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Synchronic Macrophage Response and *Plasmodium falciparum* Malaria

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Abstract: This study describe the levels of plasma Chitotriosidase (CHIT) activity in 62 African children with severe and uncomplicated *Plasmodium falciparum* malaria with respect to 140 healthy African children. Medially all children with acute malaria showed plasma CHIT activity levels higher than healthy control subjects (140 nmol/mL/h, range 13-521, versus 72 nmol/mL/h, range 14-150, $p < 0.0001$). Although the distribution curve of plasma CHIT activity showed a bimodal behavior, both children with severe malaria ($n=22$) and with uncomplicated malaria ($n=40$) showed elevated levels of plasma CHIT activity (median 138 nmol/mL/h, range 13-521 and median 151 nmol/mL/h, range 13-491, respectively). The difference between the two groups was not significant. On the contrary a significant correlation was found between plasma CHIT activity and serum ferritin only in children with uncomplicated malaria, but not in children with severe malaria. Since it is generally accepted that *P. falciparum* infection is mediated by the immune system, only a synchronic macrophage response guarantees the favourable outcome of this infection. We do not know how the expression of the CHIT gene is regulated, but the lack of correlation between plasma CHIT activity and serum ferritin in African children with severe *P. falciparum* malaria suggests in a failure of the synchronic macrophage response an important determinant of disease outcome.

Key words: Chitotriosidase activity, *Plasmodium falciparum* malaria

INTRODUCTION

Human Chitotriosidase (CHIT), produced by activated macrophage, is a member of the chitinase family, a group of enzymes with the capability to hydrolyze chitin.

The CHIT gene is localized on chromosome 1q31-q32 and consists of 12 exons spanning about 20 kilobases^[1] and a 24-base pair duplication in exon 10 of the CHIT gene has been described^[2] with an in frame deletion of 87 nucleotides. This CHIT mutant allele has been found in different populations and the presence of the 24 bp duplication is associated with a recessive inherited deficiency of plasma CHIT activity.

Homozygous CHIT deficiency was found in 5.5% of the population in Sicily and 3.7% of the population in Sardinia, with a further 44 and 33% of the populations heterozygous for the 24 bp duplication, showing Hardy-Weinberg equilibrium for both populations^[3].

In contrast, in Benin and in Burkina Faso, both mesoendemic regions for *P. falciparum* malaria and other parasitic diseases, no homozygous subject for 24 bp duplication has been found, while heterozygous subjects were 0 and 2%, respectively^[3].

Recently high plasma CHIT activity was found in children with acute *P. falciparum* malaria^[4], compared with healthy African children, as a consequence of macrophage activation due to the presence of parasites. In this study we measured the levels of plasma CHIT activity in African children with *P. falciparum* malaria distinct in severe and uncomplicated and in 140 healthy African children

MATERIALS AND METHODS

Subjects: We recruited 62 African children (30 males and 32 females, aged 2-140 months; median 16.5 months), affected by acute *P. falciparum* malaria, born and living in

Burkina Faso (Table 1). Control subjects included 140 healthy African children (79 males and 61 females) with age ranging from 10 to 100 months (median 22 months) at evaluation time. They did not show signs of acute infectious disease and smears for *P. falciparum* were negative. All collected samples were negative for HIV antibody testing.

Study area: The study started in July and concluded in October 2003 in Burkina Faso. In this country, *P. falciparum* malaria is a major cause of morbidity and mortality in children <5 years (38.5%, all causes). It is hyperendemic, perennial transmission peaking August-October (>90% *P. falciparum*, 1% *P. malariae*). Primary malaria vectors are *A. gambiae* and *A. funestus* (150 infective bites/year). Children with *P. falciparum* malaria and control subjects were evaluated and enrolled at the local Centre Medical Saint Camille (CMSC) of Ouagadougou, the capital of Burkina Faso. This study was approved by the local Ethical Committees of CMSC. Parents of the participating children in the study were orally informed of the scope of this research.

Methods: Personal data (age, sex), clinical findings (history, symptoms, temperature) and parasitological data (thin and thick blood films) were collected from all subjects. Blood samples were collected in sterile tubes containing EDTA from patients and controls at beginning of study for evaluation of complete blood count, serum ferritin and plasma CHIT activity.

Patients were classified based on symptoms, physical signs and laboratory findings of malaria at the time of first presentation and diagnosis of malaria was confirmed by microscopic detection of asexual *P. falciparum* in the peripheral blood. According to the WHO criteria, children had severe malaria, when showing neurological impairment, respiratory distress, oliguria, cardiovascular shock, jaundice, diffuse hemorrhages. In addition, they suffered from severe anaemia (Ht<15%), parasitemia degree $>2.5 \times 10^5 \mu\text{L}^{-1}$ or $>2.5\%$ in non immune subjects and hypoglycemia (serum glucose less than 2.2 mmol L^{-1} corresponding to 40 mg dL^{-1}). Mild malaria (uncomplicated malaria) was established by microscopic confirmed parasitemia degree $<2.5 \times 10^5 \mu\text{L}^{-1}$ or $<2.5\%$ with fever, headache, myalgias or gastrointestinal symptoms without any findings of severe malaria.

Chitotriosidase activity determination: For plasma CHIT assay, 3 mL of EDTA-blood was centrifuged and plasma samples were stored at -40°C until determination by fluorimetric method^[5] at the Center for Metabolic Diseases-University of Catania, Italy. Plasma CHIT

activity was measured by incubating 5 μL of undiluted plasma with 100 μL of a solution containing $22 \mu\text{mol L}^{-1}$ of the fluorescent substrate 4-methylumbelliferyl- β -D-N,N',N''-triacetylchitotriose (Sigma Chemical CO) in 0.5 M citrate-phosphate buffer pH 5.2, for 15 min at 37°C . The reaction was stopped by using 2 mL of $0.5 \text{ mol L}^{-1} \text{Na}_2\text{CO}_3\text{-NaHCO}_3$ buffer, pH 10.7. The fluorescence was read by a PerkinElmer fluorimeter, using 365 nm excitation and 450 nm emission wavelength. Chitotriosidase activity was measured as nanomoles of substrate hydrolyzed per mL per h (nmol/mL/h). Samples with a chitotriosidase levels $>110 \text{ nmol/mL/h}$ were measured again after a dilution of 10 fold or 50 fold with distilled water.

Statistical analysis: Since haematological parameters are normally distributed, we calculated for each group, mean \pm SD. Plasma CHIT activity and serum ferritin were also reported as median and range. Student's t-test was used for evaluate the difference between normally distributed data. Wilcoxon rank test was used for the non parametric data. Bonferroni test was used as a corrective for multiple observations. $p<0.01$ was considered significant.

RESULTS

Based on their clinical and hematological parameters, 22 children (35.48%) were affected by severe malaria, while 40 children (64.51%) were affected by uncomplicated malaria (Table 1).

Medially all children with acute *P. falciparum* malaria had levels of plasma CHIT activity higher (median 140 nmol/mL/h , range 13-521) than healthy controls (median 72 nmol/mL/h , range 14-150; $p<0.0001$) and the distribution of plasma CHIT activity in these children showed a bimodal behavior (Fig. 1). Also Hb level, red cell count, leukocytes count, platelet count and serum ferritin

Table 1: Haematological data of 62 children with acute *Plasmodium falciparum* malaria, according to the clinical severity

	Severe (N. 22) (A)	Uncomplicated (N. 40) (B)	Healthy controls (N.140) (C)
Age (months)	12.50 (2-70)	23.00 (9-140)	22.00 (10-100)
Leukocyte X $10^3 \text{ (mm}^3\text{)}$	$17.50 \pm 12.6^*$	$17.10 \pm 6.97^{\wedge}$	8.53 ± 2.74
Red cells X $10^6 \text{ (mm}^3\text{)}$	$1.40 \pm 0.61^{**}$	$3.10 \pm 0.70^{\wedge}$	3.95 ± 0.78
Hb (g dL $^{-1}$)	$3.75 \pm 1.26^{**}$	$8.30 \pm 1.58^{\wedge}$	9.60 ± 2.28
Ht (%)	$11.50 \pm 3.8^{**}$	24.60 ± 5.01	26.40 ± 4.38
MCV (μ^3)	$84.50 \pm 18.8\$$	$73.80 \pm 13.4^{\wedge}$	81.55 ± 11.48
MCH (pY)	$27.60 \pm 5.94\%$	25.40 ± 4.43	24.52 ± 4.40
MCHC (%)	$32.40 \pm 4.41\text{f}$	$33.38 \pm 3.48^{\wedge}$	30.21 ± 2.66
Platelets (mm 3)	$141.85 \pm 61.96^{**}$	$262.04 \pm 157.13^?$	324.12 ± 102.25
Ferritin (ng mL $^{-1}$)	$394 (35-2500)^*$	$441 (28-2500)^{\wedge}$	$120 (15-140)$
Plasma Chitotriosidase Activity (nmol/mL/h)	$138 (13-491)^*$	$151 (13-521)^{\wedge}$	$72 (14-150)$
A-B ° $p<0.0001$ \$ $p = 0.012$; A-C * $p<0.0001$; % $p=0.004$; f $p=0.001$; B-C ^ $p<0.0001$; ? $p = 0.003$; N: Number of children			

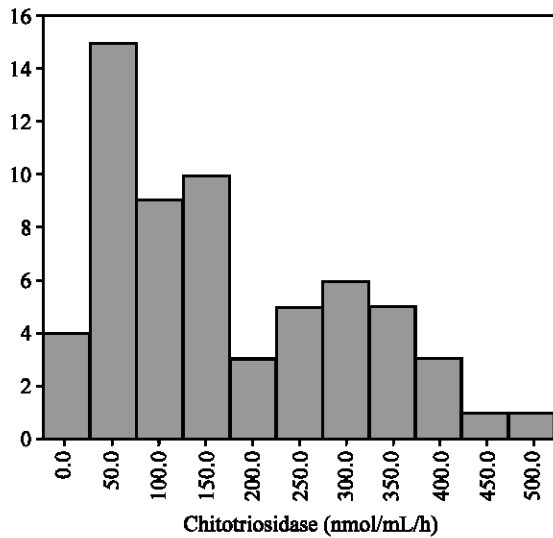


Fig. 1: Distribution of plasma Chitotriosidase activity in 62 children with acute malaria

were found statistically different from the healthy controls ($p < 0.0001$) in all children with acute *P. falciparum* malaria (Table 1).

The children with severe malaria showed hemoglobin levels below 6 g dL^{-1} and elevated parasitemia degree ($365,000 \mu\text{L}^{-1}$, range $2.5\text{--}5.0 \times 10^5$). In the children with uncomplicated malaria severe abnormalities of hematological parameters were not encountered (haemoglobin levels >7 to $<9 \text{ g dL}^{-1}$), while the parasitemia degree was $250,000 \mu\text{L}^{-1}$ below $2.5 \times 10^5 \mu\text{L}^{-1}$. Platelet count was significantly ($p < 0.0001$) lower ($141.85 \pm 61.94 \text{ mm}^3$) in children with severe malaria against children with uncomplicated malaria ($262.04 \pm 157.13 \text{ mm}^3$). The levels of serum ferritin were comparable in children with severe malaria (394 ng mL^{-1} , range 35-2500) than in children with uncomplicated malaria (441 ng mL^{-1} , range 28-2500) (Table 1). The level of CHIT activity in children with severe malaria was also comparable (median 138 nmol/mL/h , range 13-521) than in children with uncomplicated malaria (median 151 nmol/mL/h , range 13-491) and the difference between the two groups was not significant. In children affected by malaria and in healthy controls, we did not encounter subjects with very low ($< 2 \text{ nmol/mL/h}$) or undetectable plasma CHIT activity.

The correlation between Hb levels, platelet count and CHIT activity both in children with severe and uncomplicated malaria was not significant. A significant correlation ($R^2 = 0.47$, $p < 0.01$) between plasma CHIT activity and serum ferritin ($p < 0.01$) was found only in children with uncomplicated malaria (Fig. 2), while this correlation was not significant ($R^2 = 0.04$) in children with severe malaria (Fig. 3). Viewed from a statistical

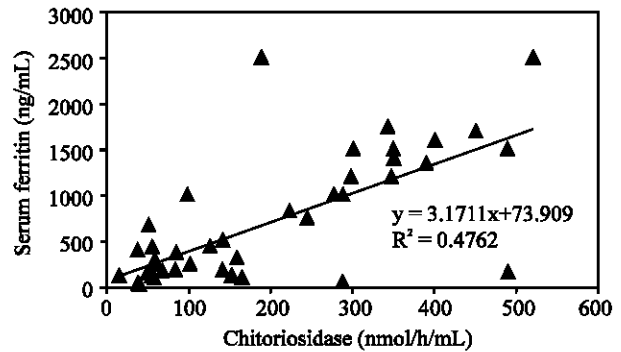


Fig. 2: Correlation between plasma chitotriosidase activity and serum ferritin in 40 children with uncomplicated malaria

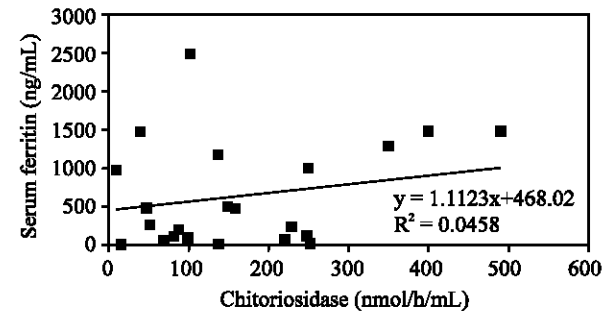


Fig. 3: Correlation between plasma chitotriosidase activity and serum ferritin in 22 children with severe malaria

perspective, this observation might simply reflect the lower sample size of the severe malaria group, but when we made a correction for 'multiple observations' using Bonferroni test the results convinced us that this is not the sort of chance effect.

DISCUSSION

In humans, CHIT is synthesized by activated macrophages and plasma CHIT activity has been proposed as a surrogate marker of macrophage activation^[6]. This has been also confirmed in patients with beta-thalassaemia and other hematological disorders^[5,7]. Odunukwe *et al.*^[8] found in 100 apparently normal children living in Lagos (Nigeria) (2 to 12 years old), a positive correlation between serum ferritin concentration and malaria parasite density ($R^2 = 0.85$, $p < 0.05$). They concluded that the high serum ferritin level in malaria is an expression of macrophage activation as well as the high serum ferritin concentration represents a specific marker of macrophage activation in patients with Still's disease^[9]. In the previous study children affected by acute malaria^[4],

showed a correlation between plasma CHIT activity and the level of serum ferritin confirming that these two parameters might be considered as markers of macrophage activation in presence of *P. falciparum*. In this study both children with severe and uncomplicated malaria had increased CHIT activity respect to healthy controls, but the correlation between plasma CHIT activity and serum ferritin was found only in children with uncomplicated malaria. This initial observation suggests that in the immune response to *P. falciparum* the function of the CHIT gene plays an important role in the cascade of events in which the macrophages activation provide a crucial mechanisms in the defense against malaria^[10]. In fact the difference between uncomplicated and severe malaria suggests two patterns of macrophage response: 1) a group with uncomplicated malaria where the plasma CHIT activity and serum ferritin levels correlated positively; 2) a group with severe malaria, where the CHIT levels did not synchronize with the increase of serum ferritin (Fig. 2 and 3).

If the correlation between serum ferritin and plasma CHIT activity does indeed break down in severe malaria, there are many possible explanations at a biological level. It could be due to a pathological breakdown of gene regulation within the macrophage with regard to the production of these two proteins, or it could also reflect a different metabolism [metabolic role] of these substances in the case of severe sickness. We have previously showed that macrophages of normal subjects stimulated *in vitro* with IFN- γ , TNF and LPS and prolactin secrete an elevated quantity of CHIT^[11,12] and that plasma level of IL-12, modulated by IL-18 and TGF- β 1, has a prognostic significance in the evolution of *P. falciparum* malaria infection^[13,14]. Since in an chimpanzee model it has been demonstrated that IL-12 injection is associated with an enhanced plasma CHIT activity^[15], we cannot rule out the possibility that the elevated levels of IL-12 modulate the macrophage activity by stimulating their anti-parasitic function through a pathway involving plasma CHIT activity.

In such conditions the outcome of malaria infection and of other parasitic disease could be dependent from the CHIT allele composition^[16], which influences the levels of plasma CHIT activity, but the lower CHIT activity observed in 7/22 (31.8%) and in 14/40 (35%) children with uncomplicated and severe malaria (Fig. 2 and 3) cannot be considered a consequence of allele mutation, since in Burkina Faso the percentage of heterozygotes for 24-base pair duplication is only 1-2%^[3]. Thus from these data we can rule out a genetic factor for the diversity in plasma CHIT activity, even if the CHIT activity may be related to

previous exposure to parasite, so that the confounding effect of age in the results presented can not be excluded^[17].

It is generally agreed that *P. falciparum* infection is mediated by the immune system and that a synchronic macrophage response facilitates the favourable outcome of infection with this parasite^[10], but we do not know how the expression of CHIT gene is regulated. The positive correlation between plasma CHIT activity and serum ferritin only in children with uncomplicated *P. falciparum* malaria, suggests a failure of the synchronic macrophage response, which may be an important determinant of disease outcome.

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