Influence of malaria on markers of iron status in children: implications for interpreting iron status in malaria-endemic communities

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To investigate Fe nutritional indices in malaria infection in children, haematology (blood haemoglobin, plasma ferritin, transferrin, Fe, and transferrin saturation), acute phase markers (albumin and caeruloplasmin) and liver function tests were studied in fifty consecutive cases of severe and mild falciparum malaria, fifty matched controls and twenty-three cases of asymptomatic malaria. Blood haemoglobin and transferrin were lower, while ferritin and transferrin saturation were higher, in groups with symptomatic malaria in comparison with the control group. The differences were greatest with the severest form of the disease. There were no differences between any of the groups in plasma Fe. Plasma transferrin correlated directly with albumin in asymptomatic, mild and severe malaria groups (r 0·48, 0·65 and 0·83; $P < 0·05$, $P < 0·01$ and $P < 0·001$ respectively), and inversely with caeruloplasmin (r −0·65, −0·34 and −0·43; $P < 0·01$, $P < 0·05$ and $P < 0·01$ respectively). For ferritin, the correlation was inverse with albumin (r −0·65, −0·57 and −0·64; $P < 0·01$, $P < 0·001$ and $P < 0·001$ respectively and direct with caeruloplasmin (r 0·83, 0·21 and 0·49, $P < 0·001$, NS and $P < 0·001$ respectively). Multiple regression analysis on data from all patients combined indicated that albumin, and to a lesser extent alanine aminotransferase (*EC* 2.6.1.2) activity, explained 62 % of the variance in transferrin. Caeruloplasmin, parasite count and albumin explained 59 % of the variance in ferritin, and transferrin and unconjugated bilirubin explained 62 % of the variance in Fe values. In conclusion, these data suggest that low transferrin and high ferritin values are primarily due to the acute phase response. High transferrin saturation and lack of differences in plasma Fe between the groups are probably due to Fe released from lysed erythrocytes. Finally, in both symptomatic and asymptomatic malaria, indices of Fe status can be misleading and may be especially problematic in community studies in malaria-endemic areas where asymptomatic malaria may be common.

Anaemia: Iron: Malaria: Acute-phase response

Both *Plasmodium falciparum* malaria and anaemia are highly prevalent in and around Rourkela, a city in the eastern part of India. The prevalence of anaemia and Fe deficiency is commonly estimated from the blood haemoglobin level (DeMaeyer, 1989). However, low Fe status is not as easily quantified, for even with a significantly depleted body Fe store, blood haemoglobin may still be acceptable. Serum ferritin concentration, therefore, is taken as a more specific indicator of body Fe status (Lipschitz *et al.* 1974). However, ferritin concentrations can rise following an inflammatory response irrespective of Fe status and, therefore, the combined use of several indicators of body Fe status is expedient in identifying Fe deficiency in such a population (Dallman *et al.* 1981). Currently, haemoglobin, ferritin, transferrin, Fe and transferrin saturation in the blood are commonly measured to assess Fe status.

Malaria may cause severe anaemia due to erythrocyte lysis and there is a consequent fall in blood haemoglobin, even though body Fe stores may not be significantly depleted (Abdalla, 1990). Extensive haemolysis is considered to be one of the important causes of anaemia in falciparum malaria (Abdalla *et al.* 1980; Weatherall & Abdalla, 1982); therefore, the haemoglobin level may not indicate true Fe status. Other markers of Fe status, e.g. serum transferrin, Fe, and ferritin, are also reported to be influenced by the malaria infection (Phillips *et al.* 1986; Aremu, 1989; Adelekan & Thurnham, 1990). However, a complete profile of indicators of Fe status in malaria of varying severity is fairly lacking in the literature and the effects exerted by malaria on the body Fe status remain incompletely understood.

The initial intention of the present study was to examine the influence of clinically mild and severe malaria disease on Fe nutritional indices in a hospital-based study of children suffering from falciparum malaria. However, in the surrounding community, the presence of moderate to severe anaemia (haemoglobin less than 100 g/l) was recorded in 48·8 % of children, and malarial parasites in peripheral blood were detected in 27·6 % of children, with 81 % of them having *P. falciparum*. The great majority (about 75 %) of those infected with malaria were asymptomatic at the time of examination (Ghosh *et al.* 1995). In view of the presence of widespread subclinical malarial infection in the locality around the hospital, the study was extended to include these asymptomatic malaria cases from the community, to examine whether the asymptomatic presence of *P. falciparum* parasites in peripheral blood influence Fe nutritional indices.

SUBJECTS AND METHODS

Subjects

Children aged 2–11 years who were found to have asexual forms of *P. falciparum* in their peripheral blood were considered for the present study. There were three groups of malaria patients, i.e. fifty severe, fifty mild and twenty-three with asymptomatic malaria and fifty control children. Severe and mild malaria cases were recruited from the Paediatric ward of Ispat General Hospital, Rourkela, Orissa, India, during the months October–December 1992 and 1993 and categorized according to the criteria of the World Health Organization, Division of Control of Tropical Disease (1990). The study was approved by the hospital ethical committee.

Severe malaria

Patients were classified as having severe falciparum malaria when one more of the following complications were present.

Cerebral malaria. Unrousable coma not attributable to any other cause and which persisted for at least 30 min after a generalized convulsion to distinguish it from a transient post-ictal coma. The level of consciousness was assessed by the Molyneux *et al.* (1989) modified Glasgow coma scale.

Severe anaemia. Normocytic anaemia with haemoglobin less than 50 g/l. Where a hypochromic microcytic anaemia was present, thalassaemia and common locally prevalent haemoglobinopathies were excluded.

Renal failure. Urine output less than 12 ml/kg per 24 h with a serum creatinine of 265 μmol/l or more.

Circulatory collapse. Systemic blood pressure less than 50 mm Hg with cold clammy skin.

Repeated generalized convulsions. Two or more convulsions within 24 h despite cooling.

Jaundice: Serum bilirubin > 50 μmol/l.

In the present study the severe group comprised the following numbers of cases: cerebral malaria (twenty-one), severe anaemia (eight), haemolytic jaundice (two), peripheral circulatory failure (six) and multiple complications (thirteen).

Mild malaria

This group included children who presented at the hospital with clinical symptoms of malaria (fever, headache, vomiting etc.) and were found to have smear-positive falciparum malaria. They were admitted to the hospital but had none of the previously described complications.

Asymptomatic malaria

This group included twenty-three children with asexual forms of *P. falciparum* in their peripheral blood but without any apparent clinical manifestations at the time of collecting the blood. These cases were recruited from Bisra block near Rourkela, during malaria surveillance conducted by Malaria Research Centre (ICMR) Field Station, Rourkela in September–October 1994.

Control subjects

The controls were fifty healthy children who were matched for age, sex and socio-economic status with the cases of clinical malaria. They were recruited from patients attending the Outpatient Clinic at the hospital for problems related to behaviour disorders like enuresis, anxiety, school phobia, hyperactivity etc. or those attending for routine immunization or for health education and counselling.

Biochemical methods

Venous blood was collected on admission, or, in the case of the field study samples, from subjects after the detection of a falciparum-positive blood smear. In all cases, blood was collected before anti-malarial therapy. The sample was transferred to a heparinized tube for biochemical investigations and into an EDTA-containing tube for haematological investigations. Samples from the field were transported immediately to the laboratory in screw-capped tubes. Malaria parasite density was determined by counting the number of parasitized erythrocytes per 2000 erythrocytes from a thin smear and expressed as percentage parasitaemia. All tests were done immediately except for ferritin, transferrin and Fe. The latter were done every 2 weeks on samples stored at $-20°$.

Haemoglobin, plasma glucose and cholesterol, renal and liver function tests (bilirubin and alanine aminotransferase (*EC* 2.6.1.2; AlAT) were done using test kits (Boehringer Mannheim, Mannheim, Germany) on a Hitachi-705 autoanalyser (Hitachi Ltd, Tokyo, Japan). Caeruloplasmin was estimated from its oxidase activity using *o*-dianisidine dihydrochloride as substrate (Schosinsky *et al.* 1974). Plasma transferrin was estimated by immunoturbidimetry, ferritin by ELISA two-step sandwich assay, Fe by reducing Fe^{3+} to Fe^{2+} with sodium dithionite and developing the colour complex with bathophenanthroline disulfonate. All these tests were done using test kits (Boehringer Mannheim). Precision of

these assays (CV) in our hands was as follows: ferritin 5·3 and 3·4 % at 28 and 467 μg/l, transferrin 6·8 % at 2·87 g/l and Fe 3·1 % at 17·6 μmol/l. Reference ranges for children in this community are close to those shown in Table 1 for the control group. Reference ranges supplied with the manufacturer's test kits are usually for adults and are inappropriate for children.

Statistical analysis

The significance of difference between the groups was assessed by one-way ANOVA, followed by a Student's Newman–Keuls test to assess differences between groups. Data on several of the analytes (see tables) were found to be skewed, and log transformation (to the base 10) was done before any statistical analysis. Correlations were tested using regression analysis for normally distributed data and after log transformation for skewed data.

RESULTS

There were twenty-two female and twenty-eight male children in the control, mild malaria and severe malaria groups and twelve male and eleven female children in those with asymptomatic malaria. The mean ages of the groups did not differ (Table 1). There was no difference in anthropometric measurement of nutritional status between the groups when assessed using percentage of ideal weight-for-age, although all the group means were lower than the corresponding ideal weight-for-age using standards from National Center for Health Statistics (Trowbridge, 1988). Neither hypoglycaemia nor renal failure were present in any of the patients or controls. Plasma albumin was significantly lower in all the malaria groups than in the controls, including the asymptomatic group. Similarly, caeruloplasmin was increased in all the malaria groups, although the difference between the control and the asymptomatic malaria group was not significant. Table 1 shows results for age, percentage of ideal weight, albumin, caeruloplasmin, bilirubin and AlAT, Fe and Fe-carrying proteins in the different groups.

Anaemia, as assessed by blood haemoglobin levels, was present in a significant number of children in all the malaria groups, but it was more common in those with severe malaria. Haemoglobin less than 100 g/l was present in 92 % of children with severe malaria, 60 % of those with mild malaria, 52 % of those with asymptomatic malaria and 18 % in the controls. Plasma Fe and Fe-binding proteins, i.e. haemoglobin, ferritin, transferrin and transferrin saturation, are also shown in Table 1. Mean plasma concentration of ferritin was significantly higher in all malaria groups than in the controls. Transferrin saturation was higher in those with clinical malaria but not in the asymptomatic group. Similarly, although transferrin was lower in all malaria groups, it was not significantly lower in the asymptomatic group. Considered together, the severe group showed the greatest number of abnormalities, but plasma Fe was not significantly different between any of the groups.

In the group with asymptomatic malaria, twenty-one children had haemoglobin concentrations less than 120 g/l, of whom thirteen (61·9 %) had plasma ferritin values more than 30 μg/l. Similarly, twenty-six of the control children had haemoglobin levels less than 120 g/l, of whom eight (30·8 %) children had ferritin values more than 30 μg/l.

To evaluate the mechanism of altered concentration of Fe nutritional indices, correlations between these indices and markers of (1) disease severity indicated by parasite

Table 1. *Nutritional and biochemical profile, iron and iron-binding proteins in Indian children with malaria and in controls**

(Values are means with their standard errors for arithmetic values or geometric means with their standard errors for log (base 10)-transformed values)

Experimental group...	Control		Asymptomatic malaria		Mild malaria		Severe malaria		Statistical significance of difference between groups (ANOVA)
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	P <
Age (years)	7·76[a]	0·42	7·52[a]	0·46	7·66[a]	0·38	7·74[a]	0·41	NS
Percentage of ideal weight	77·04[a]	1·17	77·16[a]	1·72	78·16[a]	1·16	74·49[a]	1·19	NS
Albumin (g/l)	45·5[a]	0·4	39·3[b]	0·8	37·0[b]	1·1	31·3[c]	1·0	0·0001
Caeruloplasmin (U/l)	196·5[a]	6·2	218·0[a]	11·8	273·4[b]	8·0	310·9[c]	9·6	0·0001
Bilirubin (μmol/l)†	6·89[a]	0·37	7·88[a]	0·65	13·89[b]	1·34	19·89[c]	2·26	0·0001
Unconjugated bilirubin (μmol/l)†	3·08[a]	0·20	3·55[a]	0·29	7·39[b]	1·10	11·76[c]	1·83	0·0001
AlAT (U/l)	16·7[a]	0·7	20·7[a,b]	1·4	22·9[a,b]	2·0	25·0[b]	2·4	0·001
Haemoglobin (g/l)	120·7[a]	2·6	98·5[b]	3·5	97·0[b]	2·9	73·3[c]	2·8	0·0001
Ferritin (μg/l)†	27·3[a]	2·0	39·1[b]	7·4	165·2[c]	16·0	410·0[d]	33·3	0·0001
Transferrin (g/l)	3·126[a]	0·097	2·784[a,b]	0·150	2·523[b]	0·127	1·963[c]	0·123	0·0001
Fe (μmol/l)	12·81[a]	0·70	11·55[a]	1·02	14·60[a]	0·86	14·59[a]	0·88	NS
Percentage transferrin saturation†	18·21[a]	0·95	17·86[a]	0·94	26·37[b]	1·37	31·98[c]	1·43	0·0001

[a,b,c,d] Mean values in the same row with unlike superscript letters were significantly different (*post hoc* Student's Newman–Keuls test; $P < 0·05$).

AlAT, alanine aminotransferase (*EC* 2.6.1.2).

* For details of subjects and procedures, see pp. 752–754.

† Log transformation done before statistical analysis for ANOVA test.

Table 2. *Correlations between transferrin and ferritin and markers of disease severity and the acute-phase response in Indian children with malaria*†

	Transferrin			Ferritin		
	Asymptomatic	Mild	Severe	Asymptomatic	Mild	Severe
n...	23	50	50	23	50	50
Parasite count‡	0·289	0·197	−0·287*	−0·210	−0·38**	0·280*
Haemoglobin	0·323	0·005	−0·075	−0·245	0·009	0·135
Transferrin	–	–	–	−0·792***	−0·504***	−0·587***
Ferritin‡	−0·792***	−0·504***	−0·587***	–	–	–
Albumin	0·477*	0·645***	0·834***	−0·645**	−0·574***	−0·642***
Caeruloplasmin	−0·647**	−0·336*	−0·430**	0·826***	0·210	0·487***
AlAT‡	0·300	−0·276*	−0·297*	−0·156	0·131	0·174
Bilirubin‡	−0·119	−0·261	−0·111	0·207	0·109	−0·031
Unconjugated bilirubin‡	−0·074	−0·293*	−0·200	0·091	0·098	−0·034

AlAT, alanine aminotransferase (*EC* 2.6.1.2).
* $P < 0·05$, ** $P < 0·01$, *** $P = 0·001$.
† For details of subjects and procedures, see pp. 752–754.
‡ Transformed variable used to determine the Pearson correlation coefficients.

count, (2) hepatic involvement, e.g. bilirubin and AlAT, (3) Fe nutrition indices such as haemoglobin, ferritin and transferrin, (4) acute-phase proteins (APP; both negative and positive), e.g. albumin and caeruloplasmin, were examined in the different malaria groups separately (Table 2).

Transferrin did not correlate with haemoglobin in any of the malaria groups and was only significantly related to the parasite count in the severe malaria group, unconjugated bilirubin in the mild malaria group, and to AlAT in the groups with clinical malaria. In contrast, transferrin was consistently inversely correlated with ferritin and caeruloplasmin and directly correlated with albumin in all the malaria groups (Table 2). Ferritin did not correlate with haemoglobin, AlAT or bilirubin in any of the malaria groups and was inconsistently correlated with parasite count in those with clinical malaria. Ferritin was, however, consistently inversely correlated with albumin and transferrin in all the malaria groups, and positively correlated with caeruloplasmin, although did not reach significance in those with mild malaria (Table 2).

Plasma Fe did not differ between the groups despite progressively lower transferrin concentration with increasing malaria severity. However, the absence of any difference in plasma Fe between the groups was due to the higher levels of transferrin saturation in the patients with clinical malaria, particularly in those with severe malaria, which compensated for the lower Fe-transport capacity (Table 1).

Since transferrin, Fe and ferritin were correlated with several markers of acute-phase response and disease severity, multiple regression analysis was done to determine the relative importance of the different biochemical indices in explaining the variance in these variables in all malaria patients combined (Table 3). Using this method, all variables listed in Table 3 explained 62 % variance in transferrin, but this was mainly attributable to albumin with a small contribution from AlAT. Similarly, 59 % of the variance in ferritin was explained mainly by caeruloplasmin, parasite count and albumin. In the case of Fe, 62 % of the variance was explained more or less equally by transferrin-unconjugated bilirubin.

Table 3. *Biochemical variables explaining the variance in transferrin, ferritin and iron in Indian children with malaria**†*

Dependent and independent variables‡	β-Intercept					Total variance explained (%)
	Mean	SE	β	T	σ T	
Transferrin						62
Albumin	70·30	9·219	0·584	7·63	0·0000	
AlAT§	−459·28	220·301	−0·125	−2·09	0·0393	
Ferritin§	−310·49	175·770	−0·157	−1·77	0·0800	
(Constant)	2141·30	902·446		2·37	0·0193	
Ferritin§						59
Caeruloplasmin	0·002	4·91 E-04	0·320	4·34	0·0000	
Parasitaemia§	0·208	0·07	0·205	2·96	0·0038	
Albumin	−0·015	0·006	−0·241	−2·53	0·0127	
Transferrin	−9·53 E-05	5·07 E-05	−0·188	−1·88	0·0627	
(Constant)	2·767	0·419		6·597	0·0000	
Fe						62
Transferrin	0·004	6·18 E-04	0·568	6·03	0·0000	
Unconjugated bilirubin§	9·281	0·896	0·677	10·36	0·0000	
Percentage of ideal weight	0·074	0·043	0·104	1·74	0·0847	
(Constant)	−4·475	6·103		−0·733	0·4649	

AlAT, alanine aminotransferase (*EC* 2.6.1.2)

* Multiple regression analyses were carried out on models which included the following variables: percentage of ideal weight, parasite count, haemoglobin, transferrin, transferrin saturation, ferritin, iron, albumin, caeruloplasmin, unconjugated bilirubin, AlAT, except in case of: 'transferrin' where Fe and transferrin saturation are excluded, and 'Fe', where transferrin saturation was excluded.

† For details of subjects and procedures, see pp, 752–754.

‡ Those variables not appearing in the table did not reach significance at $P = 0{\cdot}10$.

§ Transformed variable (log base-10) was used in the regression models.

DISCUSSION

The occurrence of Fe deficiency and anaemia in many tropical countries has been reported to affect 20–30 % of the population (Hercberg *et al.* 1987; DeMaeyer, 1989; Taylor *et al.* 1993). A variety of markers have been used to assess Fe status, including haemoglobin, ferritin and transferrin saturation. In the present study, we have examined some of the difficulties in interpreting markers of Fe status in a malaria-endemic area.

Plasma ferritin concentrations are significantly higher in all the malaria groups than in the controls despite associated anaemia. Ferritin is a positive APP and is known to increase in infections and injury (Harju *et al.* 1984; Fitzsimons & Govostis, 1986). Marked increases in serum ferritin are reported in malaria patients (Phillips *et al.* 1986; Adelekan & Thurnham, 1990). In community-based studies, it is not uncommon for serum ferritin to be high, even in the presence of anaemia. In a recent survey of Indian preschool children, high serum ferritin was positively associated with erythrocyte protoporphyrin and negatively related to the mean corpuscular haemoglobin concentration (Raman *et al.* 1992). These indicators probably reflected Fe deficiency in addition to subclinical infection. Similarly, Taylor *et al.* (1993) observed that 57 % of anaemic Venezuelan children had apparently normal ferritin values, which suggested the presence of subclinical infection since no infection was detected during the medical examination.

Serum ferritin is also increased in chronic diseases and, in order to assess Fe status correctly, the possibility of using another APP, not influenced by changes in Fe metabolism, has been suggested (Witte *et al.* 1988). However, this method for predicting

Fe status was criticized by Coenen *et al.* (1991), and others have suggested that the problem might be better overcome by using a higher cut-off point to identify Fe deficiency where chronic inflammation may also be occurring (Ahluwalia *et al.* 1995).

In the present study a significant number of children with asymptomatic malaria and even a few in the control group had high ferritin concentrations (> 30 μg/l) associated with haemoglobin < 120 g/l. In all the malaria groups, ferritin correlated excellently with markers of acute-phase response, i.e. albumin as well as caeruloplasmin. Albumin has also been shown to predict risk of chronic disease in large studies such as the British Men's Heart Study (Phillips *et al.* 1989) or NHANES Study (Gillum & Makuc, 1992), even though the values are within the normal adult reference range. However, albumin and caeruloplasmin as APP tend to lack specificity and are generally too insensitive or slow-reacting to be useful markers of disease (Thompson *et al.* 1992). In contrast, C-reactive protein, α_1-antichymotrypsin or α_1-acid glycoprotein increase rapidly with infection, and if monitored in combination with ferritin might make it possible to identify inappropriately high ferritin values in such communities. 'Inappropriate' is used here to indicate a plasma ferritin concentration that is greater than expected from the liver Fe stores.

Unlike ferritin, the usefulness of transferrin and transferrin saturation values in monitoring infections, as well as in malaria, has remained controversial. While a study from Nigeria reported elevated transferrin concentration with a decreased saturation with Fe in malaria patients (Aremu, 1989), another in Gambian children did not notice any change in Fe-binding capacity, an indicator of transferrin concentration (Snow *et al.* 1991). Others have reported low transferrin values in acute illness (Fleck & Myers, 1985; Rajamaki *et al.* 1979) as well as in anaemia of chronic diseases (Punnonen *et al.* 1994).

Plasma transferrin in the present study was significantly lower in the groups with mild and severe malaria than in the controls. Similarly, in asymptomatic malaria it was also lower than that in the control group, but the difference was not significant. The low transferrin concentrations are probably a consequence of the acute-phase response, as shown by the correlations with albumin, caeruloplasmin and ferritin, and represent an increase in the permeability of the vascular endothelial barrier to facilitate the movement of small-molecular-weight proteins to move into the extracellular fluid compartment (Fleck & Myers, 1985; Das *et al.* 1996). Such movements on the part of transferrin may facilitate the binding of Fe released from damaged tissues and prevent Fe toxicity. The increased transferrin saturation in clinical malaria despite lower transferrin levels in the present study (Table 1) may be evidence of this possibility.

Plasma Fe concentration was not altered to any significant extent in the malaria patients in comparison with the controls. A low Fe content of plasma is commonly noticed in infection (Keusch, 1990) and surgical stress (Fitzsimons & Govostis, 1986) and is attributed to decreased transferrin saturation (Keusch, 1990). In the present study, raised transferrin saturation encountered in malaria patients is probably due to continuing erythrocyte lysis. Extensive haemolysis is considered to be one of the important causes of anaemia in falciparum malaria (Abdalla *et al.* 1980). Fe released from the haemoglobin of ruptured erythrocytes is taken up by the macrophages and incorporated into transferrin, increasing its saturation with Fe. This is supported by the observation that transferrin saturation was positively correlated with unconjugated bilirubin in the malaria-infected subjects (r 0·82, n 123; $P < 0·001$), a commonly-used marker of haemolysis. In addition, there was also a positive correlation between percentage transferrin saturation and haemoglobin in the control group (r 0·827; $P < 0·001$) and a negative correlation in patients with severe malaria (r −0·555; $P < 0·01$), indicating that while in control

subjects transferrin saturation indicates Fe availability, in severe malaria patients the inverse correlation indicates haemolysis.

In conclusion, in both acute and asymptomatic malaria infection, assessment of Fe status using the present cut-off points for ferritin, transferrin and transferrin saturation appears unreliable. However, measurement of ferritin concentrations in conjunction with another APP may be a practical alternative to indicate the possible cause of high ferritin values.

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