World Health Organization. Stratégie de coopération de l'OMS avec le Bénin: 2016-
51
52 486 2019. Available at: <http://apps.who.int/iris/handle/10665/246191>
53

54 487 5. Mallewa M, Vallely P, Faragher B, *et al*. Viral CNS infections in children from a
55
56 488 malaria-endemic area of Malawi: a prospective cohort study. *Lancet Glob Health*. 2013;1:e153-
57
58 489 160.
59
60

- 1
2
3 490 6. Beare NAV, Taylor TE, Harding SP, *et al.* Malarial retinopathy: a newly established
4
5 491 diagnostic sign in severe malaria. *Am J Trop Med Hyg* 2006;75:790–7.
6
7 492 7. Dondorp AM, Fanello CI, Hendriksen ICE, *et al.* Artesunate versus quinine in the
8
9 493 treatment of severe falciparum malaria in African children (AQUAMAT): an open-label,
10
11 494 randomised trial. *Lancet Lancet* 2010;376:1647–57.
12
13
14 495 8. World Health Organization. La prise en charge du paludisme grave - guide pratique.
15
16 Troisième édition. Available at:
17 496 <http://www.who.int/malaria/publications/atoz/9789241548526/fr/>
18
19 497
20
21 498 9. Severe malaria. *Trop Med Int Health TM IH.* 2014;19 Suppl 1:7–131.
22
23
24 499 10. Christensen SS, Eslick GD. Cerebral malaria as a risk factor for the development of
25
26 500 epilepsy and other long-term neurological conditions: a meta-analysis. *Trans R Soc Trop Med*
27
28 501 *Hyg*;109:233–8.
29
30
31 502 11. Wernsdorfer WH. The development and spread of drug-resistant malaria. *Parasitol*
32
33 503 *Today* 1991;7:297–303.
34
35 504 12. Mengesha T, Makonnen E. Comparative efficacy and safety of chloroquine and
36
37 505 alternative antimalarial drugs: a meta-analysis from six African countries. *East Afr Med J*
38
39 506 1999;76:314–9.
40
41
42 507 13. World Health Organization. WHO calls for an immediate halt to provision of single-
43
44 508 drug artemisinin malaria pills. Available at:
45
46 509 <http://www.who.int/mediacentre/news/releases/2006/pr02/en/>
47
48
49 510 14. Noedl H, Se Y, Schaecher K, *et al.* Evidence of artemisinin-resistant malaria in western
50
51 511 Cambodia. *N Engl J Med* 2008;359:2619–20.
52
53
54 512 15. Dondorp AM, Nosten F, Yi P, *et al.* Artemisinin resistance in *Plasmodium falciparum*
55
56 513 malaria. *N Engl J Med* 2009;361:455–67.
57
58 514 16. World Health Organization. Status report on artemisinin resistance and ACT efficacy.
59
60

- 1
2
3 515 Available at: [http://www.who.int/malaria/publications/atoz/artemisinin-resistance-](http://www.who.int/malaria/publications/atoz/artemisinin-resistance-august2018/en/)
4
5 516 [august2018/en/](http://www.who.int/malaria/publications/atoz/artemisinin-resistance-august2018/en/)
6
7
8 517 17. World Health Organization. Global report on insecticide resistance in malaria vectors:
9
10 518 2010-2016. Available at: <http://www.who.int/malaria/publications/atoz/9789241514057/en/>
11
12 519 18. Kraemer SM, Smith JD. A family affair: var genes, PfEMP1 binding, and malaria
13
14 520 disease. *Curr Opin Microbiol* 2006;9:374–80.
15
16
17 521 19. Tuikue Ndam NG, Salanti A, Bertin G, *et al.* High level of var2csa transcription by
18
19 522 *Plasmodium falciparum* isolated from the placenta. *J Infect Dis* 2005;192:331–5.
20
21 523 20. Moussiliou A, Alao MJ, Denoed-Ndam L, *et al.* High plasma levels of soluble
22
23 524 endothelial protein C receptor are associated with increased mortality among children with
24
25 525 cerebral malaria in Benin. *J Infect Dis* 2015;211:1484–8.
26
27
28 526 21. Miller LH, Baruch DI, Marsh K, *et al.* The pathogenic basis of malaria. *Nature*
29
30 527 2002;415:673–9.
31
32
33 528 22. Tuikue Ndam N, Deloron P. Towards a vaccine against pregnancy-associated malaria.
34
35 529 *Parasite* 2008;15:515–21.
36
37
38 530 23. Bertin GI, Sabbagh A, Argy N, *et al.* Proteomic analysis of *Plasmodium falciparum*
39
40 531 parasites from patients with cerebral and UM. *Sci Rep.* 2016;6:26773.
41
42 532 24. White NJ, Turner GDH, Day NPJ, *et al.* Lethal malaria: Marchiafava and Bignami were
43
44 533 right. *J Infect Dis* 2013;208:192–8.
45
46
47 534 25. Berendt AR, Tumer GD, Newbold CI. CM: the sequestration hypothesis. *Parasitol*
48
49 535 *Today* 1994;10:412–4.
50
51 536 26. Clark IA, Cowden WB, Rockett KA. The pathogenesis of human cerebral malaria.
52
53 537 *Parasitol Today* 1994;10:417–8.
54
55
56 538 27. Beare NAV, Harding SP, Taylor TE, *et al.* Perfusion abnormalities in children with
57
58 539 cerebral malaria and malarial retinopathy. *J Infect Dis* 2009;199:263–71.
59
60

- 1
2
3 540 28. Dorovini-Zis K, Schmidt K, Huynh H, *et al.* The neuropathology of fatal cerebral
4
5 541 malaria in malawian children. *Am J Pathol* 2011;178:2146–58.
6
7 542 29. McDonough A, Weinstein JR. Neuroimmune Response in Ischemic Preconditioning.
8
9 543 *Neurother J Am Soc Exp Neurother* 2016;13:748–61.
10
11
12 544 30. Kim E, Cho S. Microglia and Monocyte-Derived Macrophages in Stroke. *Neurother J*
13
14 545 *Am Soc Exp Neurother* 2016;13:702–18.
15
16
17 546 31. Xia C-Y, Zhang S, Gao Y, *et al.* Selective modulation of microglia polarization to M2
18
19 547 phenotype for stroke treatment. *Int Immunopharmacol* 2015 Apr;25:377–82.
20
21
22 548 32. Kumar A, Barrett JP, Alvarez-Croda D-M, *et al.* NOX2 drives M1-like
23
24 549 microglial/macrophage activation and neurodegeneration following experimental traumatic
25
26 550 brain injury. *Brain Behav Immun* 2016;58:291–309.
27
28
29 551 33. Pino P, Taoufiq Z, Nitchou J, *et al.* Blood-brain barrier breakdown during cerebral
30
31 552 malaria: suicide or murder? *Thromb Haemost* 2005;94:336–40.
32
33
34 553 34. Postma NS, Mommers EC, Eling WM, *et al.* Oxidative stress in malaria; implications
35
36 554 for prevention and therapy. *Pharm World Sci PWS* 1996;18:121–9.
37
38 555 35. Eisenhut M. The evidence for a role of vasospasm in the pathogenesis of cerebral
39
40 556 malaria. *Malar J*;14:405.
41
42
43 557 36. Eisenhut M. Vasospasm in cerebral inflammation. *Int J Inflamm* 2014;2014:509707.
44
45 558 37. O’Brien NF, Mutatshi Taty T, Moore-Clingenpeel M, *et al.* Transcranial Doppler
46
47 559 Ultrasonography Provides Insights into Neurovascular Changes in Children with cerebral
48
49 560 malaria. *J Pediatr* 2018;203:116-124.e3.
50
51
52 561 38. Bertin GI, Lavstsen T, Guillonneau F, *et al.* Expression of the domain cassette 8
53
54 562 *Plasmodium falciparum* erythrocyte membrane protein 1 is associated with cerebral malaria in
55
56 563 Benin. *PloS One* 2013;8:e68368.
57
58
59 564 39. Ponts N, Chung D-WD, Le Roch KG. Strand-specific RNA-seq applied to malaria
60

- 1
2
3 565 samples. *Methods Mol Biol Clifton NJ* 2012;883:59–73.
4
5 566 40. Bertin GI, Sabbagh A, Guillonneau F, *et al.* Differential protein expression profiles
6
7 567 between *Plasmodium falciparum* parasites isolated from subjects presenting with pregnancy-
8
9 568 associated malaria and uncomplicated malaria in Benin. *J Infect Dis* 2013;208:1987–97.
10
11 569 41. Otto TD, Wilinski D, Assefa S, *et al.* New insights into the blood-stage transcriptome
12
13 570 of *Plasmodium falciparum* using RNA-Seq. *Mol Microbiol* 2010;76:12–24.
14
15 571 42. Varet H, Brillet-Guéguen L, Coppée J-Y, *et al.* A DESeq2- and EdgeR-Based R Pipeline
16
17 572 for Comprehensive Differential Analysis of RNA-Seq Data. *PloS One* 2016;11:e0157022.
18
19 573 43. van Buuren S, Boshuizen HC, Knook DL. Multiple imputation of missing blood
20
21 574 pressure covariates in survival analysis. *Stat Med* 1999;18:681–94.
22
23 575 44. Idro R, Kakooza-Mwesige A, Asea B, *et al.* Cerebral malaria is associated with long-
24
25 576 term mental health disorders: a cross sectional survey of a long-term cohort. *Malar J*
26
27 577 2016;15:184.
28
29
30
31
32
33 578
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3 1 Identification of *Plasmodium falciparum* and host factors associated with cerebral
4
5 2 malaria. Description of the protocol for a prospective, case-control study in Benin (NeuroCM)
6
7
8 3

9
10 4 Valentin Joste¹, Laurine Maurice^{1,2}, Gwladys I. Bertin¹, Agnès Aubouy², Farid Boumédiène³,
11
12 5 Sandrine Houzé^{1,4,9}, Daniel Ajzenberg³, Nicolas Argy^{1,4,9}, Achille Massougbodji⁵, Ida Dossou-
13
14 6 Dagba⁶, Jules Alao⁷, Michel Cot¹, Philippe Deloron¹ on behalf of the NeuroCM group, Jean
15
16 7 François Faucher^{3,8}
17
18
19 8

20
21 9 ¹. MERIT, IRD, Université Paris 5, Sorbonne Paris Cité, Paris, 75006, France
22

23
24 10 ². PHARMADEV, IRD, Université Paul Sabatier Toulouse III, Toulouse, France
25

26
27 11 ³. NET, INSERM, Université de Limoges, Limoges, France
28

29
30 12 ⁴. Laboratoire de Parasitologie-Mycologie, AP-HP, Hôpital Bichat, Paris
31

32
33 13 ⁵. Institut de Recherche Clinique du Bénin (IRCB), Calavi, Benin
34

35
36 14 ⁶. Pediatric Department, Calavi Hospital, Calavi, Benin
37

38
39 15 ⁷. Pediatric Department, Mother and Child University and Hospital Center (CHUMEL),
40
41 16 Cotonou, Benin.

42
43 17 ⁸. Department of Infectious Diseases, Limoges University Hospital, Limoges, France
44

45
46 18 ⁹. National French Malaria Reference Center, Bichat-Claude Bernard hospital, Paris
47

48
49 20 **NeuroCM group:** Dissou Affolabi⁶, Hélène Authier², Linda Ayedadjou⁴, Bibiane Biokou⁷,
50
51 21 Agnès Coste², Jean-Eudes Degbelo⁵, Latifou Dramane⁴, Sayeh Jafari-Guemouri¹, Claire
52
53 22 Kamaliddin¹, Elisée Kinkpe⁴, Anaïs Labrunie³, Yélé Ladipo⁷, Thomas Lathiere³, Audrey
54
55 23 Mowendabeka³, Jade Papin¹, Bernard Pipy², Pierre-Marie Preux³, Marie Raymondeau³, Jade
56
57 24 Royo², Darius Sossou⁴, Brigitte Techer¹, Bertin Vianou⁴.

58
59 25
60

1
2
3 26 **Corresponding author:**
4

5 27 Joste Valentin, MERIT, IRD, Université Paris 5, Sorbonne Paris Cité, Paris, 75006, France

6
7 28 Phone: +33617435543

8
9 29 Email: valentinjoste@gmail.com
10
11
12
13
14

15 31 **Abstract**

16
17 32 **Introduction:** In 2016, an estimated 216 million cases and 445,000 deaths of malaria occurred
18
19 33 worldwide, in 91 countries. In Benin, malaria causes 26.8% of consultation and hospitalization
20
21 34 motif in the general population and 20.9% in children under five years old.

22
23
24 35 The goal of the NeuroCM project is to identify the causative factors of neuroinflammation in
25
26 36 the context of cerebral malaria. There are currently very few systematic data from West Africa
27
28 37 on the etiologies and management of non-malarial non-traumatic coma in small children, and
29
30 38 NeuroCM will help to fill this gap. We postulate that an accurate understanding of molecular
31
32 39 and cellular mechanisms involved in neuroinflammation may help to define efficient strategies
33
34 40 to prevent and manage cerebral malaria.

35
36
37 41 **Methods and analysis:** This is a prospective, case-control study comparing cerebral malaria to
38
39 42 uncomplicated malaria and non-malarial non traumatic coma. This study takes place in Benin,
40
41 43 precisely in Cotonou for children with coma and in Sô-Ava district for children with
42
43 44 uncomplicated malaria. We aim to include 300 children aged between 24 and 71 months and
44
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
48
49 48 research results will be collected and managed using CS online-Ennov clinical.
50
51 49

52
53 48 **Ethics and dissemination:** Ethics approval for the NeuroCM study has been obtained from
54
55 49 *Comité National d’Ethique pour la Recherche en santé* of Benin
56
57 50 (n°67/MS/DC/SGM/DRFMT/CNERS/SA; 10/17/2017). NeuroCM study has also been
58
59
60

1
2
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
8
9 54 meetings and peer-reviewed publications in scientific or medical journals, and through
10
11 55 guidelines and booklets.
12
13
14
15
16

17 **Strengths and limitations of this study**

- 18
19 58 ➤ This case-control study aims to identify the causative factors of neuroinflammation in
20
21 59 the context of cerebral malaria
- 22
23 60 ➤ This study will inform on the etiologies and management of non-malarial non-traumatic
24
25 61 coma in small children
- 26
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
30
31 64 malaria outcome
- 32
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
36
37 67 non-traumatic coma admissions, and no certainty on the number of children who will
38
39 68 included in the non-malarial non-traumatic group.
- 40
41 69 ➤ According to the limited number of patients, conclusions will further need to be
42
43 70 confirmed in larger studies
44
45
46
47
48

49 71
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3 97 currently debated, and it has been highlighted that a *P. falciparum* parasitaemia can be observed
4
5 98 in comatose children with coma related to a non-malarial central nervous system disease⁵,
6
7 99 leading to a possible overestimation of CM cases. A recent study in Malawi found that 25% of
8
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.
52
53
54
55
56
57
58
59
60

1
2
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.

20
21
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
54
55
56
57
58
59
60

1
2
3 146 receptor. In a previous study conducted in Benin, we identified several proteins associated with
4
5 147 CM²³.

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.

17
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
54
55
56
57
58
59
60

1
2
3 171 study, we intend to better understand mechanisms of neuroinflammation and its resolution in a
4
5 172 context of CM, by comparing data collected in children presenting with CM, in children
6
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
12
13 176 response through urine and blood samples analysis at inclusion, 3 and 21 to 28 days post-
14
15 177 inclusion.
16
17
18
19
20
21
22

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.
35
36
37
38
39

40 187 There are three distinct objectives in this study.
41
42
43

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.
51
52
53

54 193
55
56 194 *II. To identify immune host factors associated with fatal or favorable outcome of CM*
57
58
59
60

1
2
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.
10
11
12 199

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.
21
22
23
24 204

25 205 **Methods and analysis**

26 206 Design

27
28
29
30 207 This is a prospective, case-control study comparing CM to UM and non-malarial non-traumatic
31
32 208 coma. Patients will be recruited in South Benin, in two different hospitals for coma and in a
33
34 209 dispensary for UM, as UM is rarely detected in hospitals where children with coma are
35
36 210 managed. This study is conducted by one Beninese research team (CERPAGE, Centre d'Etude
37
38 211 et de Recherche sur le Paludisme Associé à la Grossesse et à l'Enfance) and three French
39
40 212 research teams (UMR D216 MERIT in Paris carrying the project, UMR D152 PHARMADEV
41
42 213 in Toulouse, UMR S1094 NET in Limoges).
43
44
45
46
47
48

49 215 Study environment

50
51 216 This study takes place in Benin, precisely in Cotonou and Calavi for the hospital's recruitment.
52
53 217 UM recruitment takes place in Sô-Ava district. Cotonou is the largest city and economic centre
54
55 218 of Benin, with an estimated population of 679,012 habitants in 2013.
56
57
58
59
60

1
2
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.
14
15
16
17
18

19 226 Participants

20
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
46
47 240 per microliter.
48
49
50
51
52

53 241

54 242 Inclusion and exclusion criteria

1
2
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
40
41 262 UM samples obtained by SARTools, and finally with the overall funding request of the project.
42
43
44
45
46
47
48
49
50

264 Recruitment process

265 *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
268

1
2
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
7
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
18 275 detection.
19
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

32
33 281 In order to allocate children to their respective groups, biological analyses according to severe
34
35 282 malaria are needed. For coma's inclusion: thick and thin blood smear analysis, complete blood
36
37 283 count (CBC) (Sysmex KX-21N, Sysmex XT-1800i and ABS Micro ES 60), biochemistry
38
39 284 analysis (Na⁺, K⁺, Cl⁻, Ca⁺⁺, HCO₃⁻, albumin, urea, creatinine, glucose, lactate) with Piccolo
40
41 285 Sysmex and ALAT plus bilirubine (Biolabo Kenza Max biochemistry and Mindray BS-200)
42
43 286 are performed on site. Blood culture, Gram staining and bacterial culture for CSF are realized
44
45 287 in a university hospital reference laboratory. Biomérieux Biofire™ FilmArray™
46
47 288 Meningitis/Encephalitis Panel multiplex PCR (looking for *E. coli*, *H. influenzae*, *L.*
48
49 289 *monocytogenes*, *N. meningitidis*, *S. agalactiae*, *S. pneumoniae*, cytomegalovirus, enterovirus,
50
51 290 Epstein-Barr virus, herpes simplex virus (HSV) 1, HSV2, human herpesvirus 6, paraechovirus,
52
53 291 varicella zona virus and *Cryptococcus neoformans* and *C. gattii*) will be further performed in
54
55 292 France. The required following samples are needed: one EDTA tube (2 mL) for CBC and
56
57
58
59
60

1
2
3 293 malaria diagnostic, one heparin tube (2 mL) for biochemistry analysis, one cerebrospinal
4
5 294 sample (1 mL), one blood sample for blood culture (5 mL) for routine analyses, two additional
6
7
8 295 EDTA tubes (6 mL) and 50 mL of urine for research analyses.

9
10 296 For UM inclusions: severe malaria was ruled out according to results from blood cell count
11
12 297 (Sysmex XS500i), biochemistry analysis (bilirubin, glucose, creatinine) on Selectra pro
13
14 298 automate (Elitech group) and thick and thin blood smear. The following samples are needed:
15
16 299 one EDTA tube (2 mL), one heparin tube (2 mL) and one fluorinated tube (2mL) for routine
17
18
19 300 analyses, two additional EDTA tubes (6 mL) and 50 ml of urine for research analyses.

21 301 *Step 3: Research analyses*

22
23
24 302 A part of research analyses is realized in Cerpage lab. One to 1.5 mL of blood is cultured in
25
26 303 supplemented Roswell Park Memorial Institute (RPMI) 1640 medium with Albumax (Gibco)
27
28 304 for less than 48 hours until parasites reach the mature stage (from young trophozoite to
29
30 305 schizont), then purified using magnetic-activated cell sorting (MACS; Milteny Biotec, Bergisch
31
32 306 Gladbach, Germany) for binding and endothelial cell activation assay. The resulting mature
33
34 307 stage are stored at -80°C for further mass spectrometry protein analysis. Two hundred µL of
35
36 308 whole blood samples are conserved at -20°C for DNA analysis, 200 µL are transferred in
37
38 309 TRIZol reagent (Life technologies, France) and stored at -80°C for further RNA extraction³⁹,
39
40 310 and 200 µL in liquid nitrogen for parasite cryoconservation. Plasma samples are conserved at -
41
42 311 20°C and -80°C respectively for immune response analysis and dosage of biomarkers.
43
44
45 312 Peripheral blood mononuclear cells (PBMC) are separated from red blood cells by Ficoll
46
47 313 density gradient and stored in liquid nitrogen. Finally, urines are stored at -80°C for further
48
49 314 analysis. See table 3 for detailed research planning.

50
51
52 315 Parasite factors analyses will be performed in several ways. We will compare CM and UM
53
54 316 isolates with whole genome DNA sequencing; RNA-sequencing and by quantitative MS
55
56 317 analysis. Highly polymorphic *var* genes will be assembled and BLASTed against peptide hits
57
58
59
60

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*

47
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
55
56 342 for children with UM.
57
58
59
60

343

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

1
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
6
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
16 374 neurological symptoms will be compared using variance analysis in samples from children from
17
18 375 the three groups, CM, UM and non-malarial non-traumatic group. The groups will be compared
19
20 376 two by two with a linear regression, with a special attention to CM/UM comparison. Adjustment
21
22 377 variables such as age, sex, ethnical group, time to hospital transfer, body temperature, and co-
23
24 378 morbidities will be taken into account in the model. The threshold for significance level will be
25
26 379 0.05, and a Bonferroni correction will be applied to take into account multiple testing. It will
27
28 380 be further determined if a global comparison between the three groups will be made. Generally
29
30 381 speaking, the non-malarial non-traumatic coma group will be used as a comparator to analyze
31
32 382 specific effect of malaria in neuroinflammation development. The second major question to be
33
34 383 answered to is, within the CM group, whether the changes of the inflammation markers between
35
36 384 D0 (admission) and D3 are predictive of the outcome (survival/death). A logistic model
37
38 385 (univariate then multivariate) will be used for this analysis. The same adjustment variables will
39
40 386 be used as in the comparison between groups. The dependent variable will be the outcome
41
42 387 survival/death.

43
44 388 The last model (also a logistic regression) will study the changes in inflammation markers
45
46 389 between D3 and D21 in the survivors in order to determine if they are predictive of a favorable
47
48 390 evolution. The dependent variable will be the outcome, here the discharge from the hospital
49
50 391 without apparent sequelae.
51
52
53
54
55
56
57
58
59
60

1
2
3 392 Missing data are not expected to affect more than 10% of the records for the main factors that
4
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.
10
11
12 396
13
14 397

16 17 398 Patient and public involvement

18
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.
32
33
34
35 406

36 37 407 **Ethics and dissemination**

38 39 408 Ethics and safety considerations

40
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
51
52 414 opportunity to ask question and refuse the protocol. Patient's confidentiality will be ensured
53
54 415 and anonymity guaranteed by anonymous coding given at the inclusion.
55
56
57
58 416
59
60

1
2
3 417 Dissemination
4

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.
22
23
24
25

26 427

27
28 428 **Discussion**
29

30 429 CM is the most life-threatening form of malaria with high mortality rate in young children.
31
32 430 Mortality related to malaria is still high in children population and accurate CM diagnosis
33
34 431 remains challenging. Among CM surviving children, up to 25% have long-term neuro-cognitive
35
36 432 deficits (visual/hearing/cognitive/language impairment/ataxia/hemiparesis/motor deficit...),
37
38 433 and 10% show evidence of mental health disorders⁴⁴. As CM might be one of the more common
39
40 434 causes of epilepsy in malaria-endemic regions, the burden of CM neurological sequelae may be
41
42 435 largely underestimated, but difficult to estimate because diagnosis is challenging in malaria-
43
44 436 endemic regions. Bacterial or viral central nervous system infections may occur in children with
45
46 437 malarial infection; this may not only originate overdiagnosis of CM, but also may overlook
47
48 438 potential bacterial and viral central nervous system infections.
49

50
51 439 Patients were included in different areas reflecting the health care system in Benin. UM patients
52
53 440 could not be included in hospital centers such as the CHU-MEL (Cotonou) hospital, and
54
55 441 Calavi's hospital, because outpatients with UM rarely seek care in these centers. In 2014, a pilot
56
57
58
59
60

1
2
3 442 study aimed to include UM patients in the Cotonou CHU-MEL, and highlighted the absence of
4
5 443 UM cases in hospitals. However, patients from the So-Ava areas are referred to the main
6
7 444 hospital centers when patients present severe malaria (or any severe illness that cannot be
8
9 445 monitored and managed in dispensary). In 2016, we aimed to include patients suffering from
10
11 446 cerebral malaria in the So-Ava, and realized that first, patients were directly sent to the main
12
13 447 hospitals, and second, that it would not be ethical to include severe malaria cases in these health
14
15 448 structures due to the facility itself. A multi-center study for UM cases inclusion, using the main
16
17 449 patient's origin from the corresponding hospital, would have been more even accurate. This
18
19 450 represents a possible limitation of our study.

20
21
22
23 451 The expected impact of NeuroCM is of great magnitude. First, NeuroCM is an attempt to
24
25 452 propose improvements for the diagnosis of CM. It will provide as far as possible, for the first
26
27 453 time in West Africa, an identification of the causes of coma in the study area. Second, thanks
28
29 454 to DNA, RNA and protein analyses, NeuroCM will allow to identify new parasite targets for a
30
31 455 vaccine to prevent CM. Third, NeuroCM will provide data on the kinetics of appearance of
32
33 456 inflammatory and pro-resolving molecular and cellular events in brain during CM. The role of
34
35 457 endogenous mediators in neuroinflammation resolution during CM will be clarified, with
36
37 458 emphasis on pro-oxidant components and lipid mediators. NeuroCM will also identify markers
38
39 459 allowing the definition of an immunological state in the process of neuroinflammation
40
41 460 resolution in CM patients. Our experimental murine model will allow the formulation of new
42
43 461 hypothesis while proof of concept will be achieved through the correlation of our proposed
44
45 462 targets with patient morbidity and mortality parameters. In the future, it may allow clinicians to
46
47 463 better manage CM, with specific pro-resolving drugs for instance.

48
49
50
51 464 The final products of NeuroCM are expected to feed the pipeline of new therapeutic (immune
52
53 465 intervention) and preventive (vaccine) strategies to improve CM outcome, as well as other
54
55 466 diseases involving neuroinflammation.
56
57
58
59
60

1
2
3 4674
5 468 **Authors contributions**

6
7 469 VJ and JFF drafted the manuscript. GB, AA, SH, DA, NA, MC, PD and JFF revised the
8
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.

17 474

18
19
20
21 475 **Collaborators**

22
23 476 NeuroCM group: Dissou Affolabi, H el ene Authier, Linda Ayedadjou, Bibiane Biokou, Agn es
24
25 477 Coste, Jean-Eudes Degbelo, Latifou Dramane, Sayeh Jafari-Guemouri, Claire Kamaliddin,
26
27 478 Elis ee Kinkpe, Ana ıs Labrunie, Y el e Ladipo, Thomas Lathiere, Audrey Mowendabeka, Jade
28
29 479 Papin, Bernard Pippy, Pierre-Marie Preux, Marie Raymondeau, Jade Royo, Darius Sossou,
30
31 480 Brigitte Techer, Bertin Vianou.

32
33 48134
35
36
37 482 **Funding**

38
39 483 This work was supported by the French Agence Nationale de la Recherche, under contract
40
41 484 ANR-17-CEI 7-0001-01.

42
43 48544
45
46
47 486 **Competing interests**

48
49 487 No competing interest.

50
51 48852
53
54 489 **Data availability statement**

55
56 490 There are no data in this work.

57
58 491

1
2
3 492 **Word Count**
4

5 493 4,696 words
6
7
8 494

9
10 495 **References**
11

- 12 496 1. World Health Organization. World malaria report 2018. Available at:
13
14 497 <https://www.who.int/malaria/publications/world-malaria-report-2018/en/>
15
16
17 498 2. Black RE, Cousens S, Johnson HL, *et al.* Global, regional, and national causes of child
18
19 499 mortality in 2008: a systematic analysis. *Lancet* 2010;375:1969–87.
20
21 500 3. Beninese health department. Annuaire des statistiques sanitaires 2016. Available at:
22
23 501 http://www2.sante.gouv.bj/IMG/pdf/annuaire_stat_pas_2016.pdf
24
25
26 502 4. World Health Organization. Stratégie de coopération de l'OMS avec le Bénin: 2016-
27
28 503 2019. Available at: <http://apps.who.int/iris/handle/10665/246191>
29
30
31 504 5. Mallewa M, Vallely P, Faragher B, *et al.* Viral CNS infections in children from a
32
33 505 malaria-endemic area of Malawi: a prospective cohort study. *Lancet Glob Health*. 2013;1:e153-
34
35 506 160.
36
37 507 6. Beare NAV, Taylor TE, Harding SP, *et al.* Malarial retinopathy: a newly established
38
39 508 diagnostic sign in severe malaria. *Am J Trop Med Hyg* 2006;75:790–7.
40
41
42 509 7. Dondorp AM, Fanello CI, Hendriksen ICE, *et al.* Artesunate versus quinine in the
43
44 510 treatment of severe falciparum malaria in African children (AQUAMAT): an open-label,
45
46 511 randomised trial. *Lancet Lancet* 2010;376:1647–57.
47
48
49 512 8. World Health Organization. La prise en charge du paludisme grave - guide pratique.
50
51 513 Troisième édition. Available at:
52
53 514 <http://www.who.int/malaria/publications/atoz/9789241548526/fr/>
54
55
56 515 9. Severe malaria. *Trop Med Int Health TM IH*. 2014;19 Suppl 1:7–131.
57
58 516 10. Christensen SS, Eslick GD. Cerebral malaria as a risk factor for the development of
59
60

- 1
2
3 517 epilepsy and other long-term neurological conditions: a meta-analysis. *Trans R Soc Trop Med*
4
5 518 *Hyg*;109:233–8.
6
7
8 519 11. Wernsdorfer WH. The development and spread of drug-resistant malaria. *Parasitol*
9
10 520 *Today* 1991;7:297–303.
11
12 521 12. Mengesha T, Makonnen E. Comparative efficacy and safety of chloroquine and
13
14 522 alternative antimalarial drugs: a meta-analysis from six African countries. *East Afr Med J*
15
16 523 1999;76:314–9.
17
18
19 524 13. World Health Organization. WHO calls for an immediate halt to provision of single-
20
21 525 drug artemisinin malaria pills. Available at:
22
23 526 <http://www.who.int/mediacentre/news/releases/2006/pr02/en/>
24
25
26 527 14. Noedl H, Se Y, Schaecher K, *et al.* Evidence of artemisinin-resistant malaria in western
27
28 528 Cambodia. *N Engl J Med* 2008;359:2619–20.
29
30
31 529 15. Dondorp AM, Nosten F, Yi P, *et al.* Artemisinin resistance in *Plasmodium falciparum*
32
33 530 malaria. *N Engl J Med* 2009;361:455–67.
34
35
36 531 16. World Health Organization. Status report on artemisinin resistance and ACT efficacy.
37
38 532 Available at: [http://www.who.int/malaria/publications/atoz/artemisinin-resistance-](http://www.who.int/malaria/publications/atoz/artemisinin-resistance-august2018/en/)
39
40 533 [august2018/en/](http://www.who.int/malaria/publications/atoz/artemisinin-resistance-august2018/en/)
41
42
43 534 17. World Health Organization. Global report on insecticide resistance in malaria vectors:
44
45 535 2010-2016. Available at: <http://www.who.int/malaria/publications/atoz/9789241514057/en/>
46
47 536 18. Kraemer SM, Smith JD. A family affair: var genes, PfEMP1 binding, and malaria
48
49 537 disease. *Curr Opin Microbiol* 2006;9:374–80.
50
51
52 538 19. Tuikue Ndam NG, Salanti A, Bertin G, *et al.* High level of var2csa transcription by
53
54 539 *Plasmodium falciparum* isolated from the placenta. *J Infect Dis* 2005;192:331–5.
55
56
57 540 20. Moussiliou A, Alao MJ, Denoed-Ndam L, *et al.* High plasma levels of soluble
58
59 541 endothelial protein C receptor are associated with increased mortality among children with
60

- 1
2
3 542 cerebral malaria in Benin. *J Infect Dis* 2015;211:1484–8.
4
5 543 21. Miller LH, Baruch DI, Marsh K, *et al.* The pathogenic basis of malaria. *Nature*
6
7 544 2002;415:673–9.
8
9 545 22. Tuikue Ndam N, Deloron P. Towards a vaccine against pregnancy-associated malaria.
10
11 546 *Parasite* 2008;15:515–21.
12
13 547 23. Bertin GI, Sabbagh A, Argy N, *et al.* Proteomic analysis of *Plasmodium falciparum*
14
15 548 parasites from patients with cerebral and UM. *Sci Rep.* 2016;6:26773.
16
17 549 24. White NJ, Turner GDH, Day NPJ, *et al.* Lethal malaria: Marchiafava and Bignami were
18
19 550 right. *J Infect Dis* 2013;208:192–8.
20
21 551 25. Berendt AR, Tumer GD, Newbold CI. CM: the sequestration hypothesis. *Parasitol*
22
23 552 *Today* 1994;10:412–4.
24
25 553 26. Clark IA, Cowden WB, Rockett KA. The pathogenesis of human cerebral malaria.
26
27 554 *Parasitol Today* 1994;10:417–8.
28
29 555 27. Beare NAV, Harding SP, Taylor TE, *et al.* Perfusion abnormalities in children with
30
31 556 cerebral malaria and malarial retinopathy. *J Infect Dis* 2009;199:263–71.
32
33 557 28. Dorovini-Zis K, Schmidt K, Huynh H, *et al.* The neuropathology of fatal cerebral
34
35 558 malaria in malawian children. *Am J Pathol* 2011;178:2146–58.
36
37 559 29. McDonough A, Weinstein JR. Neuroimmune Response in Ischemic Preconditioning.
38
39 560 *Neurother J Am Soc Exp Neurother* 2016;13:748–61.
40
41 561 30. Kim E, Cho S. Microglia and Monocyte-Derived Macrophages in Stroke. *Neurother J*
42
43 562 *Am Soc Exp Neurother* 2016;13:702–18.
44
45 563 31. Xia C-Y, Zhang S, Gao Y, *et al.* Selective modulation of microglia polarization to M2
46
47 564 phenotype for stroke treatment. *Int Immunopharmacol* 2015 Apr;25:377–82.
48
49 565 32. Kumar A, Barrett JP, Alvarez-Croda D-M, *et al.* NOX2 drives M1-like
50
51 566 microglial/macrophage activation and neurodegeneration following experimental traumatic
52
53
54
55
56
57
58
59
60

- 1
2
3 567 brain injury. *Brain Behav Immun* 2016;58:291–309.
- 4
5 568 33. Pino P, Taoufiq Z, Nitchou J, *et al.* Blood-brain barrier breakdown during cerebral
6
7 569 malaria: suicide or murder? *Thromb Haemost* 2005;94:336–40.
- 8
9
10 570 34. Postma NS, Mommers EC, Eling WM, *et al.* Oxidative stress in malaria; implications
11
12 571 for prevention and therapy. *Pharm World Sci PWS* 1996;18:121–9.
- 13
14 572 35. Eisenhut M. The evidence for a role of vasospasm in the pathogenesis of cerebral
15
16 573 malaria. *Malar J*;14:405.
- 17
18 574 36. Eisenhut M. Vasospasm in cerebral inflammation. *Int J Inflamm* 2014;2014:509707.
- 19
20 575 37. O'Brien NF, Mutatshi Taty T, Moore-Clingenpeel M, *et al.* Transcranial Doppler
21
22 576 Ultrasonography Provides Insights into Neurovascular Changes in Children with cerebral
23
24 577 malaria. *J Pediatr* 2018;203:116-124.e3.
- 25
26
27 578 38. Bertin GI, Lavstsen T, Guillonneau F, *et al.* Expression of the domain cassette 8
28
29 579 *Plasmodium falciparum* erythrocyte membrane protein 1 is associated with cerebral malaria in
30
31 580 Benin. *PloS One* 2013;8:e68368.
- 32
33
34 581 39. Ponts N, Chung D-WD, Le Roch KG. Strand-specific RNA-seq applied to malaria
35
36 582 samples. *Methods Mol Biol Clifton NJ* 2012;883:59–73.
- 37
38 583 40. Bertin GI, Sabbagh A, Guillonneau F, *et al.* Differential protein expression profiles
39
40 584 between *Plasmodium falciparum* parasites isolated from subjects presenting with pregnancy-
41
42 585 associated malaria and uncomplicated malaria in Benin. *J Infect Dis* 2013;208:1987–97.
- 43
44
45 586 41. Otto TD, Wilinski D, Assefa S, *et al.* New insights into the blood-stage transcriptome
46
47 587 of *Plasmodium falciparum* using RNA-Seq. *Mol Microbiol* 2010;76:12–24.
- 48
49
50 588 42. Varet H, Brillet-Guéguen L, Coppée J-Y, *et al.* A DESeq2- and EdgeR-Based R Pipeline
51
52 589 for Comprehensive Differential Analysis of RNA-Seq Data. *PloS One* 2016;11:e0157022.
- 53
54
55 590 43. van Buuren S, Boshuizen HC, Knook DL. Multiple imputation of missing blood
56
57 591 pressure covariates in survival analysis. *Stat Med* 1999;18:681–94.
- 58
59
60

1
2
3 592 44. Idro R, Kakooza-Mwesige A, Asea B, *et al.* Cerebral malaria is associated with long-
4
5 593 term mental health disorders: a cross sectional survey of a long-term cohort. *Malar J*
6
7 594 2016;15:184.
8
9

10 595
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For peer review only

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