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Raised plasma insulin level and homeostasis model assessment (HOMA) score in cerebral malaria: evidence for insulin resistance and marker of virulence

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Objective: To study the glycaemic profile of patients with severe malaria (SM). Methods: For this purpose, 110 SM patients were recruited. Pre-treatment random blood glucose and plasma insulin were measured in a subset of donors. An *ex-vivo* experiment was developed for estimation of glucose consumption by parasitized erythrocytes. Results: Hyperglycaemia was frequent in SM but more commonly associated with cerebral malaria (CM), while hyperinsulinaemia was recognized in severe-malarial-hypotension (median, 25 %–75 %, 188.2, 93.8–336.8 pmol/L). The plasma insulin level was positively correlated with age (CC = 0.457, $P < 0.001$) and negatively with parasitaemia (CC = –0.368, $P = 0.045$). Importantly, fatal-CM was associated with hyperglycaemia (12.22, 6.5–14.6 mmol/L), hyperinsulinaemia (141.0, 54.0–186.8 pmol/L) and elevated homeostasis model assessment (HOMA) values. However, there was a trend of higher glucose consumption by parasites in CM compared with that in uncomplicated malaria (UM). Conclusion: Hyperglycaemia, hyperinsulinaemia and elevated HOMA are evidence for insulin resistance and possibly pancreatic B-cell dysfunction in fatal-CM.

Keywords: cerebral malaria, hyperglycaemia, HOMA, insulin resistance, virulence

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INTRODUCTION

Plasmodium falciparum is the most virulent species of malaria parasites, which is responsible for diverse clinical symptoms with varying degree of severity; at one extreme it may cause asymptomatic infection and at the other it may lead to severe malaria (SM) (WHO, 2000; Cox *et al.*, 2007). Severe malaria includes clinically and pathologically heterogeneous complications that vary in incidence, age and geographical distributions and other parameters (WHO, 2000). Cerebral malaria (CM) is the most fatal form of SM, and in sub-Saharan Africa severe malarial anaemia (SMA) ranks the second. Other forms of SM include**:** convulsions, hypotension, hypoglycaemia, renal failure, respiratory distress, pulmonary oedema, acidosis, bleeding/clotting disturbances and jaundice, which vary in prevalence. The pathology and consequences of infection in the individual complications are indeed variable, but a common finding in all complications is the metabolic disturbance (English *et al.*, 1997), importantly, of glucose. The glucose metabolism in malaria infections is affected by several factors including drug treatment, fever, parasite metabolism, hormonal changes, cytokines, fasting and gastrointestinal disturbances (Davis *et al.*, 1993; 2002). The major hormone that is involved in blood glucose homeostasis is insulin which is counterbalanced by most of the other hormones.

The glucose homeostasis during malaria infection is in part influenced by the intra-erythrocytic malaria parasite. The erythrocytes are classified as insulin independent tissue because they have no plasma membrane insulin receptors, and glucose transport across the membrane is facilitated by glucose transporter 1 (GLUT1). Once the parasite invades the erythrocyte, it makes significant alteration in the structure of the parasitized erythrocyte membrane, by placing parasite-derived proteins in the membrane (Deitsch & Wellems, 1996). These alterations facilitate the movement of nutrients into and waste products out of the infected cells, to meet the needs of the growing intra-erythrocytic parasites. Glucose is the main source of energy for the parasite; it is transported to the parasite by *P. falciparum* hexose transporter (PfHT), which is different from GLUT1, the former transports both glucose and fructose (Krishna *et al.*, 2000; Woodrow *et al.*, 2000). The intra-parasitic glucose concentration may exceed that of the parasitized erythrocyte (Kirk *et al.*, 1996).

Hyperglycaemia reflects insulin dysfunction either due to a lack or a deficit of insulin activity, which leads to intracellular glucose deficiency in insulin-dependent tissues, e.g. muscles and adipose tissues. Alternatively, hyperglycaemia could be due to increased levels of the counter-regulatory hormones such as glucagon, epinephrine and steroids. In SM, hyperinsulinaemia might lead to hypoglycaemia while the levels of the counter-regulatory hormones may remain normal (White *et al.*, 1883; Phillips *et al.*, 1993). Previously, we reported the disturbance of glucose metabolism during SM (Giha *et al.*, 2005), here; we expanded the data, analysis and inter-

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Abbreviations: HOMA, homeostasis model assessment; CM, cerebral malaria; GI, gastrointestinal; GLUT, glucose transporter; HOMA-IR, homeostasis model assessment of insulin resistance; MC, multiple complications; MOI, multiplicity of infection; MSP2, merozoite surface protein-2; PfHT, *P. falciparum* hexose transporter; RBG, random blood glucose; SM, severe malaria; SMA, severe malarial anaemia; SMC, severe malarial convulsions; SMH, severe malarial hypotension; UM, uncomplicated malaria.

pretation of the results for explanation of the phenomenon of "hyperinsulinaemic hyperglycaemia" in fatal CM.

MATERIALS AND METHODS

Study area, population and design. This study was conducted during the malaria transmission seasons in the period between October 2000 and January 2002, in Gedarif Teaching Hospital (Gedarif, Eastern Sudan). The area is meso-endemic and characterized by unstable and seasonal malaria transmission. The populations are multiethnic in origin; the majority work at farms. Individuals in all age groups are prone to develop symptomatic malaria infection, although older ones are relatively more protected. This study is part of a project investigating SM epidemiology and pathogenesis. The details of the study design, the clinical pattern and epidemiology of SM were described before (Giha *et al.*, 2005). The study received Ethical Clearance from the Ministry of Health (Sudan), and consents were obtained from each patient or guardian before inclusion in the study.

Clinical data collection. A specialized malaria clinic was established in the hospital. All patients who had or suspected to have malaria were referred to this clinic, where they were clinically examined after the disease history was obtained from them or from their guardians. Thereafter, the malaria diagnosis was carried out as mentioned below. The SM patients were identified by using the WHO criteria for SM (WHO, 2000).

Diagnosis of malaria. From patients suspected to have malaria, thin and thick blood smears were prepared from their finger prick blood samples. The blood smears were stained with Giemsa and examined microscopically for detection of malaria parasites, using ×100 oil immersion lens as described by the WHO (1991). The blood film was declared negative after examination of 200 fields. The number of asexual parasites per microlitre of blood was obtained by counting the number of parasites per 300 leukocytes, considering the normal leukocyte count to be 6000 per μL of blood. Patients who had SM were treated by quinine injections/tablets or artemether injections, the full details were published before (Giha *et al.*, 2005).

Sample collection. About 5 mL of venous blood sample was collected into sterile EDTA-containing vacutainers from each malaria patient (uncomplicated malaria — UM, and SM) before treatment and from apparently healthy malaria-free controls. The blood samples were centrifuged at 2000 rounds per minute for 8 min to separate plasma from parasitized erythrocytes. The plasma was then collected in cryotubes and stored at –20 °C, and the parasitized erythrocytes were mixed with glycerol freezing solution and stored in liquid nitrogen until used.

Measurement of blood glucose. The random blood glucose level was estimated by using 'One Touch Strip' glucose meter, Accutrend alpha (Boehringer, Germany) (Marks & Dawson, 1965). Briefly, one drop of blood was normally blotted on strip containing glucose oxidase. Glucose in presence of oxygen and enzyme converts into gluconic acid and hydrogen peroxide which is subsequently oxidized by peroxidase producing a blue colour. The result was read by electronic colorimeter. The blood drop was taken simultaneously with the venous blood sample which was used for other investigations, including insulin measurement. The blood samples were taken before commencement of any type of treatment or administration of intravenous fluid. In SM, random blood glucose likely represented fasting blood glucose level, since all patients were anorexic and the CM patients were comatose for several hours. The fasting reference range for blood glucose was considered to be 3.3–3.9 mmol/L (60–70 mg/dL), and the post-absorptive level as 4.5–5.5 mmol/L (80–100 mg/dL) (Murray *et al.*, 2000).

Measurement of plasma insulin level. The insulin level was estimated in a subset of 99 plasma samples obtained from individuals with uncomplicated malaria (n. 20), severe malaria (n. 30) and apparently healthy controls (n. 49). The plasma insulin was estimated by radioimmunoassay (kit IMK-414, isotope Beijing China, measurement limit is 160 mIU/L, the fasting reference range is 4–16.8 mIU/L). The assay depends on a competition between I^{125}-labelled insulin and patient's plasma insulin for a limited number of binding sites on insulin specific antibodies (Morgan & Lazarow, 1962). First, 100 μL of each, a buffer and standard or plasma sample, were pipetted into test tubes, then, 100 μL of antibody solution was added to all tubes except those labelled as 'non-specific binding' and 'total binding'. Thereafter, 100 μL of iodine-labelled solution was added to all tubes, mixed and incubated at 37 °C for 2 h, then 500 μL of separating reagent was added to all tubes except one labelled 'total'. The tubes were then mixed thoroughly and incubated; all tubes were centrifuged, except 'total', at 1500×*g* for 15 min. The reagent was then removed and results were read by a programmed gamma counter.

Calculation of HOMA. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as follows**:** fasting serum insulin (μU/mL) × fasting plasma glucose (mg/dL)/405 and was used as an index for insulin resistance. Clinically, the insulin resistance definition is based on repeated measurements of fasting blood glucose and insulin levels and estimation of HOMA score (Inchiostro, 2005). Although it was practically not feasible and ethically unacceptable to request severely ill patients to fast, there were many reasons to consider the measured random blood glucose and insulin levels as fasting levels. That is because almost all SM patients had anorexia and gastrointestinal upset for a few days before presentation to hospital. In addition, the CM patients were in coma at the time of blood collection. This assumption is supported by findings of several studies which showed "fasting an unrecognized and insufficiently emphasized risk factor in malaria" (van Thien *et al.*, 2006)

***Ex-vivo* experiment for estimation of glucose consumption by infected erythrocytes.** We developed this experiment to estimate glucose consumption of parasites obtained from patients with UM and SM. Five parasite isolates obtained from patients with acute malaria were grown in culture as described by Trager and Jensen (1976), in 96-well microtitre plate. Three isolates were successfully grown and used in this part of the study. One isolate (frozen for approx. 2 years) was obtained from a patient who died of cerebral malaria (SG22). The other two parasite isolates were obtained from patients with UM, one obtained from Gedarif (frozen for approx. 2 years), and the other obtained from Khartoum (cultured immediately after being obtained from the patient). We used erythrocytes from the same donor for culture of all parasite isolates. Each parasite line was grown in two wells. Approximately 10 μL of human insulin (equivalent to 0.66 mLU/mL, Novo Nordisk, Denmark) was added to one of the two wells. Then, the culture plate was put in a candle jar and incubated at 37 °C for 24 h. The culture medium was changed every day

Table 1. Age and random blood glucose and insulin levels in patients with severe malaria, uncomplicated malaria and malaria-free donors

All donors	Age in years Median, 25–75% Mean ± STD	Random blood glucose Median, 25–75% Mean ± SD (mmol/L).	Plasma insulin Median, 25–75% Mean ± STD (pmol/L)
Malaria-free donor (n. 49)	8.0, 5.0–16.5 15.1 ± 15.4	Not done	59.7, 43.8–86.1 89.6 ± 103.0
Uncomplicated malaria (n. 20)	4.0, 2.3–12.8 11.3 ± 15.5	Note done	46.5, 27.4–77.1 76.7 ± 90.0
Severe malaria (n. 30)	5.5, 4.0–11.0 12.8 ± 15.3		59.4, 45.8–115.3 95.8 ± 88.4
Severe malaria patients (individual complications)			
Severe malarial convulsions (n. 8)	4.4 ± 3.0	5.78, 5.00–6.96 5.92 ± 1.51	54.52, 47.58–62.50 61.90 ± 24.43
Sever malarial anemia (n. 8)	6.3 ± 3.8	5.75, 5.17–6.36 5.98 ± 1.08	45.84, 39.93–59.04 72.66 ± 78.11
Cerebral malaria (n. 7)	18.4 ± 19.2	11.50, 6.49–13.35 10.52 ± 4.36	63.20, 44.28–165.46 104.77 ± 75.95
Severe malarial hypotension (n. 4)	39.5 ± 8.8	5.45, 5.14–6.11 5.63 ± 0.65	188.21, 93.76–336.83 215.30 ± 150.16
Multiple complications (n. 3)	3.7 ± 0.6	5.78, 5.74–5.95 5.83 ± 0.15	56.25, 37.50–100.53 67.60 ± 43.15
Severe malaria patients (died *vs* survived)			
Fatal severe malaria (n. 5)	23.0 ± 21.4	12.22, 6.52–14.61 11.14 ± 4.85	140.98, 53.99–186.82 126.39 ± 80.80
Nonfatal severe malaria (n. 25)	10.7 ± 13.5	5.78, 5.14–6.50 6.13 ± 1.53	55.56, 45.15–88.55 89.67 ± 90.07

after estimation of glucose and parasitaemia. The cultures of the three parasite lines were maintained for different periods. The glucose consumption of the parasites was estimated by calculating the difference in the glucose level in each well at 0 time and 24 h thereafter. The final result of glucose consumption was obtained as a mean and S.D. of all series of experiment for each parasite line separately.

Statistical analysis. Sigma-stat software was used for statistical analysis. For differences in age and random blood glucose between study groups, and for the difference in *ex-vivo* glucose consumption by the parasites, we used Kruskal-Wallis One Way Analysis of Variance on Ranks. For comparison of age, random blood glucose and plasma insulin levels between two groups, we used Mann-Whitney Rank Sum Test. Pearson Product Moment Correlation was used in testing all correlations, while for differences in rates and proportions chi-square was used.

RESULTS

Clinical data

The general clinical data was presented before (Giha *et al.*, 2005). In brief, more than 16000 patients suspected to have malaria had been screened for malaria infection, a total of 110 patients with SM were identified. The SM patients were grouped into five clinical categories**:** severe malarial convulsions (n. 23 [median age, 4 years; 25–75 percentile, 3–7 years]) SMA (n. 50 [age 3, 2–6]), CM (n. 8 [age 9.5, 6–13]), severe malarial hypotension (n, 13 [age 35, 25–44.8]) and multiple complications (n. 6 [age 3.5, 3–4]). The age difference between the clinical groups was significant, $P < 0.001$. Five patients died of CM (age, 23.0 ± 21.4 years).

Plasma insulin level in SM, UM and malaria-free donors

The subset of donors (n. 99) for whom insulin level was measured included 30 patients with SM (median age, 5.5 years; 25–75 percentile, 4.0–11.0 years, [mean age ± S.D.], 12.8 ± 15.3 years), 20 with UM (age 4.0, 2.3–12.8 years; 11.3 ± 15.5 years) and 49 malaria-free donors (age 8.0, 5.0–16.5 years; 15.1 ± 15.4 years), (Table 1). The age of the SM patients was comparable to that of UM patients ($P = 0.303$) and malaria-free donors ($P = 0.128$). The plasma insulin level in SM (59.4, 45.8–115.3 pmol/L) was comparable to that in UM (46.5, 27.4–77.1 pmol/L) and malaria-free donors (59.7, 43.8–86.1 pmol/L), $P = 0.145$ and $P = 0.805$, respectively.

The SM patients frequently complained of symptoms suggestive of gastrointestinal upset (Table 2). For the subset of the SM patients for whom random blood glucose and insulin levels were measured (n. 30), 66% complained of anorexia, 83% of vomiting and 33% of diarrhoea. However, the random blood glucose and plasma insulin levels were not significantly different between patients with and without any of these symptoms. The values of other parameters in this sub-group of patients are shown in Table 2.

Random blood glucose and insulin levels in individual complications of severe malaria

For all SM patients, the random blood glucose level was significantly different between patients with different complications (Fig. 1). For the subset of the SM patients shown in Table 1, the random blood glucose level was

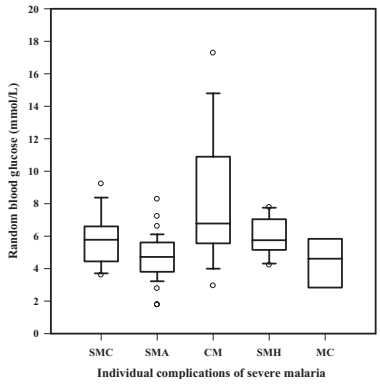

Figure 1. Random blood glucose levels at the time of malaria diagnosis and before treatment in patients with different complications of severe malaria
The SM patients are sub-grouped into patients with: severe malarial convulsions (SMC), severe malarial anemia (SMA), cerebral malaria (CM), severe malarial hypotension (SMH) and multiple complications (MC). The horizontal lines stand for median levels, the boxes limit the 25–75 percentile, bar errors are the 5–95% confidence interval, and open circle are outliers. Note: This figure is taken and modified with permission from Giha *et al.* (2005)

highest in CM (median, 11.50 mmol/L; 25–75 percentile, 6.49–13.35 mmol/L), but the difference was not significant between the individual complications ($P = 0.078$). The levels in the other complications were as follows: severe malarial convulsions (5.78, 5.00–6.96 mmol/L), multiple complications (5.78, 5.74–5.95 mmol/L), SMA (5.75, 5.17–6.36 mmol/L) and severe malarial hypotnesion (5.45, 5.14–6.11 mmol/L). The random blood glucose was significantly higher in fatal SM (12.22, 6.52–14.61 mmol/L) compared with non fatal SM (5.78, 5.14–6.50 mmol/L), $P = 0.016$, (Table 1). The random blood glucose of individual patients is presented in Table 2. In the same subset of patients, the levels of the plasma insulin in severe malarial hypotension (188.21, 93.76–336.83 pmol/L), CM (63.20, 44.28–165.46 pmol/L), multiple complications (56.25, 37.50–100.53 pmol/L), severe malarial convulsions (54.52, 47.58–62.50 pmol/L) and SMA (45.84, 39.93–59.04 pmol/L) were highly variable, but not significantly different ($P = 0.096$) (Table 1). The plasma insulin levels in fatal SM (140.98, 53.99–186.82 pmol/L) was higher than in non-fatal SM (55.56, 45.15–88.55 pmol/L), but the difference was not significant ($P = 0.290$).

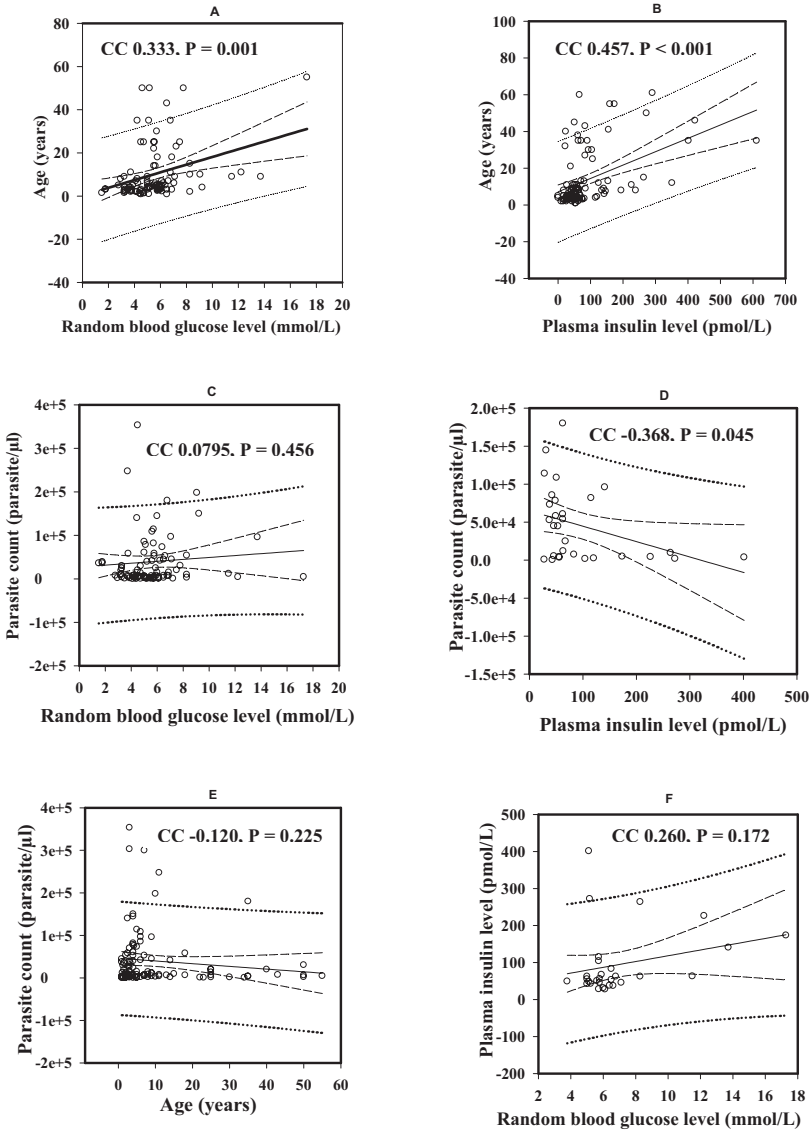

Figure 2. Correlations of random blood glucose and plasma insulin levels with parasite count, age, and of each pair with each other
Blood glucose was measured for 110 patients with severe malaria including 30 patients for whom plasma insulin was measured, while plasma insulin was measured for 99 donors including patients with uncomplicated malaria and healthy malaria-free donors. Statistically significant positive correlations were the correlations of age with random blood glucose and insulin levels, and a significant negative correlation was of insulin level with parasite count. Other correlations were not significant.

Table 2. Description of the severe malaria (SM) patients
The following parameters are given: age, SM types, gastrointestinal (GI) upset, random blood glucose (RBG, mmol/L), plasma insulin level (pmol/L) and resistance index (HOMA), and chloroquine-resistance genotypes of parasites.

No.	Donors	Age	SM	GI-upset	RBG	Insulin	HOMA	pfcrt	pfmdr1	Fate
1	SG10	4.5	SMC	Ö	ND	120.15	ND	M	M	
2	SG24	9	SMC	Ö	5.00	55.56	1.78	M	M	
3	SG30	4	SMC	Ö	5.00	62.50	2	M	W	
4	SG34	2.5	SMC	Ö	3.78	49.31	1.19	M	M	
5	SG40	0.9	SMC	Ö	6.50	53.48	2.22	W	W	
6	SG42	9	SMC	Ö	7.11	45.84	2.09	M	M	
7	SG44	2	SMC	Ö	8.28	62.50	3.31	M	M	
8	SG47	3.6	SMC	X	5.78	45.84	1.69	M	M	
9	SG50	55	CM	Ö	17.28	173.62	19.2	M	M	died
10	SG13	11	CM	Ö	12.22	226.40	17.71	W	W	died
11	SG23	9	CM	Ö	13.72	140.98	12.38	M	M	died
12	SG22	35	CM	X	6.78	62.50	2.71	W	W	died
13	SG43	5	CM	Ö	5.72	28.47	1.04	W	W	died
14	SG45	9	CM	Ö	11.50	63.20	4.65	M	M	
15	SG21	5	CM	Ö	6.39	38.20	1.56	M	M	
16	SG17	4	MC	Ö	5.72	115.29	4.22	W	**W**/M	
17	SG19	4	MC	Ö	6.00	31.25	1.2	M	M	
18	SG39	3	MC	Ö	5.78	56.25	2.08	M	M	
19	SG11	3	SMA	Ö	5.89	67.37	2.54	M	M	
20	SG15	7	SMA	Ö	6.61	37.50	1.59	M	M	
21	SG18	15	SMA	X	8.28	263.91	13.98	W	W	
22	SG20	6	SMA	Ö	5.61	50.70	1.82	M	M	
23	SG28	4	SMA	Ö	5.22	43.06	1.44	W	W	
24	SG29	5	SMA	Ö	6.11	27.78	1.09	W	W	
25	SG38	6	SMA	Ö	5.00	42.36	1.36	M	M	
26	SG02	4	SMA	Ö	5.11	48.61	1.59	ND	ND	
27	SG03	35	SMH	Ö	5.11	401.42	13.13	ND	ND	
28	SG05	50	SMH	Ö	5.17	272.24	9	ND	ND	
29	SG12	30	SMH	Ö	5.72	104.17	3.81	M	M	
30	SG32	43	SMH	Ö	6.50	83.34	3.47	M	M	

CM, cerebral malaria; HOMA, 'homeostatic model assessment' for insulin resistance; M, mutant; MC, multiple complications; ND, not done; Pfcrt, *P. falciparum* chloroquine resistance transport; pfmdr, a homolog of the human multi-drug resistance; SMA, severe malarial anemia; SMC, severe malarial convulsions; SMH, severe malarial hypotension; W, wild. Fatal CM highlighted in bold.

Correlations of donor's age, random blood glucose, plasma insulin level and parasite density

As shown in Fig. 2, there was an overall statistically significant positive correlation between donor's age and random blood glucose level (CC 0.333, $P=0.001$, n. 94). The association of insulin level with age was strongly statistically significant in SM, UM and malaria-free groups, whether each group was considered separately or all donors were taken together (CC 0.457, $P<0.001$, n. 98). In SM, the median (25%–75%) insulin level in children (>0–10 years), 50.0 (42.4–62.5) pmol/L, was significantly lower than in adolescents and adults (>10 years) 200.0 (93.8–268.1) pmol/L, $P<0.001$, unlike the RBG, which was comparable between the two age groups (5.8, 5.2–6.5 *vs* 6.6, 5.4–10.3), $P=0.222$. The overall correlation between RBG and plasma insulin level in SM (UM data was not available) was not significant (CC 0.260, $P=0.172$, n. 29), however, in children (≤ 10 years) but not in older patients, there was a positive correlation (CC 0.587, $P=0.005$). The insulin level was inversely significantly associated with the parasite density in SM (CC 0.368, $P=0.045$, n. 30), but there was no correlation between the parasite density and random blood glucose (CC 0.0795, $P=0.456$, n. 90) or age (CC –0.120, $P=0.225$, n. 104).

Characterization of parasites from fatal CM patients

In the first malaria season, five patients died because of CM (ages 5, 9, 11, 35, and 55 years). Using *merozoite surface protein-2* (*MSP2)* gene for parasite typing, the parasites causing fatal CM were characterized by having low multiplicity of infection (MOI, 1.2), no parasite was found to carry the FC27 genotype as a single clone (0/5) (not shown) (A-Elbasit *et al.*, 2007). Three of the five parasites carried the wild-type variant of the chloroquine resistance genes, *pfcrt*-76T (*P. falciparum* chloroquine resistance transporter) and *pfmdr*1-86Y (a homolog of the human multi-drug resistance p-glycoprotein) (Table 2) (Giha *et al.*, 2006). Patients who died of CM had normal or high random blood glucose level (5.7, 13.7, 12.2, 6.8 and 17.3 mmol/L, respectively) and their plasma insulin levels (28.5, 141, 226.4, 62.5 and 173.6 pmol/L, respectively) were mostly higher than normal (Giha *et al.*, 2005). However, the proportion of donors with HOMA >5.0 was significantly higher in the fatal CM group (60%, 3/5) compared with the other patients with SM (12.5%, 3/24), $P=0.046$ (Table 2).

Parasite virulence and *ex-vivo* glucose consumption

The consumption of glucose by SG22 parasite line (fatal CM) in nine experiments was consistent over a month (median, 0.167 mmol/L per 24 h; 25–57 percentile, 0.139–0.239). The glucose consumption of the parasites obtained from patients with UM were lower: UM-fresh isolate (median of 4 experiments, 0.111 mmol/L per 24 h; 25–74 percentile, 0.111–0.200) and the UM-frozen isolate (median of 10 experiments, 0.139 mmol/L per 24 h; 25–74 percentile, 0.111–0.167), but the differences between the three parasite lines were not significant, $P=0.267$. The glucose consumption of the parasites obtained from patients with UM was consistent for each isolate over time and comparable between the two isolates (not shown). The glucose consumption of the culture to which insulin was added was insignificantly slightly higher than that of the culture to which no insulin was added regardless of the origin of the isolate.

DISCUSSION

In severe malaria, peculiar glucose homeostasis is recognized as an isolated complication or as part of a clinical scenario. Hypoglycaemia is frequently recognized as isolated complication (WHO, 2000), on the other hand, hyperglycaemia is frequently associated with other SM complications, mostly CM. Unlike hyperglycaemia, the malarial hypoglycaemia has been thoroughly investigated before (Phillips, 1989; Kawo *et al.*, 1990a; Zijlmans *et al.*, 2008). In this study, the hyperglycaemia was associated with CM, as the mean blood glucose level was found to be above the fasting and post-absorptive levels (3.3–5.6 mmol/L) and the levels were even higher in the fatal cases of CM. Although the method we used for blood glucose estimation is not the most accurate one, the differences between the methods are not expected to be significantly large. The used method gave consistent and quick results, and helped in successful management of the patients; none of the patients died because of hypoglycaemia in this study.

The association of hyperglycaemia with SM was not a novel observation, as it was previously reported (van Thien *et al.*, 2001). Also, the high mortality rate of CM associated with hyperglycaemia was recognized before in Kenya (Osier *et al.*, 2003). However, the hyperinsulinaemic hyperglycaemia that associates with CM and indicates bad prognosis and increased mortality, was not reported before. The malarial hyperglycaemia is due to gluconeogenesis (Phillips, 1989). The gluconeogenesis process increases in the presence of gluconeogenic precursors, such as lactate and alanine, which are produced by both parasite and host. On the other hand, insulin secretion is stimulated by plasma glucose and potentially inhibited by pancreatic B cell dysfunction. Thus, increased plasma insulin with normal plasma glucose indicates insulin resistance, while increased plasma glucose in combination with increased plasma insulin indicates insulin resistance associated with relative B-cell failure. Here, the hyperglycaemia, hyperinsulinemia and high HOMA scores in fatal CM were likely indicative of insulin resistance associated with B-cell dysfunction. The SM-associated insulin resistance is in line with a previous report (Binh *et al.*, 1997).

Generally, we observed that the plasma level of insulin increased with increasing age irrespective of the malaria severity; similarly, the blood glucose level increased with age up to 18 years. Furthermore, we found that the level of insulin varied with the type of SM complication; it was within a normal range and associated with normoglycaemia or hypoglycaemia in SMA and severe malarial convulsions. In CM, the hyperinsulinaemia was associated with hyperglycaemia, while the relatively high level of insulin in severe malarial hypotension was associated with normal levels of glucose (Giha *et al.*, 2005). Although the number of patients was small, there were still significantly higher levels of insulin and random blood glucose in fatal compared with non-fatal SM. The co-existence of hyperinsulinaemia and hyperglycaemia in fatal CM can not be explained by the age factor, as hyperinsulinaemia in the severe malarial hypotension group (eldest group of patients) was not associated with hyperglycaemia. Hyperinsulinaemic hypoglycaemia was previously recognized in CM (White *et al.*, 1983; Planche *et al.*, 2005); in other studies hypoinsulinaemia was associated with hypoglycaemia (White *et al.*, 1987; Kawo *et al.*, 1990a). van Thein *et al.* (2001) found no significant change in insulin levels during SM. The discrepancies in the association of insulin levels with blood glucose levels in SM in different studies might be a reflection for the differences in the epidemiological settings, definitions of SM, age of the patients, feeding status before the sampling (Kawo *et al.*, 1990b) or for other reasons. The hyperinsulinaemia in malaria might be due to TNF-α release (Manish *et al.*, 2003).

In *ex-vivo* experiments, confounding host factors such as cytokines, hormones, immunity and others are excluded, however, insulin was added in this experiment, as it is the key hormone in glucose metabolism. Our results revealed a trend of increased glucose consumption by the more virulent parasites (CM) compared with the less virulent parasites (UM). Interestingly, the former parasite (SG22) carried the wild-type variants of the drug resistance-associated genes *pfcrt*-76T and *pfmdr1*-86Y. The mutant variants of these genes are known to incur fitness cost on the parasites. Previously, we showed that the above-mentioned wild-type gene variants are associated with increased parasite virulence (Giha *et al.*, 2006). Taken together, the increased glucose uptake by SG22 parasite might indicate a higher metabolic activity of the wild-type and more virulent strains. Higher turnover of glucose in SM as compared with UM was previously re-

ported (Agbenyega *et al.*, 2000). However, the increased parasite glucose consumption is expected to contribute to hypoglycaemia, as recognized in other studies (Davis *et al.*, 1993; Agbenyega *et al.*, 2000). The paradox of having hyperglycaemia coupled with hyperinsulinaemia in fatal CM in this study could be explained by a temporal status of insulin-resistance associated with pancreatic B-cells dysfunction induced by infection with virulent malaria strains. It would be of interest to follow the surviving CM patients who had high HOMA scores, to see if they developed diabetes mellitus thereafter. The relation between diabetes mellitus and *P. falciparum* malaria is poorly understood, although malaria can results in severe metabolic disturbances (Planche *et al.*, 2005). The abnormal glucose homeostasis in SM could be due to abnormal liver or renal functions which are both common in SM; however, the latter conditions are expected to contribute to the hypoglycaemia, although delayed insulin catabolism is a possibility. Further studies in this line of research should be undertaken.

Another interesting observation in this study was that patients who developed severe malaria-associated hypotension also had high insulin levels, in line with a previous report (Porcellati *et al.*, 1993). The effect of insulin on blood pressure is thought to be due to peripheral vasodilatation, especially in skeletal muscles (Hall *et al.*, 1995), which is probably mediated through nitric oxide (Kawasaki *et al.*, 2000). However, other factors may contribute to the development of hypotension during SM, such as dehydration. In this study, all patients who had severe malarial hypotension had completely and quickly recovered after resuscitation.

In conclusion, glucose and insulin levels were highly variable between individual complications of SM. We reported here the occurrence of hyperinsulinaemic hyperglycaemia and high HOMA score in fatal CM, and hyperinsulinaemic normoglycaemia in hypotension associated with SM. The former can be explained by induction of insulin resistance and B-cell dysfunction by virulent parasites. Although hyperglycaemia was recognized in CM, the parasites causing CM consumed relatively more glucose than those causing UM in an *ex-vivo* experiment. This study should add to our understanding of the malaria parasite metabolism and association of the latter with parasite virulence and SM pathogenesis.

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REFERENCES

A-Elbasit IE, Elghazali G, A-Elgadir TM, Hamad AA, Babiker HA, Elbashir MI, Giha HA (2007) Allelic polymorphism of MSP2 gene in severe *P. falciparum* malaria in an area of low and seasonal transmission. *Parasitol Res* **102:** 29–34.

Agbenyega T, Angus BJ, Bedu-Addo G, Baffoe-Bonnie B, Guyton T, Stacpoole PW, Krishna S (2000) Glucose and lactate kinetics in children with severe malaria. *J Clin Endocrinol Metab* **85:** 1569–1576.

Binh TQ, Davis TM, Johnston W, Thu LT, Boston R, Danh PT, Anh TK (1997) Glucose metabolism in severe malaria: minimal model analysis of the intravenous glucose tolerance test incorporating a stable glucose label. *Metabolism* **46:** 1435–1440.

Cox J, Hay SI, Abeku TA, Checchi F, Snow RW (2007) The uncertain burden of *Plasmodium falciparum* epidemics in Africa. *Trends Parasitol* **23:** 142–148.

Davis TM, Looareesuwan S, Pukrittayakamee S, Levy JC, Nagachinta B, White NJ (1993) Glucose turnover in severe falciparum malaria. *Metabolism* **42:** 334–340.

Davis TM, Binh TQ, Thu le TA, Long TT, Johnston W, Robertson K, Barrett PH (2002) Glucose and lactate turnover in adults with falciparum malaria**:** effect of complications and antimalarial therapy. *Trans R Soc Trop Med Hyg* **96:** 411–417.

Deitsch KW, Wellems TE (1996) Membrane modifications in erythrocytes parasitized by *Plasmodium falciparum*. *Mol Biochem Parasitol* **76:** 1–10.

English M, Sauerwein R, Waruiru C, Mosobo M, Obiero J, Lowe B, Marsh K (1997) Acidosis in severe childhood malaria. *Q J Med* **90:** 263–270.

Giha HA, Elghazali G, A-Elgadir TM, A-Elbasit IE, Eltahir EM, Baraka OZ, Khier MM, Adam I, Troye-Blomberg M, Theander TG, Elbashir MI (2005) Clinical pattern of severe *Plasmodium falciparum* malaria in Sudan in an area characterized by seasonal and unstable malaria transmission. *Trans R Soc Trop Med Hyg* **99:** 243–251.

Giha HA, Elbashir MI, A-Elbasit IE, A-Elgadir TM, ElGhazali GE, Mackinnon MJ, Babiker HA (2006) Drug resistance-virulence relationship in *Plasmodium falciparum* causing severe malaria in an area of seasonal and unstable transmission. *Acta Trop* **97:** 181–187.

Hall JE, Brands MW, Zappe DH, Alonso-Galicia M (1995) Cardiovascular actions of insulin**:** are they important in long-term blood pressure regulation? *Clin Exp Pharmacol Physiol* **22:** 689–700.

Inchiostro S (2005) Measurement of insulin sensitivity in Type 2 diabetes mellitus**:** comparison between KITT and HOMA-%S indices and evaluation of their relationship with the components of the insulin resistance syndrome. *Diabet Med* **22:** 39–44.

Kawasaki H, Kuroda S, Mimaki Y (2000) Vascular effects of insulin, *Nippon Yakurigaku Zasshi* **115:** 287–294 (in Japanese).

Kawo NG, Msengi AE, Swai AB, Chuwa LM, Alberti KG, McLarty DG, Orskov H (1990a) Hypoglycaemia and cerebral malaria. *Lancet* **336:** 1128–1129.

Kawo NG, Msengi AE, Swai AB, Chuwa LM, Alberti KG, McLarty DG (1990b) Specificity of hypoglycaemia for cerebral malaria in children. *Lancet* **336:** 454–457.

Kirk K, Horner HA, Kirk J (1996) Glucose uptake in *Plasmodium falciparum*-infected erythrocytes is an equilibrative not an active process. *Mol Biochem Parasitol* **82:** 195–205.

Krishna S, Woodrow CJ, Burchmore RJ, Saliba KJ, Kirk K (2000) Hexose transport in asexual stages of *Plasmodium falciparum* and kinetoplastidae. *Parasitol Today* **16:** 516–521.

Manish R, Tripathy R, Das BK (2003) Plasma glucose and tumor necrosis factor-alpha in adult patients with severe falciparum malaria. *Trop Med Int Health* **8:** 125–128.

Marks V, Dawson A (1965) Rapid stick method for determining blood-glucose concentration. *Br Med J* **5430:** 293–294.

Morgan CR, Lazarow A (1962) Immunoassay of insulin using a two-antibody system. *Proc Soc Exp Biol Med* **110:** 29–32.

Murray RK *et al.* (2000) Appendix. *Harper's Biochemistry.* **25**th edn, 869.

Osier FH, Berkley JA, Ross A, Sanderson F, Mohammed S, Newton CR (2003) Abnormal blood glucose concentrations on admission to a rural Kenyan district hospital**:** prevalence and outcome. *Arch Dis Child* **88:** 621–625.

Phillips RE (1989) Hypoglycaemia is an important complication of falciparum malaria. *Q J Med* **71:** 477–483.

Phillips RE, Looareesuwan S, Molyneux ME, Hatz C, Warrell DA (1993) Hypoglycaemia and counter regulatory hormone responses in severe falciparum malaria: treatment with Sandostatin. *Q J Med* **86:** 233–240.

Planche T, Dzeing A, Ngou-Milama E, Kombila M, Stacpoole PW (2005) Metabolic complications of severe malaria. *Curr Top Microbiol Immunol* **295:** 105–136.

Porcellati F, Fanelli C, Bottini P, Epifano L, Rambotti AM, Lalli C, Pampanelli S, Scionti L, Santeusanio F, Brunetti P *et al.* (1993) Mechanisms of arterial hypotension after therapeutic dose of subcutaneous insulin in diabetic autonomic neuropathy. *Diabetes* **42:** 1055–1064.

Trager W, Jensen JB (1976) Human malaria parasites in continuous culture. *Science* **193:** 673–675.

van Thien H, Ackermans MT, Dekker E, Thanh Chien VO, Le T, Endert E, Kager PA, Romijn JA, Sauerwein HP (2001) Glucose production and gluconeogenesis in adults with cerebral malaria. *Q J Med* **94:** 709–715.

van Thien H, Kager PA, Sauerwein HP (2006) Hypoglycemia in falciparum malaria: is fasting an unrecognized and insufficiently emphasized risk factor? *Trends Parasitol* **22:** 410–415.

White NJ, Warrell DA, Chanthavanich P, Looareesuwan S, Warrell MJ, Krishna S, Williamson DH, Turner RC (1983) Severe hypoglycemia and hyperinsulinemia in falciparum malaria. *N Engl J Med* **309:** 61–66.

White NJ, Miller KD, Marsh K, Berry CD, Turner RC, Williamson DH, Brown J (1987) Hypoglycaemia in African children with severe malaria. *Lancet* **8535:** 708–711.

WHO (1991) *Basic malaria microscopy*. Part 1. WHO, Geneva.

WHO (2000) Severe falciparum malaria. Communicable Diseases Cluster. *Trans R Soc Trop Med Hyg* **94:** 1–90.

Woodrow CJ, Burchmore RJ, Krishna S (2000) Hexose permeation pathways in *Plasmodium falciparum*-infected erythrocytes. *Proc Natl Acad Sci USA* **29:** 9931–9936.

Zijlmans W, van Kempen A, Ackermans M, de Metz J, Kager P, Sauerwein H (2008) Glucose kinetics during fasting in young children with severe and non-severe malaria in Suriname. *Am J Trop Med Hyg* **79:** 605–612.