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ASSESSMENT OF A SIMPLIFIED METHOD FOR COUNTING LEUKOCYTIC MALARIA PIGMENT

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Abstract. Severe and fatal malaria is associated with the increased presence of malaria hemozoin in peripheral phagocytes. Large studies of this relationship are hampered by the fact that identifying and counting phagocytes on thick blood smears is time consuming. Distinguishing which mononuclear cells are monocytes and which granulocytes are neutrophils requires time and careful training. In this study, we evaluated a simplified method in which only the proportions of hemozoin-containing mononuclear cells and granulocytes are counted. Thick blood films from 471 Gabonese children with malaria were evaluated. We found a linear relationship and a strong correlation between the proportions of hemozoin-containing monocytes versus mononuclear cells ($r = 0.85$) and neutrophils versus polymorphonuclear cells ($r = 0.93$), respectively. The two methods had similar predictive values, as estimated by receiver operating characteristics curves. This simplified method can be used to estimate the amount of extra-erythrocytic pigment in peripheral blood, and we suggest that it may be particularly suitable for very large studies.

INTRODUCTION

Although peripheral parasitemia generally correlates with disease severity in individuals infected with *Plasmodium falciparum*, the association is not strong enough to be useful in directing the treatment of individual patients. Erythrocytes containing mature *P. falciparum* trophozoites sequester in the microvasculature of various organs; for this reason, the peripheral parasitemia represents an unknown proportion of the total parasite burden in any given patient. A single measure of peripheral parasitemia, e.g., at the time of admission, is not a reliable enough marker to identify patients at high risk of a poor outcome.[1]

Malaria parasites grow and divide asexually within human red blood cells and this process is supported by the systematic digestion of hemoglobin. Malaria pigment (hemozoin) is the end product of this digestive process, during which the potentially toxic heme moiety is transformed into an insoluble polymer. When malaria parasites (schizonts/meronts) mature and rupture the host red blood cells, merozoites (the next generation of malaria parasites) are released, along with red blood cell contents, including malaria pigment. An unknown proportion of this free pigment is phagocytosed by scavenger neutrophils and monocytes; the rest is taken up by tissue macrophages to the extent that various tissues (brain, liver, spleen) are grossly discolored.[2,3] Neutrophils have a half-life of approximately seven hours, and are thus likely to be markers of recent pigment phagocytosis. Monocytes are longer lived, and the pigment contained therein reflects a more protracted time course.[4]

Previous clinical studies consistently demonstrate that elevated proportions of pigment-containing neutrophils are associated with increased severity of illness caused by *P. falciparum*, and with a worse outcome, although none of the studies evaluates both associations simultaneously.[4–8] However, the proportions of pigmented neutrophils that are associated with increased severity and increased mortality vary widely. In Vietnamese adults, all of whom had pigment-containing neutrophils circulating in their peripheral blood at the time of admission, the proportions of pigment-containing neutrophils were 3.2% and 7.7% in survivors and fatal cases, respectively.[4] In Gabon, 95% of children with severe malaria had pigment-containing neutrophils on admission, but the median proportion was only 2%.[5] Of children with mild malaria, 32% had pigment-containing neutrophils on admission, but the median proportion was 0% (range = 0–7%); half as many adults with mild malaria had pigment-containing neutrophils noted on admission, and again, the proportions noted were small (median = 0%, range = 0–1%). In contrast, while 100% of Nigerian children with cerebral malaria had neutrophils containing pigment, 27% (median) of the neutrophils counted had pigment within them.[6] Of Nigerian children with mild malaria, 95% had pigment-containing neutrophils but only 9% (median) of the neutrophils were pigmented. This was not significantly different from the 94% of children with asymptomatic parasitemia who had pigment-containing neutrophils present; in this group, the median proportion of neutrophils with pigment was 6.5%. In aparasitemic, asymptomatic Nigerian children, pigmented neutrophils were noted in 71%, and among those, 2% (median) of the neutrophils contained pigment, a result strikingly similar to that observed in the sickest Gabonese children. The original observation, in Vietnamese adults, has been corroborated by studies on African children in two epidemiologically distinct sites. However, given the extremely wide range of relevant findings, and the dearth of data relating pigment to severity and outcome simultaneously, it is not possible, on the basis of the data available, to evaluate the hypothesis properly. Comparable data, collected from epidemiologically distinct sites in Africa, and from patients with uncomplicated, severe, and fatal malaria, are required before the prognostic significance of intraleukocytic pigment in pediatric *P. falciparum* malaria can be determined.

Quantifying malarial pigment in peripheral blood can be done using thick or thin blood films. The morphology of various white blood cells is more readily observed on a thin film,

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but many more fields must be examined to assess a representative number of monocytes and neutrophils. We evaluated a method of assessing pigment using thick blood films, and quantifying pigment-containing mononuclear cells and polymorphonuclear cells, and compared the results to those made using the traditional method, in which monocytes and neutrophils are identified, and the associated pigment is quantified.

METHODS

The study was part of a series of studies within the "Severe Malaria in African Children" network, which aim to evaluate interventions to reduce malaria mortality in various sites throughout Africa. The study took place in the Albert Schweitzer Hospital in Lambaréné, Gabon, an area of moderate to high malaria transmission.[9,10]

Between December 2000 and January 2002, all children with asexual *P. falciparum* parasitemia admitted to the pediatric ward were eligible for enrollment. The study was reviewed and approved by the ethical review committees of the International Foundation of the Albert Schweitzer Hospital, Michigan State University, and the International Center for Infectious Diseases Research protocol review committee within the National Institute of Allergy and Infectious Diseases, U.S. National Institutes of Health. Informed consent was obtained from parents/guardians of all participants before enrollment.

Demographic data (age and sex) and clinical data (temperature, Blantyre coma score, respiratory status) were recorded on admission. Laboratory data recorded on admission included parasitemia, hemoglobin, hematocrit, and glucose.

Among parasitemic children, severe malaria was defined as having one or more of the following: cerebral malaria (Blantyre coma score ≤ 2), hyperparasitemia (parasitemia $\geq 250{,}000/\mu L$), severe malarial anemia (hemoglobin level ≤ 5 g/dL or hematocrit $\leq 15\%$) or hypoglycemia (glucose level ≤ 2.2 mmol/L).

Thick blood films were stained for 15 minutes using 20% Giemsa stain. This preparation is suitable for counting both peripheral parasitemia and pigmented leukocytes on the same slide (Missinou MA, unpublished data). On each thick blood film, 100 monocytes, 200 mononuclear leukocytes, 100 neutrophils, and 200 polymorphonuclear cells were examined for malaria pigment, and expressed in proportions of the respective cell type. One observer made all of the observations of a single slide. Parasitemia (parasites per microliter) was quantified by counting the number of parasites per microscopic field from a defined volume of blood (10 μL) spread on a defined area (1.8 cm^2).[11]

Since the measurements for the pigment counts were highly skewed, the relationship between the proportion of pigmented monocytes and neutrophils and pigmented mononuclear and polymorphonuclear cells was assessed after arcsine square root transformation. Linear regression using the transformed mononuclear and polymorphonuclear cells to predict the monocytes and neutrophils was done using these transformed proportions and the root mean square error was used to calculate the 95% confidence intervals.

The agreement between the ability of pigmented monocytes and mononuclear cells to predict cerebral malaria, severe malarial anemia, and hyperparasitemia was assessed by comparing the areas under the receiver operating characteristic (ROC) curve for different diagnostic tests applied to the same sample.[12] A P value < 0.05 was considered significant.

RESULTS

A total of 471 children were enrolled in the study, and their clinical features are summarized in Table 1. Paired measurements of pigment-containing mononuclear cells and polymorphonuclear cells were available for 468 and 386 children, respectively. The relationship between arcsine-root transformed measurements is shown in Figure 1. There was a strong correlation between transformed values for both monocytes versus mononuclear cells ($r = 0.85$, $P < 0.001$) and neutrophils versus polymorphonuclear leukocytes ($r = 0.93$, $P < 0.001$). The 95% confidence intervals suggest that the relationship between arc-sine square root transformed data shows more variability for the mononuclear cells than for the polymorphonuclear cells.

The ROC curves were used to compare differences in predictive ability between types of pigmented white blood cells and syndromes of severe malaria (cerebral malaria, severe malarial anemia, and hyperparasitemia). The area under the ROC curves was low in most cases. Overall, for pigmented monocytes, the area under the curve was 0.65, 0.68, and 0.67 for cerebral malaria, severe malarial anemia, and hyperparasitemia, respectively. For pigmented neutrophils, the area under the curve was 0.67, 0.51, and 0.76 for cerebral malaria, severe malarial anemia, and hyperparasitemia, respectively. Figure 2 shows four examples (of six possible associations); two associations had ROC lines too near the diagonal for a meaningful comparison. There were no significant differences in terms of type or severity of syndrome, except for severe malarial anemia (area under the curve $= 0.68$, $P = 0.01$). In this case, the proportion of pigmented mononuclear cells was more strongly associated with severe malarial anemia than was the proportion of pigmented monocytes.

DISCUSSION

Identifying children who are at a high risk of dying when they initially present with malaria is an important step in

TABLE 1
Characteristics of patients on admission

Demographic factors	
Age in years (mean ± SD)	3.2 ± 3.0
Sex (% male)	55%
Clinical presentation	
Any severe disease	42% (198/471)
Cerebral malaria	6% (26/468)
Severe malarial anemia	16% (72/464)
Hyperparasitemia	24% (111/471)
Hypoglycemia	8% (33/417)
Monocytes	
Patients*	85% (398/471)
Pigmented cells†	8% (2–16)
Mononuclear cells	
Patients*	83% (393/471)
Pigmented cells†	2% (0.5–4)
Neutrophils	
Patients*	49% (190/389)
Pigmented cells†	0% (0–20)
Polymorphonuclear cells	
Patients*	57% (266/469)
Pigmented cells†	0.5% (0–2)

* Percentage of subjects positive for pigmented leukocytes.
† Median (interquartile range).

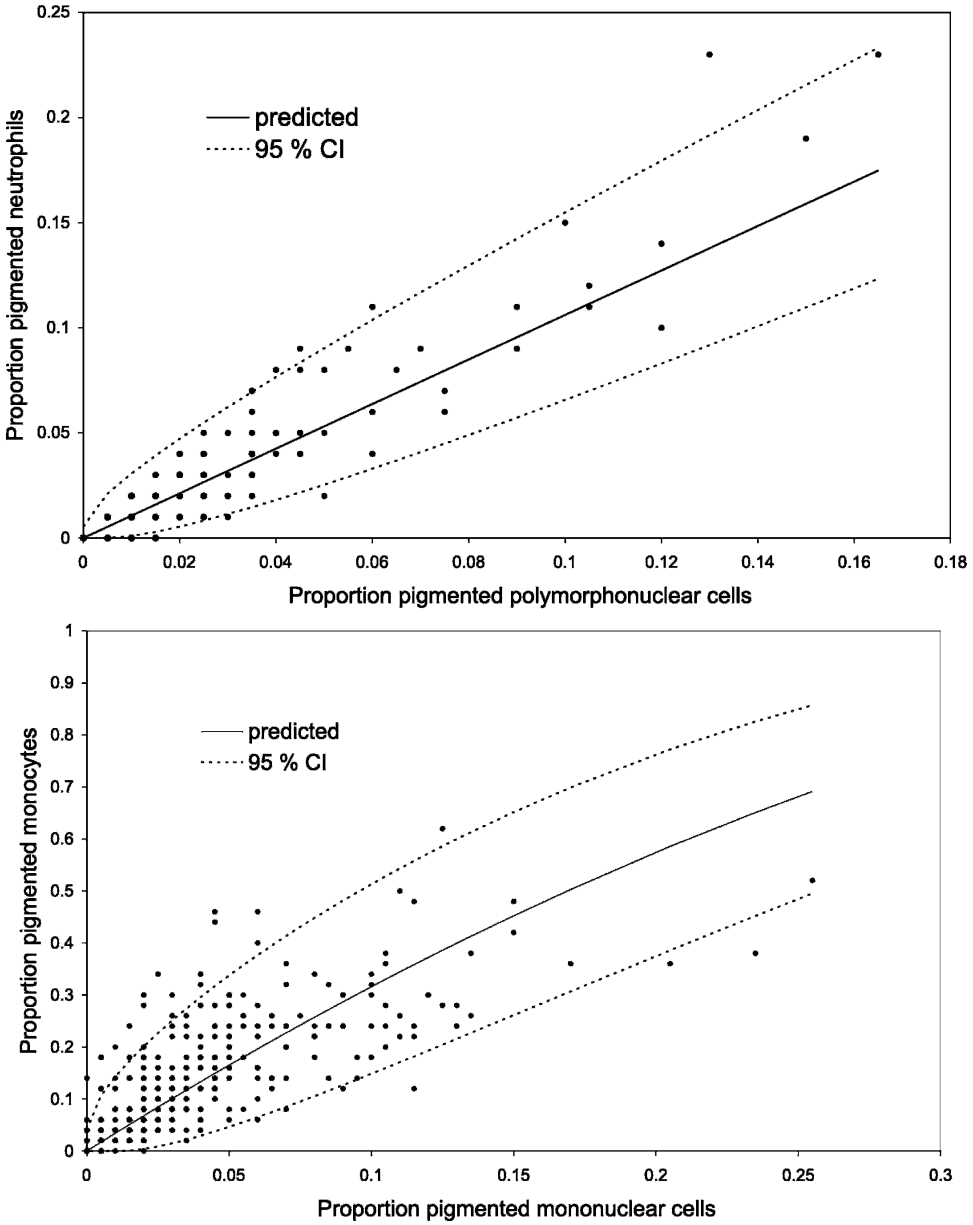

FIGURE 1. Scatterplots of the proportion of pigmented monocytes versus pigmented mononuclear cells (**top**) and of the proportion of pigmented neutrophils versus pigmented polymorphonuclear cells (**bottom**). The linear regression and pointwise 95% confidence intervals (CIs) are based on arc-sine square root transformed proportions. Points may represent overlapping values

reducing malaria mortality in Africa. To be useful in disease-endemic areas, a measure should be simple, inexpensive, and easy to implement. The amount of malaria pigment in peripheral leukocytes has the potential to be such a measure, but a simplified counting procedure would increase its utility. Here, we evaluated a simpler method to measure peripheral pigment load by counting pigmented mononuclear cells rather than pigmented monocytes and pigmented polymorphonuclear cells rather than pigmented neutrophils. The simplified method requires 10 minutes; the traditional approach needs 30 minutes. An additional advantage is that all counts in the simplified method can be made on the same slide as the thick blood smear.

The present study confirms the association between pigmented leukocytes and severity of malaria. It also corroborates the hypothesis that pigmented phagocytes with long half-lives (i.e., monocytes) are better markers for cerebral malaria and severe malarial anemia than are phagocytes with short half-lives. Peripheral parasitemia, in contrast, is more strongly correlated with pigment-containing neutrophils than with pigment-containing monocytes.[5,13] These findings are consistent with some[4,8] but not all previous studies.[7]

In our study, the capacity of the two methods to identify patients with more severe forms of malaria, assessed via ROC curves, was similar and not particularly high. Larger populations, including a significant number of fatal outcomes, are required to assess these measures as outcome predictors.

The simpler approach to assessing phagocytosed pigment in peripheral blood can be used to estimate the amount of extra-erythrocytic pigment in peripheral blood, and this approach is

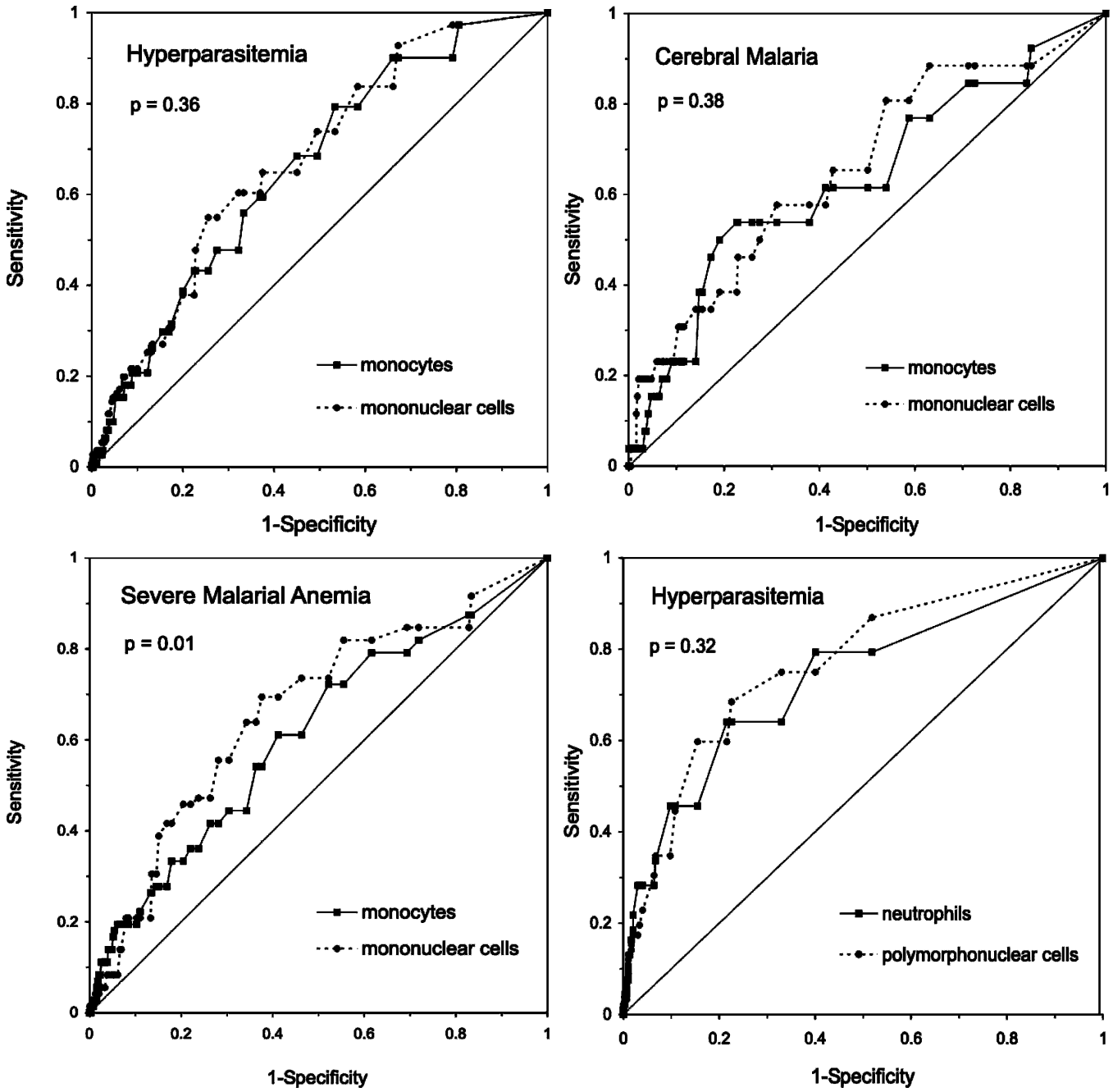

FIGURE 2. Comparision of receiver operating characteristics of pigmented leukocytes for prediction of severe malaria.

particularly suitable for the large studies which are required to elucidate the prognostic significance of extra-erythrocytic pigment.

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