POTENTIAL ROLE OF NITRIC OXIDE IN THE NORMAL HUMAN IMMUNE RESPONSE TO MALARIA AND IN THE IMMUNOPATHOGENESIS OF CEREBRAL MALARIA.

BY

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ABSTRACT

Introduction

The pathogenesis of cerebral malaria, a clinical syndrome with a high mortality rate despite the best available chemotherapy, is complex and imperfectly understood. Both parasite sequestration and cytokines have been shown to be important. It has been hypothesized, but not proven, that high levels of tumor necrosis factor alpha induce nitric oxide synthase, and increased nitric oxide contributes to the pathogenesis of cerebral malaria by inhibiting neurotransmission.

Objectives:

To assess the role of nitric oxide in the immune response to *P. falciparum* and its role in the immunopathogenesis of cerebral malaria, and to look for clinical and laboratory prognostic indicators in children with cerebral malaria.

Setting:

Children hospitalized into wards in general paediatric and paediatric surgery at Muhimbili Medical Centre, Dar es Salaam, Tanzania.

Patients and study design:

A prospective cross sectional case control study was done. Markers of nitric oxide production was compared in 86 children with cerebral malaria with those in 45 children with non-cerebral malaria and 41 healthy controls. Urine was collected from all these participants on isopropanol. Urinary nitrates was measured by reducing

nitrates to nitrites with bacterial nitrate reductase coupled with Greiss reaction. Urinary creatinine was measured using creatinine sigma diagnostic kit, and then the spot urine nitrate: creatinine ratio was computed. Cerebrospinal fluid was obtained from all children with cerebral malaria and analysed for nitrates.

Simple bed side clinical and laboratory prognostic indicators was assessed in 88 children with cerebral malaria. History of witnessed convulsions within 3 hrs of admission was obtained and presence of decerebration and corneal reflex was looked. Level of conciosness was assessed by Blantyre coma score. Total white blood cell count was obtained using a coulter counter, and differential count was obtained manually. The parasite count was calculated on a thick film stained with Fields A and B stain. Methaemoglobin was calculated by using the method of Evelyn and Malloy. Cerebrospinal fluid opening pressure was measured using a spinal fluid manometer.

Results:

Nitric oxide production correlated inversely with the severity of the disease, the levels being lowest in fatal cerebral malaria, and highest in healthy controls. There was no evidence of increased nitric oxide production in the cerebrospinal fluid. The three groups were different from each other (ANOVA; $P < 0.0001$). By multiple comparison test, patients with cerebral malaria and those with non-cerebral malaria differed from healthy controls ($P < 0.05$). Furthermore, patients with cerebral malaria differed from those with non-cerebral malaria ($P < 0.05$).

The clinical and laboratory features associated with poor prognosis were:-

witnessed convulsions, presence of decerebration, absent corneal reflex, low level of

coma and increased total white blood cell count.

Conclusions:

It appears that nitric oxide production is not increased in cerebral malaria. Rather, it is suppressed in both cerebral and non-cerebral malaria. The production is below the baseline levels of healthy controls, suggesting that both the constitutive and inducible isoforms are suppressed. These results suggest that nitric oxide is protective rather than harmful in malaria.

Five clinical and laboratory features were associated with poor prognosis as mentioned above. However, differential total white blood cell count, level of parasitemia, methaemoglobin, and raised cerebrospinal fluid opening pressure did not correlate with poor outcome. Thus early recognition of the factors for poor prognosis and good medical and nursing care may reduce mortality.

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DECLARATION

I declare that the whole of this work has not been submitted for a degree in any other university.

A.A. Amaral — 24.10.95

Signature — Date

Passed by

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ABBREVIATIONS

AP	-	Asymptomatic parasitemia
CSF	-	Cerebrospinal fluid
CM	-	Cerebral malaria
eNOS	-	Endothelial nitric oxide synthase
HC	-	Healthy controls
iNOS	-	Inducible nitric oxide synthase
IL-1	-	Interleukin-1
IL-2	-	Interleukin-2
IL-6	-	Interleukin-6
IL-10	-	Interleukin-10
INF-gamma	-	Interferon gamma
nNOS	-	Neuronal nitric oxide synthase
NO synthase	-	Nitric oxide synthase
NO	-	Nitric oxide
NMMA	-	N^G-monomethyl L-arginine
NCM	-	Non-cerebral malaria.
TNF - alpha	-	Tumor necrosis factor-alpha
WHO	-	World Health Organization

INTRODUCTION AND REVIEW OF LITERATURE:

Malaria is one of the leading causes of illness, disability and death throughout the world. The World Health Organization (WHO) estimates that there are 270 million people infected with malarial parasites, and 110 million clinical cases occur every year causing 1-2 million deaths a year. Across Sub-Saharan Africa, malaria is one of the five most important causes of mortality and morbidity particularly in infants, young children and pregnant women. The causes of mortality in malaria are severe forms such as cerebral malaria and anaemia which account for over 1 million deaths in children each year[1,2]. Approximately 1% of all clinical episodes of *P. falciparum* infection result in cerebral malaria[3], a severe form of malaria with a mortality rate of 10 - 40%[4,5,6]. Since cerebral malaria is the major cause of mortality, more emphasis is being made to determine its pathogenesis which is complex and imperfectly understood. While sequestration of malarial parasites within the cerebral microvasculature is the major finding at necropsy, the relative importance of cytokine production and parasite sequestration in human cerebral malaria continues to be debated[7]. Serum levels of cytokines, particularly tumor necrosis factor alpha (TNF-alpha) are increased in childhood cerebral malaria compared with non-cerebral malaria, with levels reported to be significantly greater in fatal compared with non-fatal cerebral malaria[5,6]. Experimental data also support the postulate that excessive TNF-alpha production contributes to the pathogenesis of cerebral malaria. Lipids released at schizogony trigger release of TNF-alpha from macrophages in both mouse and human malaria[8]. It is likely that both microvascular obstruction and local production of TNF-alpha within the cerebral vasculature are responsible for much of

the pathology of cerebral malaria[9].

Because cytokines such as TNF-alpha, interferon gamma (INF-gamma), interleukin-1 (IL-1), interleukin -2 (IL-2) and interleukin-6 (IL-6) activate the enzyme nitric oxide synthase (NO synthase) and because levels of these cytokines are elevated in cerebral malaria, it is possible that nitric oxide (NO) production is also increased in cerebral malaria. Documenting increased NO production in proportion to the severity of malaria and correlating NO turnover with other metabolic abnormalities and also with clinical parameters and outcome, would help elucidate the role of NO production in the immunopathogenesis of this frequently fatal disease of major public health importance. Should the magnitude of NO synthesis be correlated with the severity of complicated malaria, it would suggest the potential for pre-clinical and clinical studies exploring the use of NO synthase inhibitors such as N^G-Monomethyl L-arginine (NMMA) in inhibiting a possible final common pathway of cytokine-mediated immunopathology. Alternatively, failure of NO production in cerebral malaria, would suggest that, in cerebral malaria, increased parasite replication and subsequent cerebral sequestration result from failure of NO induction. Given the 10-40% mortality rate from cerebral malaria with the best currently available antimalarial therapy, new approaches to the therapy of cerebral malaria are urgently needed. A greater understanding of its pathogenesis should assist in this goal.

Normal physiological role of nitric oxide:

NO is a small molecule composed of one atom each of nitrogen and oxygen. It is an uncharged molecule with an unpaired electron; these characteristics of NO

make it an ideal messenger molecule. Uncharged NO can diffuse freely across membranes and after spontaneously transmitting a signal, it decays into nitrite and nitrate, the measurement of which allow quantitation of NO turnover. The formation of NO requires an enzyme NO synthase in a reaction that converts the substrate L-arginine and oxygen into citrulline and NO[10,11]. NO synthesis is inhibited by NMMA, its competitative inhibitor.

Two major classes of NO synthase have been isolated. The constitutive isoforms which are found in endothelial cells and neurons [endothelial nitric oxide synthase (eNOs) and neuronal nitric oxide synthase (nNOs) respectively][11,12] and the inducible isoform (iNOs) which are found in macrophages and hepatocytes. The constitutive NO synthase isoforms which are physiologically present in the body are calcium and calmodulin dependent and release picomoles of NO in response to receptor stimulation[13]. The endothelial NO (eNOS) maintains the normal vasodilator tone that is essential for regulation of blood flow and pressure while the neuronal NO (nNOS) is essential for transmission of signals between neurons and thus acts as a neurotransmitter[13]. In contrast, the inducible NO synthase isoform is calcium independent and is normally absent from macrophages and hepatocytes. However, when these cells are activated by specific cytokines such as INF-gamma, TNF-alpha, IL-1 or IL-2, the inducible NO synthase enzyme is produced and once produced this results in the synthesis of large amounts of NO. The continuous production of large amounts of NO kills or inhibits pathogens, including bacteria, fungi and parasites such as *P. falciparum*[14].

The sequence of events leading to NO production begins with an infection that

induces immune cells to release cytokines, which in turn signal macrophages to make NO synthase. For example, a viral infection induces CD4+ helper T-lymphocytes or natural killer lymphocytes to produce INF-gamma, TNF-alpha or lipopolysaccharides (LPS) from the cell wall of infecting bacteria. Specific combination of cytokines, such as INF-gamma along with either TNF-alpha or IL-1, activate intracellular transcription factors inside macrophages, such as nuclear factor-*k*B to initiate production of NO synthase[15]. The activated macrophages must eventually curtail NO production to avoid damaging the host. This is probably achieved by other inhibitory cytokines such as transforming growth factor-ß and mRNA-destablising sequences[16].

Nitric oxide as a neurotransmitter:

Abundant evidence indicates that NO is a neurotransmitter in the peripheral and central nervous systems. Immunohistochemical studies have localized NO synthase to discrete neuronal populations, such as those in the myenteric plexus throughout the gastrointestinal tract[17]. Electric stimulation of gut segments produces relaxation corresponding to the relaxant phase of peristalsis and this is associated with NO release. NO synthase inhibitors block this relaxation, indicating that NO is the neurotransmitter of these neurons[18].

In the brain, NO synthase occurs in discrete neuronal groups but the specific pathways in which it is involved is unclear[13].

Nitric oxide and the transmission of signals between neurons:

Neurons release NO which diffuses into adjacent neurons in a series of steps. The presynaptic neuron releases glutamate which binds to the N-methyl D-aspartate subtype receptor on the postsynaptic neuron. This receptor possesses a calcium channel that opens and the resulting influx of calcium binds to calmodulin to activate neuronal nitric oxide synthase. The NO thus produced binds to the heme group of guanylate cyclase, activating it to produce cyclic guanosine monophosphate (cGMP). Some of the NO that is produced also diffuses back to the presynaptic neuron and releases more glutamate: therefore it has a positive feedback effect[13]. Given such physiologic conditions, small amounts of NO allow glutamate to increase cGMP levels in the brain (Fig.1).

Animal models on the role of nitric oxide in normal immune response:

The role of NO in the immune system is better defined in animals than in humans. In the murine macrophages, neutrophils, hepatocytes, fibroblast, glial cells, smooth muscle cells and endothelial cells, NO synthase is induced by lymphokines during the cell mediated immune response. NO has been shown to be an important mediator in mammalian host defense against intracellular pathogens[19,20]. Its synthesis is L-arginine dependent and inhibited by NMMA[21,22].

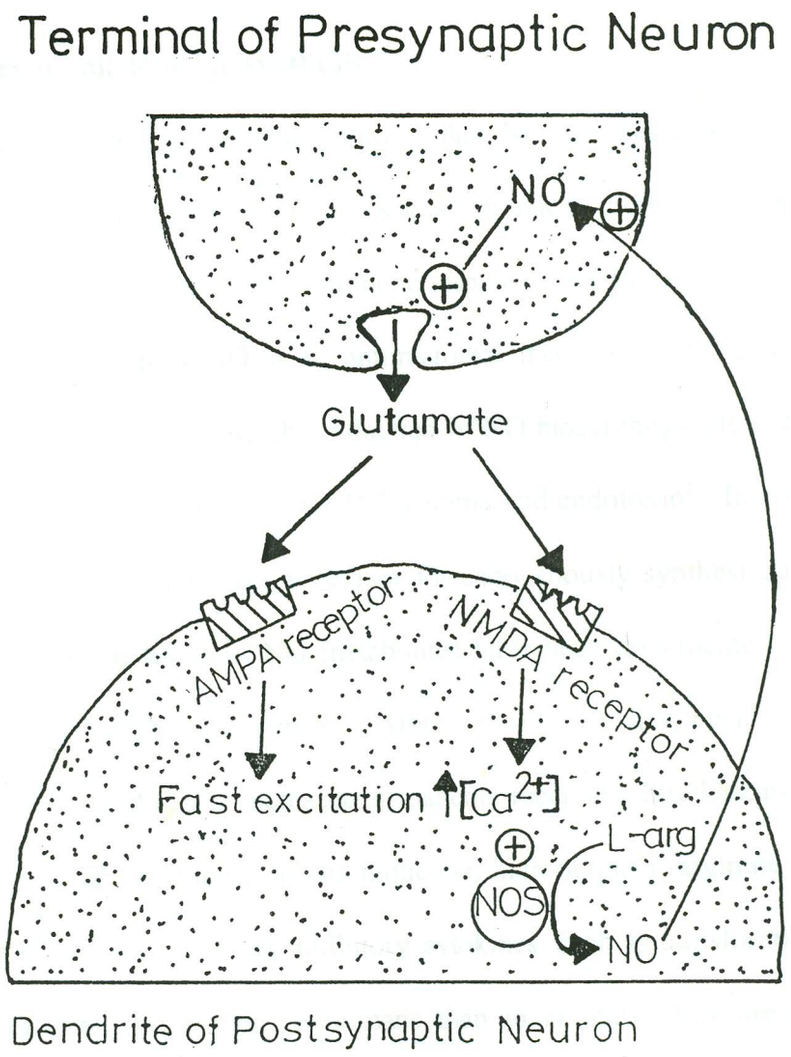


Fig:1 The Role of Nitric Oxide in the Long-Term Potentiation of Neuronal Activity.

Glutamate released from the presynaptic nerve terminal activates different types of receptors on the dendrites of the postsynaptic neuron. Under normal conditions the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors mediate most of the effects of glutamate. During high-frequency synaptic transmission, however, the activation of *N*-methyl-D-aspartate (NMDA) receptors results in an increase in intracellular calcium, which stimulates the constitutive nitric oxide synthase (NOS). The nitric oxide (NO) that is produced diffuses back to the presynaptic neuron, where it enhances the release of glutamate. The increased glutamate release leads to greater activation of postsynaptic glutamate receptors, thereby increasing the effectiveness of that synapse. Plus signs indicate stimulation, and L-arg denotes L-arginine. **(Ref:13)**

Human studies on nitric oxide synthesis:

Whereas a wealth of evidence shows that NO synthesis is an important mediator of the cell mediated immune response in mice, there has been far less documentation of its role in the human immune response. It is still not known whether human macrophages employ NO as an antimicrobial mechanism. Studies in human hepatocytes, however, have shown the induction of NO biosynthesis after stimulation with a combination of IL-1, TNF-alpha, INF-gamma and endotoxin[23]. In 1989, Leaf[24] demonstrated that L-arginine was a precursor for endogenously synthesized nitrate in human adults. Elevated levels of NO metabolites have been documented in humans critically ill from trauma and sepsis[25]. More recently, it has been shown that endogenous nitrate synthesis is increased in patients receiving Interleukin-2 therapy, demonstrating for the first time cytokine-inducible, high output L-arginine dependent NO synthesis in humans[26]. Perhaps inhibitory cytokines, such as transforming growth factor ß, are present more often in humans than in animals, thus preventing the widespread induction of NO synthase. Studies are now required to extend these observations and to identify the full range of pathogens and disease processes that involve a NO-mediated immune response in humans.

Nitric oxide in the protective immune response to malaria:

Cytokines have been shown to be important in the protective immune response to malaria. TNF-alpha, IL-1 and IFN-gamma have all been shown to inhibit intrahepatic malarial parasites in animals[27,28] via NO production. TNF-alpha causes L-arginine-dependent inhibition of intrahepatic parasite development in vitro[29,30,31].

Increased NO turnover in non-cerebral malaria would provide important supporting evidence that NO induction occurs as part of the normal human immune response to malaria.

Possible role of nitric oxide in cerebral malaria:

NO has recently been shown to inhibit the activity of glutamate-controlled calcium channels in the post excitatory neurons[32]. Despite very high serum levels of TNF-alpha in cerebral malaria, cerebrospinal fluid levels are not elevated[5]. Clark *et al*[33] have suggested that although cytokines such as TNF-alpha cannot pass the blood brain barrier, they alter central nervous system (CNS) function via induction of NO synthesis in cerebral endothelial cells. They postulate that NO diffuses across the blood brain barrier resulting in inhibition of glutamate induced Ca^{++} entry and NO formation in postsynaptic neurons, thus altering normal neurotransmission and subsequent coma. Alternatively low levels of NO will fail to activate the presynaptic neurons, and, thus, the formation of glutamate which is essential for normal physiological neurotransmission. Nevertheless, because appropriate studies have not been performed, there is no evidence at present in humans that NO induction is increased in either non-cerebral malaria or cerebral malaria.

Clinical and laboratory prognostic indicators of cerebral malaria in paediatric practice:

Simple clinical and laboratory parameters are useful in assessing prognosis in children with cerebral malaria. The largest study to date that looked at prognostic

indicators was performed at Queen Elizabeth Central hospital, Blantyre, Malawi[4]. It was found that no component of the clinical history was useful in assessing prognosis. However, eight clinical and laboratory features were prognostically useful. These were witnessed convulsions (R.R. 1.38), age below 3 years (R.R. 1.72), decerebration (R.R. 1.91), absent corneal reflex (R.R. 2.69) and coma score `0' (R.R. 5.99). The laboratory features were increased total white blood cell count (R.R. 3.19), parasitemia (R.R. 3.64) and hypoglycemia (R.R. 6.25) in ascending order of importance. Walker *et al* in Nigeria[34] found only some of the above parameters, such as level of coma, decerebrate rigidity and hypoglycemia were useful in assessing prognosis. In the above studies, only 50% and 69% respectively of the study patients had received antimalarial treatment prior to admission. However, since different geographical variations exist, it is not clear whether these criteria are reliable in assessing prognosis in patients admitted to a tertiary referral hospital like Muhimbili Medical Centre, where almost all children have received some form of antimalarial treatment prior to admission. In a study done by Kawo *et al*[35] in Tanzanian children, it was found that hypoglycemia is not a specific complication of cerebral malaria, but it is also found in all severely ill children irrespective of the cause of the illness and therefore will not be studied in this study.

There is also need to assess other markers of prognosis in cerebral malaria. None of the above authors included cerebrospinal fluid (CSF) opening pressure as a measure of prognosis. High CSF opening pressure was found to predict mortality by Newton *et al*[36] in Kenya. However, Waller *et al* in The Gambia[37], did not find any correlation between raised CSF opening pressure and outcome. Thus, it is not clear

whether raised CSF pressure is a measure of poor prognosis.

Other potential laboratory markers of prognosis that have not been well documented in the literature are methemoglobin and white cell differential count. However, complex laboratory measures such as serum and CSF lactate, alanine aminotransferase, 5-nucleotidase and serum TNF-alpha have already been shown in several studies throughout Subsaharan Africa to be correlated with outcome[2] and will not be measured in this study. Furthermore, they are expensive and are not widely available practical markers for routine clinical use.

OBJECTIVES AND HYPOTHESIS

Broad objectives or Goals

1. To assess the role of nitric oxide in the immune response to *P.falciparum* and its role in the immunopathogenesis of cerebral malaria in children.

2. To look for clinical and laboratory prognostic indicators in children with cerebral malaria.

Specific objectives:

1. To determine if there is any evidence that the normal human immune response to *P.falciparum* involves NO-mediated mechanism.

2. To determine if the amount of nitric oxide production correlates with the severity of malaria.

3. To look for evidence of NO production in cerebral malaria by quantitating the NO metabolites in CSF.

4. To determine which clinical features and laboratory findings in Tanzanian children with cerebral malaria can be used to predict outcome.

Hypothesis:

1. Nitric oxide production will be part of the human immune response to *P. falciparum* and the production of NO oxide will be higher in patients with non-cerebral malaria than in healthy controls.

2. The pathogenesis of cerebral malaria includes, not only, sequestration of parasitized red cells within cerebral vessels, but also TNF-alpha mediated induction of NO synthesis. It is hypothesized that NO production will correlate with clinical severity of malaria being highest in patients with fatal cerebral malaria and lowest in those with non-cerebral malaria, and that this will support the role of inducible NO synthesis in the pathogenesis of cerebral malaria in children.

3. For reasons outlined above, it is hypothesized that nitrate levels in CSF will be elevated in cerebral malaria.

Confounding effect of diet on nitrate:

Nitrate excretion in humans arises from both exogenous intake as well as endogenous production of nitrate. The exogenous nitrate refers to inhaled nitrate and dietary nitrate ingestion and this may effect the nitrate excretion in the three study groups. The dietary influence of nitrate will be established from the healthy control group of children. Exogenous nitrate excretion falls quickly with fasting. The half life of exogenous nitrate in human volunteers commencing a nitrate free diet is

approximately 6-8 hours[44].

Children with cerebral malaria have significant anorexia prior to progressing to the severe form of the disease. Molyneux *et al*[4], in a study of 131 Malawian children with cerebral malaria, found that all of them were unable to eat or drink prior to losing consciousness. The mean fasting time was 23.4 hours. Thus the amount of dietary nitrate excretion in this group will be minimal. Similarly, children with non-cerebral malaria have anorexia and thus likely to have lesser nitrate intake than healthy controls. Therefore, the confounding effect of dietary ingestion and its contribution to total urinary nitrate excretion is likely to be small.

Non-cerebral malaria (NCM):

NCM was defined as a febrile illness with *P. falciparum* parasitemia of > 2500/µl, a level likely to be associated with clinical rather than subclinical asymptomatic parasitemia[3], no history of convulsions, no other causes of fever evident, haemoglobin > 5gm/dl, fully alert and no other major complication of malaria.

Healthy controls (HC):

Comprised of children who had no history of or documented fever within the last two weeks, no parasites on thick blood film, normal white cell count ($\leq 15.5 \times 10^9$ /litre) to exclude subclinical infection, and no other acute illness

Informed consent was obtained from parents or guardians. The information obtained was recorded on a standardized data collection sheet (see appendix I).

Sample size:

The sample size was calculated from the following formula:[39]

$$n = \left(\left[\frac{Z\alpha - Z\beta}{U_1 - U_o} \right] \times \sigma \right)^2$$

where:

n = number of patients in each group.

$Z\alpha$ = Denotes the upper percent point of the normal distribution under the null hypothesis. ($U_1 = U_0$)

$Z\beta$ = Denotes the lower percent point of the normal distribution under the alternate hypothesis. ($U_1 > U_0$)

σ = Standard deviation.

U_o = Mean of nitrate:creatinine ratio among healthy controls.

U_1 = Mean of nitrate:creatinine ratio among each group.

Since no published studies are available on nitrate:creatinine ratios in children, calculations of mean fasting nitrate:creatinine ratios were obtained from extrapolations from values available in the literature on studies in adults[26]. In this study the mean urine nitrate: creatinine ratio among healthy adults controls on low nitrate diet was taken as 0.5 µmol/mg. In another study done by Kwiatkowski *et al*[6], in The Gambia on TNF-alpha among healthy children with non-cerebral malaria and cerebral malaria, there was a six fold increase in TNF-alpha levels in cases of cerebral malaria survivors compared with healthy controls and a three and half fold increase in cases of non-cerebral malaria versus healthy controls. Since we hypothesized TNF-alpha mediated NO synthesis, we assume that there will be three and half fold increase between patients with cerebral malaria and healthy controls, and also those with non-cerebral malaria and healthy controls. At 80% power shown at 5% significance level, we have:

$$n = \left[\left(\frac{1.96 + 0.84}{1.75 - 0.5} \right) \times 2.6 \right]^2$$

where n = 34 in each group

In the TNF-alpha study[6] there was a five fold difference in TNF-alpha levels between fatal cerebral malaria and non-fatal cerebral malaria. To detect a 300% difference between fatal and non-fatal cases of cerebral malaria in our study, 24 fatal cases were

required. Assuming a mortality rate of 30% in children with cerebral malaria[2], 80 children were recruited to obtain 24 fatal cases.

History and physical examination:

A detailed history was obtained from the parent(s) or guardian(s), of each patient and thorough physical examination done on admission. Important points in the history included name of the child, sex, exact age from the mother if she remembered or from the maternal and child health clinic card if otherwise. The weight of the child in kilogram (Kg) was recorded. Other points included in the history and clinical examination are shown in appendix I.

Collection of specimens:

In children with cerebral malaria and non-cerebral malaria , a thick blood film for malaria parasites was obtained by a finger prick on all patients recruited in the study and stained by using Fields Stain A and B[40]. The malaria parasites were counted as a number of asexuals per 200 white blood cells. Venous blood was collected in an EDTA tube for determination of haemoglobin, total white blood cell and differential count. The determination of the level of haemoglobin and total white blood cell count was done by using a coulter counter. The differential white blood cell count was done manually on a thin film stained by Giemsa stain.

Methaemoglobin was calculated within 1 hour after blood collection by using the method of Evelyn and Malloy[41]. This is an indirect and non-specific method of nitric oxide quantitation (nitric oxide combines with oxyhaemoglobin forming

methaemoglobin).

In children with cerebral malaria, blood glucose was checked by using Reagent Strips for blood glucose (Glucostix Miles Inc. Ames) for the purpose of correcting hypoglycemia where necessary. Lumbar puncture was done on admission by using a 22G spinal needle to exclude meningitis. Opening CSF pressure was measured using a paediatric spinal fluid manometer. Half a millilitre of CSF was sent for cell count, gram stain, protein, sugar and, culture and sensitivity at the Central Pathological Laboratory, Muhimbili Medical Centre. In addition another half a millilitre of the CSF was frozen at -80°C in liquid nitrogen for subsequent nitrate quantitation.

The patients in the healthy control group were hospitalized in paediatric surgical ward for simple closed fractures, correction of congenital malformations such as talipes, genu valgum, post-surgical contractures and patent ductus arteriosus. This group also included siblings who, for one reason or another, had to be with the mother in the ward. Mothers were advised not to feed their children after the last meal at night except breast fed babies who were allowed to continue breast feeding. An early morning urine specimen was obtained from each of these children. Venous blood was obtained for haemoglobin and total white cell count. A thick film was prepared in order to exclude those with asymptomatic parasitemia.

A urine bag in which isopropanol (isopropyl alcohol) was added to prevent reduction of nitrate to N_2 or NH_3 by bacteria was placed on all children for purpose of collecting urine. Urine microscopy was done on all children to exclude those with pyuria(> 5 Leukocytes/HPF). A sample of the urine was frozen at -80°C for measurement of urinary nitrate and creatinine concentrations.

Processing of the urinary and CSF specimens for nitrate and creatinine assays:

Measurements of urinary and CSF nitrate was carried out at the Joint Duke-Muhimbili Clinical Research Laboratory, Muhimbili, Dar es Salaam, by reducing nitrate to nitrite with a bacterial nitrate reductase using Pseudomonas oleovorans as recommended in the literature[42,43,44].

Nitrite was then measured using the Greiss reagent[45,46]. Granger *et al*[44] have recently shown that a spot urine nitrate:creatinine ratio can be used accurately to quantitate nitric oxide production in human volunteers, correlating closely with 24 hour urinary nitrate measurements. The urinary creatinine measurements was made by using creatinine Sigma diagnostic kit utilising the principles of Slots[47] and Heinegard *et al*[48].

Clinical and laboratory prognostic parameters:

In addition to normal admission history and physical examination, all children with altered state of consciousness were assessed and scored as per Blantyre coma score[2], and a thick film for malaria parasite obtained by a finger prick as mentioned earlier.

The following details were obtained for the study children from their parents or guardians:

1. Time interval since admission to the time of clinical examination.
2. Time elapsed since the child last ate or/and drank.
3. Time since the onset of drowsiness.
4. Antimalarial treatment received prior to admission.
5. Convulsions in the last 48 hours.

The following were specifically looked for:

1. Decerebration
2. Corneal reflex
3. History of convulsions witnessed by the ward staff within three hours of admission.

Venous blood was obtained for determination of full blood count, and methaemoglobin. Lumbar puncture was done, and opening CSF pressure measured by using a paediatric spinal fluid manometer as mentioned above. The blood pressure was obtained by auscultatory method using a cuff of a size appropriate to each child.

Each child was reassessed at least once daily by the author, in addition to the standard observation undertaken by the nursing and medical staff of the ward teams under which they were admitted. In addition, they were examined neurologically by the author prior to discharge and subsequently divided into 3 groups for analysis of outcome:

1. Deaths
2. Neurological sequelae on discharge
3. Discharge without sequelae

Statistical analysis:

The results were analyzed using one way analysis of variance (ANOVA). Scheffe's test was used for multiple comparison among the study groups, using the Statistical Package for Social Sciences (SPSS). For prognostic indicators the differences between the relative frequencies of certain clinical features in fatal and

non-fatal cases were compared using Kruskal-Wallis test for comparing two means using non-parametric technique and one way analysis of variance for comparing three means. P- value of 0.05 or less was taken to indicate significant association.

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RESULTS

During the 9 month period, 186 children were studied. Of these, 88 children had cerebral malaria, 46 non-cerebral malaria , and 52 were healthy controls. Ten of the healthy controls were excluded from the analysis as they were found to have asymptomatic parasitemia.

Tables Ia and Ib show the baseline characteristics of the study population. No significant difference in the distribution of age and sex was observed between the study groups. The time interval since the patient last fed and/or drank among non-cerebral malaria patients was half that of healthy controls and cerebral malaria, indicating that patients with non-cerebral malaria had food more recently than healthy controls or those with cerebral malaria. The difference observed was statistically significant ($F(3,169)=6.615$, $P < 0.0001$). Almost all those in the healthy control group (95%) did not have any antimalarial treatment prior to admission thus showing a sharp contrast when compared to the other groups ($X^2=93.22$, $df=3$, $P < 0.0001$).

The laboratory findings of the patients in the study population are shown in Table II. No difference was observed in the parasite load between the study patients. However, a statistically significant difference was observed between the groups in the level of haemoglobin, white blood cell count, and methaemoglobin ($P < 0.0001$). As expected, lower haemoglobin levels were found in proportion to the severity of illness, the haemoglobin being highest in healthy controls and least in patients with cerebral malaria with a fatal outcome. The total white cell count correlated with the severity of the disease. It was highest in patients with cerebral malaria with a fatal outcome and least in healthy controls.

TABLE Ia: BASELINE CHARACTERISTICS OF THE STUDY POPULATION

VARIABLE		HEALTHY CONTROLS	PATIENTS WITH NCM	PATIENTS WITH CEREBRAL MALARIA (ALIVE AND WELL)	PATIENTS WITH CEREBRAL MALARIA (DIED OR RECOVERED WITH SEQUELAE)	P value
Age (years)	n	42	46	51	37	
	Mean	3.9	3.2	3.5	3.4	NS[1]
	(Range)	(0.5 - 9.6)	(1 - 8)	(0.5 - 7.2)	(0.5 - 7.2)	
	SD	2.6	2.1	1.9	1.8	
Duration since last fed (hours)	n	40	45	51	37	
	Mean	13.0	6.0	15.7	17.3	< 0.0001
	(Range)	(11 - 38)	(1 - 48)	(1 - 96)	(1 - 48)	
	SD	4.1	9.3	18.8	12.1	
Duration since last drank (hours)	n	31	44	51	37	
	Mean	12.4	2.7	11.9	15.4	< 0.0001
	(Range)	(11 - 15)	(1 - 10)	(1 - 48)	(1 - 48)	
	SD	0.9	2.5	12.8	10.6	

NCM = Non-cerebral malaria .
1. (F (3,172)=0.962, P>0.5)

TABLE Ib: BASELINE CHARACTERISTICS OF THE STUDY POPULATION

VARIABLE	HEALTHY CONTROLS	PATIENTS WITH NCM	PATIENTS WITH CEREBRAL MALARIA (ALIVE AND WELL)	PATIENTS WITH CEREBRAL MALARIA (DIED OR RECOVERED WITH SEQUELAE)	
	n = 42	n = 46	n = 51	n = 37	P value
Sex Ratio M:F	27:15	23:23	22:29	23:14	NS[1]
No (%) of patients with weight for age > 80%	41 (97.6)	45 (97.8)	50 (98)	37 (100)	NS[2]
No (%) of patients who received antimalarial treatment before admission	2 (4.9)	25 (54.3)	50 (98)	31 (83.8)	< 0.001

P: Significance of difference between study patients.

1. (X^2=5.5, df = 3, P> 0.1)

2. (X^2=0.8368, df = 3, P> 0.8)

TABLE II: LABORATORY FINDINGS OF THE PATIENTS IN THE STUDY POPULATION

VARIABLE		HEALTHY CONTROLS	PATIENTS WITH NCM	PATIENTS WITH CEREBRAL MALARIA (ALIVE AND WELL)	PATIENTS WITH CEREBRAL MALARIA (DIED OR RECOVERED WITH SEQUELAE)	P value
Malaria (Count/200 WBC)	n	41	46	51	37	
	Mean	-	1472.5	1392	1837.7	
	(Range)	-	(128-6266)	(2-5330)	(2-7000)	NS[1]
	SD	-	1315.6	1508	1953	
Haemoglobin (gm/dl)	n	37	46	51	32	
	Mean	11.2	7.9	6.5	6.3	
	(Range)	(8.1-14.5)	(5.2-12.5)	(2.5-13.6)	(3.2-11.1)	<0.0001[2]
	SD	1.4	1.8	2.4	1.9	
Total White Blood Cell Count $X10^9$/Liter	n	37	44	50	32	
	Mean	8.6	10.8	13	19.7	
	(Range)	(5-14.8)	(3.6-32)	(2.6-28.6)	(4.8-51.5)	<0.0001[3]
	SD	2.2	6.0	6.4	11.9	
Methaemogobin	n	35	37	33	19	<0.0001[4]
	Mean	1.9	4.1	4.7	5.7	
	(Range)	(0-3.6)	(0-10.2)	(0-16.4)	(1.2-15.2)	
	SD	0.8	2.6	4.2	4.1	

1. (x^2 = 1.2=1.27, df = 2, P>0.5)
2. (x^2 = 75, df = 3, P <0.0001)
3. (x^2 = 25.12, df = 3, P <0.0001)
4. (x^2 = 22.3, df = 3, P < 0.0001)

Methaemoglobin increased with severity of disease, signifying that some oxidants may be produced with increasing disease severity thus causing the increase in methaemoglobin production.

Table III shows the mean Urine Nitrate:creatinine expressed as ratio, per kilogram of body weight and also per body surface area. The same is shown in figure 2, 3 and 4 in that order. The mean urine nitrate:creatinine ratio is inversely correlated with the disease severity, being higher in healthy controls and least in cerebral malaria ($F_{(2,169)}=11.766$, $P < 0.0001$). The same trend is observed when the urine nitrate:creatinine ratio is expressed per body weight ($F_{(2,158)}=17.38$, $P < 0.0001$) or per body surface area ($F_{(2,156)}=15.52$, $P < 0.0001$). Using Scheffe's multiple comparison test, statistically significant differences were demonstrated between the three groups ($P < 0.05$). Table IV compares the urine nitrate:creatinine ratio in patients with cerebral malaria who were alive and well to those who died or recovered with sequelae. The same is shown is Fig.5. The urine nitrate:creatinine ratio is inversely correlated with the disease severity among patients with cerebral malaria, being highest in those who were alive and well and least in those who died. However, no statistically significant difference was observed in the nitrate:creatinine ratio among those who were alive and well when compared with those who died or recovered with sequelae ($F_{(2,83)}=1.3594$, $P > 0.2$). The same trend is observed when urine nitrate:creatinine ratio is expressed per body weight ($F_{(2,80)}=1.37$, $P > 0.2$) or per body surface area ($F_{(2,78)}=1.334$, $P > 0.2$).

TABLE III: URINE NITRATE:CREATININE RATIO OF PATIENTS IN THE STUDY GROUPS

VARIABLE		HEALTHY CONTROLS	PATIENTS WITH NCM	PATIENTS WITH CEREBRAL MALARIA	P value
Nitrate: Creatinine Ratio	n	41	45	86	
	Mean	0.461	0.224	0.144	<0.0001
	(Range)	(0.088-3.582)	(0.026-1.658)	(0.003-1.644)	
	±SEM	0.089	0.043	0.021	
Nitrate: Creatinine Ratio/Kg	n	33	45	83	
	Mean	5.461	2.563	1.554	<0.0001
	(Range)	(1.080 -30.800)	(0.364-18.500)	(0.26-17.260)	
	±SEM	0.896	0.485	0.219	
Nitrate: Creatinine Ratio/M^2	n	33	45	81	
	Mean	0.253	0.116	0.075	<0.0001
	(Range)	(0.07-1.54)	(0.02-0.76)	(0.01-0.81)	
	±SEM	0.045	0.022	0.01	

NCM = Non-cerebral malaria.

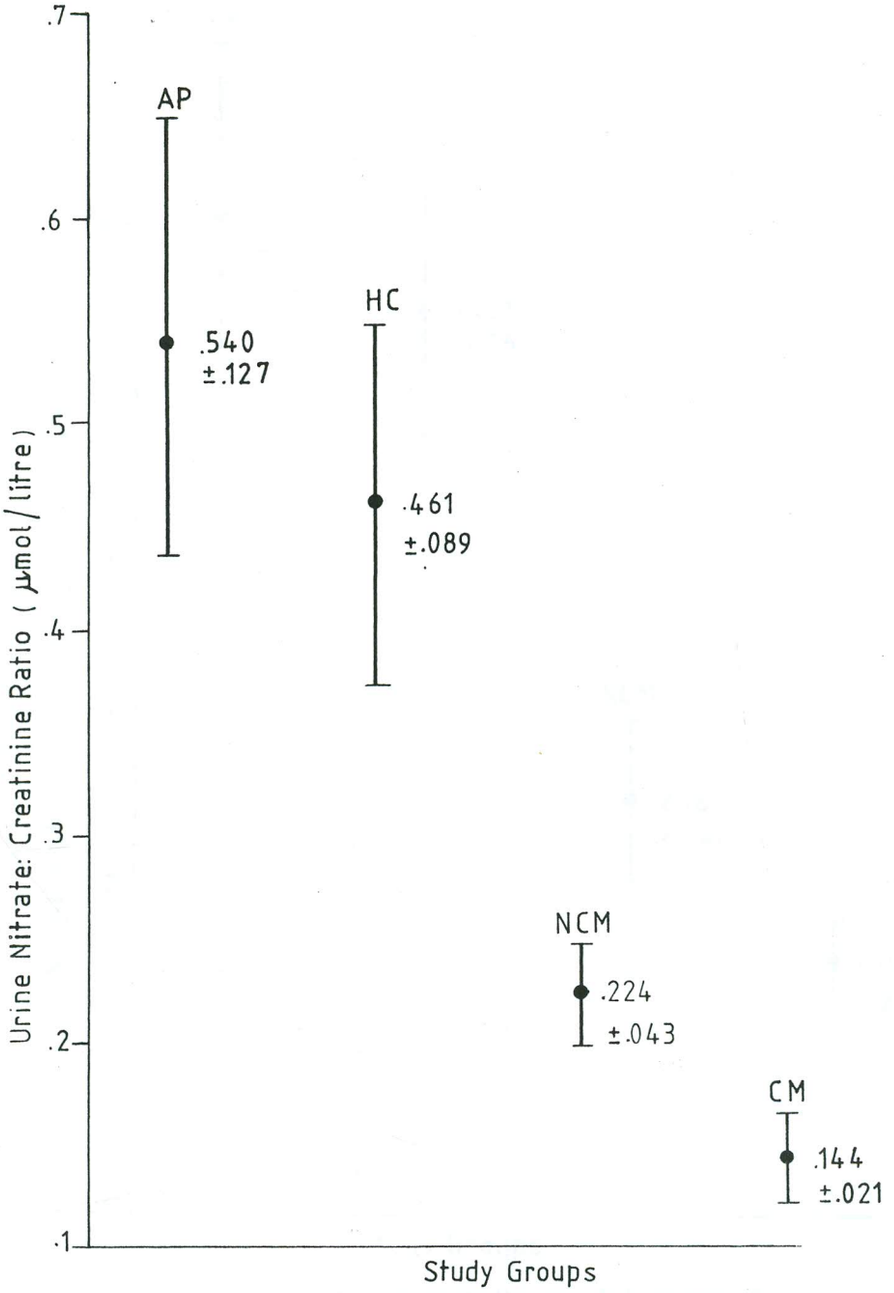


Fig. 2: Urine Nitrate: Creatinine Ratio in patients with cerebral malaria (CM; n=86) and non-cerebral malaria (NCM; n=45) versus healthy controls (HC; n=41). Bars show mean ± SEM.
(AP = Asymptomatic parasitemia; n = 10).

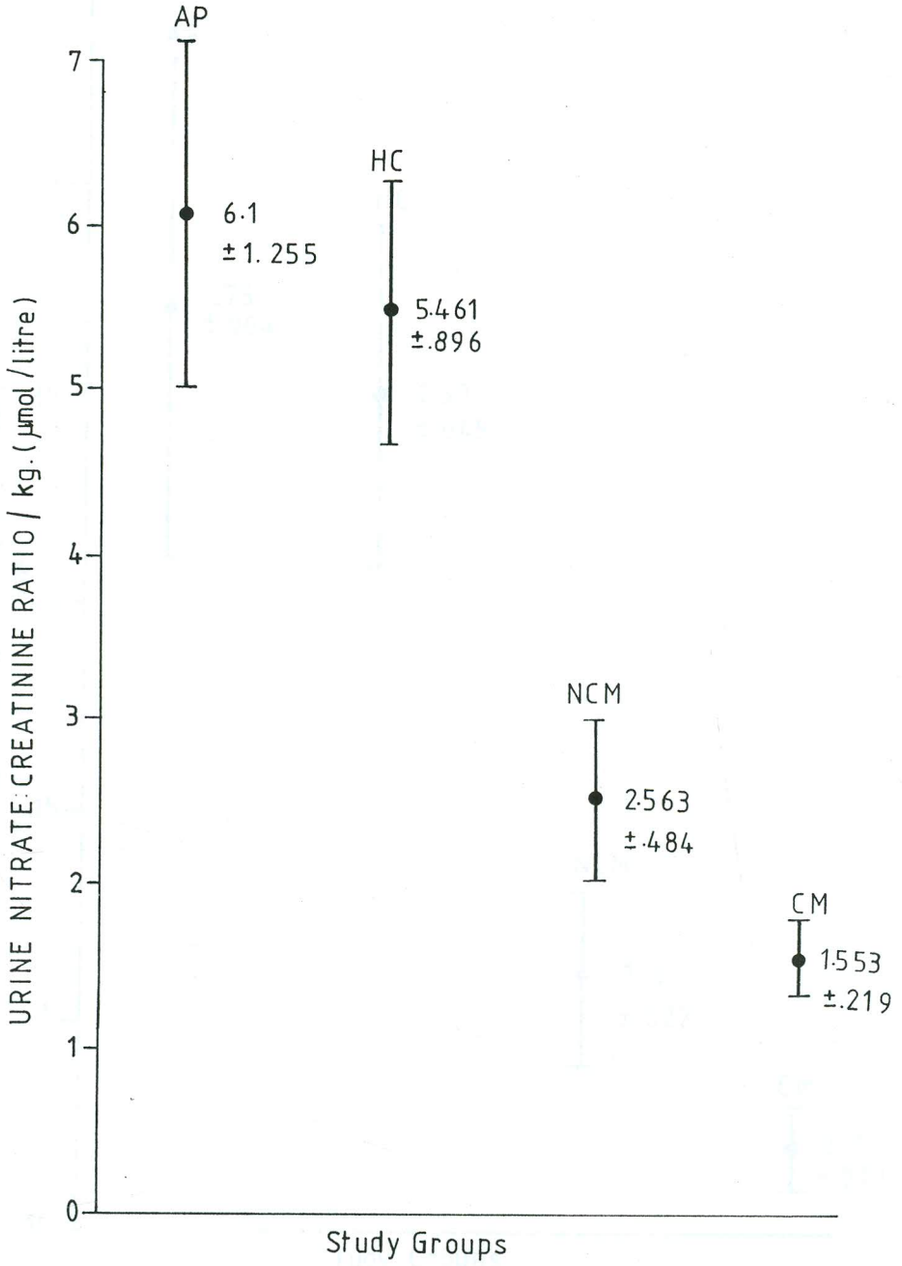


Fig. 3: Urine Nitrate: Creatinine Ratio per Kilogramme of body weight in patients with cerebral malaria (CM; n=83) and non-cerebral malaria (NCM; n=45) versus healthy controls (HC; n=33). Bars show mean ± SEM.
(AP = Asymptomatic parasitemia; n = 10).

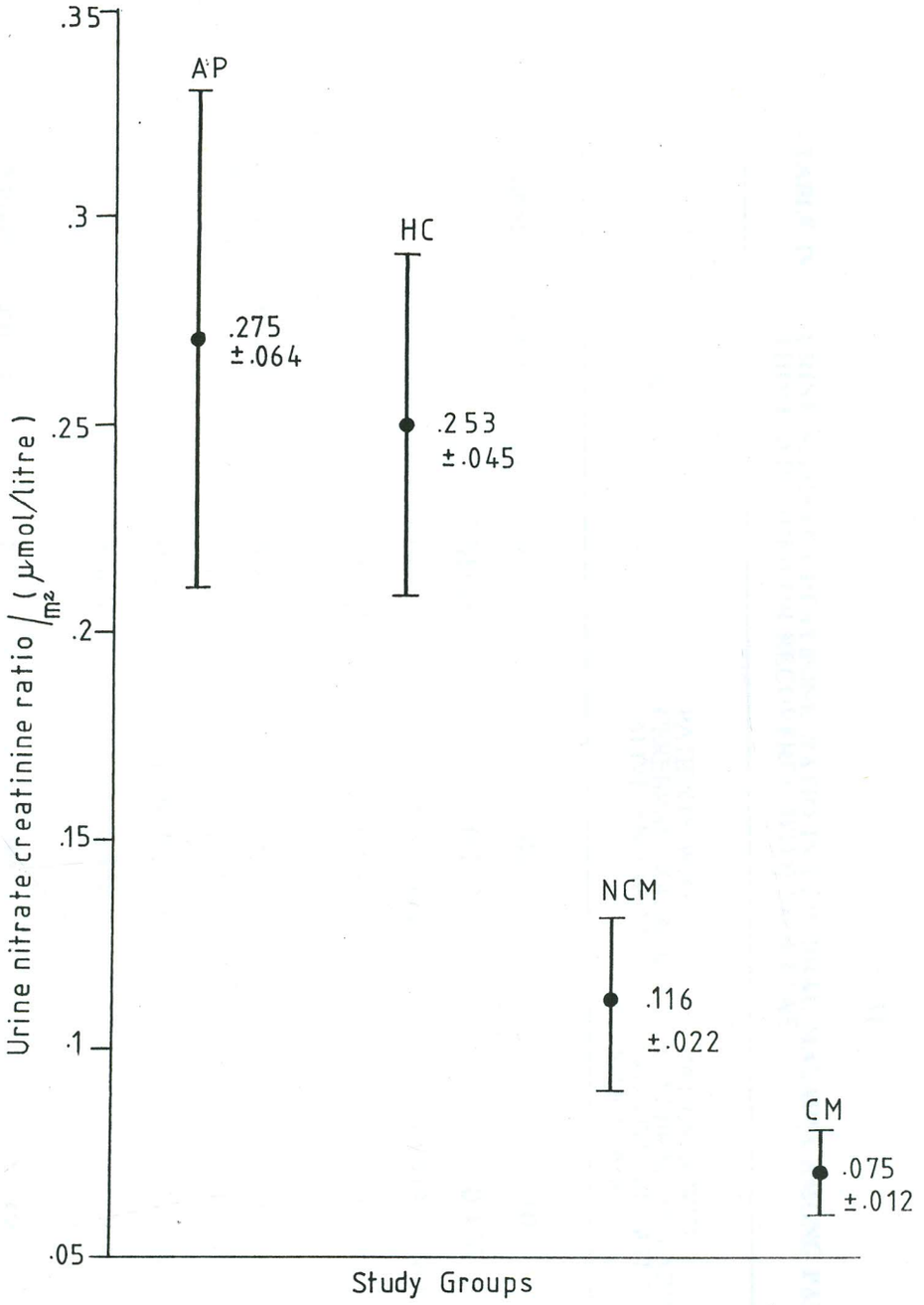


Fig. 4: Urine Nitrate: Creatinine Ratio per body surface area in patients with cerebral malaria (CM; n=81) and non-cerebral malaria (NCM; n=45) versus healthy controls (HC; n=33). Bars show mean ± SEM.
(AP = Asymptomatic parasitemia; n = 10).

TABLE IV: URINE NITRATE:CREATININE RATIO IN CEREBRAL MALARIA AMONG PATIENTS WHO WERE ALIVE AND WELL AND THOSE WHO DIED OR RECOVERED WITH SEQUELAE.

VARIABLE		PATIENTS WITH CEREBRAL MALARIA (ALIVE AND WELL)	PATIENTS WITH CEREBRAL MALARIA (RECOVERED WITH SEQUELAE)	PATIENTS WITH CEREBRAL MALARIA (DIED)	Pvalue
Nitrate:Creatinine Ratio	n	49	10	27	NS
	Mean	0.172	0.136	0.095	
	(Range)	(0.005-1.644)	(0.013-0.389)	(0.002-0.490)	
	±SEM	0.034	0.036	0.018	
Nitrate:Creatinine Ratio/Kg	n	47	10	26	NS
	Mean	1.836	1.578	1.033	
	(Range)	(0.077-17.260)	(0.117-3.89)	(0.026-3.43)	
	±SEM	0.366	0.346	0.151	
Nitrate:Creatinine Ratio/M^2	n	46	10	25	NS
	Mean	0.087	0.074	0.049	
	(Range)	(0.010-0.810)	(0.010-0.190)	(0.010-0.180)	
	±SEM	0.018	0.017	0.007	

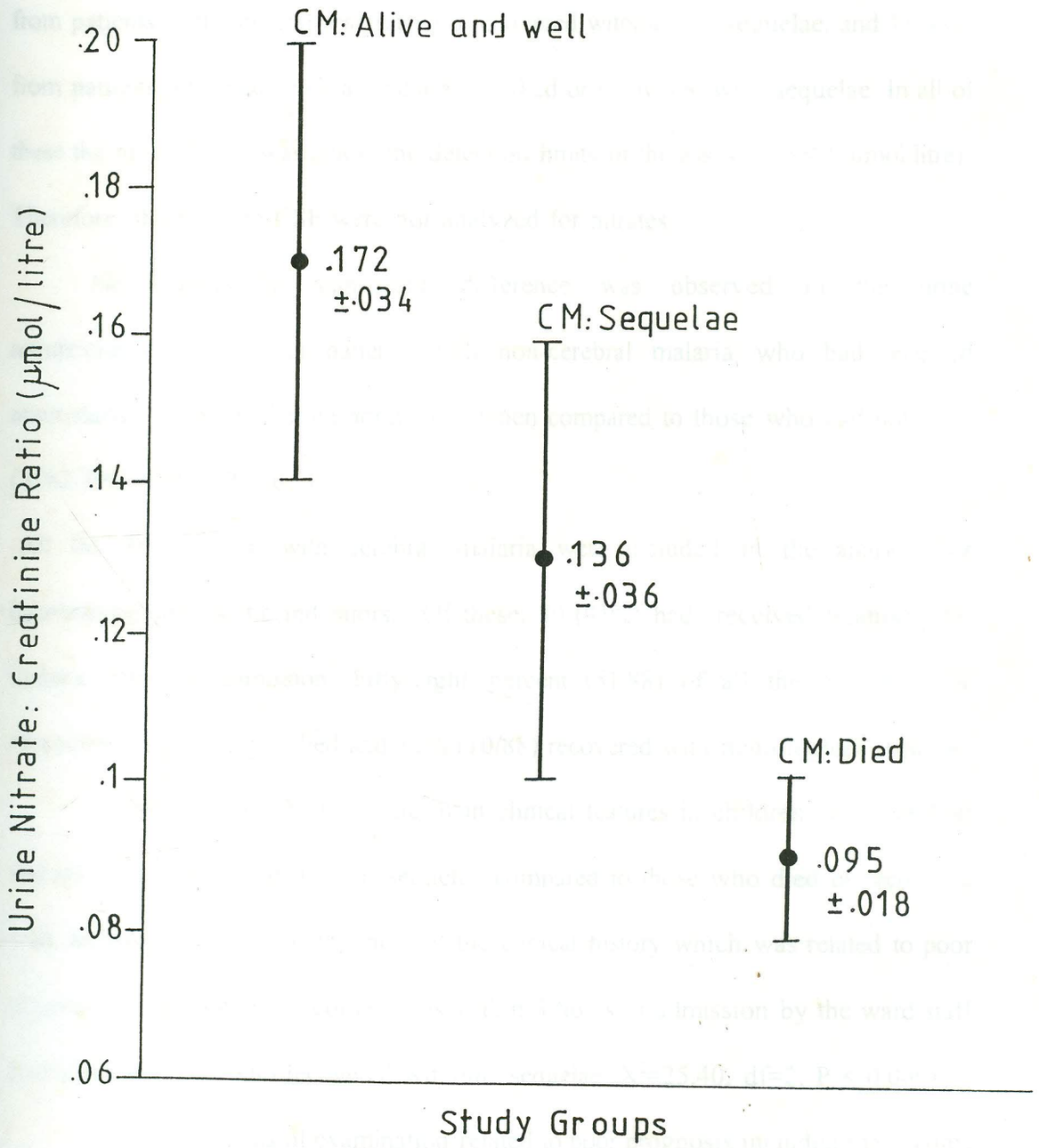


Fig. 5: Urine Nitrate: Creatinine Ratio in patients with cerebral malaria (CM); a comparison between those who fully recovered (n = 49) versus those who died (n = 27) or recovered with sequelae (n = 10). Bars show mean ± SEM.

Twenty-five specimen of the CSF were analyzed for nitrates. Of these, 14 were from patients with cerebral malaria who recovered without any sequelae, and 11 were from patients who had cerebral malaria and died or recovered with sequelae. In all of these the nitrate level was below the detection limits of the assay, (i.e < 5 μmol/litre). Therefore, the rest the CSF were not analyzed for nitrates.

No statistically significant difference was observed in the urine nitrate:creatinine ratio in patients with non-cerebral malaria who had received antimalarial treatment before admission, when compared to those who had not (X^2=2.395, df=1, P > 0.1).

All the 88 children with cerebral malaria were included in the analysis for determining prognostic indicators. Of these, 80 (91%) had received treatment for malaria prior to admission. Fifty-eight percent (51/88) of all the patients fully recovered, 30% (27/88) died and 11% (10/88) recovered with neurological sequelae.

Table Va and Vb shows the main clinical features in children with cerebral malaria who recovered without sequelae compared to those who died or recovered with sequelae. The only component of the clinical history which was related to poor prognosis was witnessed convulsions within 3 hours of admission by the ward staff compared to those who recovered without sequelae (X^2=25.40, df=2, P < 0.0001).

The components of examination related to poor prognosis included mean coma score (F (1,86)=3.987, P < 0.05). The lower the coma score the worse the outcome and vice versa. However, the number of patients with a coma score equal to zero did not have a statistically significant difference in those who died or recovered with sequelae compared to those who recovered without sequelae.

TABLE Va: PRESENTING CLINICAL FEATURES IN RELATION TO OUTCOME OF THE PATIENTS WITH CEREBRAL MALARIA

CHARACTERISTIC	PATIENTS WITH CEREBRAL MALARIA (ALIVE AND WELL)				PATIENTS WITH CEREBRAL MALARIA (DIED OR RECOVERED WITH SEQUELAE)				P value
	n	Mean	(Range)	SD	n	Mean	(Range)	SD	
Duration of coma (hours)	48	12.9	(1-72)	14.9	36	16.7	(2-96)	17.1	NS[1]
Duration since last fed (hours)	51	15.6	(1-96)	18.8	37	17.3	(1-48)	12.1	NS[2]
Duration since last drank (hours)	51	11.9	(1-48)	12.1	37	15.4	(1-48)	10.6	NS[3]
Total coma score	51	1.5	(0-2)	0.7	37	1.2	(0-2)	0.8	< 0.05

1. ($F(1, 82)=1.154$, $P > 0.2$)
2. ($x^2 = 2.915$, $df=1$, $P > 0.08$)
3. ($F(1, 86 = 2.076$, $P > 0.1$)

Table Vb: PRESENTING CLINICAL FEATURES IN RELATION TO OUTCOME OF PATIENTS WITH CEREBRAL MALARIA.

CHARACTERISTIC	PATIENTS WITH CEREBRAL MALARIA (ALIVE AND WELL)	PATIENTS WITH CEREBRAL MALARIA (DIED OR RECOVERED WITH SEQUELAE)	P value
	n = 51	n = 37	
No (%) of patients age < 3 years	20 (38.5)	15 (42.9)	NS[1]
No (%) of patients with history of vonvulsions.	50 (98)	37 (100)	NS[2]
No (%) of patients with witnessed convulsions	19 (37.3)	33 (89.2)	< 0.0001
No (%) of patients with coma score=0	6 (37.5)	10 (62.5)	NS[3]
No (%) of patients with decerebration.	2 (3.9)	6 (16.2)	< 0.05
No (%) of patients with absent comeal reflex.	10 (22.7)	13 (48.1)	<0.05

1. (X^2=0.17, P > 0.6)
2. (X^2=0.73, P > 0.3)
3. (X^2=3.32, P > 0.06)

Decerebration was present in 8 patients, six of whom died or recovered with neurological sequelae (X^2=3.88, P < 0.05). Twenty-three patients had an absent corneal reflex. Of these, 13 died or recovered with sequelae (X^2=6.31, df=2, P < 0.05). Thus decerebration and absence of corneal reflex was correlated with poor prognosis among those who died or recovered with sequelae compared to those who recovered without sequelae.

Table VIa and VIb shows the laboratory findings in relation to outcome. The only laboratory marker of poor prognosis was increased total white blood cell count which showed an inverse correlation (X^2=5.743, df=1, P < 0.05). The higher the white cell count the worse the prognosis and vice versa. For the other laboratory markers, there was no significant difference between those who died or recovered with sequelae compared to those who recovered without sequelae.

TABLE VIa: LABORATORY FINDINGS IN RELATION TO OUTCOME

VARIABLE	PATIENTS WITH CEREBRAL MALARIA (ALIVE AND WELL)				PATIENTS WITH CEREBRAL MALARIA (DIED OR RECOVERED WITH SEQUELAE)				Pvalue
	n	Mean	(Range)	SD	n	Mean	(Range)	SD	
Malaria parasites (count per 200 white blood cells).	51	1392	(2-5330)	1508	37	1837.7	(2-7000)	1953	NS[1]
Haemoglobin (gm/dl).	49	6.5	(2.5-13.6)	2.5	32	6.3	(3.2-11.1)	1.9	NS[2]
White blood cell count x 10^9/litre.	48	13.1	(2.6-28.6)	6.5	32	19.8	(4.8-51.5)	11.9	<0.05
Methaemoglobin.	32	4.8	(0.8-16.4)	4.1	19	5.8	(1.2-15.2)	4.1	NS[3]
Opening pressure (in Cm of water)	37	18.1	(6-47.5)	7.1	22	19.8	(9.8-48)	7.1	NS[4]

1. $(F(1,86) = 1.460, P > 0.2)$
2. $(F(1,79) = 0.163, P > 0.6)$
3. $(F(1,49) = 0.689, P > 0.5)$
4. $(F((1,57) = 0.824, P > 0.6)$

TABLE VIb: LABORATORY FINDINGS IN RELATION TO OUTCOME

CHARACTERISTICS	PATIENTS WITH CEREBRAL MALARIA (ALIVE AND WELL) n = 38	PATIENTS WITH CEREBRAL MALARIA (DIED OR RECOVERED WITH SEQUELAE) n = 27	P value
No (%) of patients with absolute neutrophilia.	13 (34.2)	14 (51.8)	NS[1]
No (%) of patients with absolute lymphocytosis.	23 (60.5)	22 (81.5)	NS[2]

1. (X^2=1.99, P > 0.2)
2. (X^2=3.2, P > 0.07)

DISCUSSION

Nitric oxide and malaria

Contrary to our hypothesis, our study has shown that NO production is not increased in cerebral malaria. Instead it is decreased. Our study shows that NO production is actually suppressed and that the production is inversely proportional to the severity of the disease. Patients with non-cerebral malaria showed a lower production of NO but the production was much lower in the patients with cerebral malaria when compared with healthy controls. If malaria results in only a failure to induce NO one would have expected the nitrate:creatinine ratio in all the three groups to be roughly equal. However, because NO production in both cerebral malaria and non-cerebral malaria was below the baseline levels found in healthy controls, it appears that there was suppression of both the constitutive isoforms of NO synthase (eNOs and/or nNOs) as well as the inducible isoform of NO synthase (iNOs).

Possible effects of suppression of constitutive NO synthase:

Endothelial NO synthase (eNOs): eNOs is essential for maintaining the normal vasodilator tone of blood vessels, and thus maintaining the regulation of blood flow and pressure[13]. Its suppression may result in failure to maintain normal cerebral blood flow, perhaps due to vasoconstriction and decreased cerebral perfusion. Recent studies from Kenya have confirmed a critical decrease in cerebral perfusion pressure (CPP) associated with neurological sequelae, as evidenced by CT scan[49]. Similarly, our study has shown that patients with cerebral malaria have CPP below normal and that the CPP correlates inversely with disease severity. The decrease in CPP and possible

vasoconstriction may aggravate the ischaemia caused by sequestration of the parasitized red blood cells in cerebral blood vessels. These may contribute to seizures and coma in patients with cerebral malaria.

Neuronal NO synthase (nNOs): nNOs is essential for transmission of signals between neurons. Its suppression may result in decreased formation of glutamate in the postsynaptic neuron. The low levels of NO may fail to induce a positive feedback effect on the presynaptic terminal and, therefore, further decrease in the formation of glutamate and NO, thus impairing normal neurotransmission. This too may contribute to coma in cerebral malaria.

Possible effects of suppression of inducible NO synthase (iNOs):

NO has been shown to have a protective immune response in mouse models of malaria by inhibiting the exoerythrocytic stages of P. Berghei in hepatocyte[27]. As a consequence, there is inhibition of the intrahepatic development of malaria parasites in a dose dependent manner[28].

Suppression of NO in those with non-cerebral malaria and cerebral malaria may cause possible suppression of the protective immune response to malaria parasites allowing increased parasite replication and more severe disease. In a subgroup of healthy controls, who had asymptomatic parasitemia, NO production was higher than in healthy controls. It is possible that NO production occurred as part of the normal protective immune response to parasite infection, and that subsequent suppression of NO production may result in loss of protection and progression from asymptomatic

parasitemia to non-cerebral malaria. However, this cannot be stated with certainty as the difference between NO production among healthy controls and asymptomatic parasitemia was not statistically significant. Further studies looking at NO production in these two groups need to be done with a larger number of patients to see if the apparently higher NO production in asymptomatic parasitemia is indeed true.

No studies in malaria have, so far, examined the metabolites of NO in urine, which more accurately reflects NO production. Recent studies by Cot *et al*[50] on NO production in African children with cerebral malaria have reported low levels of NO by quantitating its metabolites in plasma, which correlated inversely with disease severity. However, the study population was very small. Furthermore, healthy controls or patients with non-cerebral malaria were not included, and there was no control for diet, thus making the results inconclusive.

Cause and effect or only an association:

Suppression of NO may be directly involved in the pathogenesis of cerebral malaria or it may be only an association between the two, not necessarily a cause and effect. Whilst there are many theoretical reasons why suppression of NO may have deleterious effects in cerebral malaria, as outlined above, association does not prove causation. It is also possible that combination of sequestration and cytokine production that results in cerebral malaria also happens to cause suppression of NO. This suppression may be a by-product rather than a cause of cerebral malaria, that is, an epiphenomenon.

Possible mechanisms of suppression of nitric oxide:

1. Studies have shown that TNF-alpha production shows variation to different parasite strains and the host[51,52]. On similar lines there may also be variations in different parasite strains and the host in suppressing NO production. The variation in TNF-alpha production by the parasite and the host factors have been hypothesized as the explanation why only 1 - 2% of patients with non-cerebral malaria develop severe disease[52]. A similar hypothesis can be postulated for variations in suppression of NO synthase.

2. Cytokines such as interleukin-10 have very recently been shown to be elevated during malaria illness in proportion to the severity of the disease[53]. Interleukin-10 has been shown to strongly inhibit the stimulatory effect of cytokines such as TNF-alpha, interleukin-2, and interleukin-6 on NO production[54]. It is also known to have a direct inhibitory effect on NO synthase. So even though TNF-alpha, interleukin-2 and interleukin-6 are markedly elevated in cerebral malaria, their inhibitory effect on NO synthase may explain why we have shown that the production of NO is not increased in cerebral malaria.

Possible confounding effects:

No difference was observed in the distribution of age, sex and nutritional status between the study and the control groups. The confounding effect of diet on NO production is very unlikely to bias the study as mean fasting hours among healthy controls and cerebral malaria were similar. Non-cerebral malaria patients had a

shorter duration of fasting than healthy controls and cerebral malaria. One would expect this to result in higher mean NO production if diet was an important confounding factor. The fact that NO production was suppressed in non-cerebral malaria despite more recent dietary ingestion suggest that the effect of diet was minimal. Furthermore, breast milk was obtained from mothers in the healthy control group of children and was analysed for nitrates, which was found to be very low, and it is therefore unlikely to bias the study in few children on breast milk in these group.

Prognostic indicators

Cerebral malaria is the most common severe and potentially fatal complication of P.falciparum infection with a mortality rate of 10 - 40%[2]. In our study the mortality rate was 30%. Over 16% of survivors (or 11% of the total number of patients) developed neurological sequelae. Ninety-one percent of all patients had received some form of treatment for malaria, mainly chloroquine injections or tablets, prior to admission to hospital. This finding contrasts with findings in Malawi[4] and Nigeria[34] where only 50% and 69%, respectively, had received antimalarial chemotherapy before admission.

Clinical Prognostic features:

Age:

This study did not show any correlation between age and poor outcome. This is in sharp contrast with the study by Molyneux *et al*[4] who reported that age less than 3 years had a poor prognosis.

Convulsions:

History of convulsions on admission was common among children with cerebral malaria with a fatal and non-fatal outcome. However, witnessed convulsions, were associated with delayed recovery of consciousness and adverse outcome. This is consistent with findings from another study done in African children with cerebral malaria[4]. Thus, it becomes necessary to prevent convulsions. However, it is not possible to conclude that convulsions are harmful or are merely associated with the severity of the disease. In order to elucidate this, a controlled trial is needed to determine the value of prophylactic anticonvulsant drugs in cerebral malaria in children. In a controlled trial in which prophylactic phenobarbitone was administered in adults with cerebral malaria, the incidence of convulsions was successfully reduced, although the effect of this on outcome could not be assessed[55].

Depth of Coma:

Mean coma score was related to poor outcome, indicating that patients who are more comatose have higher chances of dying or developing a sequelae. This is consistent with findings from another study done in African children with cerebral malaria[56]. In contrast, Molyneux *et al*[4] showed that patients who have a coma score of zero on admission have a poor outcome compared with those with a coma score above zero. In our study no significant difference was observed among patients with a coma score of zero and those above zero on admission, although 62.5% of the deaths occurred in patients with a coma score of zero. In the study done by Molyneux *et al*[4] the patients recruited had coma scores up to 4, while in our study, only patients

with a score of 2 or less were recruited. Therefore, all patients in our study were in deep coma. This may probably explain why we did not show a statistically significant difference between those with a coma score of zero and those with a score above zero. However, there was a statistically significant difference observed between the mean coma scores of the two groups.

Decerebration and absent corneal reflexes:

This study showed that absent corneal reflex and decortication correlates with poor outcome. This is consistent with other studies [4,36,57,58].

Laboratory Prognostic factors:

Total white blood cell and differential count:

The only laboratory marker of poor prognosis was total white blood cell count. This was also reported by Molyneux *et al*[4] in children and by Warrel *et al*[58] in adults. However, no difference was observed in the absolute neutrophil or lymphocyte counts among fatal and non-fatal cases.

Total Parasite count:

Our study did not show any difference between the parasite count in fatal when compared with non-fatal cases. This is consistent with findings from another study done in Nigeria[34]. However, it contrasts with findings by Molyneux *et al*[4] who showed that the parasite load correlated with poor outcome. The possible explanation of this findings in our study may be that almost all patients had received antimalarial

treatment prior to admission to the hospital compared to only half of the patients in the Malawi study. This may have decreased the parasite load. It is not clear from this study if the level of parasitemia was related to the pathogenesis of the disease as we had some patients with a parasite count as low as 2/200 WBC.

Haemoglobin and Methaemoglobin:

Anaemia is a common feature of children who live in malarious areas. This study did not show any relationship between anaemia and mortality. This is consistent with the findings from other studies done in Malawi[4] and Nigeria[34] . It is possible that the effect of anaemia on the outcome may have been masked by transfusion of all severely anaemic patients with packed red cells.

Methaemoglobin, a reduced form of haemoglobin which is not able to carry oxygen, was higher among those with a poor outcome. Although, this was not statistically significant, it suggests that those children with a poor outcome have increased levels of reduced haemoglobin which is unable to deliver oxygen to the brain thus aggravating the ischaemia caused by parasitized red blood cells. If this is true, then early blood transfusion, in severely anaemic patients, may improve the outcome.

CSF opening pressure:

Mean CSF opening pressure was raised in all patients, except one who fully recovered. In this study the mean CSF opening pressure did not correlate with poor outcome. This is consistent with the findings in another study done in The Gambia[37].

However, it contrasts with findings by Newton *et al*[36] who showed that high CSF opening pressure correlated with poor outcome. We suggest that measures to reduce the level of intracranial pressure should be investigated to assess their beneficial effects. Cerebral perfusion pressure was obtained by a difference in mean arterial pressure and CSF opening pressure. This method is not very accurate but it was the only option available to us. The mean CPP was below normal in patients with cerebral malaria. Furthermore, it was even lower in fatal compared to non-fatal cases. The possible explanation of reduced CPP has been described above.

CONCLUSION

Nitric oxide and malaria

1. There is no evidence of increased NO production in cerebral malaria.
2. It appears that NO production is suppressed in both non-cerebral malaria and to a greater extent in cerebral malaria.
3. It appears that the suppression involves both the constitutive and inducible isoforms of NO synthase.
4. The suppression of constitutive isoform of NO could possibly impair cerebral blood flow and neurotransmission which may contribute to seizures and coma.
5. The suppression of the inducible isoform may suppress the protective immune response to malaria parasites allowing parasite replication and more severe disease.
6. The suppression of NO may be due to variations in the parasite and the host in suppressing NO synthesis or it may be only an association between disease severity and NO synthesis.

Prognostic indicators

7. This study has shown five clinical and laboratory markers of poor prognosis. These are:-witnessed convulsions, low level of coma score, presence of decerebration, absent corneal reflex and increased total white blood cell count.
8. Age, total parasite count, haemoglobin, methaemoglobin, cerebrospinal fluid opening pressure and cerebral perfusion pressure did not correlate with poor prognosis.

RECOMMENDATIONS

Nitric oxide and malaria

1. Animal studies using NO donors should be done to look for protective effects of NO in malaria.
2. Laboratory studies should explore, in vitro, the white cell, cytokines, parasite and endothelial interaction that appears to suppress NO synthesis.
3. Further studies are needed to look for NO production in larger number of patients with asymptomatic parasitemia and the possible protective role of NO in this patients.
4. It will be useful to confirm the results of this study in different settings where different parasite strains and hosts exist.

Prognostic indicators

5. Early intervention, good medical and nursing care become necessary in a patient who has symptoms and/or signs of poor prognosis. This may reduced mortality.

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