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Tissue distribution of migration inhibitory factor and inducible nitric oxide synthase in falciparum malaria and sepsis in African children

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Abstract

Background: The inflammatory nature of falciparum malaria has been acknowledged since increased circulating levels of tumour necrosis factor (TNF) were first measured, but precisely where the mediators downstream from this prototype inflammatory mediator are generated has not been investigated. Here we report on the cellular distribution, by immunohistochemistry, of migration inhibitory factor (MIF) and inducible nitric oxide synthase (iNOS) in this disease, and in sepsis.

Methods: We stained for MIF and iNOS in tissues collected during 44 paediatric autopsies in Blantyre, Malawi. These comprised 42 acutely ill comatose patients, 32 of whom were diagnosed clinically as cerebral malaria and the other 10 as non-malarial diseases. Another 2 were non-malarial, non-comatose deaths. Other control tissues were from Australian adults.

Results: Of the 32 clinically diagnosed cerebral malaria cases, 11 had negligible histological change in the brain, and no or scanty intravascular sequestration of parasitised erythrocytes, another 7 had no histological changes in the brain, but sequestered parasitised erythrocytes were present (usually dense), and the remaining 14 brains showed micro-haemorrhages and intravascular mononuclear cell accumulations, plus sequestered parasitised erythrocytes. The vascular walls of the latter group stained most strongly for iNOS. Vascular wall iNOS staining was usually of low intensity in the second group (7 brains) and was virtually absent from the cerebral vascular walls of 8 of the 10 comatose patients without malaria, and also from control brains. The chest wall was chosen as a typical non-cerebral site encompassing a range of tissues of interest. Here pronounced iNOS staining in vascular wall and skeletal muscle was present in some 50% of the children in all groups, including septic meningitis, irrespective of the degree of staining in cerebral vascular walls. Parasites or malarial pigment were rare to absent in all chest wall sections. While MIF was common in chest wall vessels, usually in association with iNOS, it was absent in brain vessels.

Conclusions: These results agree with the view that clinically diagnosed cerebral malaria in African children is a collection of overlapping syndromes acting through different organ systems, with several mechanisms, not necessarily associated with cerebral vascular inflammation and damage, combining to cause death.

Background

Falciparum malaria is a complex multi-organ disease that is often fatal. It is not yet clear how *Plasmodium falciparum* causes the seizures, coma, cerebral oedema, hypoglycaemia and respiratory failure seen in a proportion of infected African children. Intense sequestration of parasitised erythrocytes in brain microvessels in fatal malarial coma was first described over a hundred years ago [1], and the mechanisms by which this phenomenon might contribute to coma or death have been the subject of speculation since then. More recently data have accumulated to suggest that in systemic infections such as malaria, excessive production of pro-inflammatory cytokines may play an important role in the generation of symptoms and disease [2]. Evidence supporting this concept includes the ability of experimental malaria to prime for tumour necrosis factor (TNF) production [2], the close similarity to malaria of the side effects observed when TNF is administered to tumour patients [3], and clinical studies showing a correlation between disease severity and the circulating levels of TNF in African [4,5] and Melanesian [6] children and European adults [7]. A large body of experimental and clinical evidence has now accumulated [8] suggesting that excessive inflammatory cytokine production, and its ensuing consequences, could play a crucial role in the pathogenesis of severe falciparum malaria. The inability of anti-TNF antibody to reverse severe malarial disease is sometimes cited against this concept [9], although subsequently evidence emerged that preliminary administration of anti-TNF can prevent the characteristic febrile reaction to treatment in rickettsial infection [10]. This is consistent with the existing evidence that TNF is an early link in the chain of events causing acute disease.

The literature on the range of cytokines involved in the pathogenesis of sepsis is now complex, and has taken some unexpected turns over recent years, none more so than the central involvement of migration inhibitory factor (MIF). This was the first cytokine to be described, and was so named because of its function in delayed hypersensitivity [11,12]. A decade later MIF was detected, by its original functional assay, in experimental malaria [13]. MIF is now recognised to potentiate lethal endotoxaemia through enhancing TNF release [14], to be a pituitary-derived antagonist of glucocorticoids, and to be released from a wide range of cell types, including macrophages [15]. Circulating levels of MIF are raised in human sepsis [16–18], and its importance in this disease state is implied by the remarkable degree of protection given by MIF-neutralizing antibody in the caecal ligation and puncture model of sepsis [16]. While MIF can be viewed as a pro-inflammatory cytokine, it also has properties characteristic of a hormone, and can be regarded as largely acting through antagonising the ability of glucocorticoids to inhibit the generation of pro-inflammatory cytokines [19]. A role for MIF in dyserythropoiesis has been suggested in a mouse model of malaria [20], and it has been localised by immunohistochemistry in the human kidney [21] cornea [22], endothelial cell [22], synovium [23] and endometrium.[24]. Since the glucocorticoid-antagonising activity of MIF [19] implies it is a major enhancer of inflammation, and circulating inflammatory mediators are increased in malaria [4,5], we have investigated, through immunohistochemistry, its distribution in fatal human malaria and sepsis. The cellular location of systemic MIF generation has not been explored in either of these conditions, or any other cause of acute systemic inflammatory disease.

TNF and other pro-inflammatory cytokines have the capacity to generate the inducible form of nitric oxide synthase (iNOS), and thus generate a continuous, potentially large, supply of nitric oxide (NO) in tissues that normally experience only low, tightly controlled, levels of this ubiquitous cellular messenger. The potential roles for NO in malaria and other parasitic diseases are many, and have effects both protective and harmful to the host [25]. It has been argued, for instance, that excess NO, generated in critical locations by iNOS, could be functionally important in falciparum malaria, accounting for some of the reversible cerebral symptoms [26,27] and malarial tolerance [28], and there is evidence for its involvement in the immunosuppression [29] and weight loss [30] seen in experimental malaria. A parallel set of arguments has been developing for sepsis [31,32]. It is still uncertain whether overproduction of NO in acute illness helps or harms the host. Nevertheless, as the signalling roles of NO are defined better, the possibility that it may contribute to the pathogenesis of disease is being increasingly recognised.

In several studies, evidence of increased NO production in severe malaria has been sought by assaying plasma, urine or cerebrospinal fluid for nitrites and nitrates, the oxidation products of NO. These data were difficult to interpret, since the presence of nitrate in a body fluid gives no

Table 1: CM(A), no apparent brain pathology

Patient no.	Age (mo)	Sex	Parasitae-mia (Pf) &/or Organism cultured	Admitting coma score	Coma duration Before/After admission (h)	Brain vessel iNOS (n)	Brain vessel MIF (n)	Parasites in brain vessels	Chest Wall vessel iNOS	Chest Wall vessel MIF	Chest Wall muscle iNOS
4	20	M	18.6%	2	missing/6	0-1+ (6)	0 (3)	1+	0-1+	4+	2+
7	29	F	4+	0	1/1	0-1+ (6)	0 (3)	1+	0-1+	3+	4+
31	39	M	49%	1	7/22	0-1+ (9)	0 (3)	1+	0-1+	3+	0-1+
33	25	M	0.26%	0	2/15	0-1+ (8)	0 (3)	1+	2+	2+	0-1+
37	6	M	34% ¶ mal. trace	2	missing/14	0-1+ (9)	0 (3)	1+	3+	3+	3+
47	22	F	11%	1	6/44	0-1+ (13)	0 (3)	1+	0-1+	3+	0-1+
49	17	M	0.09%	1	2/34	0-1+ (12)	0 (3)	1+	2+	3+	4+
22	18	M	1.75%	0	1/3	2+ (8)	0 (3)	1+	3+	ND	2+
38	84	F	22%	0	5/35	2+ (9)	0 (3)	1+	0-1+	2+	0-1+
45	34	M	2.5%	0	8/28	2+ (13)	0 (3)	1+	0-1+	3+	0-1+
23	30	F	29%	0	5/7	3+ (9)	0 (3)	1+	3+	3+	0-1+
CM(B), sequestered parasites only											
5	14	M	3.5%	1	NA/18	0-1+ (3)	0 (3)	4+	4+	3+	4+
21	25	F	0.33%	2	2/12	0-1+ (3)	0 (3)	4+	4+	4+	4+
16	51	F	5.5%	1	22/6	0-1+ (3)	0 (3)	2+	2+	4+	2+
39	18	M	0.27%	2	6/18	0-1+ (9)	0 (3)	4+	2+	3+	2+
42	37	F	20%	0	missing	0-1+ (11)	0 (3)	4+	3+	4+	2+
25	44	F	0.02%	2	1/11	2+ (8)	0 (3)	4+	3+	3+	0-1+
1	27	F	31.1%	0	7/18	3+ (3)	0 (3)	2+	2+	ND	2+
CM(C), sequestered parasites plus local intravascular inflammation											
13	22	M	36.52%	0-1	15/1	0-1+ (3)	0 (3)	3+	2+	2+	2+
27	20	M	53.4% & Sal. typhi-m	0	missing/3	2+ (3)	0 (3)	3+	4+	3+	0-1+
28	61	F	23.6%	0	12/0	2+ (9)	0 (3)	2+	0-1+	2+	0-1+
29	43	M	33%	2	18/11	3+ (9)	0 (3)	3+	0-1+	3+	0-1+
32	18	F	47%	0	24/3	3+ (9)	0 (3)	3+	0-1+	4+	0-1+
34	70	M	2.4%	1	36/3	3+ (9)	0 (3)	4+	3+	3+	2+
35	114	M	0.21%	1	12/15	3+ (9)	0 (3)	3+	2+	2+	2+
36	21	F	0.88%	2	3/10	3+ (8)	0 (3)	2+	3+	3+	3+
48	48	M	0.03%	2	4/22	3+ (13)	0 (3)	3+	2+	2+	3+
6	17	F	5.8%	0	19/1	4+ (9)	0 (3)	3+	4+	3+	4+
9	16	M	0.03%	1	11/23	4+ (3)	0 (3)	3+	3+	3+	4+
11	29	F	29.5% & H. influ. B	1	28/30	4+ (3)	0 (3)	2+	4+	4+	4+
15	8	F	0.94%	2	8/15	4+ (3)	0 (3)	2+	3+	2+	3+
26	30	M	19%	1	1/31	4+ (9)	0 (3)	4+	4+	4+	missing
COMA (clinically judged not cerebral malaria)											
* 8	41	F	negative	3	3/9	3+ (3)	0 (3)	negative	0-1+	4+	0-1+
10	66	F	0.01%	0	5/4	0-1+ (3)	0 (3)	negative	2+	0-1+	2+
12	6	M	Sal. enteritidis	2	1/4	0-1+ (3)	0 (3)	negative	2+	4+	2+
14	9	F	E. coli	0	missing	0-1+ (3)	0 (3)	negative	4+	4+	4+
17	51	F	negative	0	2/9	0-1+ (3)	0 (3)	negative	0-1+	0-1+	0-1+
18	7	F	H. influenzae B	1	48/14	0-1+ (3)	0 (3)	negative	3+	3+	4+
20	96	F	Tuberculosis	0	missing	0-1+ (3)	0 (3)	negative	4+	2+	0-1+
24	48	F	Strep. pneu-moniae	3	missing	0-1+ (3)	0 (3)	negative	2+	4+	2+
40	26	F	0.02%	1	24/63	2+ (9)	0 (3)	negative	0-1+	4+	2+
44	21	F	negligible	1	7/3	0-1+ (13)	0 (3)	negative	2+	2+	2+
MALAWIAN CONTROLS (without terminal coma)											

Table 1: CM(A), no apparent brain pathology *(Continued)*

41	29	F	Sal. typhimurium	5	NA/NA	0-1+ (13)	0 (3)	negative	0-1+	0-1+	0-1+
* 50	144	F	Strep. pneumoniae	5	NA/NA	0-1+ (13)	0 (3)	negative	0-1+	3+	0-1+
AUSTRALIAN CONTROLS											
n = 5		F/M	NA	NA	NA/NA	NA	NA	negative	0-1+	0-1+	0-1+
n = 7		F/M	NA	5	NA/NA	0-1+ (3)	0 (3 ea)	negative	NA	NA	NA

* Serologically positive for HIV (n) Number of brains areas examined ND = not done NA = not applicable

indication of where its parent NO was generated, an important issue when investigating the functional relevance of a short-lived molecule that can be formed in many tissues, and acts very close to its cell of origin. We have approached this problem by employing immunohistochemical staining for iNOS on the same range of formalin-fixed, wax-embedded tissues collected at autopsy from cases of falciparum malaria and other acute systemic diseases, including sepsis, as we stained for MIF. Several patterns were observed in the brain of children. The outcome is consistent with the view that clinically diagnosed cerebral malaria in African children is best viewed as a collection of overlapping syndromes, with several mechanisms potentially combining to cause coma and death.

Methods

Case Tissues

All 44 subjects (26 females) were children who had been admitted to the Malaria Project wards in the Department of Paediatrics at the Queen Elizabeth Central Hospital in Blantyre, Malawi. Ages ranged from 6 months to 12 years (Table 1). Subjects had been comprehensively evaluated during life, through clinical history and examination and by laboratory tests. They were treated as clinically indicated with intravenous fluids, glucose, antimalarial drugs, antibiotics, anticonvulsant and antipyretic drugs and blood transfusion. Thirty-two patients were clinically diagnosed as cerebral malaria because they (a) died with coma (Blantyre coma score 2/5 or less, lasting for at least 2 hours before death), showing no improvement with 50% dextrose or within the first 2 hours of treatment for seizures, (b) had P. falciparum asexual parasitaemia, and (c) had no evidence of pyogenic meningitis in the initial CSF sample and no alternative explanation for coma. Another 10 patients were comatose on admission and before death, had very low or no peripheral malaria parasitaemia, and were considered on clinical grounds to be suffering from a disease other than malaria. The case fatality rate of children admitted to the ward was 16%. Family members were asked for their permission for the autopsy to be conducted: this request was made by one of the Malawian physicians who had been involved in the management of the patient. Autopsies were performed as quickly after death as possible, with post-mortem intervals ranging from 2 to 14.5 hr. Tissue samples were placed into 10% neutral buffered formalin for fixation. The project was approved by ethics and research boards of Malawi, the Liverpool School of Tropical Medicine and the Australian National University.

Control tissues

Various adult controls from Australian sources were studied. These comprised sections of 5 blocks of tissue, trimmed from the periphery of tumour excisions from adult chest wall, and containing skeletal muscle, adipose tissue and small blood vessels. Mid-brain sections from 4 adults who had died of coronary artery disease, and from another 3 who died of non-infectious, non-cerebral conditions (Brain Bank for Sydney Central Area Health Science Approval X980216), were also stained. In addition, tissues from 2 Malawian children who were enrolled in this study served as local controls, in the sense that no coma was present at any stage. One of these children grew *Salmonella typhimurium* from cerebrospinal fluid and blood, and died after an acute gastrointestinal haemorrhage, and the other grew scanty *Streptococcus pneumoniae* from the cerebrospinal fluid.

Immunohistochemistry

Formalin-fixed tissue samples were embedded in paraffin, sectioned (3–6 microns) on to polylysine-coated slides, and stained with haematoxylin and eosin (H&E) for routine morphology. A goat polyclonal antibody against MIF, raised against an *Escherichia. coli*-expressed recombinant human MIF, was purchased from R & D Systems (Cat. No. AF289-PB). Anti-iNOS antibodies were purchased from Transduction Laboratories (N32020, a monoclonal directed against bases 772–787 of mouse iNOS), Santa Cruz (Cat. SC-650, a polyclonal against bases 1126–1144) and StressGen (Cat. KAP-NO001, a polyclonal against bases 1131–1144). Anti-myoglobin was used as irrelevant pri-

mary control antibody, and in other controls the primary antibody was omitted. The procedure was modified from Cobbs and co-workers [33]. Antigen retrieval was performed by immersion in 0.01 M citrate buffer, pH 6.0, in a waterbath at 95°C for 20 min and then cooling to room temperature while still immersed in buffer. After quenching with 3% H_2O_2 and treating with primary antibody (dilution of the stock solution within the range 1:100 to 1:2,500) at room temperature for 1–4 hr, biotin-conjugated secondary antibody and streptavidin-conjugated horse radish peroxidase from an LSAB+ kit (DAKO) were applied to sections for 20 min at room temperature to amplify the antigen signal for subsequent 3,3'-diaminobenzidine (DAB) staining. All three anti-iNOS antibodies detected the same antigen distribution, although the StressGen polyclonal required a higher concentration of protein to stain to the same degree as the other two antibodies. Across a range of tissues, optimal dilutions of the Transduction monoclonal anti-iNOS and the R&D polyclonal anti-MIF were determined by using 1:100 to 1:10,000 as primary antibodies on control and case tissues. Known positive controls were stained in each run, and runs were often duplicated on different days to confirm repeatability. Sections were counterstained with haematoxylin, and outcomes with a dilution of 1:2000 (MIF) or 1:1000 (iNOS) are shown to illustrate the observed changes.

Statistical analysis

The differences between the relative densities of iNOS staining, combining 0/1+ and 2+ as low, and 3+ and 4+ as high, were compared using Fischer's exact test, on Instat software. Probability values less than 0.05 were regarded as significant. For the frequency counts in a 4 × 4 table classified by MIF score and iNOS score, Cohen's kappa (pages 395–397) of Bishop [34] was used to test whether the frequencies on the diagonal were greater than would be expected by chance. The table was also tested for symmetry using G^2, the goodness of fit statistic given on p. 283 (reference equation 8-2-1) of Bishop [34].

Histological examination of brain

Depending on availability, sections from 3 to 13 blocks (see Table 1) of a range of brain regions were examined. Samples were from frontal lobe, parietal lobe, occipital calcarine fissure, hippocampus, basal ganglia, thalamus, mid-brain, pons and cerebellum, with mid-brain and pons almost always included. All were stained with hematoxylin and eosin (H&E). The overall density of parasites was described as 1 when only widely scattered individual parasites were present, as 2 when low numbers of parasites were seen, but more frequently, as 3 when most vessels contained parasites, and as 4 when exceptional numbers were present. Scores from cases clinically diagnosed as cerebral malaria were confirmed by examining more blocks, up to 13, with a median of 9, for each case. The additional blocks came from the medulla, cerebellar tonsils, cerebellar dentate, spinal cord and choroid plexus. Histopathological changes in tissue were documented including presence of micro-haemorrhages, intravascular inflammatory infiltrates and fibrin deposition.

Quantification of staining

The degree of staining in tissues was approximately quantified into categories 0-1+, 2+, 3+ and 4+ by evaluating stain density alone (see Figures), not the proportion of positively stained cells in a field. Observers (IAC and MMA) blinded to the clinical diagnoses examined the sections initially. To further establish objectivity, and to meet the challenge of a number of collaborators in different parts of the world being able to access and compare the same material from so many sets of tissues, the brain and other sections were comprehensively photographed. An additional observer (CGH) independently monitored that the tissue sections were fairly represented by the range of photographs taken of each. A large number (>1500) of digital images, graded and in their original groupings, were distributed on CD-ROM to all members of the investigating team for independent examination.

Cases were allocated into the categories used in the Results section purely on clinical and histological grounds. The 32 patients with *clinically diagnosed cerebral malaria* were grouped according to cerebral light microscopic changes into three categories:

• Category A (n = 11) had no or scanty (score 1) intracerebral parasites and no tissue pathology;

• Category B (n = 7) had sequestered parasites in brain vessels (score 2, 3 or 4) without tissue pathology;

• Category C (n = 14) had both sequestered parasites (score 2, 3 or 4) and inflammatory pathology in the form of intravascular inflammatory infiltrates, fibrin deposition and/or microhaemorrhages.

Several groups of patients clinically diagnosed as *non-malarial illnesses* were used as comparison cases:

• Coma of other causes (n = 10). These included patients presenting in coma but not clinically diagnosed as cerebral malaria.

• Non-comatose illness (n = 2).

The individual diagnoses in these patients with non-malarial illnesses are listed in the Table 1.

Results

MIF staining

Tissues exposed to the outside environment

Immunohistochemical evidence of MIF was strongly present (3+ – 4+) in control tissue from a range of locations exposed to the outside environment. This included cells such as keratinocytes of the epidermis and cells forming sebaceous glands and hair follicle sheaths, bronchial epithelial cells, and alveolar macrophages. The pattern was the same in these locations for iNOS, and serve as positive staining controls.

Brain

MIF staining proved to be identical in all brains, and thus results for this mediator, in contrast to those for iNOS, are described together. It was amply detectable in inflamed meninges (not shown), but not in more than occasional trace amounts in blood vessel walls within the brain parenchyma in any brain, including those from controls. In contrast, it was present widely and uniformly in astrocytes (cytoplasm, but not in nuclei), and ependymal cells (cytoplasm, and often nuclei), to a degree that did not vary significantly between brains. This was true for the total of the 53 human brains of all origins sectioned and stained for both MIF and iNOS. These comprised 7 from adults who had died in Australia of non-infectious, non-cerebral conditions (represented by Fig. 1A), the 2 childhood controls from Malawi (Fig. 1C), 32 cases of cerebral malaria (Fig. 1E), the 10 with non-malarial fever and coma, including the 5 with culture-positive bacterial septic meningitis (*E. coli* illustrated in Fig. 1G). We also examined brains from 17 other children dying of various causes in Malawi and again found no evidence of MIF in cerebral vascular walls (data not shown).

Blood vessels in chest wall

MIF staining in chest wall blood vessels (Table 1) was negligible in sections from Australian control adults (Fig. 1B) and the non-febrile one of the pair of non-comatose Malawian control children (Fig. 1D), and was strong (3+ or 4+ on a 0–4 scale) in chest wall vessels from 24 of 31 cerebral malaria patients (Fig. 1F), and 4 of 5 cases of culture-positive sepsis (*S. pneumoniae* illustrated in Fig. 1H). Both cytoplasm and nuclei stained in endothelial cells and smooth muscle. By Kruskal-Wallis non-parametric ANOVA there was no difference in MIF staining in chest wall blood vessels in the three categories of cerebral malaria.

Skeletal muscle in chest wall

MIF was not detectable in normal skeletal muscle from 2 control Australian adult samples (Fig. 2A), nor samples from 2 control Malawian children (Fig. 2B), but was present in the nuclei of skeletal muscle from almost all of the malaria and culture-positive sepsis cases (12 of 14) that showed high iNOS (3+ or 4+ on a 0–4 scale) in the surrounding myofibrils. Staining was rarely present in cytoplasm. A representative malaria example is shown in Fig. 2C, and sepsis (Haemophilus influenzae) in Fig. 2D. The corresponding iNOS immunostaining is shown in Figs 4H,4F,4E, and 5D respectively. MIF staining was largely confined to nuclei, but even with the strongest staining only some two-thirds of nuclei were positive in any section.

iNOS staining

Coma with falciparum malaria

Brain blood vessel walls

The 32 cases clinically diagnosed as cerebral malaria were examined after staining with H&E and anti-iNOS antibody. Differences in degree of staining between the various parts of the brain in any single individual were slight to negligible. All iNOS staining was in cytoplasm only. It was detectable in some glial cells and some motor neurons across the brain, but the strongest staining, and differences, were in the walls of small blood vessels, both smooth muscle and endothelial cells. iNOS staining was analysed according to histological categories A, B, and C (see Methods). Category A (11 cases with no discernible histological changes and only the occasional rare malarial pigment or parasites within vessels). Seven of these 11 scored nil to trace for vascular wall iNOS (0-1+, see Table 1, and Fig. 3A). Another 3 scored 2+, and one scored 3+. Category B (seven cases with no histological changes but with sequestered parasitised erythrocytes). Sequestration in this group usually consisted of young parasites (trophozoites), and was commonly intense (Fig. 3B). Five of these 7 scored 0-1+ for vascular wall iNOS, one 2+, and one 3+ (Table 1). Category C (14 cases showing micro-haemorrhages, intravascular mononuclear cell accumulations, and also sequestered parasites). iNOS staining was stronger in this group, with one case scoring 0-1+, 2 scoring 2+, 6 scoring 3+, and 5 scoring 4+ (Table 1, and Fig. 3C). iNOS staining was conspicuously concentrated in areas of microhaemorrhage. iNOS staining in vessel walls in Category C was significantly more likely to be scored as 3+ or 4+ than in Categories A and B combined ($p = 0.0005$), and when compared to A ($p = 0.002$) or B ($p = 0.015$) separately. Immunostaining in Categories A and B were not significantly different ($p = 1.000$).

Where mononuclear cellular accumulations were present they stained in parallel with the vessel walls (Fig. 3D). The strongest (4+) cerebral vascular iNOS staining was almost exclusively restricted to vessels that showed evidence of inflammatory activation including deposition of intravascular fibrin. In samples obtained from the Sydney Brain Bank none showed more than trace amounts of iNOS staining (example shown in Fig. 3E). The low level of iNOS staining of the frontal lobe of the Malawi control with the fatal gut haemorrhage is illustrated in Fig. 3F.

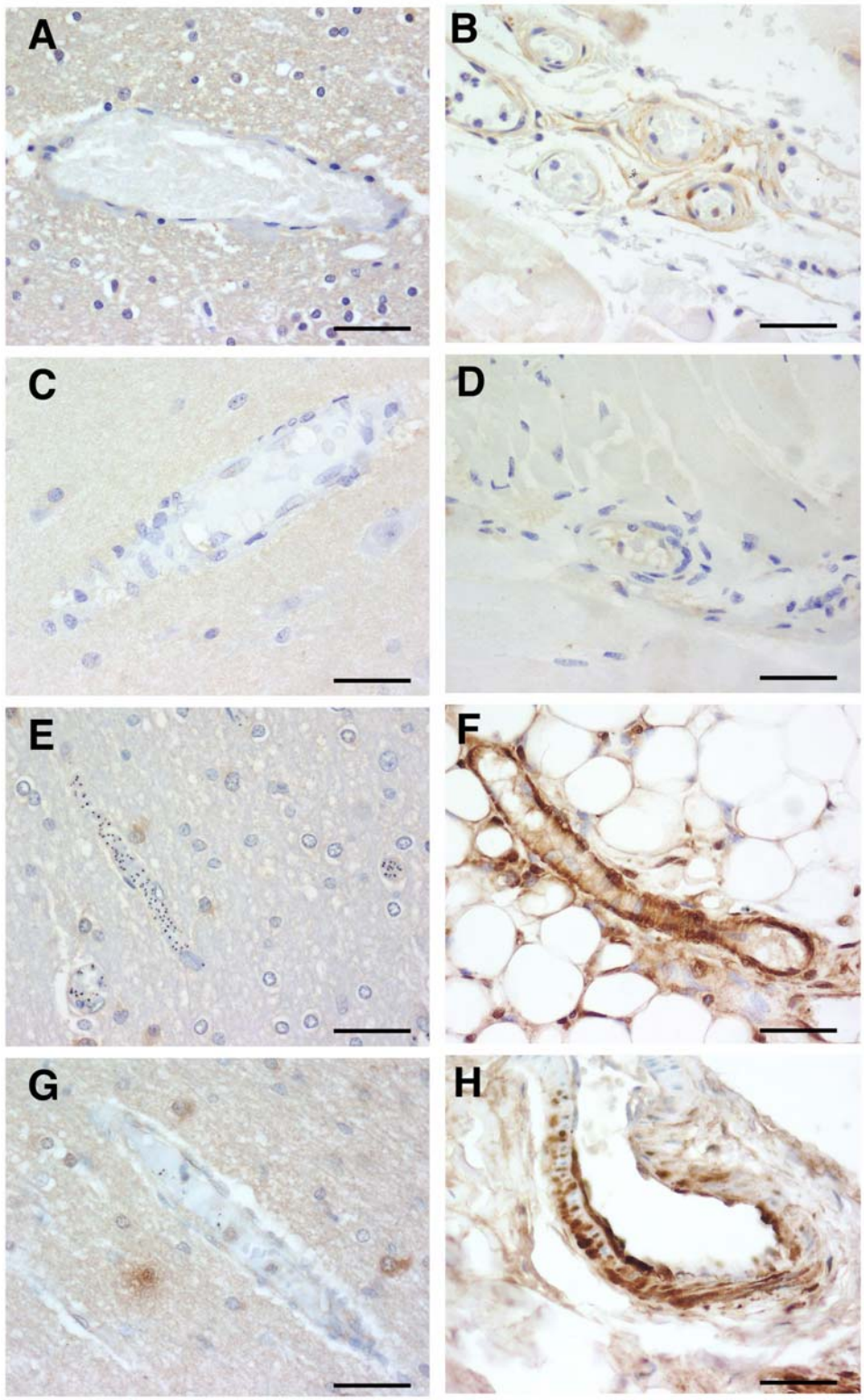

Figure 1
MIF staining of blood vessels of control, cerebral malaria and sepsis brains and chest wall. A. Brain and **B.** chest wall of Caucasian adult controls, **C** brain and **D** chest wall of African paediatric control, **E** Brain and **F** chest wall of cerebral malaria case, and **G** brain and **H** chest wall of sepsis case (*Streptococcus pneumoniae*). Scale bar, 100μm.

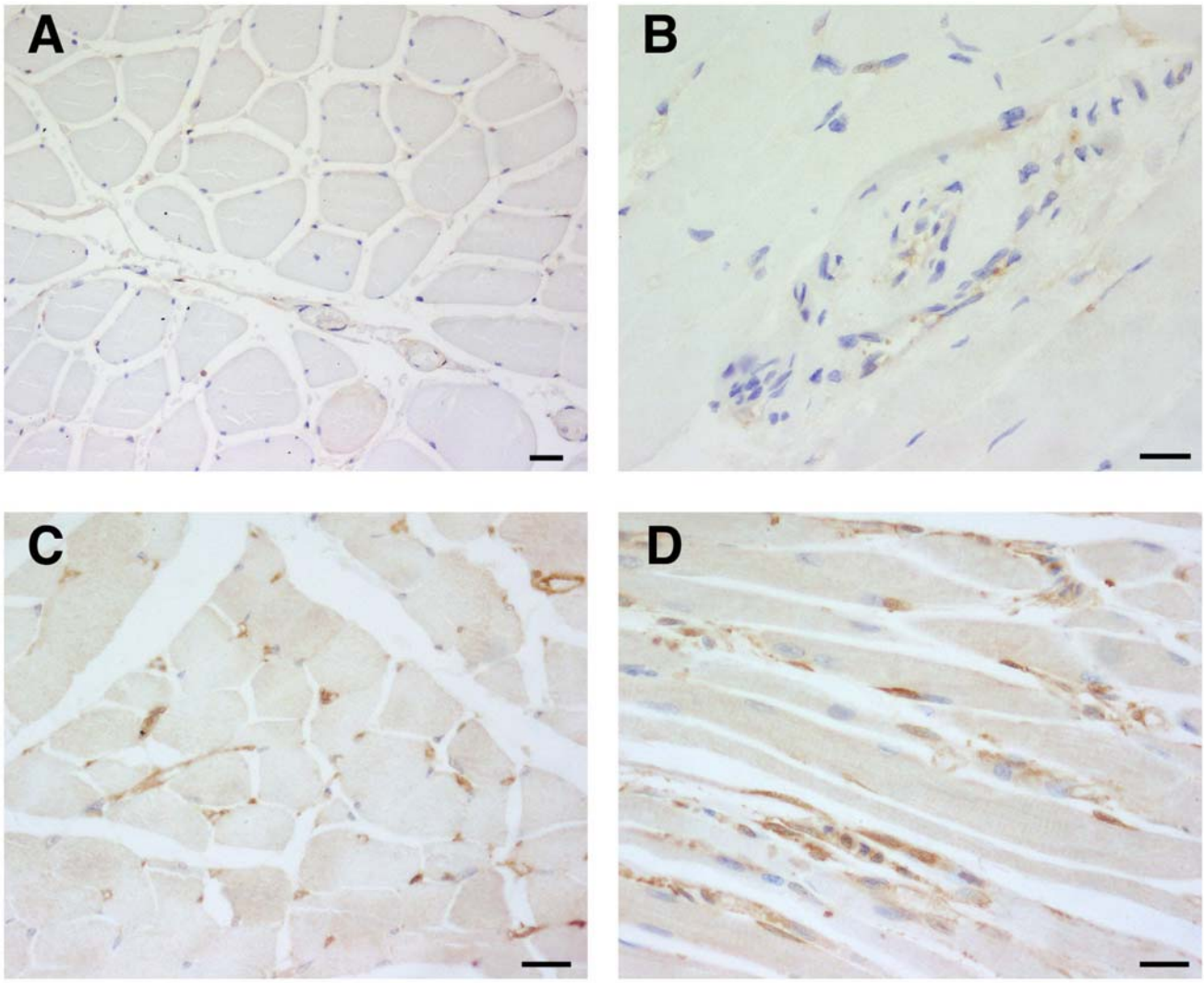

Figure 2
MIF staining in chest wall skeletal muscle. A. Caucasian adult control, **B.** African paediatric control, **C.** cerebral malaria case, and **D.** sepsis case (*Haemophilus influenzae*). Scale bar, 100 μm.

Blood vessels and skeletal muscle in chest wall

In contrast to MIF, iNOS was even across the cytoplasm, and not present in nuclei of any cells. As shown in Table 1, most of the cases of clinically diagnosed cerebral malaria in Category A showed low levels of iNOS in chest wall small blood vessels and skeletal muscle myofibrils (Fig. 4A). The remainder of category A and the 7 cases in Category B had high levels of iNOS staining in chest wall small blood vessels (Fig. 4B) and skeletal muscle (Fig. 4C), but cases in which this was observed did not include the few in these groups with significant brain vessel iNOS. Most of the 14 cases in Category C had high levels of iNOS in vessel walls (Fig. 4D) and skeletal muscle (Fig. 3E) of the chest wall, although a few stained weakly (Table 1). There was no apparent association between the presence or intensity of iNOS staining in muscle and that in brain in any of the groups. Where iNOS was appreciable in cerebral vessels (eg Fig. 3C) it was never as strong as that seen in vessels in chest walls (eg Fig. 4D) and elsewhere (not shown). Parasites (whether sequestered or not) or pigment were rare to absent, even in those chest wall sections that stained heaviest for iNOS. Both Malawi coma controls (Figs 4F and 4G), and the Canberra controls (example in Fig. 4H) had no detectable iNOS in vessels or skeletal muscle of chest wall sections.

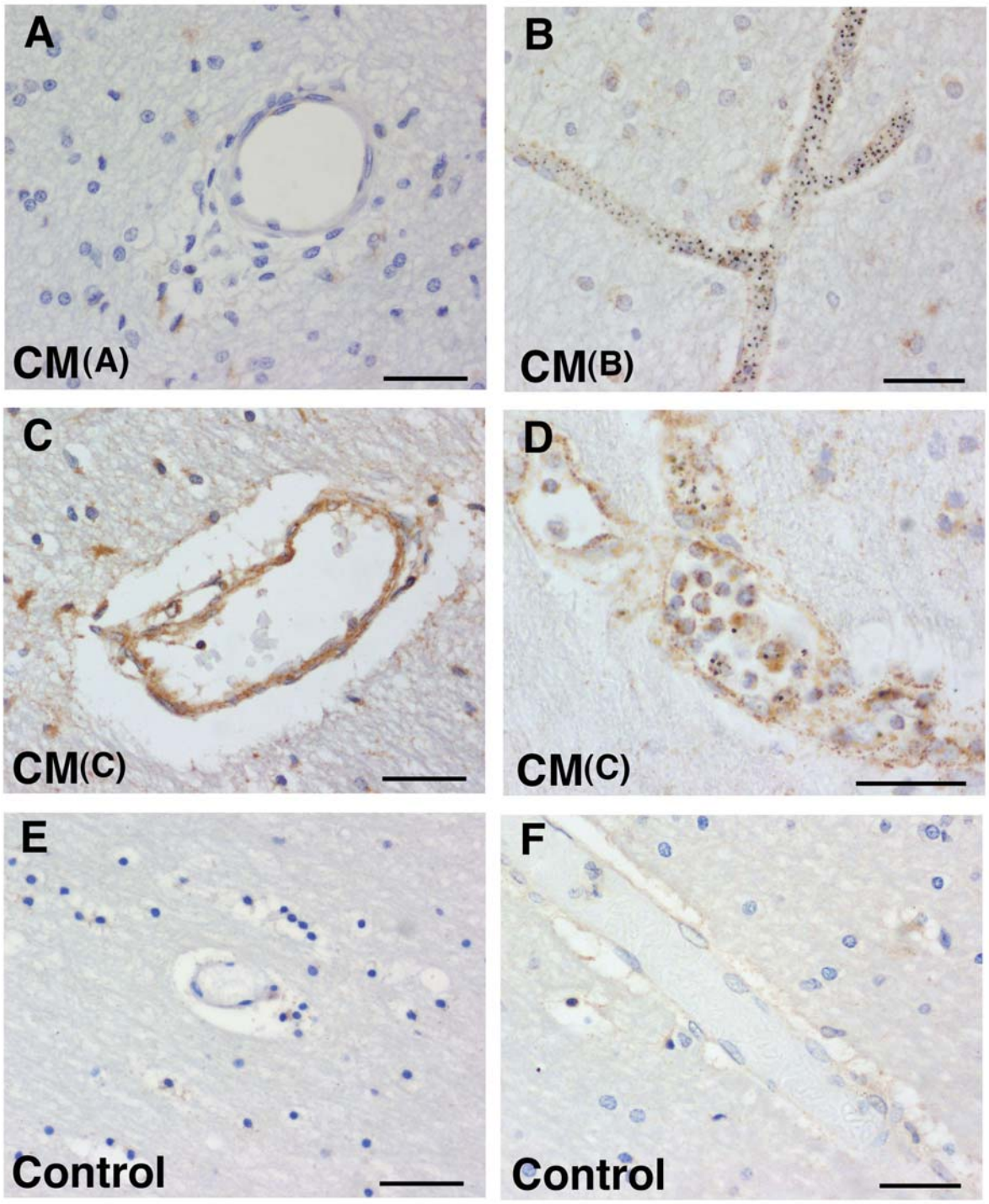

Figure 3
iNOS staining in cerebral malaria brains and in control brains. A: Category A cerebral malaria (no discernible histological changes except the occasional rare malarial pigment within vessels) frontal lobe showing negligible vascular wall iNOS. **B**: Category B (no discernible changes except significant sequestration of parasitised erythrocytes) occipital region showing negligible vascular wall iNOS. **C**: Category C (microhemorrhages, significant mononuclear cell accumulations, and also sequestered parasites) frontal lobe showing notably more vascular wall iNOS than seen in categories A or B. **D**: Mononuclear cells in Category C frontal lobe staining in parallel with the vessel walls for iNOS. **E**: iNOS immunostaining in mid-brain of Caucasian control brain. **F**: iNOS immunostaining in pons region of a childhood Malawi control brain. Scale bar, 100 μm.

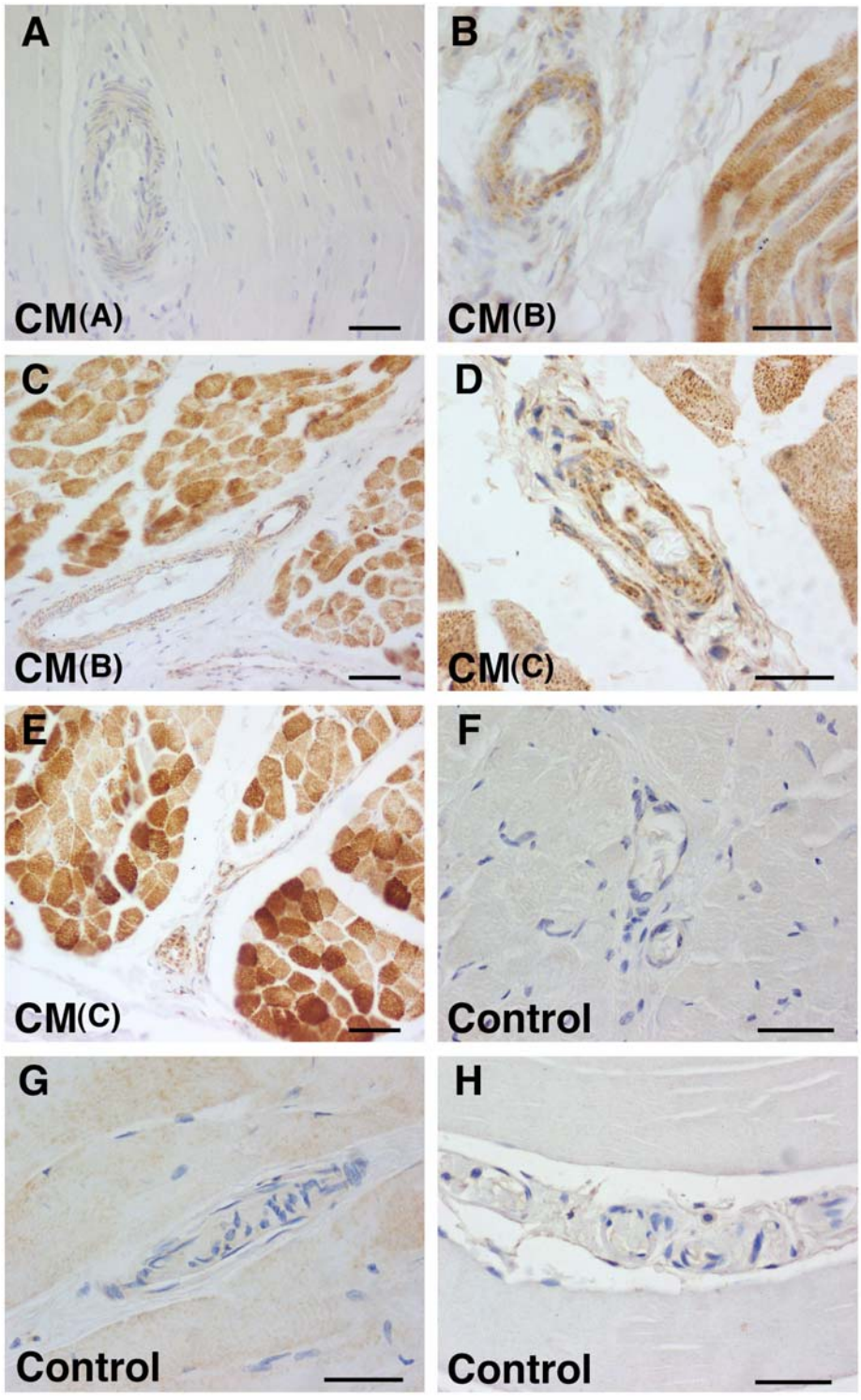

Figure 4
iNOS staining in chest wall in cerebral malaria and in controls. A: Category A cerebral malaria chest wall and skeletal muscle both showing negligible vascular wall iNOS. **B**: Category B cerebral malaria chest wall showing high vascular wall iNOS, and, in **C**, skeletal muscle. **D**: Category C cerebral malaria chest wall showing high vascular wall iNOS and, in **E**, skeletal muscle. **F and G**: Negligible iNOS in chest wall blood vessel walls and skeletal muscle in two Malawi childhood controls, and in **H**, same in Caucasian control. Scale bar, 100 μm.

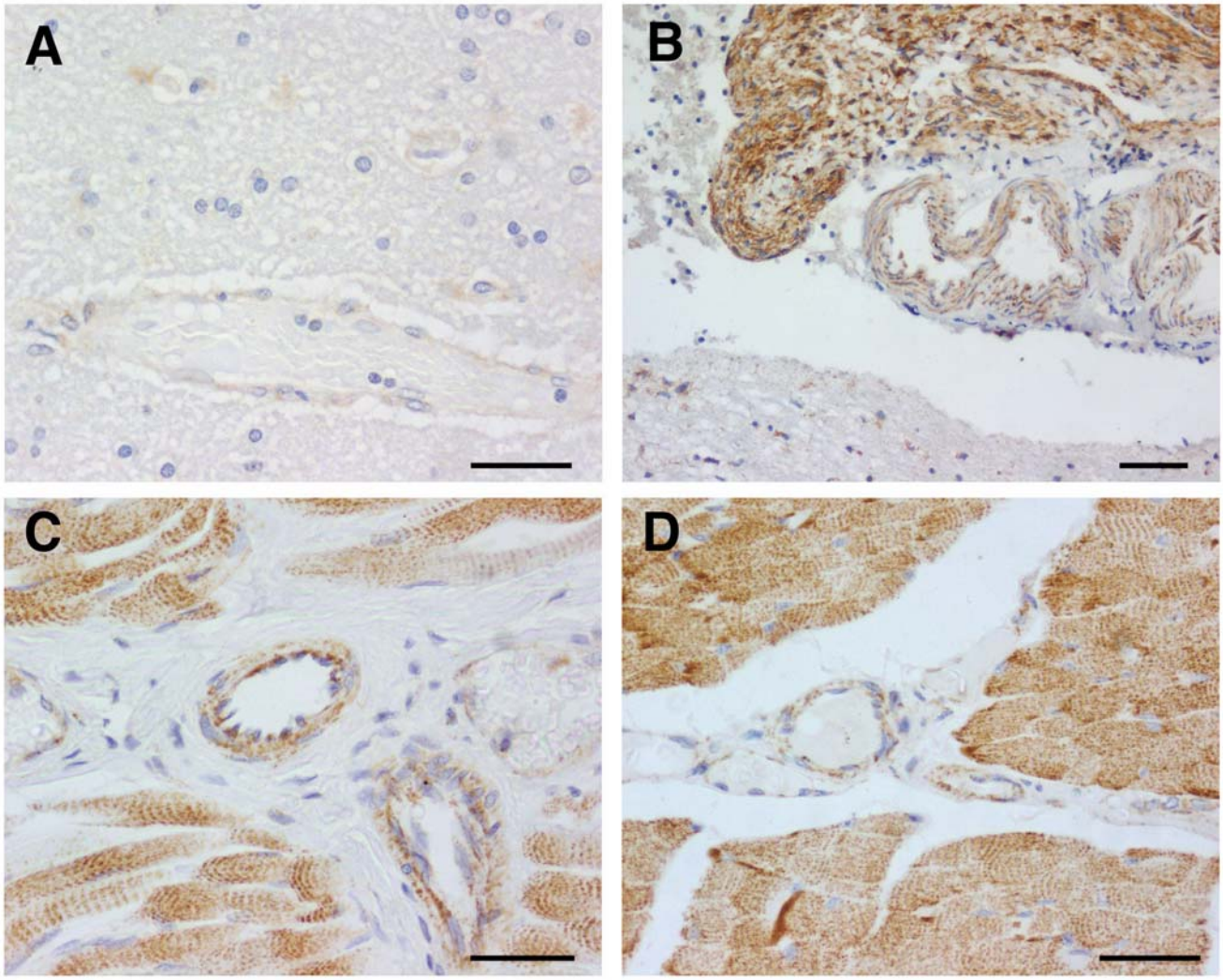

Figure 5
iNOS staining in brain and chest wall from culture-defined cases of septis. Pattern shown was the same in all cases illustrated. **A**: Frontal lobe of brain from *Streptococcus pneumoniae* infection, showing negligible vascular wall iNOS. **B**: Meninges from pons region from *Salmonella enteriditis* infection, with strong iNOS staining. **C**: Chest wall vessel and muscle from *E coli* infection showing strong staining for iNOS. **D**: Chest wall vessel and muscle from *Haemophilus influenzae* infection showing strong staining for iNOS. Scale bar, 100 μm.

Non-malaria coma

iNOS staining in brain blood vessels

A further 10 comatose children had very few (3) or no detectable (7) malaria parasites in thick smears and had a clinical diagnosis other than malaria to account for their disease. Two of these 10 patients had bacterial meningitis (one each culture positive for *H. influenzae* and *S. pneumoniae*), two had a positive blood culture (one *S. enteritidis*, one *E. coli*) and one, with a lymphocytic infiltrate in the cerebrospinal fluid, was diagnosed histologically, including the presence of acid-fast organisms, as tuberculous meningitis. The causes of death of the other 5 were judged, on clinical grounds, to be organophosphate poisoning (1), salicylate overdose (1) (both with low grade parasitaemia), hyponatraemia in a child with severe brain damage from previous bacterial meningitis (with transient low-grade parasitaemia), and hypoglycaemia of unknown cause (2) (both aparasitaemic). As available, sections from 3 to 13 areas from the brain regions listed earlier, (always including cerebrum, mid-brain or pons),

were stained for iNOS. Most cerebral vascular walls, including those from all the bacterially defined cases of septic meningitis, scored 0-1+ for iNOS (Fig. 5A; Table 1). iNOS staining of these vessels was significantly less likely to score 3+ or 4+ than in cerebral vessels in Category C cerebral malaria ($p = 0.005$), but was not different from that seen in Categories A or B (each $p = 1.0$), or the cerebral malaria group as a whole ($p = 0.066$). Meningeal vessel walls from the septic meningitis cases stained conspicuously more strongly for iNOS than did vessel walls within the brain (Fig. 5B).

iNOS staining in blood vessels and skeletal muscle in chest wall

Staining for iNOS in blood vessels ($p = 0.730$) and skeletal muscle ($p = 0.282$) was not significantly different, in degree, from that seen in these locations in the 32 cerebral malaria cases (Table 1). In all groups except the controls there were patients with intense muscle iNOS staining who had little or no iNOS evident in the cerebral vasculature walls. Examples of chest wall microvessel and myofibril iNOS staining in patients with non-malarial coma and bacteraemia are shown in Figs 5C and 5D.

When MIF and iNOS expression in chest blood vessel walls were compared, those in 16 samples stained strongly (3+ or 4+) for both MIF and iNOS, and 12 showed strong (3+ or 4+) MIF plus low (0-1+ or 2+) staining iNOS, 11 showed low (0-1+ or 2+) staining for both MIF and iNOS, and only 4 had low MIF and high iNOS. Thus, of the 20 with high iNOS, only four had low MIF, whereas 11 of the 22 with low iNOS had low MIF. The value of Cohen's kappa as a measure of agreement in the MIF and iNOS classifications was 0.25 with a standard error of 0.085. Using a Normal approximation, this is significantly different from zero at the 0.1% level. The G^2 for lack of symmetry was 18.08, which is significant at the 1% level in relation to the chi-squared distribution with six degrees of freedom, showing a strong tendency for the MIF score to be greater than the iNOS score.

Discussion

Attempts to explain the pathogenesis of severe falciparum malaria have, in recent years, been dominated by two approaches now generally considered complementary: poor tissue oxygen supply due to the obstructive effects of microvascular sequestration [35] and the local and systemic effects of the excessive release of the cytokines that regulate cell-mediated immunity [36]. In this study we investigated these interactions further, through immunohistochemical staining for MIF and iNOS in brain and other tissues from paediatric autopsies in Malawi. iNOS protein expression in the brain in fatal malaria has been reported in one previous study, of adults in south-east Asia [37]. Sampling the cerebrum only, these authors reported immunohistochemical staining for iNOS in a range of cell types, most strongly in endothelial cells, in 13 cases of adult cerebral malaria. Bacterial septicemia cases were not studied. Judging from the universal presence of microhemorrhages reported in all brains examined, these adult brains correspond closest to the Category C subgroup of our paediatric cerebral malaria cases.

Tissues from systemic human disease have not previously been examined for MIF. The normal presence of MIF protein in bronchial epithelial cells, epidermis and subcutaneous glandular structures, as we have found in these human tissues, has previously been reported, by this same technique, in the rat [38]. It has not previously been reported in skeletal muscle. A function of MIF possibly relevant to the pathology of severe malaria and sepsis, and consistent with its presence in skeletal muscle, is its ability to increase synthesis of fructose 2,6-bisphosphate (F2,6BP), a positive allosteric regulator of glycolysis [39]. A neutralising anti-MIF mAb prevents the hypoglycaemia and increased muscle F2,6BP levels caused in mice by TNF [39]. In African children with severe malaria, hypoglycaemia is associated with very high circulating concentrations of TNF [4]. These observations are consistent with a role for MIF in the pathogenesis of malarial hypoglycaemia through the excessive enhancement of glycolysis in skeletal muscle. The presence of MIF in skeletal muscle would, by inhibiting the suppressive effects of glucocorticoids on inflammatory cytokine production [19], allow the observed high iNOS induction (Fig. 4C).

MIF protein has previously been reported in ependymal cells, astrocytes, and subpopulations of neurons in the bovine brain [40], but no comment was made on its presence or absence in cerebral blood vessel walls. These authors also noted its intranuclear location in several cell types, as we have in skeletal muscle, ependymal cells, and non-cerebral endothelial cells in malaria and sepsis. Nuclear staining of skeletal muscle for MIF was commonly associated with high iNOS in the cytoplasm of these cells. The roles of MIF in nuclei have not yet been determined, the only functional association of its expression in this organelle so far reported being in pulmonary adenocarcinoma cells, where is it is associated with a better clinical outcome [41]. As noted, MIF had an asymmetrical association with iNOS in chest vessel walls. This is consistent with MIF appearing before iNOS, as this literature predicts.

MIF protein has been reported in the endothelial cells of human umbilical vein [22], but to our knowledge neither the neuropil nor blood vessels of human brains have previously, except for our recent conference proceedings report [42], been examined for its presence. The absence of MIF staining in the walls of the cerebral vasculature during malaria and bacterial sepsis (Figs 3F &3H), despite evi-

dence for its strong presence in the rest of the vascular tree, is remarkable. It was also absent in all other brains examined, including an additional 17 from other children dying of various causes in Malawi (data not shown). This absence could help explain previous observations of others. For example, the brain could thereby be innately protected from the excesses of systemic inflammation, in the same way as neutralising anti-MIF antibody protects the whole organism in experimental sepsis [16]. Absence of local vascular wall MIF would mean that glucocorticoids would be locally unopposed, leading to much less local transcription of inflammatory responses in the brain than elsewhere in the body, as reported in DNA microarray studies of the caecal ligation and puncture model of sepsis [43], another example of systemic inflammation. This is consistent with cerebral vasculature iNOS never, in our experience, achieving densities seen in peripheral vessels (Figs 3C vs 4D).

We suggest two possible reasons why low MIF, and thus, through unrestricted glucocorticoid activity, relatively low iNOS, should be biologically advantageous in the cerebral vasculature. First, since normal brain function requires a subtly triggered network of calcium-controlled generation of NO from constitutive NOS, relatively uncontrolled release of NO through iNOS could override this, with loss of function. Second, we propose that dangerous increases in brain perfusion pressure are minimised through MIF being absent from the cerebral vasculature. The brain is protected from capillary rupture because resistance of larger cerebral arteries is remarkably high, keeping the pressure in intracranial arterioles lower than in similar vessels elsewhere [44]. Endotoxin-induced iNOS diminishes the normal constrictor responses that maintain the normally low cerebral perfusion pressure [45]. Were cerebral vascular iNOS to increase as much during a severe systemic infection as elsewhere in the body, the resistance of cerebral arterioles would decrease accordingly, and the hydrostatic pressure of cerebral capillaries would increase, exposing these vessels to pressure they normally do not experience and cannot withstand, and microhemorrhages would ensue. This could account for the co-localisation of vascular wall iNOS and microhemorrhages in Category C brains. An inability to induce MIF in the cerebral vasculature, leading in turn to less induction of iNOS, may thus have a survival advantage in preventing excessive increases in cerebral perfusion pressure, and the risk of intracranial haemorrhage during systemic infections. This may also explain the observation that endotoxin infusion into human volunteers reduces systemic vascular resistance, but leaves cerebral vascular resistance unaltered [46].

Since MIF opposes the function of glucocorticoids, and is thus able to upregulate iNOS [47,48], it was of interest to document the change in iNOS as well as MIF in fatal human malaria and sepsis. Except for its routine presence in human airway epithelium [49], iNOS is rarely detectable by immunological methods in healthy tissues. For example iNOS has been reported to be absent by immunohistochemistry in normal human blood vessel wall [50] and by reverse transcriptase-driven *in situ* polymerase chain reaction none could be detected in human brain [51,52], and very little in cerebellum or skeletal muscle [53]. Most of these authors have used the main commercial antibody source, Transduction Laboratories, that we have employed. Our argument that we are indeed detecting authentic iNOS is strengthened by detecting the same antigen distribution with three unrelated anti-iNOS antibodies described in Methods.

A third (11/32) of the Malawian children diagnosed as cerebral malaria on the basis of established clinical criteria, including peripheral *P falciparum* parasitaemia, had neither histological changes, nor more than scant sequestered parasites, in the brain sections we stained for iNOS (Category A in the Table 1). The relative absence of parasites from these tissue sections, even though they were prominent on peripheral blood smears on admission, cannot be explained by their clearance from the brain by antimalarial treatment, since very similar average times (mean +/- SEM 21.2 +/- 4.6 hr for Category A, and 27.0 +/- 6.0 hr for Category B) had lapsed from admission, when blood smears were taken and treatment began, to time of death in both these cases and those in Category B, in which parasites were common in brain sections. In the majority of cases in Category A, iNOS was scanty or undetectable in brain vessels (Fig. 3A). In each patient group there was evident diversity in the quantity of iNOS detectable in both brain and muscle (Table 1). As discussed, MIF differences could contribute to this. Since the local release of infected red cell contents during post-schizogony rupture of erythrocytes is accepted to trigger the inflammatory cascade in malaria, the intensity of staining for iNOS would vary throughout the 48 hr schizogony cycle. Since the tissue sections were collected at variable times during the cycle, some variation in iNOS staining intensity between patients with otherwise similar clinical syndromes is therefore inevitable.

About half of the cerebral malaria cases (Category C, 14/32) showed significant microhemorrhages, intravascular accumulations of mononuclear cells, and at times fibrin. Mononuclear cell accumulations (Fig. 2H) have been described previously in cerebral malaria brains from Indian adults [54] and Thai adults [55,56], but not, so far as we are aware, African children. These features were negligible in the brain sections examined from the other cerebral malaria categories. In contrast to the low iNOS seen in cerebral vessel walls in Categories A and B, all except three of the 14 brains in Category C scored 3+ or 4+. It is possible

that mononuclear cells, fibrin and parasitised red cells could all have contributed to local ischaemia by reducing blood flow through affected vessels, and the consequent ischaemia could, in turn, have contributed to iNOS increase [57]. However, in view of the negligible iNOS induction in Category B brains, despite intense sequestration, it seems more likely that the iNOS induction in vascular walls uniquely observed in Category C brains has resulted from the strong local stimulus provided by recent rupture of schizont-infected red cells. Nevertheless, histological staining cannot establish a chain of cause and effect; this awaits other types of experimentation.

High iNOS concentrations in cerebral vessel walls in Category C was almost invariably associated with equally strong, and often stronger, staining in chest wall vasculature and skeletal muscle (and elsewhere; unpublished data). The virtual absence of sequestered parasites in chest wall sections with intense iNOS staining (Figs 2D and 2E) suggests that a circulating iNOS inducer, such as TNF, may be responsible. The intense iNOS staining in striated muscle from Category B patients who had extensive brain sequestration but no or little iNOS, may similarly indicate a circulating stimulus to muscle iNOS in severe malaria in the absence of a local stimulus in the brain. This stimulus could have systemic metabolic consequences that contribute to altered consciousness through mechanisms other than the local cerebral events observed histologically in Category C cases. Our data thus support the observation that the syndrome diagnosed clinically as cerebral malaria may be the result of a variety of different underlying events [58], some being local within the brain, and others a metabolic disorder resulting from the systemic infection [59].

The intense staining for iNOS in cerebral vessel walls that characterise Category C brains could have local pathological consequences. NO generated by the constitutive forms of NOS performs many essential signalling and regulatory roles in the brain, and these normally are precisely controlled by cytoplasmic calcium levels. Examples include the regulation of cerebral blood flow [60], N-methyl-D-aspartate (NMDA) receptors [61], and the induction of sleep when release occurs in the pedunculopontine tegmentum [62]. NO also inhibits the Na^+/K^+ ATPase-driven pump [63,64] that prevents sodium and therefore water accumulation, or oedema, in various tissues, including brain. These neuronal homeostatic functions of NO can be expected to be altered when iNOS is induced in the brain in high concentrations, as we describe in some of these cases. For example, it has been proposed that the reduced uptake of glutamate by astrocytes when inflammatory cytokine levels are high, allowing this excitotoxin to build up to levels that can cause seizures, is mediated by the NO that these cytokines induce [65]. In addition, vasodilation from the NO so generated could, from animal studies [45], lower the high cerebral vascular resistance that normally protects the cerebral capillaries from rupture [44].

Levels of nitrogen oxides, indirect measures of total NO production, in plasma [66,67], urine or cerebrospinal fluid [68] have not been associated with degree of coma in severe falciparum malaria. However, total circulating nitrite plus nitrate merely gives an indication of total NO production in the whole body, and is too blunt an instrument to measure the local concentration of a molecule that acts within a cell or two of its site of production. The total NO production in the body's large mass of skeletal muscle, as implied by the degree of iNOS induction in muscles we have observed, could be sufficient to obscure the contribution to plasma levels by the cerebral vasculature. Likewise, our arguments for pathogenic roles for iNOS-induced NO in severe falciparum malaria do not conflict with studies on an iNOS promoter polymorphism associated with increased NO production and protection from severe malaria in East African children [69], since a population study gives no evidence against these molecules being deleterious in individual fatal cases. In an additional study to that reported here we have found nitrotyrosine where iNOS is detectable, suggesting that NO is actually generated by this enzyme.

Non-malarial illnesses

A group of 10 comatose children were found, after admission into the study, to be carrying only few or no malaria parasites (and thus were not diagnosed as cerebral malaria), and pathogenic bacteria were cultured from the blood of 5 of them (Table 1). As noted, their cerebral vascular walls contained little iNOS, although iNOS was evident in skeletal muscle and the walls of blood vessels within it. In Category A cerebral malaria cases, the brain vessels were similarly devoid of parasites or iNOS staining. These patients also suffered a fatal comatose illness, suggesting that cerebral vascular wall NO is not necessary for encephalopathy to develop in systemic infections. The high iNOS in the chest wall vessels and skeletal muscle of some of the culture-positive sepsis cases (Figs 3C and 3D), as well as our Category A cerebral malaria cases (Figs 2B,2C,2D,2E), suggests that death may have been due to systemic inflammation rather than intracerebral mechanisms. It is also consistent with the high nitrite/nitrate levels recently reported in skeletal muscle of fatal, but not non-fatal, sepsis cases [70].

The considerable induction of iNOS in skeletal muscle in a number of our malaria and sepsis patients is consistent with parallels in their pathophysiology [71]. Lanone and co-workers [72] recently made the first report of iNOS in

skeletal muscle in human sepsis. We can confirm their findings (Fig. 3D), and extend it to falciparum malaria (Fig. 2E). From the recognised effects of iNOS-induced NO in skeletal muscle and diaphragm [73], such NO could cause poor contractility of the ventilatory muscles, and thus contribute to the terminal respiratory arrest observed in severe falciparum malaria by others in African children [74]. Because of a need to hyperventilate to compensate for metabolic acidosis, a common condition in severe malaria in Malawian children [75], their respiratory muscles would be particularly vulnerable to the effect of this iNOS.

Conclusions

We have demonstrated iNOS in the walls of microvessels in the brain and in skeletal muscle in children with fatal malarial and bacterial infections, and observed considerable diversity in the intensity of this phenomenon, iNOS being particularly associated with other features of intravascular inflammation in the brain, but not in muscle. MIF has been detected in a pattern that is compatible with this, and also offers fresh insights into differences between the brain and elsewhere. An autopsy-based study of this kind cannot demonstrate that iNOS-generated NO plays a role in the pathogenesis of disease or death in fatal malaria [26], but it moves towards that conclusion, broadening the range of tissues involved. These results also agree with the view that cerebral malaria in African children is a collection of overlapping syndromes acting through different organ systems, with several mechanisms potentially combining to cause coma and death.

Authors' contributions

IC helped plan the study, read histology slides, performed the statistical analyses, and wrote the text. MA developed and carried out the immunohistochemistry staining, and read histology slides. RW carried out autopsies, and read histology slides. CH carried out control tissue sections, and read histology slides. NL provided clinical expertise. MM provided clinical expertise, helped plan and coordinate the study, and helped write the text. TT provided clinical expertise, and helped plan and coordinate the study. All authors read and approved the final manuscript.

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