

## LOW PREVALENCE OF AN ACUTE PHASE RESPONSE IN ASYMPTOMATIC CHILDREN FROM A MALARIA-ENDEMIC AREA OF PAPUA NEW GUINEA

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**Abstract.** Levels of C-reactive protein (CRP), a classic marker for the acute phase response (APR), were measured in children with asymptomatic malaria infection in the Amele region of Papua New Guinea (PNG). Despite the presence of parasitemia, the prevalence of CRP levels consistent with an APR (CRP > 10 (xg/mL) was very low (< 10%). Splenomegaly was significantly associated with increased parasitemia ( $P < 0.001$ ) and CRP levels ( $P < 0.001$ ), highlighting the importance of splenomegaly as an indicator of recent high density infection in this population. Multivariate analysis showed that CRP levels were significantly associated with splenomegaly, fever, hemoglobin, and age ( $P < 0.002$ ). CRP levels also increased with increasing parasitemia ( $P < 0.001$ ) but remained < 3.5 (xg/mL). The low levels of CRP indicate that children in the Amele modulate inflammation associated with malaria.

### INTRODUCTION

Acute phase proteins (APPs) are serum proteins, the concentrations of which change during the acute phase response (APR) to a variety of stimuli, such as infection and other inflammatory reactions. APPs are predominantly synthesized by the liver with interleukin (IL)-6 as the principal cytokine inducing production.<sup>1</sup> IL-1, IL-8, tumor necrosis factor- $\alpha$ , interferon $\gamma$ , leukemia inhibitory factor, and transforming growth factor- $\beta$  can also regulate APP production.<sup>1</sup> APPs are classified on the basis of how they change during the APR. Proteins that decrease are classified as negative APPs, whereas proteins that increase are classified as positive APPs. Albumin is a negative APP because its synthesis is significantly reduced by IL-6 during the APR.<sup>2</sup> Positive APPs include C-reactive protein (CRP),  $\alpha$ -antitrypsin, ferritin, and haptoglobin (Hp). During acute inflammation, CRP concentrations increase 100- to 1,000-fold, whereas  $\alpha$ -antitrypsin, ferritin, and Hp concentrations increase 2- to 4-fold during an APR.<sup>1</sup> CRP is therefore a classic marker for inflammation, and an APR is often defined as CRP > 10 (xg/mL).<sup>1,2</sup>

When malaria schizonts rupture, host monocytes and macrophages secrete pro-inflammatory cytokines stimulating the production of CRP.<sup>3,4</sup> Strong correlations between CRP and malaria parasitemia have been shown, even in afebrile individuals.<sup>5–8</sup> Previous studies in African populations show that children in malaria-endemic areas have a chronic inflammatory response to continued and prolonged exposure to *Plasmodium* infections as evidenced by the high prevalences of APR.<sup>9–12</sup> The APR has not been studied in malaria-endemic communities outside Africa. Consequently, we measured concentrations of CRP,  $\alpha$ -antitrypsin, and albumin in a cohort of children living in the north coastal region of Papua New Guinea (PNG). Children living in this area harbor chronic, asymptomatic malaria infections with clinical episodes associated with fever, high parasitemia, and splenomegaly.<sup>13,14</sup> We examined the relationship of the above-mentioned APPs with demographics, malariometric indices, and host polymor-

phisms in a cohort of children from the Amele region, PNG, where we have previously reported concentrations of the APP Hp.<sup>15</sup>

### MATERIALS AND METHODS

**Study site.** The study was conducted in villages of the north coastal Amele region of PNG where malaria transmission is intense.<sup>16</sup> The region has a tropical climate with high annual rainfall and year round malaria transmission, with a peak recognized between October and May. The EIR (*P. falciparum* and *P. vivax*) in this region is estimated at 0.86 infective bite/person/night.<sup>17</sup> Semi-immune children > 4 years of age from Amele harbor chronic, asymptomatic malaria infections of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, with occasional episodes of clinical malaria associated with splenomegaly, fever, and parasitemia above the fever threshold of 1,000 parasites/jxA.<sup>13,14</sup> Young children < 4 years of age have more frequent clinical episodes of malaria associated with high parasite densities.<sup>14</sup>

$\alpha$ -Thalassemia reaches very high frequencies in the Amele region, and polymorphisms such as Southeast Asian ovalocytosis (SAO) and glucose 6-phosphate dehydrogenase (G6PD) deficiency are also present in this area.<sup>18–20</sup>

**Study design and data collection.** A serial cross-sectional survey was conducted in a cohort of children (1–17 years of age in 1999) from November to December of 1999 and 2000. This study was initially designed to examine Hp levels with respect to host genotype in the Amele community, details and results of which have been published previously.<sup>21</sup> We studied these children further in relation to the APR in relation to host genotype. The dynamics of APP during the APR are such that data from a single time-point may be difficult to interpret. A serial study design would ensure that any associations observed with host genotype would be less likely to have occurred by chance. Therefore, archived samples from this previous study were deemed suitable for this retrospective analysis of APP. Sufficient samples for APP determination remained for 394 individuals in 1999 and 466 individuals in 2000 (343 pairs).  $\alpha$ -Antitrypsin concentrations were determined in samples collected in 1999 only.

Axillary temperatures were recorded for each child, and fever was defined as a temperature > 37.5°C Examination

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for splenomegaly was conducted using the Hackett grading system. Venous blood was drawn using Vacutainer tubes (Becton Dickinson, Oxford, UK) containing EDTA as an anti-coagulant. A blood smear was immediately made and later stained with Giemsa. Levels of hemoglobin (Hb) were immediately determined using an Hb photometer (HemoCue AB, Angelholm, Sweden). The remaining blood was centrifuged and separated into plasma (stored at -80°C), buffy coat, and erythrocyte pellet, stored in guanidine hydrochloride (G-HCl) at 4°C.

**Parasitology.** *Plasmodium* species and counts (number of parasites per 200 leukocytes) were analyzed by microscopy and recorded. A parasite negative slide was one on which 2,000 leukocytes were observed and no parasites seen (i.e., 10 fields). Duplicate readings were made for 20% of smears. The following age-stratified leukocyte counts were used to calculate parasites per microliter of blood: < 4 years,  $8.6 \times 10^3$  leukocytes/ $\mu$ L; 5-9 years,  $7.8 \times 10^3$  leukocytes/ $\mu$ L; > 10 years,  $7.7 \times 10^3$  leukocytes/ $\mu$ L (M. Bruce, unpublished data).

**Laboratory methods.** Plasma Hp levels were determined by ELISA.<sup>22</sup> Hypohaptoglobinemia was defined as Hp < 0.18 mg/mL.<sup>23</sup> Commercial ELISA kits were used to measure levels of CRP (American Laboratory Products, Windham, NH). An APR was defined as CRP > 10 (xg/mL).<sup>1</sup> Levels of  $\alpha_1$ -antitrypsin were determined using a commercial ELISA kit (American Laboratory Products).  $\alpha_1$ -Antitrypsin levels > 4 mg/mL are consistent with an APR.<sup>2</sup> Albumin concentrations were determined using bromocresol green reagent (Abbott Laboratories, Maidenhead, UK) and reading optical density at 630 nm. Low albumin was defined as < 35 mg/mL.<sup>23</sup>

**Statistical analysis.** Differences in APP concentrations between the 2 years were assessed using a Wilcoxon signed rank test. Within each year, the frequencies of categorical variables by splenomegaly were assessed by  $\chi^2$  tests or Fisher exact tests and continuous variables by Mann-Whitney *U* tests or analysis of variance. Linear models were used to examine the effect variables of interest on APP levels: this included a subject random effect to allow for the correlation between the repeated measures contributed by each child in CRP and albumin analysis.<sup>24</sup> This model does not require equally spaced or equal numbers of APP observations per child.<sup>25</sup> The fitted model predicts the mean APP level for the population while controlling for statistically relevant variables.

**Data considerations.** Because CRP levels showed considerable heteroscedasticity (non-constancy of the variance), the levels were log-transformed [ $\log(\text{CRP} + 0.015)$ ] before analysis by examining profile likelihoods.<sup>26</sup> *Plasmodium* densities were transformed by  $\log(\text{Plasmodium spp.} + 1)$ , in the mixed model analyses to reduce the skewness but leaving the zero values unchanged.

**Details of regression analysis.** The linear models were developed using a stepwise procedure with Akaike Information Criterion (AIC) as the criterion of goodness of fit on a dataset, which omitted all subjects for whom any data were missing. The full model examined the effect of sex, age, splenomegaly, Hb, and *Plasmodium* density temperature, together with a quadratic term for Hb,  $\alpha^+$ -thalassemia genotype, Hp genotype, SAO, G6PD deficiency, ABO blood group, and interactions between variables. The final model was refitted to a dataset excluding only those subjects with missing data for the variables that remained in the model, and variables with  $P > 0.05$  removed. After excluding missing val-

ues, 832 observations contributed to the final analysis. *P* values reported are unadjusted.

SPSS (for Windows Rel 13.0. 2004; SPSS, Chicago, IL) was used for the analysis, with the exception of the CRP, albumin, and  $\alpha_1$ -antitrypsin linear models, which were developed in R 2.0.1.<sup>27</sup>

## RESULTS

**Characteristics of children.** Data were collected from 546 children during two cross-sectional surveys that took place during November to December 1999 and 2000. APP levels were determined in 394 samples from 1999 and 466 samples in 2000 (343 pairs). The data differed from the main cohort with respect to age in 1999 only ( $P < 0.001$ ). The mean (SE) age of the original cohort was 8.58 (0.16) years compared with the subsample, which had an older mean age of 9.78 (0.15) years. This was because of a smaller volume of blood collected from some younger children, which limited the number of laboratory analyses.

Table 1 shows age, parasitologic, and APP data for the total population stratified by splenomegaly. There was no significant difference in variables between the 2 years; therefore, presented data was pooled. The proportion of children having an APR (CRP > 10 (xg/mL) was low (8.2%), despite the fact that 63.5% of children had detectable parasitemia with any species of *Plasmodium*. The prevalence of splenomegaly was 21.9% in this population. Children with splenomegaly had significantly higher prevalences of *Plasmodium* and higher parasite densities compared with children with normal spleens ( $P < 0.05$ ). Children with splenomegaly also had higher prevalences of CRP concentrations consistent with an APR ( $P < 0.001$ ), together with higher levels of CRP ( $P < 0.001$ ) and lower levels of Hp ( $P < 0.01$ ) and albumin ( $P < 0.01$ ), compared with children with normal spleens (Table 1).

**Multivariate analysis.** Linear models were used to examine the effect of a large number of variables on CRP levels. The resulting fitted model predicts the median CRP level and 95% confidence intervals (CIs) for the population, controlling for relevant variables. Although all children present in the surveys appeared healthy, for the purpose of analysis, the few children with a temperature > 37.5°C plus a parasitemia > 10,000 parasites/ $\mu$ L ( $N = 8$ ) were excluded. Median CRP concentrations in these "symptomatic" children were 7.1 (xg/mL [range, 1.4-36 (xg/mL)]. The overall CRP level in this population after controlling for relevant variables was 1.45 (xg/mL (95% CI, 1.24, 1.73)). The model predicts that CRP levels decrease with age (coefficient = -0.13; 95% CI, -0.19, -0.07;  $P < 0.001$ ) and increasing Hb levels (coefficient = -0.17; 95% CI, -0.22, -0.11;  $P < 0.001$ ). CRP levels increased with increasing parasitemia (coefficient = 0.26; 95% CI, 0.2, 0.32;  $P < 0.001$ ) but remained < 3.5 (xg/mL (Figure 1)). CRP concentrations were also elevated in children with fever (coefficient = -0.55; 95% CI, 0.26, 0.84;  $P < 0.001$ ) and splenomegaly (coefficient = 1.43; 95% CI, 0.81, 2.05;  $P < 0.001$ ). An interaction was found between age and splenomegaly. The decrease of CRP with age was less evident in those with normal spleens (coefficient = 0.11; 95% CI, 0.04, 0.17;  $P = 0.002$ ) compared with those with splenomegaly.

The overall concentration of  $\alpha_1$ -antitrypsin in the children was 3.02 mg/mL (95% CI, 2.89, 3.15) after adjusting for age (coefficient = -0.05; 95% CI, -0.09, -0.1;  $P = 0.01$ ), para-

TABLE 1  
Distribution of age, parasitology, and acute phase proteins according to splenomegaly

	All children (N = 887)	Spleen size*		P
		Normal (N = 689)	Splenomegaly (N = 193)	
Age (years)				
1-4	74 (8.3)	51 (7.4)	22 (11.4)	< 0.01
5-9	276 (31.1)	183 (26.6)	91 (47.2)	
10-17	537 (60.5)	455 (66.0)	80 (41.5)	
<i>Plasmodium</i> spp. (%)	563 (63.5)	418 (62.3)	142 (74.7)	< 0.05
Density log <sub>10</sub> value/μL	2.66 (0.03)	2.57 (0.04)	2.93 (0.7)	< 0.001
Fever	94 (10.6)	73 (10.6)	21 (11.1)	> 0.05
Acute phase proteins				
CRP (μg/mL)	0.9 [0.4-2.6]	0.29 [0.13-0.54]	2.0 [0.7-7.2]	< 0.001
CRP > 10 μg/mL	72 (8.2)	38 (5.5)	34 (17.6)	< 0.001
Hp (mg/mL)	0.26 [0.11-0.49]	0.29 [0.13-0.54]	0.16 [0.07-0.33]	< 0.01
a <sub>1</sub> -Antitrypsin (mg/mL)	3.1 (0.17)	3.07 (0.22)	3.3 (0.11)	> 0.05
Albumin (mg/mL)	45.5 (0.27)	45.9 (0.31)	44 (0.57)	< 0.01

Categorical values are N (%), continuous variables are mean (SE) or median [IQR]. Discrepancies in percentages are caused by missing values or rounding.

An acute phase response is defined as CRP > 10 μg/mL.

\* Missing spleen data N = 5.

† Density of positive slides.

‡ Data from 404 children in 1999 only (splenomegaly, N = 94).

CRP, C-reactive protein; Hp, haptoglobin.

sitemia (coefficient = -0.13; 95% CI, 0.05, 0.21;  $P = 0.002$ ), and splenomegaly (coefficient = 0.44; 95% CI, 0.17, 0.71;  $P = 0.002$ ). The overall concentration of albumin in these children was 45.35 mg/mL (95% CI, 44.77, 45.9) after adjusting for significant confounders. Albumin concentration increased with age (coefficient = 0.53; 95% CI, 0.35, 0.7;  $P < 0.001$ ) and increasing Hb concentration (coefficient = 0.54; 95% CI, 0.19, 0.89;  $P = 0.002$ ). Albumin was not significantly associated with parasitemia.

There was no significant association of a<sup>+</sup>-thalassemia genotype, Hp genotype, SAO, G6PD deficiency, or ABO blood group with either CRP, a<sub>1</sub>-antitrypsin, or albumin concentrations in this population of asymptomatic children from PNG.

## DISCUSSION

The Amele region of PNG experiences intense, year round transmission of malaria.<sup>16</sup> Episodes of clinical malaria associated with fever, splenomegaly, and parasitemia above the fever threshold of 1,000 parasites/μL occur, particularly in chil-

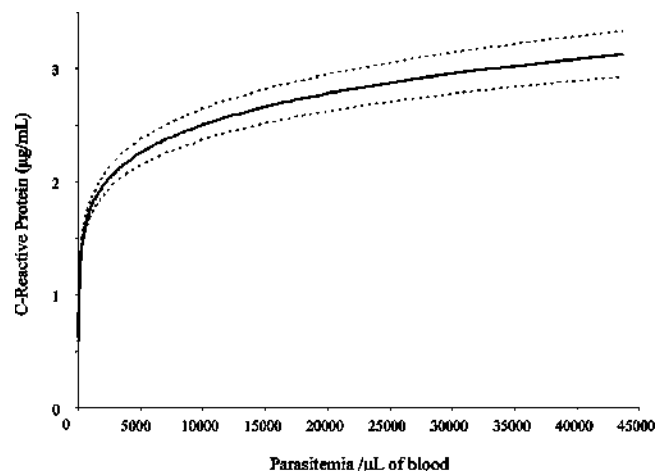


FIGURE 1. Predicted CRP levels in asymptomatic children in relation to parasitemia.

dren < 5 years.<sup>14</sup> A positive smear in a cross-sectional survey points to an infection of at least 40 parasites/μL of blood, although children in the Amele are infected with parasite densities below this detection limit, as shown by PCR and re-emergence of particular parasite genotypes.<sup>28,29</sup> Longitudinal observation of semi-immune children (> 4 years) in the Amele show that they harbor chronic, asymptomatic malaria infections with all *Plasmodium* spp.<sup>13</sup> Children who are parasite negative 1 day have a high probability of being positive the next day because *P. falciparum* infections in this population are quite synchronous.<sup>30</sup> Indeed, cumulative *Plasmodium* prevalence has been shown to be > 98.5% in children from the Amele region.<sup>30</sup>

Given the burden of chronic asymptomatic malaria infection, as well as clinical malaria, children living in Amele could be expected to have a chronic inflammatory response to continued and prolonged exposure to *Plasmodium* infections. It is therefore surprising that < 10% of children in both years had evidence of an APR, and median levels of CRP were only 1.49 (μg/mL (95% CI, 1.25, 1.78)). This does not vary considerably from the serum concentration of CRP in the healthy human population, which has a median value of 0.8 (μg/mL (interquartile range, 0.3-1.7 μg/mL) and is < 10 (μg/mL in 99% of normal processes.<sup>31,32</sup> These PNG data contrast with higher APR prevalences found in Africa; we have also found APR prevalences as high as 81% in Gabonese asymptomatic children (temperature < 37.5°C in the absence or presence of asexual parasitemia; F.J.I. Fowkes, unpublished observation).<sup>6-8</sup>

During a febrile episode of malaria, CRP levels peak 1-2 days after an inflammatory stimulus, and elevated CRP levels may increase by 30,000% and return to baseline 7-14 days later.<sup>1</sup> A previous study in asymptomatic children from The Gambia showed that the median CRP concentration in children with a parasitemia > 5,000 parasites/μL was 34.5 (μg/mL (95% CI, 29.1, 40.9)).<sup>7</sup> In our study, multivariate analysis showed that, in chronically infected Amele children with no evidence of clinical malaria, CRP levels do not vary significantly among children with high density parasitemia (> 10,000 parasites/μL in the absence of fever) and remain

< 3.5 (xg/mL). Data from children with mild malaria (fever plus parasitemia > 10,000/(xL) in this area show that the median CRP concentration was 116.4 (xg/mL (95% CI, 91.7, 147.9: F.J.I. Fowkes, unpublished observations).<sup>33</sup> It would seem that high-density parasitemia alone cannot stimulate an APR.

Children in the Amele study could be differentiated on the basis of whether they had splenomegaly, because this is particularly indicative of recent high-density parasitemia (> 10,000 parasites/jxL) in PNG.<sup>34</sup> Asymptomatic children with splenomegaly had higher prevalences and densities of *Plasmodium* spp. and higher levels of CRP. The higher levels of CRP detected most likely represent the decline of CRP after an acute episode of malaria, where an individual most likely experienced an APR.<sup>6,8</sup> CRP levels decreased with age in children with splenomegaly. Older children will have the benefit of a more effective acquired immune response so they may experience a reduction in the level of inflammatory response.

Children with splenomegaly also had lower levels of Hp. This most likely represents clearance of the Hp-Hb complex during the mass hemolysis seen in heavy malaria infection.<sup>35</sup> We have previously reported that concentrations of Hp are associated with both Hp genotype and  $\alpha^+$ -thalassemia genotype.<sup>15</sup> There was no significant association with the APP concentrations reported here and host genotype. Therefore, previous differences in Hp levels with respect to Hp and  $\alpha^+$ -thalassemia genotype were most likely caused by differences in Hp clearance rather than IL-6-dependent production.

Children living in the Amele experience chronic year round infections. We showed they modulate inflammation associated with malaria infection as evidenced by the absence of an APR. The mechanism modulating the balance between infection and disease in these children warrants further study.

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