Estimation of the sequestered parasite load in severe malaria patients using both host and parasite markers

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SUMMARY

The virulence of the malaria parasite *Plasmodium falciparum* is due, in part, to its ability to cytoadhere in deep vascular beds. Our inability to quantify the load of sequestered parasites hampers our understanding of the pathophysiological mechanisms involved in disease progression and complicates diagnosis. In this study we evaluate potential biochemical markers of sequestered load by comparing them with estimates of the sequestered load from a statistical model fitted to longitudinal patterns of peripheral parasite densities in a series of 22 patients with severe *Plasmodium falciparum* malaria. The markers comprised the host factors: haematocrit, circulating host DNA, sTNF-R75 and parasite derived products HRP2, pLDH, pigments and circulating parasite DNA. We investigated the suitability of these markers in determining sequestered loads in patients on quinine treatment. Observed peripheral parasitaemia, plasma levels of sTNF-R75 and circulating parasite DNA were most strongly correlated with estimates of sequestered loads on admission. However the dynamics of both sTNF-R75 and circulating parasite DNA during follow-up were very different from those of the estimated sequestered mass. These analyses suggest that none of the markers gave reliable estimates of the current sequestered load, though they may reflect the history of infection. Longitudinal analyses are needed that allow for the clearance rates of the marker molecules and for variations between hosts in the history of parasitaemia.

Key words: *Plasmodium falciparum*, sequestration, biochemical and parasitological markers, statistical models.

INTRODUCTION

Plasmodium falciparum is by far the most virulent malaria parasite infecting humans, being responsible for almost all malaria-related deaths. The particular virulence of *P. falciparum* is believed to be due to the ability of red blood cells containing mature stages of the parasite to sequester in deep vascular beds, through a process of cytoadherance to vascular endothelium (Aikawa, 1988; Sein *et al*. 1993; Sherman, Eda and Winograd, 2003). Sequestration of mature stages makes it difficult to estimate the true parasite burden that is likely to be an important prognostic indicator, and in extreme cases where all parasites are sequestered at a particular time-point, it may complicate diagnosis.

Both *in vitro* and *in vivo* studies have shown that parasite products including histidine-rich protein-2 (HRP2) (Parra, Evans and Taylor, 1991; Desakorn *et al*. 1997; Kilian *et al*. 1999), parasite lactate dehydrogenase (pLDH) (Makler and Hinrichs, 1993; Piper *et al*. 1999), malaria pigment (Day *et al*. 1996; Lyke *et al*. 2003), circulating parasite DNA (Gal *et al*. 2001) are released into circulation when schizonts rupture. Assays of some of these components form the basis of diagnostic tests that have been, or are being developed as alternatives to microscopic examination of blood films. Quantitation of these substances represents one possible approach for measuring total parasite burdens.

Measurement of host products released as a result of sequestration might also be used to estimate the sequestered load. Sequestration and schizont rupture precipitate a cascade of events leading to an acute phase response within the host, including up-regulation of cytokines such as the interleukins IL-1β, IL-6, IL-10, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) (Chen, Schlichtherle and Wahlgren, 2000; Vogetseder *et al*. 2004) and its receptors soluble sTNF-R75 and sTNF-R55 (Hurt *et al*. 1995; McGuire *et al*. 1998). Tissue damage leads to the release of circulating host DNA and might be a marker of pathology (Saiki *et al*. 1985). In principle, quantification of such markers might be used to estimate the sequestered parasite load. By radio-isotope labelling of erythrocytes, Davis *et al*. (1990) found that the venous haematocrit provided an indirect estimate of total sequestered volume in a small number of Thai patients. However, no studies to date have validated measures of levels of any host or parasite factors as markers of the sequestered parasite load.

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Table 1. Biochemical and parasitological markers used to assess the sequestered parasite load

(RBCs, red blood cells; IRBCs, infected red blood cells; WBCs, white blood cells; FBC, full blood count analysis; Hb, haemoglobin; PMNs, polymorphonucleated cells; mono, monocytes; PCV, packed cell volume.)

Markers	Method	Type of sample	Rationale	Reference for method
Peripheral parasitaemia	Light microscopy	Giemsa-stained thick and thin blood smears	Assess the quantity of parasites in peripheral blood for model	
Candidate parasite markers of sequestration				
HRP2	Sandwich ELISA, to quantify this protein in ng/ml (L. B. Ochola, unpublished observations)	Plasma	HRP2 is released from growing IRBCs into blood circulation. HRP2 levels are elevated during schizont stage and could relate to sequestered load.	(Parra *et al*. 1991; Desakorn *et al*. 1997)
pLDH	An immunocapture based ELISA measuring pLDH as rate of enzyme activity, OD/ min based on the method by Makler & Hinrichs, 1993 (L. B. Ochola, unpublished observations)	Plasma	*In vitro* measurement has shown that pLDH is measurable during schizont rupture and could relate to estimates of sequestered load	Makler and Hinrichs, 1993; Piper *et al*. 1999
		RBCs	pLDH enzyme activity in IRBC reflects circu-lating parasites and are thus a direct measure of parasitaemia.	
Pigmented PMNs	Count number of pigments/200 PMNs (units counts/μl)	Giemsa stained thick blood smears	The malarial parasite metabolizes haemoglobin and the by-product is pigment. At schizont rupture this is released into blood circulation and is engulfed by WBCs so quantification of this could relate to sequestered load.	(Nguyen *et al*. 1995; Day *et al*. 1996; Lyke *et al*. 2003)
Pigmented Monos	Count number of pigments/200 mono (units counts/μl)			
Circulating pDNA	Quantify using real-time PCR results in genomes/μl, using the method of (Gal *et al*. 2001)	Plasma	During schizont rupture circulating pDNA is released into supernatant of *in vitro* cultures and this could relate to the sequestered mass. Previous work has quantified parasite DNA in plasma from patients.	(Gal *et al*. 2001)
Candidate host markers of sequestration				
PCV	FBC	Whole blood	Haematocrit rapidly falls in patients with severe disease due to destruction of red blood cells. Davis and others (1990) claimed that PCV provides an indirect estimate of sequestered load	(Davis *et al*. 1990)
sTNF-R75	ELISA kit from Biosource (sTNF-RII EASIA kit, KAC 1772), in ng/ml	Plasma	Schizont rupture causes release of TNF-α and pyrogenic factors. Soluble TNFs forms complexes with TNF-α acting as an antagonist. sTNF-R75 levels could correlate with estimates of sequestered load	(Hurt *et al*. 1995)
Circulating host DNA	Quantify using real-time PCR results given as ng/ml, using the method of (Gal *et al*. 2001)	Plasma	Malarial infections can lead to damage of tissues within the host leading to pathology	Gal *et al*. (personal communication) (Saiki *et al*. 1985)

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We have studied 22 severe *P. falciparum* malaria patients in Kilifi, Kenya and made estimates of the sequestered load during therapy, using a range of candidate host and parasite markers. We have attempted to optimize these estimates by comparing them with each other and with corresponding estimates from a statistical model fitted to the longitudinal patterns of peripheral parasite densities.

MATERIALS AND METHODS

Patients and blood sampling

Twenty-two children under the age of 6 years admitted to the high dependency unit (HDU) in Kilifi District Hospital were recruited into this study. Children were defined as having severe malaria if they were malaria slide positive (*P. falciparum*), had no other detected cause for their illness and had one of the following: (1) prostration, (2) respiratory distress or (3) severe anaemia (haemoglobin less than 5 g/per dl) (Marsh *et al*. 1995). Parental consent was obtained. All patients were treated with intravenous quinine and received full supportive therapy as described elsewhere (Murphy *et al*. 1995).

Peripheral parasite densities were assessed every 4 h over 48 h and 0·5 ml of venous blood was taken every 8 h. This was separated into red blood cell (RBC) pellet and plasma and frozen initially at −20 °C before being transferred to −80 °C.

Analyses of biochemical and parasitological markers

Samples were tested for a range of potential biochemical markers of the sequestered parasite load (Table 1).

Associations between markers. We anticipated that measures of different markers of parasite sequestration should be correlated with each other, and tested the associations at admission using Spearman correlations. Since the levels of almost all the markers were correlated with admission parasitaemia, in addition we used partial (Spearman) correlations allowing for the admission parasitaemia, to test whether the levels of the remaining markers were correlated.

Estimates of sequestered load from longitudinal peripheral parasitaemia profiles. Estimates of the sequestered load were derived using the longitudinal pattern of peripheral parasitaemia, and also using the baseline levels of those parasitological and biochemical markers that were most correlated with this estimate of the sequestered load.

We fitted a discrete-time age-stage model recently used to estimate the sequestered parasite load in severe malaria patients in Kilifi (Smith *et al*. 2004). This approach uses 4 hourly peripheral parasitaemia determinations to provide estimates of the numbers of parasites during therapy of the patients (Smith *et al*. 2004). It differs from other recent statistical models used to address this problem (Gravenor, van Hensbroek and Kwiatkowski, 1998; Gravenor *et al*. 2002) in assuming a fixed 48-h duration of the asexual cycle of the parasite, and uses literature-based (ter Kuile *et al*. 1993) estimates of pharmacodynamic and pharmacokinetic parameters. The model is fitted using a Markov chain Monte Carlo approach that allows it to be fitted simultaneously to the entire data set allowing point and interval estimates for both population and individual patient parameters.

Using this model we obtained estimates (of the sequestered mass S_{1i}) for the 4-h time-period immediately after hospital admission i.e. the number of sequestered IRBCs in full for first use per unit of blood volume at baseline for patient *i*. Full details of this model have been provided by Smith *et al*. (2004).

Estimates of sequestered load from peripheral parasitaemia, circulating parasite DNA and sTNF-R75

We used the levels of the biochemical markers at baseline to make estimates of the sequestered loads (see Appendix). Estimates were made only for those markers that correlated with S_{1i}, the estimated sequestered load based on the longitudinal model in patient *i*. These comprised the levels of peripheral parasitaemia, circulating parasite DNA (pDNA), sTNF-R75 which were used to derive estimates S_{2i}, S_{3i} and S_{4i}, respectively of the sequestered mass. In addition we derived further estimates S_{5i} and S_{6i} by combining the data of S_{2i}, S_{3i} and S_{4i} and S_{3i}, S_{4i} respectively (Appendix).

RESULTS

Clinical data and marker profiles

Data were available for a total of 22 children, with median age 2·6 years (interquartile range 1–6 years), with a wide range of levels of admission parasitaemia (Table 2). All but 2 children were discharged alive from hospital. One had a final diagnosis of encephalopathy while the other patient had a co-infection of *P. falciparum* and *Salmonella* spp. and final diagnosis of septicaemia/sepsis. The measurement of the various markers in admission samples and the mean longitudinal patterns in this population during follow up are shown in Table 2 and Fig. 1 respectively. Longitudinal patterns within this population revealed the persistence of HRP2, pigment in monocytes, sTNF-R75 and host DNA over the 2 days (Fig. 1: A, F, G, H) while circulating pDNA, pLDH activity measured in RBCs and pigment

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Table 2. Admission clinical and laboratory data from 22 patients with severe malaria

(PMNs, polymorphonucleated cells; mono, monocytes.)

	Median (Inter-Quartile Range)
Parasite count (per/μl)	157 220 (217 50–526 920)
HRP2 (ng/ml)	63·08 (10·37–161·25)
pLDH in plasma (OD/min)	1·75 (0·02–4·98)
pLDH in RBCs (OD/min)	31·74 (17·5–74·52)
Total no. of pigmented PMNs/μl	0 (0–389·75)
Total no. of pigmented monos/μl	277 (74·25–861·75)
sTNF-R75 (ng/ml)	55·75 (44·45–74·6)
Circulating pDNA (genomes/μl $\times 10^5$)†	524·89 (205·58–1838·5)
Circulating host DNA (ng/ml)†	284·27 (175·02–719·29)
Venous haematocrit*	25·75 (19·78–32·1)

* As a percentage.
† Assessed in 21 patients.

in polymorphonucleated cells (PMNs) declined over this time (Fig. 1: B, C, E). pLDH activity in plasma increased 8 h after admission, probably due to release of the enzyme from rupturing schizonts from the sequestered mass. Estimates of the sequestered load declined more rapidly than peripheral parasitaemia during the 2 days (Fig. 1: I and J).

Associations between estimated sequestered parameters and markers

The analysis of the Spearman correlations between the various markers on admission confirmed that the pLDH levels in plasma were not correlated with that in erythrocytes (Table 3A), suggesting that plasma pLDH does not arise simply from lysis of a constant proportion of the infected erythrocytes. HRP2 correlated positively with all other markers except PCV. The PCV is reduced by high parasitaemia and showed negative correlations with most markers. The amounts of pigment in the two different cell types were strongly correlated with each other, but showed somewhat different patterns of correlation with the other markers, with pigment counts in PMNs most strongly correlated with the parasite density. The levels of circulating pDNA correlated with circulating host DNA.

The model fitted to the longitudinal patterns of parasitaemia gave estimates of the sequestered load at each time-point during follow-up (Fig. 1). We initially considered good candidate markers of sequestration to be those that showed a strong correlation with the estimates of sequestered load on admission. Significant correlations were observed with the level of pLDH measured in RBCs, sTNF-R75, HRP2, circulating parasite DNA and pigment in PMNs. The measured peripheral parasite density was most strongly correlated with S_{1i}. We carried out a partial correlation analysis, adjusting for the initial peripheral densities to identify those markers that provide information about the sequestered load additional to that contained in the baseline parasitaemia (Table 3B). This analysis found a significant residual correlation only with the level of sTNF-R75. The next highest correlation was with the level of pLDH in erythrocytes, and third highest with circulating pDNA.

The kinetics of HRP2 levels during follow-up are clearly very different from those of sequestered parasites (Fig. 1A), so we did not consider this marker further as a candidate indicator of sequestration. pLDH in RBCs was also eliminated as a candidate because the levels in erythrocytes directly measures the peripheral parasite density (Fig. 1C), while those in plasma show little correlation with either peripheral parasitaemia or S_{1i} (Fig. 1D). Although the levels of pigment in PMNs showed a decline with time since admission, similar to that for S_{1i} (Fig. 1E), the baseline levels were negatively correlated with S_{1i} when adjusted for the levels of parasitaemia. We therefore further considered only estimates of the sequestered load that made use of circulating pDNA, the sTNF-R75 and peripheral densities.

Concordance between different estimates of levels of sequestration at admission

The agreement between the distinct estimates of the sequestered load, S_{1i} was assessed by computing concordance correlation coefficients (CCC) between the logarithmically transformed estimates (Shoukri and Pause, 1999). This method is valuable when precision and accuracy are required in the evaluation of two methods that attempt to measure the same thing. As a graphical indication of agreement with the estimate derived from the longitudinal pattern of parasitaemia, S_{1i}, we also plotted the values of $\ln(\ln S_{1i} - \ln S_{ki})/2$ against $\ln(\ln S_{1i} + \ln S_{ki})/2$ for each of the other measures, $k = 2 \ldots 5$ (Fig. 2).

Both the peripheral density and the circulating pDNA level appear to be directly proportional to S_{1i} (Fig. 3) justifying the derivation of estimates S_{2i} and S_{3i} that assume direct proportionality. The sTNF-R75 levels were strongly correlated with S_{1i} but the relationship was not one of direct proportionality, thus requiring the regression model of equation 3 in order to obtain an estimate, S_{4i}, of the sequestered load from the sTNF-R75 levels.

Concordance correlation coefficients

The plots of the differences between the logarithmically transformed estimates S_{2i}, S_{3i}, S_{4i}, and S_{1i} against the averages, confirmed that these estimates

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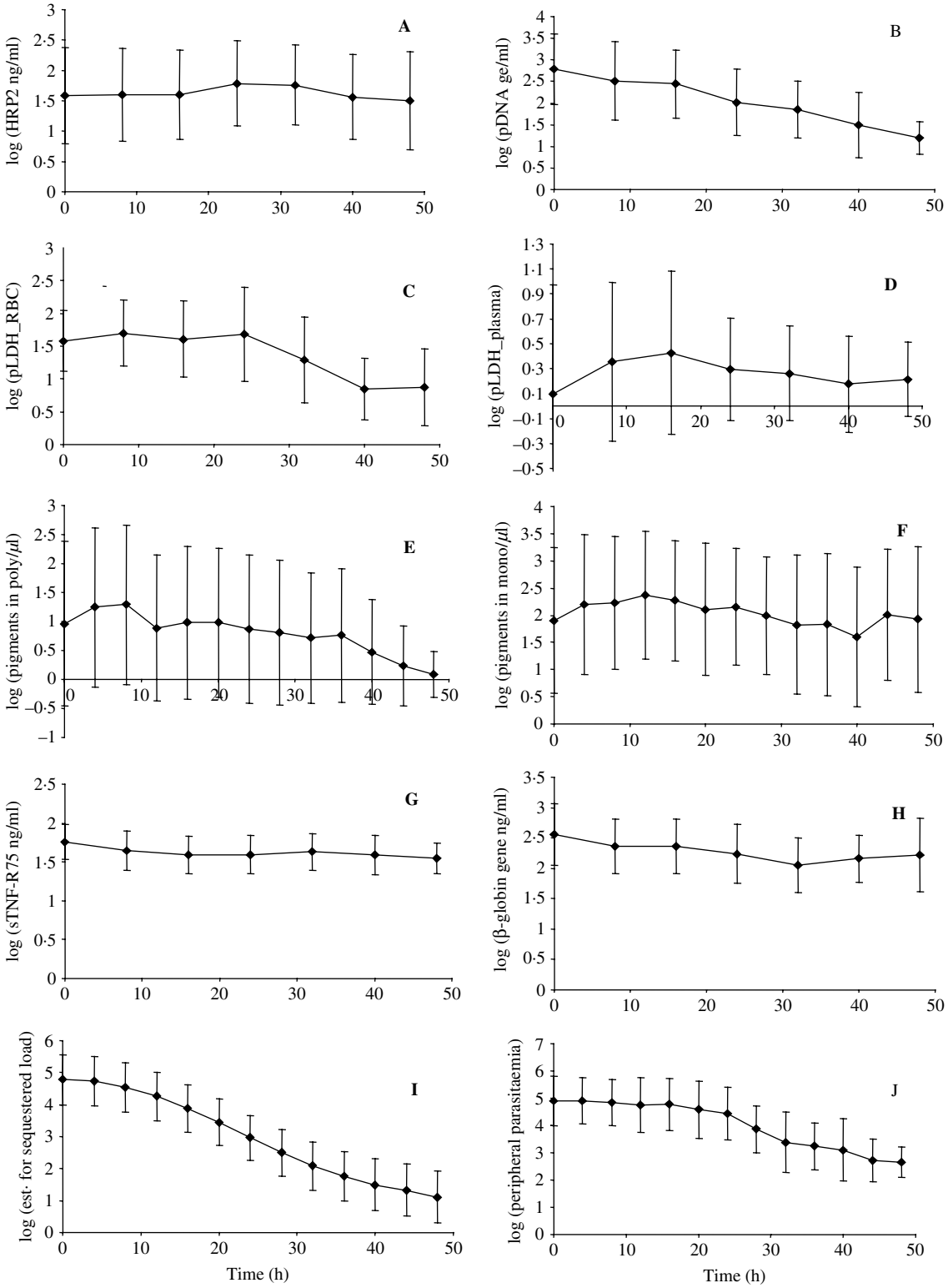

Fig. 1. Longitudinal plots for markers (A) HRP2, (B) circulating pDNA, (C) pLDH activity in RBC, (D) pLDH activity in plasma, (E) total number of pigment in PMNs, (F) total number of pigments in monocytes, (G) sTNF-R75, (H) circulating host DNA and (I) estimate of sequestered load S_{1i} and (J) peripheral parasitaemia. Error bars represent mean at each time-point and standard deviation.

of sequestered loads were approximately directly proportional to each other (Fig. 2). The analysis of levels of agreement showed that the inclusion of the sTNF-R75 data in the estimation gave stronger agreement with S_{1i} than could be obtained using just the peripheral parasitaemia. No more improvement was made by including in addition the circulating pDNA data (Table 4).

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Table 3. Spearman Correlation between levels of markers on admission

(A) Correlations unadjusted for peripheral parasite density

	Measured peripheral parasitaemia	S_{1i}	HRP2	pLDH_P	pLDH_R	PCV	Poly/μl	Mono/μl	sTNF-R75	pDNA	Human DNA
S_{1i}	0·89***	1·00									
HRP2	0·59**	0·63**	1·00								
pLDH_P	0·11	0·16	0·48*	1·00							
pLDH_R	0·69***	0·73***	0·76***	−0·04	1·00						
PCV	−0·22	−0·26	−0·38	0·07	−0·60**	1·00					
Poly/μl	0·70***	0·56**	0·60**	0·15	0·57**	−0·28	1·00				
Mono/μl	0·33	0·31	0·67***	0·16	0·54**	−0·49*	0·69***	1·00			
sTNF-R75	0·65***	0·72***	0·70***	0·40	0·63*	−0·19	0·25	0·13	1·00		
pDNA	0·46*	0·53**	0·58**	0·36	0·37	0·13	0·08	0·03	0·67**	1·00	
Human DNA	0·49*	0·42*	0·57**	0·34	0·31	0·18	0·38	0·31	0·56**	0·60**	1·00

(B) Partial correlations adjusted for admission peripheral parasite density

	S_{1i}	HRP2	pLDH in plasma	pLDH in RBC	PCV	Poly/μl	Mono/μl	sTNF-R75	pDNA	Human DNA
S_{1i}	1·00									
HRP2	0·28	1·00								
pLDH_P	0·12	0·51*	1·00							
pLDH_R	0·31	0·61**	−0·11	1·00						
PCV	−0·13	−0·32	0·09	−0·63**	1·00					
Poly/μl	−0·25	0·32	0·05	0·21	−0·22	1·00				
Mono/μl	0·01	0·61**	0·07	0·50*	−0·51*	0·66**	1·00			
sTNF-R75	0·44*	0·52*	0·48*	0·32	−0·04	−0·32	−0·05	1·00		
pDNA	0·30	0·43	0·37	0·07	0·27	−0·39	−0·15	0·57**	1·00	
Human DNA	−0·02	0·40	0·34	−0·01	0·32	0·08	0·23	0·57**	0·41	1·00

S_{1i} is the estimate of sequestered load based on analysis of longitudinal patterns of parasitaemia; pLDH_P, pLDH,in plasma; pLDH_R, pLDH in RBC PCV, packed cell volume; PMNs, polymorphonucleated cells/μl; mono/μl monocytes/μl; pDNA, circulating parasite DNA; human DNA, circulating host DNA.
* $P<0·05$, ** $P<0·01$, *** $P<0·001$.

DISCUSSION

Peripheral counts of *P. falciparum* parasitaemia are in general related to disease severity, yet in endemic areas, asymptomatic parasitaemia with high densities are common and up to 37% has been recorded (Warrell D. A., Molyneux and Beales, 1990). On the other hand, those admitted to hospital with severe disease or coma, may have parasitaemias as low as 0·01% (White, 2002). Much of this variation in outcome associated with the measured parasite load is presumed to be due to differences in the proportions of parasites that are sequestered. We have now assessed a range of biochemical markers as predictors of the extent of sequestration in a series of patients.

The parasite protein levels that we quantified are the most obvious candidates as measures of the sequestered load. As expected and in keeping with other studies, levels of the pLDH glycolytic enzyme in RBCs were strongly associated with peripheral parasite density (Makler and Hinrichs, 1993; Piper *et al.* 1999). *In vitro* studies have revealed that pLDH is released during lysis of RBCs (unpublished observations) and levels in plasma should therefore be a direct measure of the rate of schizont rupture. However, as a consequence of a short half-life of the free protein in the circulation, plasma levels of pLDH were much lower than those in RBCs, making it difficult to measure them. Plasma pLDH on admission was poorly correlated with both the observed peripheral parasitaemia and the estimates of sequestration *in vivo*. The partial correlations with S_{1i}, allowing for the peripheral parasitaemia, suggested that neither pLDH in RBCs nor in plasma added much information about sequestered loads on admission to that provided by the peripheral parasite counts. The increase in plasma pLDH in most patients following admission may reflect the fact that it is released only at the very end of the parasite cycle. Further analyses of the post-admission kinetics are needed to test this.

In vitro studies have demonstrated release of HRP2 from rings, trophozoite and schizont parasite stages (Howard *et al.* 1986; Desakorn *et al.* 1997,

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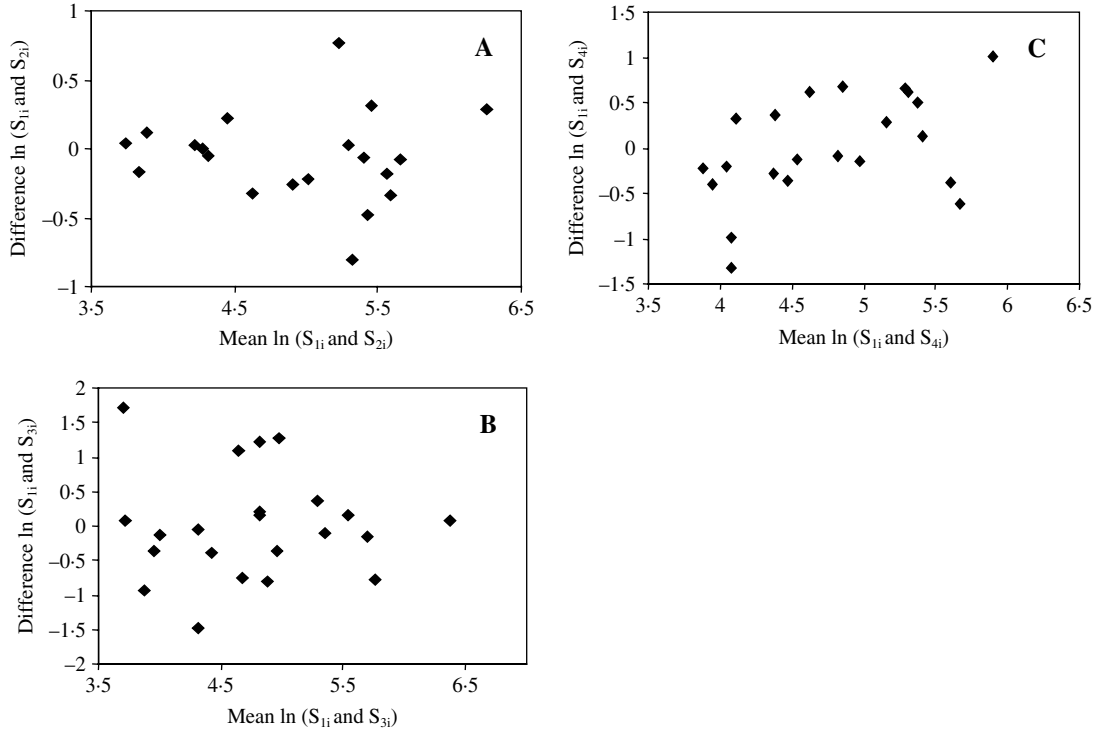

Fig. 2. Bland–Altman plots at baseline; differences between duplicate measurements plotted against their average. (A) Estimate of sequestered load (S_{1i}) and alternative estimate of sequestered using peripheral parasitaemia (S_{2i}), $\rho_c = 0{\cdot}91$. (B) Estimate of sequestered load (S_{1i}) and alternative estimate of sequestered using circulating pDNA (S_{3i}), $\rho_c = 0{\cdot}53$. (C) Estimate of sequestered load (S_{1i}) and alternative estimate of sequestered using sTNF-R75 (S_{4i}) $\rho_c = 0{\cdot}65$.

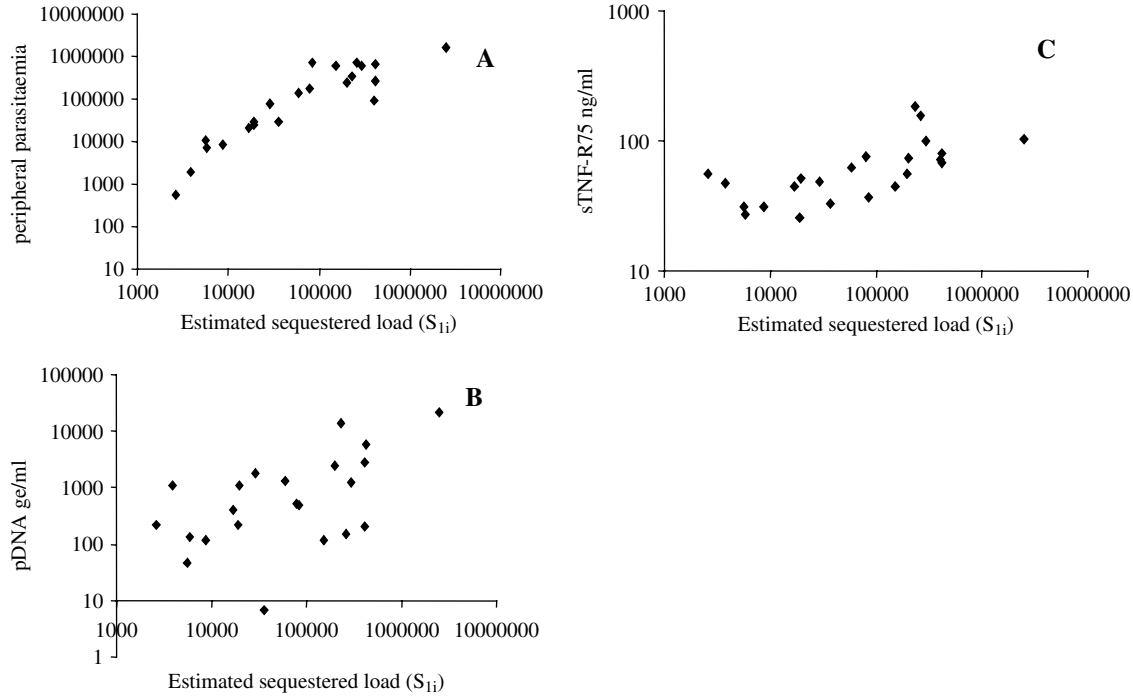

Fig. 3. Baseline data for markers plotted against estimate from longitudinal pattern of sequestered load (S_{1i}) and (A) observed peripheral parasitaemia, (B) measured circulating pDNA and (C) measured sTNF-R75.

2005) and *in vivo* studies have shown that it correlates with parasitaemia (Desakorn *et al*. 1997). It has been proposed that it is a good marker of the level of sequestration in Thai patients (Dondorp *et al*. 2005). We confirmed the correlation with levels of peripheral parasitaemia, but found only a weak relationship with the estimated sequestered load at admission. HRP2 persists in the bloodstream for at

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Table 4. Concordance correlation coefficients of estimates of the log density of sequestered parasites

(Obs. peri-observed peripheral parasitaemia; sTNF-sTNF-R75; pDNA, circulating parasite DNA.)

	sTNF	pDNA	Obs. peri	sTNF and peri	S_{1i}
sTNF	1·00				
pDNA	0·58	1·00			
Obs. Peri	0·52	0·46	1·00		
sTNF & peri	0·71	0·54	0·96	1·00	
S_{1i}	0·65	0·53	0·91	0·94	1·00

least 2 weeks (Mayxay *et al*. 2001), and in our study the average level showed no decay during the follow-up, so its dynamics indicate that it cannot provide a good estimate of concurrent levels of sequestration.

Haemozoin is released during schizont rupture, phagocytosed initially by scavenger PMNs and subsequently taken up into monocytes (Schwarzer *et al*. 1992; Day *et al*. 1996). Day and others (1996) showed that pigment measured in PMNs have clearance times of 49–95 h, while pigments in monocytes have 180–240 h. We observed a rapid decline in pigmented PMNs while there was no overall change with time since admission in the number of pigmented monocytes. Pigment levels counted in PMNs were better correlated with peripheral parasitaemia than those in monocytes. On the other hand, pigment levels were not correlated with our model-based estimates of sequestered loads in analyses in which peripheral density was adjusted. It is known that pigment can remain attached to remnants of the IRBCs, which are attached to endothelia, from where it is later cleared by circulating WBCs. Consequently, a number of factors other than the rate of schizont rupture have a major influence on the amounts that can be measured and this limits the value of pigment assessment in quantifying schizogony or sequestration.

In view of these problems with the use of established diagnostic assays to estimate sequestered loads, we considered quantitation of circulating pDNA as an alternative. Studies on circulating pDNA have focused mainly on whole blood (Lee *et al*. 2002; Richardson *et al*. 2002; Perandin *et al*. 2004) yet it can also be measured in plasma (Gal *et al*. 2001). Circulating pDNA levels in plasma were correlated with peripheral parasitaemia, with the estimate, S_{1i}, of sequestered loads. The moderate correlation with S_{1i} encouraged us to develop the estimate S_{3i}, based on the circulating pDNA level, but the scatter in the relationships between circulating pDNA levels and the other estimates raises questions as to whether this is because of variation in circulating pDNA levels, due to other causes, or because the other estimates, S_{1i} and S_{2i}, are themselves imprecise. Circulating pDNA levels in plasma declined much faster than did S_{1i} suggesting that the release of circulating pDNA from ruptured schizonts does not explain the variation in levels.

sTNF-R75 levels have previously been shown to correlate with parasite density in both symptomatic and asymptomatic children (Leeuwenberg, Dentener and Buurman, 1994; Hurt *et al*. 1995). Since TNF-α release is a key part of the whole cascade of events precipated by schizont rupture, we anticipated that the level of this receptor should correlate with the recent sequestered load. Making use of the relationships between sTNF-R75 levels and the estimates S_{1i}, S_{2i}, and S_{3i} of the sequestered load, we therefore derived the further estimates S_{4i}, and S_{6i}. As with HRP2, the average level showed no decay during the follow-up, so the dynamics of sTNF-R75 argue against its value as an indicator of concurrent levels of sequestration.

We considered a number of other host markers. Davis *et al*. (1990) found that anaemia levels could be used to measure sequestration in Thai patients. However, these patients were unlikely to have suffered a long history of repeated infections and so their PCVs reflected very recent events. Only weak relationships between PCV and levels of parasitaemia were found in our more highly endemic population. The PCV measures the extent of red cell destruction and so might be expected to correlate with sequestration over an extended period, but variations during the time course of an infection are very small in Kilifi, and so it is unsurprising that the PCV appears to be a poor marker of current sequestered loads. Gal and others (personal communications) showed that circulating host DNA measured in plasma is not elevated in severe malaria and we have further shown that it is not associated with estimates of sequestered load.

Together with peripheral parasitaemia, measures of sTNF-R75 and of circulating pDNA therefore appear most useful as predictors of the sequestered load on admission. These markers do not appear to be good indicators of changes in sequestered loads during the course of a clinical episode. We were particularly disappointed by the lack of relationship between pLDH levels in plasma and the estimated sequestered loads. However, we may well have undervalued some markers by assuming the validity of the model for longitudinal patterns of parasitaemia and ignoring the dynamics of the markers. At the price of making the analyses even more complex, it would be possible to model these dynamics using knowledge from *in vitro* studies of the stage specificity of release and of drug effects, and to allow for the estimated pre-treatment rate of expansion of the parasite population.

The various markers also need to be evaluated as measures of population average levels of sequestration in the absence of acute illness. Highly

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persistent markers, such as HRP2 may be particularly appropriate for this purpose. The identification of a suitable method for estimating sequestered loads of *P. falciparum* remains a major challenge.

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