Logistic regression analysis of PIT and HJB in qualitative spleen function

The values of 3.5% for PIT count and the 300/10^6 HJB are from the literature and back back calculation of "normal percentages of HJB in the peripheral smear." However to further explore the biomarker levels that are most predictive of determining the difference between normal and diminished spleen function we performed weighted logistic regressions.

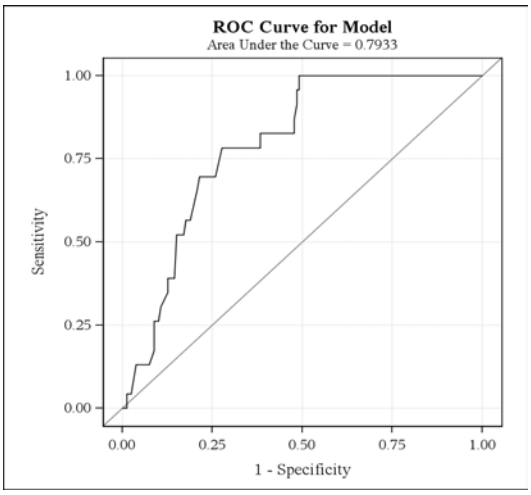
The HJB values that best separated qualitative spleen scans into the three categories were: present ≤ 42/10^6 RBC, decreased 43–677/10^6 RBC and absent ≥ 678/10^6 RBC. The correct classification rate for HJB values stated to divide spleen scans into the three categories was 78% if spleen was normal, 33% if present but decreased, and 96% if absent. However, a value of HJB ≤ 55/10^6 RBC predicts that the spleen will be normal rather than decreased or absent (area under ROC curve .7933; correct classification 83% if spleen normal, or 62% if decreased or absent). In these analyses we are trying to determine the utility of a HJB value in determining spleen scan uptake of normal so we have just placed the 83% in the table. In the other direction HJB ≥ 665/10^6 RBC predicts absent spleen function rather than normal or decreased (area under ROC curve .8704; correct classification rate for HJB is 96% if spleen function is absent and 74% if normal or decreased).

For PIT counts the cut off values that best separated qualitative spleen scans into three categories were present ≤1%, decreased >1 to < 5.2%, while absent is ≥5.2%. The correct classification rate for PIT stated into these three categories is 61% if spleen function is normal, 49% if present but decreased, and 81% if absent. PIT count ≤ 1.2% predicts that the spleen will be normal rather than decreased OR absent (area under ROC curve .7893; correct classification rate for PIT ≤ 1.2% is 65% if normal function, 76% if decreased or absent). PIT ≥ 4.5% predicts absent spleen function rather than normal OR decreased (area under ROC curve .8357; correct prediction rate 73% for normal or decreased, 81% if absent).

Figure S1. ROC curves for logistic models to separate spleen function into two categories using HJB

(a) Divide present from decreased or absent spleen function
(b) Divide absent from present or decreased spleen function

a.



b.

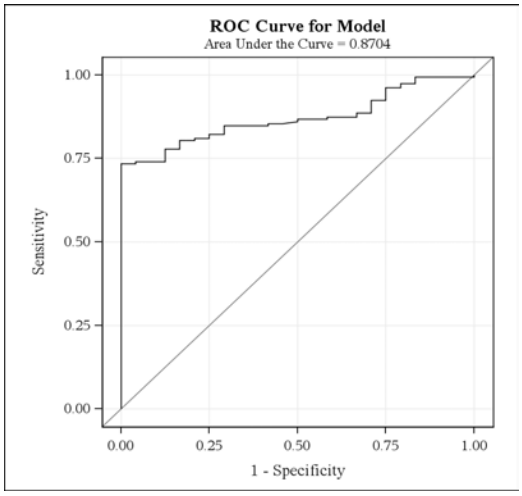
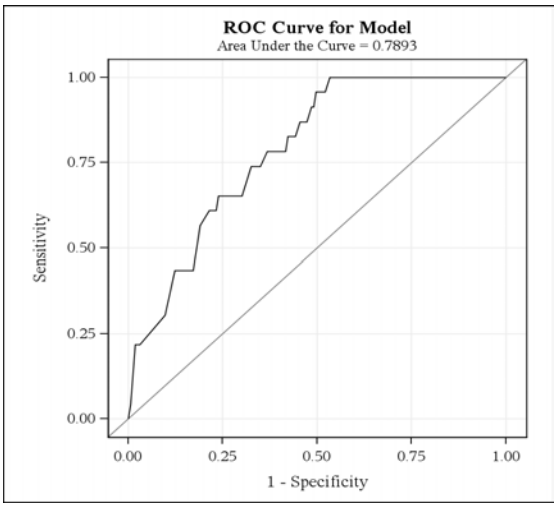


Figure S2. ROC curves for logistic models to separate spleen function into two categories using PIT

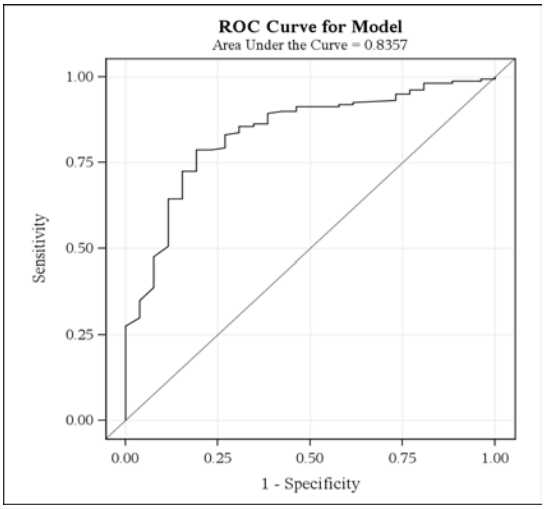
(a) Divide normal from decreased or absent spleen function

(b) Divide absent from normal or decreased spleen function

a.



b.



Method for flow cytometric analysis of HJB

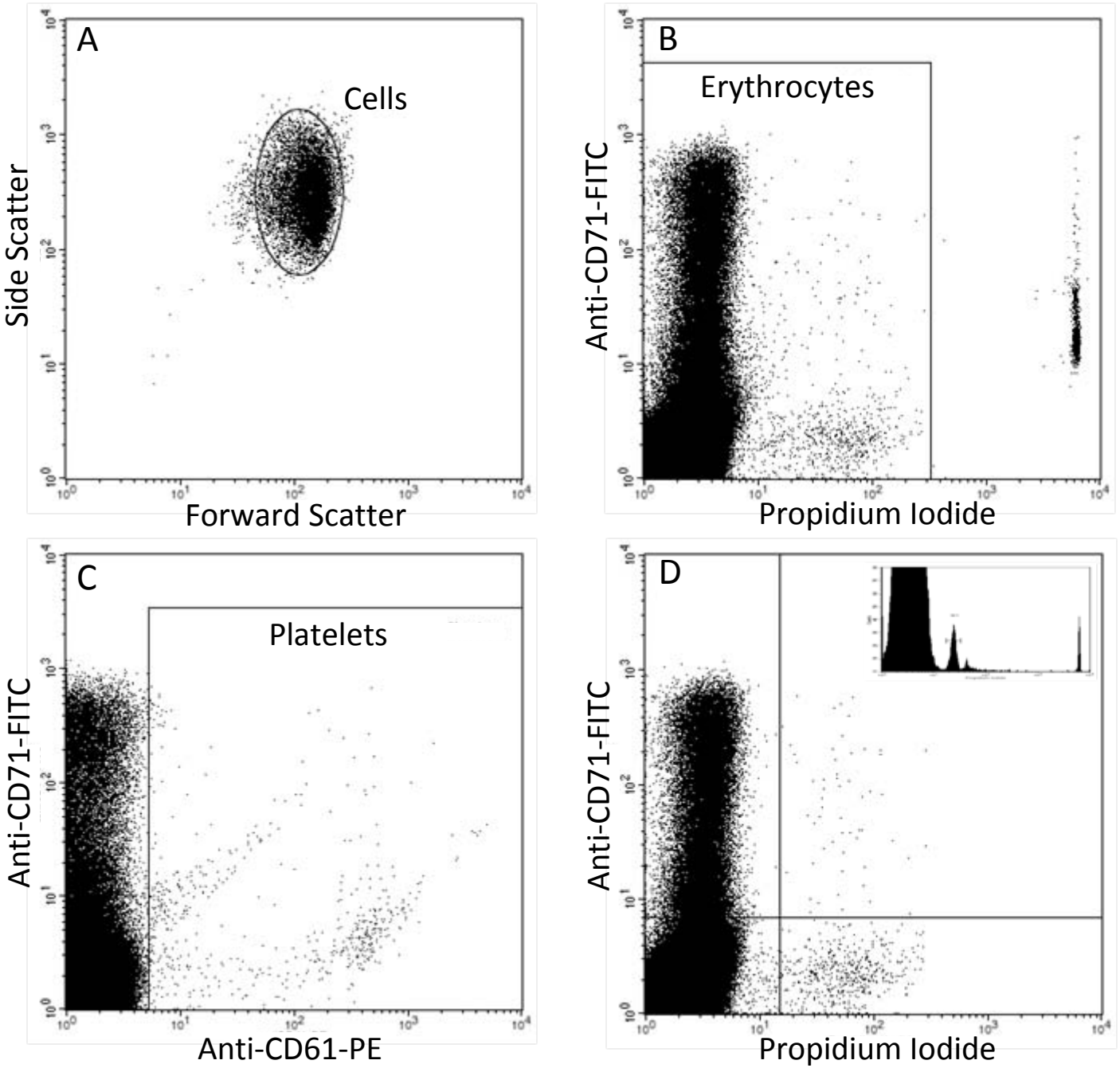
Within 24 hrs of collection, 10 µL of whole blood was added to 200 µL of heparinized phosphate-buffered salt solution and then forcefully injected into 15 mL polypropylene tubes containing 2 mL ultra-cold methanol (−80°C). Fixed samples were stored at −85°C until shipment on dry ice to Litron Laboratories (Rochester, NY). HJB measurements were performed at Litron as previously described [reference in manuscript 7]. Briefly, fixed whole blood cells were pelleted by centrifugation and then incubated with a buffer solution containing 1% fetal bovine serum, RNase, anti–CD71-FITC (to label reticulocytes), and anti–CD61-PE (to exclude platelets). Following successive incubations at 4°C and 37°C, the labeled cells were washed with 5 mL buffer with 1% fetal bovine serum and finally resuspended in propidium iodide (1.25 µg/mL) solution to stain DNA. Samples were stored at 4°C until same-day analysis by flow cytometry. In parallel to human blood specimens, a fixed *P. berghei*-infected rodent blood sample (*i.e.,* "Malaria Biostandard") was similarly stained and served as an instrument calibration standard that was useful for configuring photomultiplier voltages and compensation settings for the HJB-scoring application (new reference Dertinger 2000, added to manuscript as reference 8 and included below). See Figure 2. Data acquisition proceeded until at least 10^6 total erythrocytes and 2×10^4 CD71+ reticulocytes per sample were acquired.

New reference 8 in manuscript: Dertinger SD, Torous SD, Hall N, Tometsko CR and Gasiewicz T. Malaria-infected erythrocytes serve as biological standards to ensure reliable and consistent scoring of micronucleated erythrocytes by flow cytometry. *Mutat Res. 2000;* 464():195–200.

Figure S3. Representative flow cytometry cytograms from a hyposplenic subject analyzed for Howell-Jolly bodies (HJBs) according to the flow cytometric method described

(A–C) These bivariate plots illustrate the gating strategy whereby events are evaluated for HJBs only if they have light scatter characteristics of unaggregated cells (A), exhibit sub-2n DNA-associated fluorescence as is the case for erythrocytes (B), and do not express the platelet-specific antigen CD61 (C). (D) Bivariate plot that displays only gated events, that is, those that meet the three gating criteria described above. The lower right quadrant are mature erythrocytes (CD71-negative) that exhibit sub-2n DNA content, that is, contain HJB(s). The frequency of HJB-containing CD71-negative erythrocytes is determined upon the acquisition of at least 10^6 total CD71-negative erythrocytes per sample (lower right plus lower left quadrant events). (D) Histogram shows the prevalence and uniform propidium iodide fluorescence of the malaria biostandard. These *P. berghei*-infected erythrocyte specimens represent an instrument calibration standard that aids instrument set up, and also provides rationale for setting the propidium iodide boundary that distinguishes erythrocytes with and without HJBs.

Figure S3


A
Cells
Side Scatter
Forward Scatter
B
Erythrocytes
Anti-CD71-FITC
Propidium Iodide
C
Platelets
Anti-CD71-FITC
Anti-CD61-PE
D
Anti-CD71-FITC
Propidium Iodide