BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-062948
Article Type:	Protocol
Date Submitted by the Author:	16-Mar-2022
Complete List of Authors:	Patel, Archana; Boston Children's Hospital, Harvard Medical School, Neurology, Division of Epilepsy & Clinical Neurophysiology; University of Zambia School of Medicine, Paediatrics & Child Health Birbeck, Gretchen; University of Rochester Department of Neurology, Epilepsy Division; University of Zambia School of Medicine, Paediatrics & Child Health Mazumdar, Maitreyi; Boston Children's Hospital, Harvard Medical School, Neurology; Harvard University T H Chan School of Public Health, Environmental Health Mwanza, Suzanna; Chipata Central Hospital, Paediatrics Nyirongo, Rosemary; Chipata Central Hospital, Paediatrics Berejena, Dixon; Chipata Central Hospital, Paediatrics Kasolo, Joseph; Chipata Central Hospital, Paediatrics Mwale, Tina; Chipata Central Hospital, Paediatrics Nambeye, Violet; Chipata Central Hospital, Paediatrics Nkole, Kafula; University Teaching Hospitals- Children's Hospital Kawatu, Nfwama; University Teaching Hospitals- Children's Hospital Zhang, Bo; Harvard Medical School, Department of Neurology and ICCTR Biostatistics and Research Design Center Rotenberg, Alexander ; Boston Children's Hospital, Harvard Medical School, Neurology, Division of Epilepsy & Clinical Neurophysiology; Beth Israel Deaconess Medical Center, Harvard Medical School, Berenson-Allen Center for Noninvasive Brain Stimulation
Keywords:	Epilepsy < NEUROLOGY, Paediatric neurology < NEUROLOGY, Tropical medicine < INFECTIOUS DISEASES, NEUROPHYSIOLOGY

Identifying biomarkers for epilepsy after cerebral malaria in Zambian Children: Rationale and design of a prospective observational study

Archana A. Patel, MD MPH[1, 2], Gretchen L Birbeck, MD MPH DTMH[2,3], Maitreyi Mazumdar, MD MPH[4,5], Suzanna Mwanza, MBBS MMed[6], Rosemary Nyirongo[6], Dixon Berejena, BSc[6], Joseph Kasolo[6], Tina Mwale[6], Violet Nambeye[6], Kafula Lisa Nkole, MBChB[7], Nfwama Kawatu, MBBS[7], Bo Zhang PhD[8], Alexander Rotenberg, MD PhD[1, 8, 9]

1. Division of Epilepsy and Clinical Neurophysiology, Department of Neurology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA
2. University of Zambia School of Medicine, Department of Paediatrics and Child Health, Lusaka, Zambia
3. Epilepsy Division, Department of Neurology, University of Rochester, Rochester, NY, USA
4. Department of Neurology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA
5. Department of Environmental Health, Harvard T.H. Chan School of Public Health
6. Department of Paediatrics, Chipata Central Hospital, Chipata, Zambia
7. University Teaching Hospitals- Children's Hospital, Lusaka, Zambia
8. Harvard Medical School, Department of Neurology and ICCTR Biostatistics and Research Design Center
9. Berenson-Allen Center for Noninvasive Brain Stimulation, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

Corresponding Author:
Archana A. Patel, MD MPH
Boston Children's Hospital, Harvard Medical School
Department of Neurology, Division of Epilepsy and Clinical Neurophysiology
Boston, Massachusetts, USA
Phone: 617.919.1407
Fax: 617.730.4850
archana.patel@childrens.harvard.edu
https://orcid.org/0000-0002-1159-0128

Word count: 2806
Tables: 1
Figures: 1

Key Words:
Cerebral Malaria
Epileptogenesis
Zambia
Pediatric
EEG

Abstract

Introduction: Malaria affecting the central nervous system (CM) is a major contributor to pediatric epilepsy in resource-poor settings, with 10-16% of survivors developing epilepsy within 2 years of infection. Although a risk factor for developing post-malaria epilepsy (PME), biomarkers indicating which CM survivors will develop epilepsy are absent. Identification of such biomarkers is essential to identify those at highest risk who might benefit most from close surveillance and/or preventive treatments. Electroencephalography (EEG) contains signals, specifically, quantification of gamma frequency activity, are correlated with higher risk of PME, and provide a biomarker for the development of epilepsy. We propose to study the sensitivity of quantitative and qualitative EEG metrics in predicting PME, and the potential increased sensitivity of this measure with additional clinical metrics. Our goal is to develop a predictive PME index composed of EEG and clinical history metrics that are highly feasible to obtain in low-resourced regions.

Methods and Analyses: This study will follow Zambian children during and for two years after acute CM, to test the performance of EEG and clinical metrics in predicting development of epilepsy. Multivariate logistic regression with variable selection will be performed to identify the combination of specific EEG and clinical metrics most predictive of PME.

Ethics and Dissemination: This study has been approved by the Boston Children's Hospital Institutional Review Board, University of Zambia Biomedical Research Ethics Committee, and National Health Research Authority of Zambia (NHRA).

Strengths and Limitations of this study:

- The prospective study design and data collection will allow for better understanding of post-malaria epilepsy development
- Primary data collected is standardly accessible in low resource regions
- Both clinical and quantitative EEG metrics will be tested for post-malaria risk prediction, in combination and individually
- First study to prospectively assess autism development after CM
- Due to resource constraints, correlation to neuroimaging is not feasible

Introduction

Over 45 million people live with epilepsy globally,[1-3] 80% of whom live in lower-resource countries.[4,5] Over 25% of these epilepsy cases are acquired as a result of central nervous system (CNS) infection or trauma.[6,7] Malaria, a parasitic infection caused by *Plasmodium falciparum*, contributes significantly to the burden of acquired epilepsy when it affects the central nervous system (CM), particularly in sub-Saharan Africa (SSA), where resources are limited.[8] The risk is highest for children under the age of 5 years.[9] Over 30% of pediatric CM survivors are estimated to develop neurodevelopmental sequelae detectable within two years of acute illness; for 10-16% of survivors, these sequelae will include post-malaria epilepsy (PME).[10-17] Cerebral malaria (defined as coma and malaria parasitemia, in absence other coma etiology)[9] has been better studied than malaria that affects the central nervous system in general (manifested by alteration of consciousness or complicated seizures), yet the two conditions have been shown to have similar rates of neurodevelopmental sequelae.[10,16,18] Thus, any malaria infection affecting the central nervous system (CM) has high rates of PME development in survivors. With over half a million CM infections annually and a 20% fatality rate in those with cerebral malaria,[19] there are over 40,000 newly acquired, and potentially preventable, pediatric epilepsy cases attributable to malaria per year in regions where the rates of epilepsy are highest and where limited resources are available to tackle this burden.

In general, there remains a need for reliable biomarkers of epileptogenesis for people with risk of an epilepsy syndrome (i.e., after traumatic brain injury or brain infection).[20] As in most acquired epilepsies, PME emerges in select children after a months-long seizure-free period following acute CM infection.[10,12,14,21,22] Ascertaining predictive factors for those at highest risk of developing PME has significant potential to impact clinical care in this condition, as well as potentially advance knowledge of epileptogenesis in acquired brain injuries overall.

Identification of those who would benefit from close observation is an essential consideration in lower-resource regions, where routine follow-up for all patients is not feasible. Identification of biomarkers that predict epilepsy risk could also be used to test potential antiepileptogenic neuroprotective therapies or select appropriate children for clinical trials evaluating such interventions. As revealed from the EPISTOP trial in tuberous sclerosis, disease-modifying therapy has potential not only to impact epilepsy development but to reduce severity of neurodevelopmental impairments.[23,24]

We propose a practical approach for utilizing metrics to create a predictive model that would be feasible across settings, including low-resource regions. Specifically, we propose to use data acquired from standardly acquired electroencephalograms (EEGs), through both quantitative and qualitative analyses, in conjunction with clinical metrics of acute infection and early recovery phases during malaria for development of an individual risk prediction model. Quantitative EEG predictors of neurodisability in adults and children after cardiac arrest have been described,[25-27] and similar EEG techniques have recently been used to demonstrate frequency band metrics associated with mortality and neurologic morbidity during hospitalization for acute CM.[28]

We propose that quantitative EEG metrics hold even more promise as a biomarker for PME. Activity of fast-spiking, parvalbumin-positive (FS-PV+) GABAergic inhibitory interneurons, a cell population that progressively declines over the course of epileptogenesis,[29,30] has been shown to be reflected by low frequency EEG gamma (30-60Hz) activity.[31-33] Animal models of acquired epilepsy demonstrate an initial peak of gamma frequency activity, suggestive of initial hyperactivation mediated by acute glutamate toxicity of the FS-PV+ interneuron population, followed by a progressive *decrease* of gamma EEG power, reflecting ultimate excitotoxic injury and cell death.[34] Our group looked at 70 standardly acquired EEGs from Malawi, acquired within the first 24 hours of acute hospitalization for pediatric cerebral malaria, to test this measure. We

found that not only is extracting low frequency gamma power through spectral analyses from EEGs obtained in SSA with minimal computing requirements feasible, but notably that an initial acute increase in gamma frequency is predictive of PME.[35] Our finding of acute increase correlation of gamma EEG power with PME development matches the preclinical data from animal models,[34] suggesting promise of this technique as a biomarker for PME.

We now propose to assess this measure in a larger-scale, prospective observational study, hypothesizing that we will find the same two-phased curve as demonstrated by animal studies, with an initial peak and then slow decline of gamma frequency activity **(Figure 1).** Through serial EEG monitoring, we anticipate narrowing the window of time during which we identify the occurrence of epileptogenesis, providing a mechanism for monitoring and potential intervention.

Furthermore, while we predict that EEG gamma frequency activity will be associated with risk of PME, we hypothesize that other quantitative and qualitative EEG metrics as well as clinical features of acute CM presentation, including maximum temperature, length and severity of coma, presence of acute seizures, presence of hypoglycemia, and prior medical history risk factors (i.e., HIV, prior neurodevelopmental disability), would all increase risk of PME in this population. Therefore, we will test these various metrics both individually and in combination via logistic regression to select the combination of EEG and clinical features most predictive for PME development. Such a measure of individual PME risk would allow monitoring and management of this high-risk population.

Methods and Analyses

Study design: This observational prospective cohort study will recruit a goal sample size of 300 children admitted for CM at Chipata Central Hospital (CCH), Chipata, Zambia, over a three-year period. CCH is a 600-bed third-level provincial referral hospital in Eastern Zambia, serving

approximately 1.5 million people and encountering a high burden of severe malaria cases annually. The research will be conducted through the pediatrics department, which has a full EEG lab for research and clinical purposes, routine laboratory diagnostic services, and a CT scanner.

Recruitment: Subjects will be enrolled on a rolling basis, as malaria is endemic year-round in this region of Zambia. The local study team will be notified of any child presenting with malaria (by rapid diagnostic test and confirmatory blood smear) and neurologic symptoms (depressed level of consciousness or seizures); those aged 6 months to 11 years are further screened for inclusion in the study. This screening includes confirmation of malaria diagnosis, eligible age, and meeting criteria for either (1) 'Cerebral Malaria'- defined as impaired consciousness with Blantyre Coma Score (BCS) of ≤ 2 in children under 2 years of age, or a Glasgow Coma Score (GCS) ≤ 10 in children ≥ 2 years, without any other explanation for coma, or (2) 'CNS Malaria', defined as complicated seizures (either prolonged ≥15 minutes, focal or multiple) or impaired consciousness without frank coma (i.e., BCS >2, GCS 11-14). Any child with pre-existing epilepsy will be excluded. Additional exclusion criteria include another acute CNS infection, clinically identifiable toxin ingestion, and head trauma within twenty-four hours. If qualified, caregivers are invited to participate once the child is clinically stable and will be consented upon agreement.

Patients and Public Involvement: The study site if Chipata, Zambia was chosen due to the high rates of pediatric cerebral malaria in the region, and current limited resources for diagnosis and managing epilepsy. The study design has been developed with significant input from medical providers in the region, and Information about this study has been dispersed to the public locally for awareness.

Power and Sample Size Calculation: Power and sample size calculation was conducted based upon the hypothesis test for detecting the difference of gamma-delta power ratio in two study groups: CM survivors who develop epilepsy and CM survivors who do not develop epilepsy. Our preliminary studies assessing spectral EEG analyses between these groups revealed a significantly higher gamma-delta power ratio in CM survivors who developed epilepsy (mean: 0.23, standard deviation: 0.10) than in those who did not (mean: 0.16, standard deviation: 0.06).[35] Based on this data and reported rates of PME in CM survivors within two years (10%–16%),[10,12-15,18] our current calculation shows a sample size of 250 (25 for CM+Epi and 225 for CM-Epi; ratio is 1:9) achieves 90% power to reject the null hypothesis of equal means in gamma-delta power ratio for the two groups, with a significance level (type I error) of 0.05 using a two-sided two-sample unequal-variance t-test. Power and sample size calculations were performed using PASS 15 Power Analysis and Sample Size Software (NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/pass, 2017).

Outcomes Measured: The primary end point of interest in this study is the development of PME in CM survivors. Secondary outcomes include neurodevelopmental impairment, including autism and motor impairment. Data will be collected during acute CM infection and over the subsequent two years (**Table 1**).

	Acute Hospitalization	**1 month post admission**	**6-month post admission**	**12-month post admission**	**18-month post admission**	**24-month post admission**
Patient Characteristics						
Sex	X	X	X	X	X	X
Weight	X	X	X	X	X	X

Pre-CM illness history (HIV status, prior neurologic disease including epilepsy or ASD, family history of seizures/epilepsy)	X					
Caregiver perception of wellness/recovery		X	X	X	X	X
Sleep Quality		X	X	X	X	X
School attendance		X	X	X	X	X
Clinical Metrics						
Age	X	X	X	X	X	X
Weight	X	X	X	X	X	X
Coma score	X					
Coma duration	X					
Maximum Temperature	X					
Presence/absence of acute symptomatic seizures	X					
Use of antiseizure medications	X	X	X	X	X	X
Diagnostic Metrics						
Glucose Level	X					
Parasite Burden (HRP2 level)	X					
EEG	X	X	X	X	X	X
Developmental Impairment and ASD Screening						
Ten Questions Screen	X					
23Q Screen		X	X	X	X	X
Epilepsy Assessment						

WHO Epilepsy Screen		X	X	X	X	X
Neurologic Assessment						
Malawi Developmental Assessment Tool (MDAT) if ≤6yo, Neurologic Exam for Subtle Signs if >6yo		X	X	X	X	X

Table 1. Schedule of Outcome Measurements.

We will enroll children who present with acute CM infection. During enrollment, baseline patient characteristics, including age, sex, HIV status, prior neurologic conditions, and developmental status (by the validated and regionally used Ten Questions Questionnaire[10,36]) will be recorded. Acute CM clinical metrics of interest will be recorded throughout hospital admission, including coma score and duration, maximum temperature, blood glucose measurements, and presence or absence of acute clinical seizures. Additionally, blood sample by finger prick will be collected within 24 hours of admission to obtain a histidine-rich protein 2 (HRP2) level (by ELISA) as a marker of parasite burden.[37] The results will not be available in real time as samples are delivered to a collaborating laboratory site in Malawi, due to absence of malaria microscopy expertise at CCH; thus, these levels will be used only for study analyses purposes. Within 24 hours, a standard 30-minute electroencephalogram is recorded by trained EEG technologists, using Natus equipment, XLTEK software and a standard international 10-20 system, at a sampling rate of 512Hz will be used.

Acute CM clinical metrics of interest are recorded throughout hospital admission, including coma score and duration, maximum temperature, blood glucose measurements, and presence or absence of acute clinical seizures. Additionally, blood sample by finger prick is collected within 24 hours of admission to obtain a histidine-rich protein 2 (HRP2) level (by ELISA) as a marker of parasite burden.[37] The results will not be available in real time as samples are

delivered to a collaborating laboratory site in Malawi, due to absence of malaria microscopy expertise at CCH; thus, these levels will be used only for study analyses purposes.

We will conduct 5 follow-up visits of survivors (at 1-, 6-, 12-, 18-, and 24-month post-infection time points) after initial CM presentation. Each follow-up evaluation consists of standard 30-minute awake and sleep EEG and clinical neurodevelopmental screening. Melatonin will be used as needed for induction of sleep during the EEG. The neurodevelopmental screening consists of (1) general follow-up information including weight, any antiseizure medication use, overall caregiver impression of recovery, sleep quality, and school attendance (when applicable), (2) a standardized Epilepsy (World Health Organization) screening questionnaire used in prior pediatric cerebral malaria studies in the region,[10] (3) 23Q Developmental Screen, which consists of the Ten Questions Questionnaire with expansion to screen for Autism Spectrum Disorder (ASD), validated in Uganda,[38,39] and a neurodevelopmental assessment via either the Malawi Development Assessment Tool (MDAT)[36,39] if ≤6 years old or the Neurological Exam for Subtle Signs[40] if >6 years old. Any positive epilepsy or neurodevelopmental screens will result in confirmatory diagnosis by a board-certified pediatric neurologist (AAP).

Acquired EEG will be analyzed utilizing commercial and in-house software to assess power in each frequency band by wavelet transform, specifically looking at trends of power band ratios for delta, theta, alpha, beta, and lower range gamma frequencies (30-60Hz). Each study will be de-identified with only age available for interpretation, and remotely undergo visual interpretation via secure web-based access by a clinical neurophysiologist. A standardized form for interpretation is used for the study, documenting the presence or absence of the variables of interest. EEGs are read in real time for clinical purposes, and any impact on treatment recorded separately. All antiseizure medication use will be documented throughout study participation as an independent variable for analyses of impact on outcomes and predictive modeling.

Statistical Analysis Plan

The clinical metrics of interest will be tested for association with the primary outcome of interest, post-malaria epilepsy, by *t*-test and one-way ANOVA, and if needed by their nonparametric alternatives Mann-Whitney test and Kruskal-Wallis test. Analysis of receiver operating characteristic (ROC) will be used to investigate the prediction performance of gamma activity on epilepsy development. Relative gamma power (30-60Hz) normalized to the whole power band will be plotted against whether PME develops, using the data points for the EEGs at each time point of interest. These will be analyzed to determine which time point of assessment reveals the largest difference between groups (CM survivors with PME and those without) in gamma frequency activity. Conventional EEG metrics and spectral analyses of each power band (delta, theta, beta, gamma) will also be assessed and will be analyzed individually using chi-squared test or one-way ANOVA, and in combination by logistic regression with variable selection for association with risk of PME development.

Interim data analyses will routinely be performed to identify the most relevant metrics for risk prediction. Multivariate logistic regression analysis will be conducted with these EEG and clinical metrics ascertained at each time point and, using initial data sets, the predictive algorithm will be built upon variable selection. Prediction performance will be evaluated with the interim data sets to ultimately identify the combination of clinical and EEG metrics with highest predictive capacity for determining risk of PME, to create a feasible predictive model.

Data Management and Monitoring: Data will be stored directly into a secure, password-protected electronic database. Data quality checks of all entered forms in the electronic database will be performed on a biweekly basis to ensure accuracy and reliability. Any missing data will be reviewed by the clinical study team, and best efforts to complete accurately with hospital data files will be performed. Any missing data that cannot be directly confirmed will be

categorized as missing and excluded from analyses. Interim analyses will be performed every 6-12 months, with frequency based on rate of recruitment, with any outlying data reviewed by two study members and confirmed by source data if necessary.

Ethics and Dissemination

This study has been approved by the Boston Children's Hospital Institutional Review Board (IRB-P00038309), University of Zambia Biomedical Research Ethics Committee and National Health Research Authority of Zambia (2529/2022). All publications and reports that result from this work will be produced with involvement and approval of all key personnel of the study. Results will be shared with relevant personnel and NHRA throughout the study period and will be disseminated locally in Zambia before internationally. To optimize availability to lower resourced regions, data will be published in open access, peer-reviewed journals when feasible.

Discussion:

Affected by a single disease process with a relatively high prevalence and risk of epilepsy in survivors within a short timeframe, pediatric CM survivors present a unique population through which to study epileptogenesis and identify biomarkers forecasting PME. Identification of such biomarkers has significant implications for clinical practice because it can provide a mechanism to indicate which patients warrant closer observation due to higher risk of developing epilepsy, in addition to providing measures that can facilitate anti-epileptogenic trials in PME, with potential applicability to other forms of acquired epilepsies.

As the highest burden of highly morbid and fatal CM is in sub-Saharan Africa, it is essential that a predictive epilepsy model for PME consider the resource restrictions of the region where it is most prevalent,[9] and be feasible and applicable within this setting going forward. Therefore, we propose metrics that can be obtained with relative ease at most tertiary care centers in sub-

Saharan Africa, where children with cerebral malaria are predominantly treated due to the complexity of their care needs. This includes EEG (available in over 80% of African countries[41]), clinical metrics, and basic laboratory measures. Neuroimaging is not included in our proposed study, as the goal is to use metrics of routine care, and MRI and CT capacity is variable with inconsistent use for clinical care, even in tertiary care centers across Africa.[41]

If successful, this study has the potential to provide not only a mechanism for improved stratification of risk of epilepsy after CM, but also provide a biomarker of epileptogenesis in this population. Such a biomarker would provide the means to subsequently test antiepileptogenic therapies, including a range of inexpensive compounds whose anti-epileptogenic potential is supported by preclinical and clinical data.[42] Additionally, to the best of our knowledge, this study will be the first to look at risk of autism after CM and will further assess if quantitative EEG metrics have value in predicting risk of neurodevelopmental impairment in addition to epilepsy.

The proposed study and metrics to be studied over a two-year period will provide a better understanding of the risk factors involved for development of neurologic sequelae after CM, particularly PME, and will provide potential avenues for both improved monitoring and potential intervention, key needs for a disease that continues to affect a large number of children in low-resourced regions annually.

Author contributions: AAP, GLB, MM, and AR were involved in study conception, and design. AAP, SM, RN, DB, JK, TM, VN, KLN, and NK are involved in data collection and contributed to study design. BZ and AAP developed analyses plan. AAP drafted the initial manuscript and all authors provided revisions and final approval for publication, as well as agreed to be accountable for all aspects of the work.

Funding statement: This work is primarily supported by NIH/NINDS grant K23 NS118051 (AAP), with additional support from NIH/NINDS grants K23NS046086, R01NS111057, R01NS102176, & R35NS122265 (GLB).

Competing interests statement: All authors have completed the ICMJE uniform disclosure form at http://www.icmje.org/disclosure-of-interest/ and declare the following: AAP has received a research grant from NIH/NINDS for the proposed work and serves (without compensation) on the advisory board of the ROW foundation which supplies anti-seizure medications through a donation grant to Zambia; GLB has received research grants from NIH/NINDS to support the proposed work and is Ambassador to Zambia for the Royal Society of the Tropical Medicine and Hygiene; MM has stock in Pfizer; and AR is a founder and advisor for Neuromotion, Cofounder PrevEp. Past consultant for or received funding from Abbvie, CRE Medical, ENCODED, Epihunter, Gamify, Neuroelectrics, Neural Dynamics, NeuroRex, Roche, Takeda, and is listed as inventor on a patent related to integration of TMS and EEG, as well as drug delivery with focused ultrasound. The remaining authors have no conflicts of interest to disclose and declare that the research was conducted in the absence of any relevant commercial or financial relationships.

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Figure Legends:

Figure 1. An initial peak of EEG gamma frequency activity is predicted to be seen during malarial infection affecting the central nervous system, followed by subsequent decline during epileptogenesis, in children who will ultimately develop post-malaria epilepsy.

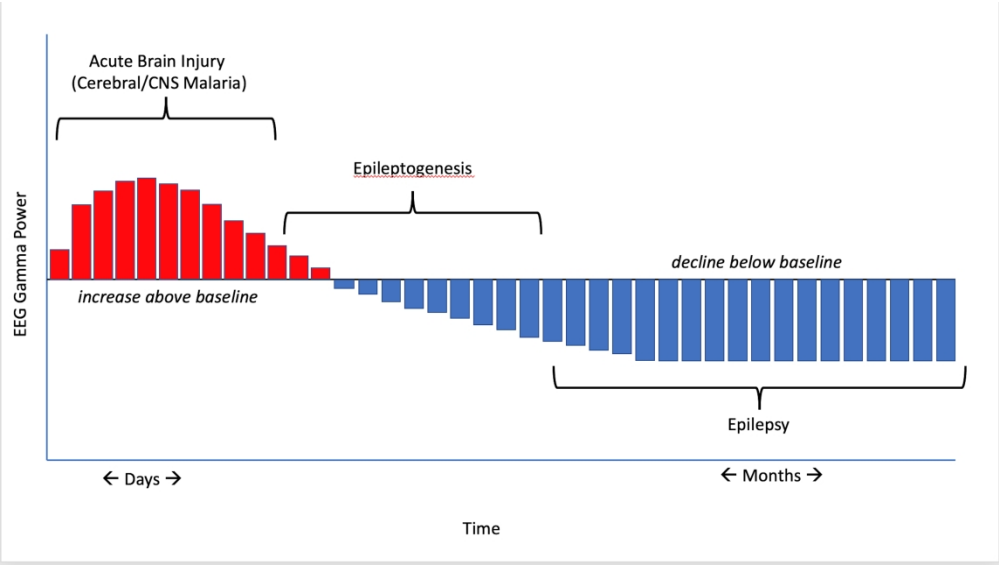

Figure 1. An initial peak of EEG gamma frequency activity is predicted to be seen during malarial infection affecting the central nervous system, followed by subsequent decline during epileptogenesis, in children who will ultimately develop post-malaria epilepsy.

396x223mm (300 x 300 DPI)

TRIPOD Checklist: Prediction Model Development

Section/Topic	Item	Checklist Item	Page
Title and abstract			
Title	1	Identify the study as developing and/or validating a multivariable prediction model, the target population, and the outcome to be predicted.	x
Abstract	2	Provide a summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions.	x
Introduction			
Background and objectives	3a	Explain the medical context (including whether diagnostic or prognostic) and rationale for developing or validating the multivariable prediction model, including references to existing models.	x
	3b	Specify the objectives, including whether the study describes the development or validation of the model or both.	x
Methods			
Source of data	4a	Describe the study design or source of data (e.g., randomized trial, cohort, or registry data), separately for the development and validation data sets, if applicable.	x
	4b	Specify the key study dates, including start of accrual; end of accrual; and, if applicable, end of follow-up.	N/A; protocol paper
Participants	5a	Specify key elements of the study setting (e.g., primary care, secondary care, general population) including number and location of centres.	x
	5b	Describe eligibility criteria for participants.	x
	5c	Give details of treatments received, if relevant.	n/a
Outcome	6a	Clearly define the outcome that is predicted by the prediction model, including how and when assessed.	x
	6b	Report any actions to blind assessment of the outcome to be predicted.	x
Predictors	7a	Clearly define all predictors used in developing or validating the multivariable prediction model, including how and when they were measured.	x
	7b	Report any actions to blind assessment of predictors for the outcome and other predictors.	n/a protocol paper
Sample size	8	Explain how the study size was arrived at.	x
Missing data	9	Describe how missing data were handled (e.g., complete-case analysis, single imputation, multiple imputation) with details of any imputation method.	n/a protocol paper
Statistical analysis methods	10a	Describe how predictors were handled in the analyses.	n/a protocol paper
	10b	Specify type of model, all model-building procedures (including any predictor selection), and method for internal validation.	x
	10d	Specify all measures used to assess model performance and, if relevant, to compare multiple models.	Planned metrics described
Risk groups	11	Provide details on how risk groups were created, if done.	N/A
Results			
Participants	13a	Describe the flow of participants through the study, including the number of participants with and without the outcome and, if applicable, a summary of the follow-up time. A diagram may be helpful.	N/A, protocol paper
	13b	Describe the characteristics of the participants (basic demographics, clinical features, available predictors), including the number of participants with missing data for predictors and outcome.	N/A protocol paper
Model development	14a	Specify the number of participants and outcome events in each analysis.	N/A protocol paper
	14b	If done, report the unadjusted association between each candidate predictor and outcome.	N/A protocol paper
Model specification	15a	Present the full prediction model to allow predictions for individuals (i.e., all regression coefficients, and model intercept or baseline survival at a given time point).	N/A protocol paper
	15b	Explain how to the use the prediction model.	N/A protocol paper
Model performance	16	Report performance measures (with CIs) for the prediction model.	N/A protocol paper
Discussion			
Limitations	18	Discuss any limitations of the study (such as nonrepresentative sample, few events per predictor, missing data).	N/A protocol paper
Interpretation	19b	Give an overall interpretation of the results, considering objectives, limitations, and results from similar studies, and other relevant evidence.	N/A protocol paper

TRIPOD Checklist: Prediction Model Development

Implications	20	Discuss the potential clinical use of the model and implications for future research.	x
Other information			
Supplementary information	21	Provide information about the availability of supplementary resources, such as study protocol, Web calculator, and data sets.	N/A protocol paper
Funding	22	Give the source of funding and the role of the funders for the present study.	x

We recommend using the TRIPOD Checklist in conjunction with the TRIPOD Explanation and Elaboration document.

BMJ Open

Identifying biomarkers for epilepsy after cerebral malaria in Zambian Children: Rationale and design of a prospective observational study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-062948.R1
Article Type:	Protocol
Date Submitted by the Author:	29-May-2022
Complete List of Authors:	Patel, Archana; Boston Children's Hospital, Harvard Medical School, Neurology, Division of Epilepsy & Clinical Neurophysiology; University of Zambia School of Medicine, Paediatrics & Child Health Birbeck, Gretchen; University of Rochester Department of Neurology, Epilepsy Division; University of Zambia School of Medicine, Paediatrics & Child Health Mazumdar, Maitreyi; Boston Children's Hospital, Harvard Medical School, Neurology; Harvard University T H Chan School of Public Health, Environmental Health Mwanza, Suzanna; Chipata Central Hospital, Paediatrics Nyirongo, Rosemary; Chipata Central Hospital, Paediatrics Berejena, Dixon; Chipata Central Hospital, Paediatrics Kasolo, Joseph; Chipata Central Hospital, Paediatrics Mwale, Tina; Chipata Central Hospital, Paediatrics Nambeye, Violet; Chipata Central Hospital, Paediatrics Nkole, Kafula; University Teaching Hospitals- Children's Hospital Kawatu, Nfwama; University Teaching Hospitals- Children's Hospital Zhang, Bo; Harvard Medical School, Department of Neurology and ICCTR Biostatistics and Research Design Center Rotenberg, Alexander ; Boston Children's Hospital, Harvard Medical School, Neurology, Division of Epilepsy & Clinical Neurophysiology; Beth Israel Deaconess Medical Center, Harvard Medical School, Berenson-Allen Center for Noninvasive Brain Stimulation
Primary Subject Heading:	Neurology
Secondary Subject Heading:	Global health, Infectious diseases, Paediatrics
Keywords:	Epilepsy < NEUROLOGY, Paediatric neurology < NEUROLOGY, Tropical medicine < INFECTIOUS DISEASES, NEUROPHYSIOLOGY

Identifying biomarkers for epilepsy after cerebral malaria in Zambian Children: Rationale and design of a prospective observational study

Archana A. Patel, MD MPH[1, 2], Gretchen L Birbeck, MD MPH DTMH[2,3], Maitreyi Mazumdar, MD MPH[4,5], Suzanna Mwanza, MBBS MMed[6], Rosemary Nyirongo[6], Dixon Berejena, BSc[6], Joseph Kasolo[6], Tina Mwale[6], Violet Nambeye[6], Kafula Lisa Nkole, MBChB[7], Nfwama Kawatu, MBBS[7], Bo Zhang PhD[8], Alexander Rotenberg, MD PhD[1, 8, 9]

1. Division of Epilepsy and Clinical Neurophysiology, Department of Neurology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA
2. University of Zambia School of Medicine, Department of Paediatrics and Child Health, Lusaka, Zambia
3. Epilepsy Division, Department of Neurology, University of Rochester, Rochester, NY, USA
4. Department of Neurology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA
5. Department of Environmental Health, Harvard T.H. Chan School of Public Health
6. Department of Paediatrics, Chipata Central Hospital, Chipata, Zambia
7. University Teaching Hospitals- Children's Hospital, Lusaka, Zambia
8. Harvard Medical School, Department of Neurology and ICCTR Biostatistics and Research Design Center
9. Berenson-Allen Center for Noninvasive Brain Stimulation, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

Corresponding Author:
Archana A. Patel, MD MPH
Boston Children's Hospital, Harvard Medical School
Department of Neurology, Division of Epilepsy and Clinical Neurophysiology
Boston, Massachusetts, USA
Phone: 617.919.1407
Fax: 617.730.4850
archana.patel@childrens.harvard.edu
https://orcid.org/0000-0002-1159-0128

Word count (including abstract): 3470
Tables: 1
Figures: 1

Key Words:
Cerebral Malaria
Epileptogenesis
Zambia
Pediatric
EEG

Abstract

Introduction: Malaria affecting the central nervous system (CM) is a major contributor to pediatric epilepsy in resource-poor settings, with 10-16% of survivors developing epilepsy within 2 years of infection. Despite high risk for post-malaria epilepsy (PME), biomarkers indicating which CM survivors will develop epilepsy are absent. Such biomarkers are essential to identify those at highest risk who might benefit most from close surveillance and/or preventive treatments. Electroencephalography (EEG) contains signals (specifically gamma frequency activity) which are correlated with higher risk of PME and provide a biomarker for the development of epilepsy. We propose to study the sensitivity of quantitative and qualitative EEG metrics in predicting PME, and the potential increased sensitivity of this measure with additional clinical metrics. Our goal is to develop a predictive PME index composed of EEG and clinical history metrics that are highly feasible to obtain in low-resourced regions.

Methods and Analyses: This prospective observational study being conducted in Eastern Zambia will recruit 250 children aged 6mo-11 years presenting with acute CM and follow them for two years. Children with pre-existing epilepsy diagnoses will be excluded. Outcome measures will include qualitative and quantitative analysis of routine EEG recordings, as well as clinical metrics in the acute and subacute period, including HRP2 levels of parasite burden, depth and length of coma, presence and severity of acute seizures, presence of hypoglycemia, maximum temperature, and one-month post-CM neurodevelopmental assessment scores. We will test the performance of these EEG and clinical metrics in predicting development of epilepsy through multivariate logistic regression analyses.

Ethics and Dissemination: This study has been approved by the Boston Children's Hospital Institutional Review Board, University of Zambia Biomedical Research Ethics Committee, and National Health Research Authority of Zambia. Results will be disseminated locally in Zambia followed by publication in international, open access, peer-reviewed journals when feasible.

Strengths and Limitations of this study:

- The prospective study design and data collection will allow for better understanding of post-malaria epilepsy development
- Primary data collected is standardly accessible in low resource regions
- Both clinical and quantitative EEG metrics will be tested for post-malaria risk prediction, in combination and individually
- Due to resource constraints, correlation to neuroimaging is not feasible

Introduction

Over 45 million people live with epilepsy globally,[1-3] 80% of whom live in lower-resource countries.[4,5] Over 25% of these epilepsy cases are acquired as a result of central nervous system (CNS) infection or trauma.[6,7] Malaria, a parasitic infection caused by *Plasmodium falciparum*, contributes significantly to the burden of acquired epilepsy when it affects the central nervous system (CM), particularly in sub-Saharan Africa (SSA), where resources are limited.[8] The risk is highest for children under the age of 5 years.[9] Over 30% of pediatric CM survivors are estimated to develop neurodevelopmental sequelae detectable within two years of acute illness; for 10-16% of survivors, these sequelae will include post-malaria epilepsy (PME).[10-17] Cerebral malaria (defined as coma and malaria parasitemia, in absence other coma etiology)[9] has been better studied than malaria that affects the central nervous system in general (manifested by alteration of consciousness or complicated seizures), yet the two conditions have been shown to have similar rates of neurodevelopmental sequelae.[10,16,18] Thus, any malaria infection affecting the central nervous system (CM) has high rates of PME development in survivors. With over half a million CM infections annually and a 20% fatality rate in those with cerebral malaria,[19] there are over 40,000 newly acquired, and potentially preventable, pediatric epilepsy cases attributable to malaria per year in regions where the rates of epilepsy are highest and where limited resources are available to tackle this burden.

In general, there remains a need for reliable biomarkers of epileptogenesis for people with risk of an epilepsy syndrome (i.e., after traumatic brain injury or brain infection).[20] As in most acquired epilepsies, PME emerges in select children after a months-long seizure-free period following acute CM infection.[10,12,14,21,22] Ascertaining predictive factors for those at highest risk of developing PME has significant potential to impact clinical care in this condition, as well as potentially advance knowledge of epileptogenesis in acquired brain injuries overall.

Identification of those who would benefit from close observation is an essential consideration in lower-resource regions, where routine follow-up for all patients is not feasible. Identification of biomarkers that predict epilepsy risk could also be used to test potential antiepileptogenic neuroprotective therapies or select appropriate children for clinical trials evaluating such interventions. As revealed from the EPISTOP trial in tuberous sclerosis, disease-modifying therapy has potential not only to impact epilepsy development but to reduce severity of neurodevelopmental impairments.[23,24]

We propose a practical approach for utilizing metrics to create a predictive model that would be feasible across settings, including low-resource regions. Specifically, we propose to use data acquired from standardly acquired electroencephalograms (EEGs), through both quantitative and qualitative analyses, in conjunction with clinical metrics of acute infection and early recovery phases during malaria for development of an individual risk prediction model. Quantitative EEG predictors of neurodisability in adults and children after cardiac arrest have been described,[25-27] and similar EEG techniques have recently been used to demonstrate frequency band metrics associated with mortality and neurologic morbidity during hospitalization for acute CM.[28]

We propose that quantitative EEG metrics hold even more promise as a biomarker for PME. Activity of fast-spiking, parvalbumin-positive (FS-PV+) GABAergic inhibitory interneurons, a cell population that progressively declines over the course of epileptogenesis,[29,30] has been shown to be reflected by low frequency EEG gamma (30-60Hz) activity.[31-33] Animal models of acquired epilepsy demonstrate an initial peak of gamma frequency activity, suggestive of initial hyperactivation mediated by acute glutamate toxicity of the FS-PV+ interneuron population, followed by a progressive *decrease* of gamma EEG power, reflecting ultimate excitotoxic injury and cell death.[34] Our group looked at 70 standardly acquired EEGs from Malawi, acquired within the first 24 hours of acute hospitalization for pediatric cerebral malaria, to test this measure. We

found that not only is extracting low frequency gamma power through spectral analyses from EEGs obtained in SSA with minimal computing requirements feasible, but notably that an initial acute increase in gamma frequency is predictive of PME.[35] Our finding of acute increase correlation of gamma EEG power with PME development matches the preclinical data from animal models,[34] suggesting promise of this technique as a biomarker for PME.

We now propose to assess this measure in a larger-scale, prospective observational study, hypothesizing that we will find the same two-phased curve as demonstrated by animal studies, with an initial peak and then slow decline of gamma frequency activity **(Figure 1).** Through serial EEG monitoring, we anticipate narrowing the window of time during which we identify the occurrence of epileptogenesis, providing a mechanism for monitoring and potential intervention.

Furthermore, while we predict that EEG gamma frequency activity will be associated with risk of PME, we hypothesize that other quantitative and qualitative EEG metrics as well as clinical features of acute CM presentation, including maximum temperature, length and severity of coma, presence of acute seizures, presence of hypoglycemia, and prior medical history risk factors (i.e., HIV, prior neurodevelopmental disability), would all increase risk of PME in this population. Therefore, we will test these various metrics both individually and in combination via logistic regression to select the combination of EEG and clinical features most predictive for PME development and develop a multivariable model for individual PME risk. Such a measure of individual PME risk would allow monitoring and management of this high-risk population.

Methods and Analyses

Study design: This observational prospective cohort study will recruit a goal sample size of 300 children admitted for CM at Chipata Central Hospital (CCH), Chipata, Zambia, over a three-year period. CCH is a 600-bed third-level provincial referral hospital in Eastern Zambia, serving

approximately 1.5 million people and encountering a high burden of severe malaria cases annually. The research will be conducted through the pediatrics department, which has a full EEG lab for research and clinical purposes, routine laboratory diagnostic services, and a CT scanner.

Recruitment: Subjects will be enrolled on a rolling basis, as malaria is endemic year-round in this region of Zambia. The local study team will be notified of any child presenting with malaria (by rapid diagnostic test and confirmatory blood smear) and neurologic symptoms (depressed level of consciousness or seizures); those aged 6 months to 11 years are further screened for inclusion in the study. This screening includes confirmation of malaria diagnosis, eligible age, and meeting criteria for either (1) 'Cerebral Malaria'- defined as impaired consciousness with Blantyre Coma Score (BCS) of ≤ 2 in children under 2 years of age, or a Glasgow Coma Score (GCS) ≤ 10 in children ≥ 2 years, without any other explanation for coma, or (2) 'CNS Malaria', defined as complicated seizures (either prolonged ≥15 minutes, focal or multiple) or impaired consciousness without frank coma (i.e., BCS 3-4, GCS 11-14). To identify children with a pre-existing epilepsy for exclusion, caregivers will be explicitly asked if the child is/has been on anti-seizure medications, has had 2 or more seizures without fever or trauma, or has been given a diagnosis of epilepsy by a clinician previously in effort to capture all pre-existing epilepsy patients. Additional exclusion criteria include another acute CNS infection, clinically identifiable toxin ingestion, and head trauma within twenty-four hours. If qualified, caregivers are invited to participate once the child is clinically stable and will be consented upon agreement.

Patients and Public Involvement: The study design has been developed with significant input from medical providers in the region, and Information about this study has been dispersed to the public locally for awareness with feedback from the community and local providers utilized to ensure methodology, and particularly enrollment procedures, is culturally appropriate. While

outcome measures were chosen based upon prior evidence, finalization of relevant measures and mechanisms was done with the local study team who are part of the community to ensure that these would have benefit. Dissemination of results will be performed locally through the hospital to the community.

Power and Sample Size Calculation: Power and sample size calculation was conducted based upon the hypothesis test for detecting the difference of gamma-delta power ratio in two study groups: CM survivors who develop epilepsy and CM survivors who do not develop epilepsy. Our preliminary studies assessing spectral EEG analyses between these groups revealed a significantly higher gamma-delta power ratio in CM survivors who developed epilepsy (mean: 0.23, standard deviation: 0.10) than in those who did not (mean: 0.16, standard deviation: 0.06).[35] Based on this data and reported rates of PME in CM survivors within two years (10%–16%),[10,12-15,18] our current calculation shows a sample size of 250 (25 for CM+Epi and 225 for CM-Epi; ratio is 1:9) achieves 90% power to reject the null hypothesis of equal means in gamma-delta power ratio for the two groups, with a significance level (type I error) of 0.05 using a two-sided two-sample unequal-variance t-test. Power and sample size calculations were performed using PASS 15 Power Analysis and Sample Size Software (NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/pass, 2017).

Outcomes Measured: The primary end point of interest in this study is the development of PME in CM survivors. Secondary outcomes include neurodevelopmental impairment, including autism and motor impairment. Data will be collected during acute CM infection and over the subsequent two years (**Table 1**).

We will enroll children who present with acute CM infection. As part of the study procedures, the local community has been made aware of this study for sensitization purposes. When a child

presents with acute CM, the caregiver will be approached when the child is deemed stable and clinical team feels that approach is appropriate. The caregiver will be taken to a designated quiet spot away from the patient to review the study and offer enrollment in the local language (Nyanja). Of note, due to the nature of acute CM presentation, assent is not feasible.

During enrollment, baseline patient characteristics, including age, sex, HIV status, prior neurologic conditions, and developmental status (by the validated and regionally used Ten Questions Questionnaire[10,36]) will be recorded. Testing for SARS-COV2 among children with malaria is not standard of care in this setting and recent research has demonstrated strong parental opposition to testing due to COVID-related stigma particularly ostracism of the parent-child dyad by other families on the inpatient service when positive tests occur. As such, SARS-COV2 testing will not be completing for research purposes. If collected for clinical care purposes, the information will be captured.

Acute CM clinical metrics of interest will be recorded throughout hospital admission, including coma score and duration, maximum temperature, blood glucose measurements, and presence or absence of acute clinical seizures. Additionally, blood sample by finger prick will be collected within 24 hours of admission to obtain a histidine-rich protein 2 (HRP2) level (by ELISA) as a marker of parasite burden.[37] The results will not be available in real time as samples are delivered to a collaborating laboratory site in Malawi, due to absence of malaria microscopy expertise at CCH; thus, these levels will be used only for study analyses purposes.

Within 24 hours, a standard 30-minute electroencephalogram will be recorded by trained EEG technologists, using Natus equipment, XLTEK software and a standard international 10-20 system, at a sampling rate of 512Hz will be used.

We will conduct 5 follow-up visits of survivors (at 1-, 6-, 12-, 18-, and 24-month post-infection time points) after initial CM presentation. Each follow-up evaluation consists of standard 30-minute awake and sleep EEG and clinical neurodevelopmental screening. Melatonin will be used as needed for induction of sleep during the EEG, with administration done prior to the beginning of setting up the EEG (1mg for children ≤ 3 years and 3mg for those >3years with option to repeat once if no signs of falling asleep within 25 minutes).[38,39] The neurodevelopmental screening consists of (1) general follow-up information including weight, any antiseizure medication use, overall caregiver impression of recovery, sleep quality, and school attendance (when applicable), (2) a standardized Epilepsy (World Health Organization) screening questionnaire used in prior pediatric cerebral malaria studies in the region,[10] (3) 23Q Developmental Screen, which consists of the Ten Questions Questionnaire with expansion to screen for Autism Spectrum Disorder (ASD), validated in Uganda,[40,41] and a neurodevelopmental assessment via either the Malawi Development Assessment Tool (MDAT)[36,41] if ≤6 years old or the Neurological Exam for Subtle Signs[42] if >6 years old. Any positive epilepsy or neurodevelopmental screens will result in confirmatory diagnosis by a board-certified pediatric neurologist (AAP). The primary outcome of epilepsy will be made by the board-certified neurologist based upon International League Against Epilepsy criteria.[43]

Of note, currently there is no standard follow-up of these patients, nor is there any pediatric neurologist available in the region for a specialist review. Any positive findings will be managed by available resources at Chipata Central Hospital, including physiotherapy for any motor impairments and pediatric/psychiatric referral for any behavioral diagnoses. Positive epilepsy diagnoses will have appropriate treatment initiated by the study neurologist in conjunction with a local pediatrician, who will then follow the child. These evaluations and interventions are above the current available standard of care.

Acquired EEG will be analyzed utilizing commercial and in-house software to assess power in each frequency band by wavelet transform, specifically looking at trends of power band ratios for delta, theta, alpha, beta, and lower range gamma frequencies (30-60Hz). Each study will be de-identified with only age available for interpretation, and remotely undergo visual interpretation via secure web-based access by a clinical neurophysiologist. A standardized form for interpretation is used for the study, documenting the presence or absence of the variables of interest. EEGs are read in real time for clinical purposes, and any impact on treatment recorded separately. All antiseizure medication use will be documented throughout study participation as an independent variable for analyses of impact on outcomes and predictive modeling.

	Acute Hospitalization	**1 month post admission**	**6-month post admission**	**12-month post admission**	**18-month post admission**	**24-month post admission**
Patient Characteristics						
Sex	X	X	X	X	X	X
Weight	X	X	X	X	X	X
Pre-CM illness history (HIV status, prior neurologic disease including epilepsy or ASD, family history of seizures/epilepsy)	X					
Caregiver perception of wellness/recovery		X	X	X	X	X

Sleep Quality		X	X	X	X	X
School attendance		X	X	X	X	X
Clinical Metrics						
Age	X	X	X	X	X	X
Weight	X	X	X	X	X	X
Coma score	X					
Coma duration	X					
Maximum Temperature	X					
Presence/absence of acute symptomatic seizures	X					
Use of antiseizure medications	X	X	X	X	X	X
Diagnostic Metrics						
Glucose Level	X					
Parasite Burden (HRP2 level)	X					
EEG	X	X	X	X	X	X
Developmental Impairment and ASD Screening						
Ten Questions Screen	X					
23Q Screen		X	X	X	X	X
Epilepsy Assessment						

WHO Epilepsy Screen		X	X	X	X	X
Neurologic Assessment						
Malawi Developmental Assessment Tool (MDAT) if ≤6yo, Neurologic Exam for Subtle Signs if >6yo		X	X	X	X	X

Table 1. Schedule of Outcome Measurements.

Statistical Analysis Plan

The clinical metrics of interest will be tested for association with the primary outcome of interest, post-malaria epilepsy, by *t*-test and one-way ANOVA, and if needed by their nonparametric alternatives Mann-Whitney test and Kruskal-Wallis test. Analysis of receiver operating characteristic (ROC) will be used to investigate the prediction performance of gamma activity on epilepsy development. Relative gamma power (30-60Hz) normalized to the whole power band will be plotted against whether PME develops, using the data points for the EEGs at each time point of interest. These will be analyzed to determine which time point of assessment reveals the largest difference between groups (CM survivors with PME and those without) in gamma frequency activity. Conventional EEG metrics and spectral analyses of each power band (delta, theta, beta, gamma) will also be assessed and will be analyzed individually using chi-squared test or one-way ANOVA, and in combination by logistic regression with variable selection for association with risk of PME development.

Interim data analyses will routinely be performed to identify the most relevant metrics for risk prediction. Multivariate logistic regression analysis will be conducted with these EEG and clinical metrics ascertained at each time point and, using initial data sets, the predictive algorithm will be built upon variable selection. Prediction performance will be evaluated with the interim data sets to ultimately identify the combination of clinical and EEG metrics with highest predictive capacity for determining risk of PME. Ultimately, a multi-variable logistic regression model will be built with the combination of metrics of highest predictive capacity.

Data Management and Monitoring: Data will be stored directly into a secure, password-protected electronic database. Data quality checks of all entered forms in the electronic database will be performed on a biweekly basis to ensure accuracy and reliability. Any missing data will be reviewed by the clinical study team, and best efforts to complete accurately with hospital data files will be performed. Any missing data that cannot be directly confirmed will be categorized as missing and excluded from analyses. Interim analyses will be performed every 6-12 months, with frequency based on rate of recruitment, with any outlying data reviewed by two study members and confirmed by source data if necessary.

Ethics and Dissemination

This study has been approved by the Boston Children's Hospital Institutional Review Board (IRB-P00038309), University of Zambia Biomedical Research Ethics Committee and National Health Research Authority of Zambia (2529/2022). All publications and reports that result from this work will be produced with involvement and approval of all key personnel of the study. Results will be shared with relevant personnel and NHRA throughout the study period and will be disseminated locally in Zambia before internationally. To optimize availability to lower resourced regions, data will be published in open access, peer-reviewed journals when feasible.

Discussion:

Pediatric CM survivors present a unique population through which to study epileptogenesis and identify biomarkers forecasting PME due to the identifiable etiology of injury and relatively high prevalence and risk of epilepsy in survivors within a relatively short timeframe. Identification of such biomarkers has significant implications for clinical practice because it can provide a mechanism to indicate which patients warrant closer observation due to higher risk of developing epilepsy, in addition to providing measures that can facilitate anti-epileptogenic trials in PME, with potential applicability to other forms of acquired epilepsies.

As the highest burden of highly morbid and fatal CM is in sub-Saharan Africa, it is essential that a predictive epilepsy model for PME consider the resource restrictions of the region where it is most prevalent,[9] and be feasible and applicable within this setting going forward. Therefore, we propose metrics that can be obtained with relative ease at most tertiary care centers in sub-Saharan Africa, where children with cerebral malaria are predominantly treated due to the complexity of their care needs. This includes EEG (available in over 80% of African countries[44]), clinical metrics, and basic laboratory measures. Neuroimaging is not included in our proposed study, as the goal is to use metrics of routine care, and MRI and CT capacity is variable with inconsistent use for clinical care, even in tertiary care centers across Africa.[44]

If successful, this study has the potential to provide not only a mechanism for improved stratification of risk of epilepsy after CM, but also provide a biomarker of epileptogenesis in this population. Such a biomarker would provide the means to subsequently test antiepileptogenic therapies, including a range of inexpensive compounds whose anti-epileptogenic potential is supported by preclinical and clinical data.[45] Additionally, this study will be one of the only to prospectively look at risk of autism after CM and will further assess if quantitative EEG metrics have value in predicting risk of neurodevelopmental impairment in addition to epilepsy.

The proposed study and metrics to be studied over a two-year period will provide a better understanding of the risk factors involved for development of neurologic sequelae after CM, particularly PME, and will provide potential avenues for both improved monitoring and potential intervention, key needs for a disease that continues to affect a large number of children in low-resourced regions annually.

Author contributions: AAP, GLB, MM, and AR were involved in study conception, and design. AAP, SM, RN, DB, JK, TM, VN, KLN, and NK are involved in data collection and contributed to study design. BZ and AAP developed analyses plan. AAP drafted the initial manuscript and all authors provided revisions and final approval for publication, as well as agreed to be accountable for all aspects of the work.

Funding statement: This work is primarily supported by NIH/NINDS grant K23 NS118051 (AAP), with additional support from NIH/NINDS grants K23NS046086, R01NS111057, R01NS102176, & R35NS122265 (GLB).

Competing interests statement: All authors have completed the ICMJE uniform disclosure form at http://www.icmje.org/disclosure-of-interest/ and declare the following: AAP has received a research grant from NIH/NINDS for the proposed work and serves (without compensation) on the advisory board of the ROW foundation which supplies anti-seizure medications through a donation grant to Zambia; GLB has received research grants from NIH/NINDS to support the proposed work and is Ambassador to Zambia for the Royal Society of the Tropical Medicine and Hygiene; MM has stock in Pfizer; and AR is a founder and advisor for Neuromotion, Cofounder PrevEp. Past consultant for or received funding from Abbvie, CRE Medical, ENCODED, Epihunter, Gamify, Neuroelectrics, Neural Dynamics, NeuroRex, Roche, Takeda, and is listed

as inventor on a patent related to integration of TMS and EEG, as well as drug delivery with focused ultrasound. The remaining authors have no conflicts of interest to disclose and declare that the research was conducted in the absence of any relevant commercial or financial relationships.

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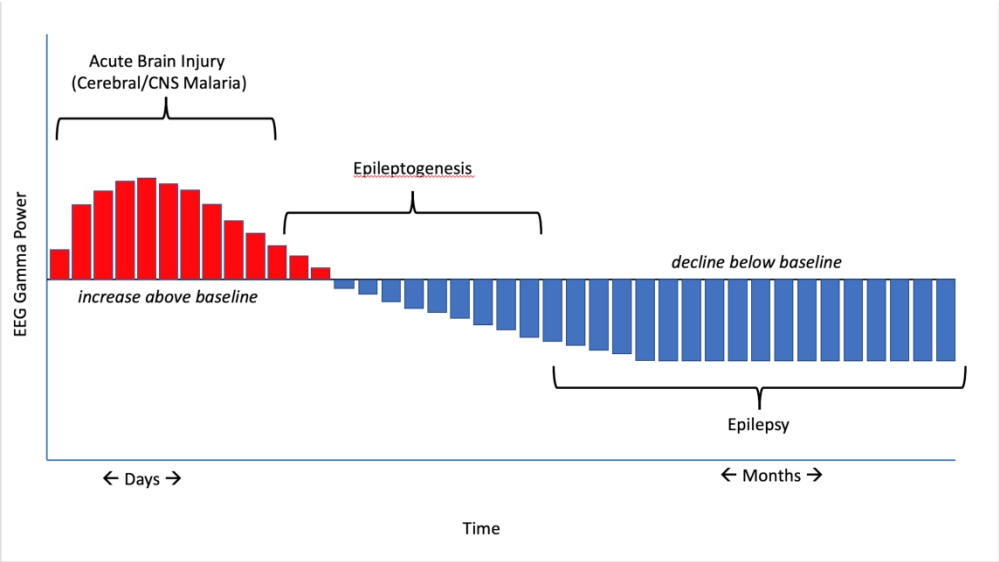

Figure 1. An initial peak of EEG gamma frequency activity is predicted to be seen during malarial infection affecting the central nervous system, followed by subsequent decline during epileptogenesis, in children who will ultimately develop post-malaria epilepsy.

396x223mm (300 x 300 DPI)

TRIPOD Checklist: Prediction Model Development

Section/Topic	Item	Checklist Item	Page
Title and abstract			
Title	1	Identify the study as developing and/or validating a multivariable prediction model, the target population, and the outcome to be predicted.	1
Abstract	2	Provide a summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions.	2-3
Introduction			
Background and objectives	3a	Explain the medical context (including whether diagnostic or prognostic) and rationale for developing or validating the multivariable prediction model, including references to existing models.	4-6
	3b	Specify the objectives, including whether the study describes the development or validation of the model or both.	6
Methods			
Source of data	4a	Describe the study design or source of data (e.g., randomized trial, cohort, or registry data), separately for the development and validation data sets, if applicable.	6
	4b	Specify the key study dates, including start of accrual; end of accrual; and, if applicable, end of follow-up.	N/A; protocol paper
Participants	5a	Specify key elements of the study setting (e.g., primary care, secondary care, general population) including number and location of centres.	7
	5b	Describe eligibility criteria for participants.	7
	5c	Give details of treatments received, if relevant.	N/A
Outcome	6a	Clearly define the outcome that is predicted by the prediction model, including how and when assessed.	10
	6b	Report any actions to blind assessment of the outcome to be predicted.	N/A protocol paper
Predictors	7a	Clearly define all predictors used in developing or validating the multivariable prediction model, including how and when they were measured.	8-11
	7b	Report any actions to blind assessment of predictors for the outcome and other predictors.	N/A protocol paper
Sample size	8	Explain how the study size was arrived at.	8
Missing data	9	Describe how missing data were handled (e.g., complete-case analysis, single imputation, multiple imputation) with details of any imputation method.	N/A protocol paper
Statistical analysis methods	10a	Describe how predictors were handled in the analyses.	N/A protocol paper
	10b	Specify type of model, all model-building procedures (including any predictor selection), and method for internal validation.	Planned methods described - 12
	10d	Specify all measures used to assess model performance and, if relevant, to compare multiple models.	Planned metrics described- 12
Risk groups	11	Provide details on how risk groups were created, if done.	N/A
Results			
Participants	13a	Describe the flow of participants through the study, including the number of participants with and without the outcome and, if applicable, a summary of the follow-up time. A diagram may be helpful.	N/A, protocol paper
	13b	Describe the characteristics of the participants (basic demographics, clinical features, available predictors), including the number of participants with missing data for predictors and outcome.	N/A protocol paper
Model development	14a	Specify the number of participants and outcome events in each analysis.	N/A protocol paper
	14b	If done, report the unadjusted association between each candidate predictor and outcome.	N/A protocol paper
Model specification	15a	Present the full prediction model to allow predictions for individuals (i.e., all regression coefficients, and model intercept or baseline survival at a given time point).	N/A protocol paper
	15b	Explain how to the use the prediction model.	N/A protocol paper
Model performance	16	Report performance measures (with CIs) for the prediction model.	N/A protocol paper
Discussion			
Limitations	18	Discuss any limitations of the study (such as nonrepresentative sample, few events per predictor, missing data).	N/A protocol paper
Interpretation	19b	Give an overall interpretation of the results, considering objectives, limitations, and results from similar studies, and other relevant evidence.	N/A protocol paper

TRIPOD Checklist: Prediction Model Development

Implications	20	Discuss the potential clinical use of the model and implications for future research.	14
Other information			
Supplementary information	21	Provide information about the availability of supplementary resources, such as study protocol, Web calculator, and data sets.	N/A protocol paper
Funding	22	Give the source of funding and the role of the funders for the present study.	15

We recommend using the TRIPOD Checklist in conjunction with the TRIPOD Explanation and Elaboration document.