Supplementary materials

Supplementary methods

Blood collection and parasite biomass

Peripheral blood was collected from eligible patients using a tourniquet that was removed upon venipuncture with a BD 21G butterfly needle. The first 2 milliliters of blood were collected into a blank BD Vacutainer® with no additives in Papua, or into a lithium-heparin BD Vacutainer® in Sabah, followed by a 4.5 milliliter BD Vacutainer® containing a mixture of citrate, theophylline, adenosine and dipyridamole (CTAD). The remaining blood in the butterfly tubing was collected into a BD Microtainer® containing EDTA for confirmation of blood film microscopy by two qualified microscopists, as well as complete blood count on a Sysmex XS-1000*i* hematology analyzer (Hyogo, Japan). Peripheral blood samples were transported immediately to the laboratory and processed within 30 min. In Papua, blood films were stained with 3% Giemsa and parasite quantitation were finalized using the Obare Method.[1] In Sabah, blood films were stained with 10% Giemsa and asexual parasite counts were calculated from thick blood film microscopic parasite count per 200 leucocytes and automated leucocyte count.[2,3] In Sabah, *Plasmodium* species was confirmed by PCR as described previously.[4,5] Platelet-free plasma was collected from CTAD-coagulated blood after two-step centrifugation and stored at -80 °C. In Papua patients with *P. falciparum*, plasma PfHRP2 was measured by ELISA as previously described.[6]

Platelet binding to iRBC and uRBC

In Papua, analysis was from fresh samples and in Sabah from cryopreservation in fixative (20 µL blood in 1 mL of 1% (w/v) formaldehyde; Cytofix™ (BD Biosciences, Australia) diluted with

phosphate-buffered saline (PBS). Fresh blood (5 µL) was stained with anti-CD45 (clone HI30) conjugated to Alexa Fluor 488, anti-CD41 (clone HIP8) conjugated to phycoerythrin (PE) and DRAQ5 nucleic acid dye (diluted 1:2000) (BioLegend, San Diego, CA) for 20 min at room temperature, washed with PBS and fixed in 1%(w/v) paraformaldehyde (PFA) in PBS. Fresh samples were acquired on a BD Accuri C6 flow cytometer using CFlow Sampler software (BD Biosciences). Fixed blood (20 µL) was washed 3 times in PBS/1% bovine serum albumin (BSA) solution, and then stained with anti-CD42b (clone HIP1) conjugated to PE, anti-CD45 (clone HI30) conjugated to peridinin chlorophyll *a* protein (PerCP), anti-CD235ab (clone HIR2) conjugated to allophycocyanin (APC) (BioLegend, San Diego, CA) and 5 µg/mL Hoechst 33342 (Sigma-Aldrich, Missouri, US) for 20 min at 4°C, then washed with PBS/1% BSA solution. Fixed samples were acquired on a LSR Fortessa flow cytometer and data analyzed using FACS Diva software (BD Biosciences). At least 500,000 events were collected per sample. All Flow cytometric data were analyzed using FlowJo software (TreeStar, Ashland, OR) or FACS DIVA software (BD Biosciences, Australia). All RBC ($CD235ab^+$) were identified as either iRBC ($DRAQ5^+$) or uRBC ($DRAQ5^-$). CD41 platelet marker was used to differentiate platelet-bound iRBC or uRBC ($CD41^+$) from platelet-free iRBC or uRBC ($CD41^-$). The frequency of platelet-iRBC/uRBC complexes was calculated by dividing the number of platelet-iRBC/uRBC events with the sum of platelet-iRBC/uRBC and free iRBC/uRBC events, then multiplying by 100. Cells expressing CD45 and CD41 were considered as platelet-bound WBC. Calculations to determine platelet-RBC and platelet-WBC per µL blood and as a ratio to free platelets are shown in **Table 3**.

Platelet-associated parasite killing

Blood samples (50 µL) collected in both the Papua and Sabah studies into anticoagulant-containing tubes (CTAD and citrate, respectively) were fixed in 1 mL of BD Cytofix™ solution (diluted 1:4 in PBS). Poly-ethylenimine-coated slides were layered with fixed blood and stained overnight with reaction mix from the Apo BrdU TUNEL Assay kit (Molecular Probes, Eugene, OR). TUNEL-labelled DNA was visualized with anti-BrdU conjugated to Alexa Fluor 488 (BioLegend, San Diego, CA). Platelets and intraerythrocytic localization of PF4 were detected with polyclonal rabbit anti-human PF4 primary Ab (Abcam, Cambridge, UK) and Cyanine-3-conjugated sheep anti-rabbit secondary Ab (Sigma-Aldrich, Missouri, US). Intraerythrocytic *Plasmodium* DNA was identified after mounting with SlowFade™ Gold Antifade Mountant with DAPI (Invitrogen, Massachusetts, US). Negative control smears were prepared identically but without terminal transferase in the TUNEL reaction. For PF4 and TUNEL labeling of reticulocytes, blood from a healthy volunteer was magnetically-enriched for reticulocytes using MACS cell separation with anti-human CD71 MicroBeads (Miltenyi Biotec, Germany). Immunofluorescent slides were kept in the dark at 4°C and analyzed within 2 days on an Axio Scope A1 fluorescent microscope (630x magnification) coupled to an Axiocam ICm-1 CCD camera for the Papua cohort, or an Axio Observer inverted fluorescence microscope (630x magnification) coupled to an Axiocam 503 monochrome camera for the Sabah cohort. Fluorescent images were processed with ZEN 2 software (all from Carl Zeiss, Germany).

Platelet activation

In Papua, 10 µL of CTAD blood diluted 1:10 in HEPES-buffered Tyrode's (HT) solution was stained with PAC-1 conjugated to FITC, anti-CD41 (clone HIP8) conjugated to PE and anti-CD62P (clone AK4) conjugated to PE-Cyanine-5, and 20 µL of undiluted CTAD blood was

stained with anti-CD14 (clone HCD14) conjugated to FITC, anti-CD41 (clone HIP8) conjugated to PE and anti-CD15 (clone W6D3) conjugated to PerCP, and incubated for 20 min at room temperature. Cells were fixed in 0.5% (w/v) PFA in HT solution or 5% (w/v) PFA in PBS followed by 10 min of RBC lysis with double deionized water and acquired on a BD Accuri C6 flow cytometer using CFlow Sampler software (BD Biosciences). Positive controls were prepared identically with addition of 20 µM thrombin receptor activating peptide (TRAP; Sigma-Aldrich, Missouri, US). Isotype controls were prepared for PAC-1 (IgM FITC) and CD62P (IgG1 PE-Cy5) to assist with gating. All Ab were purchased from BioLegend (San Diego, CA), except PAC-1 was purchased from BD Biosciences.

Soluble PF4 levels were measured in platelet-free plasma either neat or diluted up to 50 times using the IMUCLONE™ PF4 ELISA (Sekisui Diagnostics, Lexington, MA) as per manufacturer instructions and analyzed using a GloMax®-Multi+ Microplate Reader (Promega, Sunnyvale, CA).

In *in vitro* experiments, platelet activation capacity of platelet preparations was tested by flow cytometry using FITC-conjugated mouse anti-human GPIIb/IIIa (PAC-1) and FITC-conjugated mouse anti-human CD62P (both from BD Biosciences, Australia).

Parasite culture

Plasmodium knowlesi strain A1H1 and *P. falciparum* strain 3D7 were maintained in a complete culture medium (CCM) in flasks filled with $1\%O_2/3\%CO_2/96\%N_2$ gas mix, and kept in an orbital shaking incubator at 50 rpm at 37°C. Parasitemias were maintained between 0.5% and 10% and checked every 1-2 days using Giemsa-stained thin blood smears. CCM was changed every 1-2 days by pelleting cells at 1500 *g* (*P. knowlesi*) or 500 *g* (*P. falciparum*) for 5 min and

resuspending in fresh CCM. Human RBC and sera were provided by Australian Red Cross Blood Service. Blood was washed three times using CCM that lacks hypoxanthine, AlbuMAX® I, and human serum, and centrifuged at 2800 RPM for 5 min for each wash. *P. knowlesi* strain A1H1 was donated by M. Blackman (Medical Research Council National Institute for Medical Research, London, UK). *P. falciparum* strain 3D7 was donated by R. Anders (La Trobe University, Melbourne, Australia).

Platelet purification

Blood was collected from healthy volunteer donors using 21G needles into BD CTAD Vacutainers® (6-8 tubes for each preparation). The first collected tube was discarded. Platelet-rich plasma (PRP) was isolated by centrifugation at 170 *g* for 13 min. Citrate dextrose buffer was added to PRP and incubated at 37°C for 30 min. Following centrifugation at 1700 *g* for 7 min, platelets were resuspended in platelet wash buffer and rested at 37°C for 30 min. Platelets were pelleted again by centrifugation and resuspended in Tyrode's buffer supplemented with 0.02 U/mL ADPase (as apyrase from *Solanum tuberosum*, EC 3.6.1.5, Sigma-Aldrich, Missouri, US) at concentration of 400 million platelets/mL, and rested at 37°C for 60 min before addition to the parasite cultures. Platelet lysates were prepared by freeze-thawing purified human platelets for three cycles of 5 min incubations in an ethanol/dry ice bath followed by 5 min in a 37°C water bath.

Culture medium and buffers

P. knowlesi CCM (RPMI 1640 supplemented with 11.1 mM D-glucose, 25 mM HEPES, 368 nM hypoxanthine, 3.6 mM $NaHCO_3$, 12 mM NaCl (Sigma-Aldrich, Missouri, US), 46.1 nM

gentamicin, 5 g/L AlbuMAX® I, 3.4 mM L-glutamine (Life Technologies, Australia), and 10% (v/v) O+ human serum).

P. falciparum CCM (RPMI 1640 supplemented with 8.8 mM D-glucose, 22 mM HEPES, 208 nM hypoxanthine (Sigma-Aldrich, Missouri, US), 46.1 nM gentamicin, 2.1 g/L AlbuMAX® I, 2.8 mM L-glutamine (Life Technologies, Australia), and 4.2% (v/v) O+ human serum).

Citrate dextrose buffer (8.5 mM sodium citrate, 6.5 mM citric acid and 7.6 mM D-glucose).

Platelet wash buffer (5.5 mM D-glucose, 0.35% (w/v) human serum albumin, 4.3 mM K_2HPO_4, 4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, 113 mM NaCl and 10 mM theophylline).

Tyrode's buffer (5 mM D-glucose, 5 mM HEPES, 0.35% (w/v) human serum albumin, 2.9 mM KCl, 1 mM $MgCl_2$, 134 mM NaCl, 12 mM $NaHCO_3$ and 0.34 mM NaH_2PO_4).

References

1. Rosen M, Dhorda M, Bell D, Gatton M. Obare Method Calculator v1.0. www.wwarn.org; 2015.

2. Grigg MJ, William T, Menon J, et al. Efficacy of Artesunate-mefloquine for Chloroquine-resistant *Plasmodium vivax* Malaria in Malaysia: An Open-label, Randomized, Controlled Trial. *Clin Infect Dis*. 2016;62(11):1403-1411.

3. Grigg MJ, William T, Menon J, et al. Artesunate–mefloquine versus chloroquine for treatment of uncomplicated *Plasmodium knowlesi* malaria in Malaysia (ACT KNOW): an open-label, randomised controlled trial. *Lancet Infect Dis*. 2016;16(2):180-188.

4. Padley D, Moody AH, Chiodini PL, Saldanha J. Use of a rapid, single-round, multiplex PCR to detect malarial parasites and identify the species present. *Ann Trop Med Parasitol*. 2003;97(2):131-137.

5. Imwong M, Hanchana S, Malleret B, et al. High-Throughput Ultrasensitive Molecular Techniques for Quantifying Low-Density Malaria Parasitemias. *Journal of Clinical Microbiology*. 2014;52(9):3303-3309.

6. Dondorp AM, Desakorn V, Pongtavornpinyo W, et al. Estimation of the Total Parasite Biomass in Acute Falciparum Malaria from Plasma PfHRP2. *PLoS Med*. 2005;2(8):e204.

Figure S1. Representative gating strategies. A) Flow cytometry gating strategy for platelet binding to iRBC and uRBC in the Sabah cohort. Flow cytometry gating strategy for platelet

activation in the Papua cohort, including B) PAC-1 binding and P-selectin surface expression, and C) platelet-monocyte and platelet neutrophil aggregates. Abbreviations: FS, forward scatter; SS, side scatter; TRAP, thrombin receptor activating peptide.

Figure S2. Platelet binding and killing correlations with parasitemia. A) Spearman correlation of platelet-bound iRBC with parasitemia in Sabah *Pf*, Papua *Pm* and Papua mixed species infection. B) Spearman correlation of $PF4^{+}TUNEL^{+}$ iRBC with parasitemia in Papua *Pm*, Sabah *Pk* and Papua mixed species infection. Abbreviations: iRBC, infected red blood cells; PF4, platelet factor-4; *Pf, P. falciparum*; *Pm*, *P. malariae*; *Pk, P. knowlesi.*

Figure S3. Platelet binding in Sabah children. A) Frequency of platelet-bound iRBC and uRBC by flow cytometry in children with *Pv* (*n*=22) and *Pk* (*n*=6) malaria (Wilcoxon test). Frequency of B) platelet-bound iRBC and C) platelet-bound uRBC in *Pv* and *Pk*, comparing children (*Pv n*=22, *Pk n*=6) to adults (*Pv n*=63, *Pk n*=100) (Kruskal-Wallis test). D) Inverse correlation between platelet-bound iRBC with parasitemia in children with *Pv* and *Pk* malaria

(Spearman). Scatterplots indicate median ± interquartile range for each group. Parasitemia values are log transformed. Abbreviations: RBC, red blood cells; iRBC, infected RBC; uRBC, uninfected RBC; *Pv*, *P. vivax*; *Pk*, *P. knowlesi*.

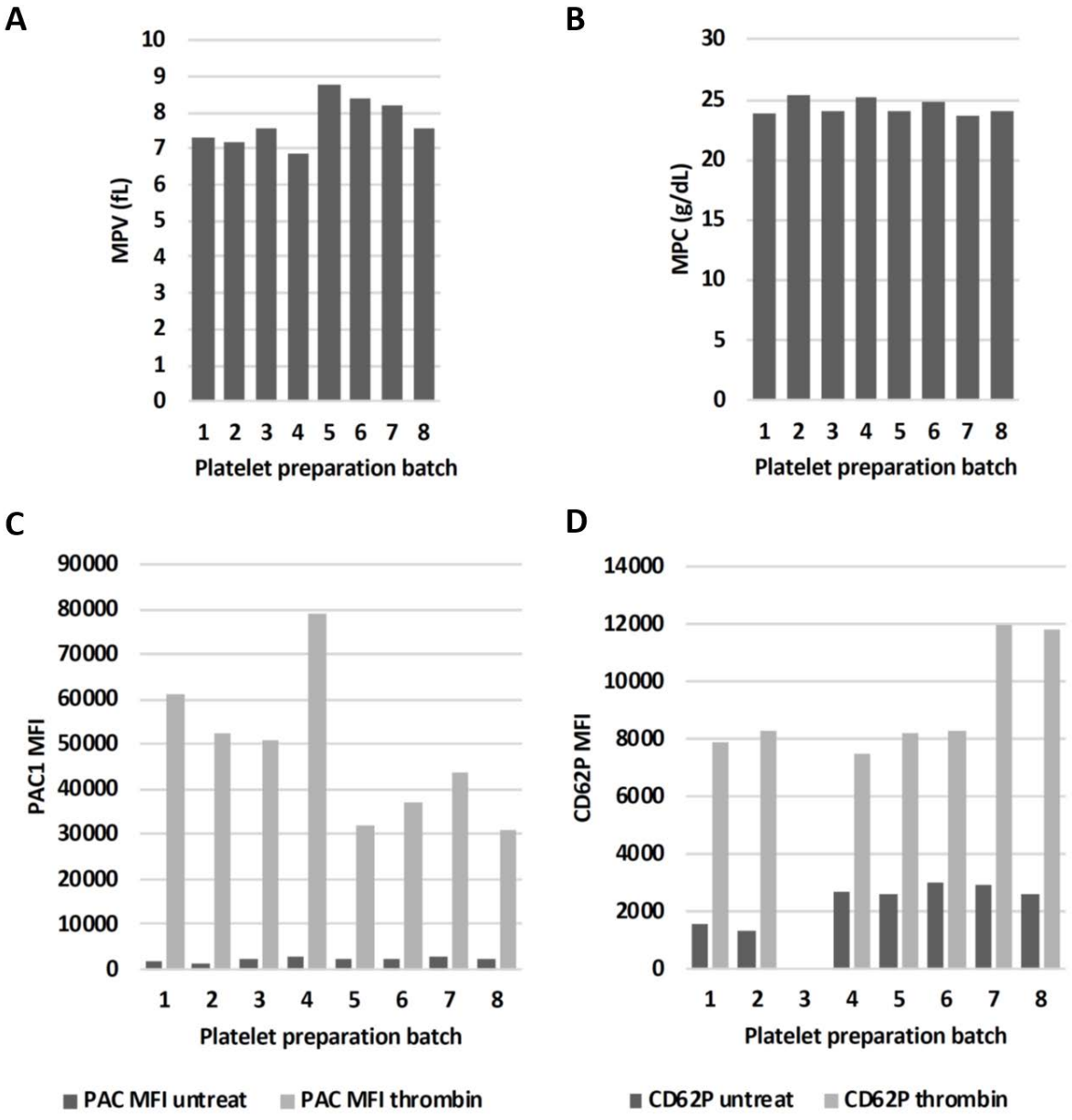

Figure S4. Analysis of purified human platelet preparations. Eight individual batches of platelets, prepared from six different donors, were used in this study. Quiescent (non-activated) platelets were determined according to MPV <9 fL (A) and MPC >23 (B). The potential for these platelets to be activated *in vitro* were assessed by flow cytometry by measuring the MFI of integrin GPIIb/IIIa (PAC-1 epitope) (C) and P-selectin (CD62P) (D) before (untreat) and after

treatment with 1U/mL thrombin for 10 min. Abbreviations: ND, not determined; MFI, mean fluorescence intensity; MPV, mean platelet volume; MPC, mean platelet component.

Table S1. Platelet-RBC complexes

Patient Cohort	***Plasmodium* species**	***n* of samples analyzed**	**Platelet-RBC complexes (median % [IQR])**		**Ratio of platelet-iRBC to -uRBC**	**Wilcoxon test *p*-value platelet-iRBC vs -uRBC**
			Platelet-iRBC	**Platelet-uRBC**		
Papua	Controls	17	-	0.28 [0.23-0.40]	-	-
	P. falciparum	23	1.54 [0.41-2.14]	0.11 [0.07-0.15]	14.0	<0.0001
	P. vivax	26	1.32 [0.64-1.99]	0.11 [0.08-0.16]	12.0	<0.0001
	P. malariae	9	1.05 [0.46-2.51]	0.13 [0.10-0.18]	8.1	0.004
	Mixed infection	7	1.15 [0.38-2.16]	0.10 [0.05-0.20]	11.5	0.02
Sabah	Controls	28	-	0.42 [0.22-0.55]	-	-
	P. falciparum	14	6.33 [2.15-8.66]	0.11 [0.04-0.25]	57.5	0.0001
	P. vivax	85	10.22 [6.09-17.91]	0.15 [0.09-0.29]	68.1	<0.0001
	P. knowlesi	106	18.07 [5.72-27.18]	0.19 [0.10-0.34]	95.1	<0.0001

Footnotes:

Abbreviations: IQR, interquartile range; iRBC, infected RBC; uRBC, uninfected RBC.

Table S2. Platelet binding to PF4-stained and PF4-unstained infected RBC.

Plasmodium species	***n* of samples analyzed**	**% platelet-bound PF4-stained**	**% platelet-bound PF4-unstained**	***P*-value [a]**
P. falciparum	9	4.2 [0-25.0]	3.2 [1.3-5.9]	0.111
P. vivax	13	6.7 [0-12.9]	0 [0-5.3]	0.002
P. knowlesi	5	6.3 [0-11.8]	4.1 [5.0]	0.343

Footnotes:

All values are median [range] unless otherwise indicated.

[a] Multiple t-test with Bonferroni-Dunn multiple comparison corrections.

Abbreviations: PF4, platelet factor-4.

Table S3. Platelet activation markers in malaria patients and healthy controls**.**

Papua Cohort	**Controls**	***P. falciparum***	***P. vivax***	***P. malariae***	**Mixed infection**		**Admission**	**Discharge**
Individuals tested, *n*	17	23	25	9	7		15	
PAC-1 binding (% platelets)	0.21 [0-0.57]	0.39 [0.04-0.88]	0.23 [0.07-0.65]	0.01 [0-0.31]	0.85 [0.48-1.66]		0.38 [0.05-0.83]	0.19 [0.02-1.09]
TRAP positive control	28.1 [14.3-45.1]	46.3 [22.9-63.8]	44.4 [31.9-65.7]	35.4 [29.7-53.7]	21.7 [19.4-54.0]		40.0 [19.6-54.5]	40.2 [31.5-70.1]
CD62P+ (% platelets)	0.07 [0-0.24]	0.16 [0.12-0.37]	0.32 [0.02-0.65]	0.10 [0-4.13]	0.55 [0-1.12]		0.14 [0.01-0.37]	0.16 [0.05-0.32]
TRAP positive control	41.8 [28.3-54.4]	67.1 [53.2-74.0]**	64.7 [53.2-75.1]**	72.8 [62.5-80.7]***	57.1 [47.9-71.2]		67.6 [52.4-76.4]	71.9 [70.3-80.8]
Individuals tested, *n*	21	43	34	12	10		15	
Platelet-monocytes (% monocytes)	16.4 [14.0-26.7]	9.7 [7.4-15.2]***	9.5 [6.9-12.5]***	10.7 [8.6-17.3]	11.9 [8.6-13.6]		7.7 [5.1-9.5]$^{\#}$	11.4 [5.9-18.9]
TRAP positive control	59.7 [33.5-77.0]	45.7 [26.6-73.2]	49.8 [29.1-69.4]	72.7 [59.4-79.2]	52.9 [19.3-65.4]		35.8 [30.7-58.1]$^{\#\#\#}$	70.8 [59.0-84.1]
Platelet-neutrophils (% neutrophils)	7.7 [6.7-9.9]	3.6 [2.2-5.0]***	3.9 [2.2-5.5]***	4.9 [3.3-6.1]*	4.3 [2.0-5.3]***		2.3 [1.7-4.0]$^{\#\#\#}$	4.5 [3.2-8.7]***
TRAP positive control	30.4 [16.4-44.5]	21.0 [7.7-53.7]	26.4 [13.0-52.2]	54.6 [36.0-60.8]	26.0 [5.4-48.4]		18.9 [5.5-26.6]$^{\#\#\#}$	46.2 [32.1-64.1]*
Individuals tested, *n*	24	55	38	14	12		15	
Soluble PF4 (ng/mL)	13.3 [9.8-24.6]	14.4 [9.7-24.1]	11.6 [8.0-19.8]	11.9 [6.7-18.3]	11.0 [8.0-18.0]		10.6 [5.8-16.2]	9.2 [6.6-23.5]
Sabah Cohort	**Controls**	***P. falciparum***	***P. vivax***	***P. knowlesi***				
Individuals tested, *n*	28	14	85	106				
Platelet-white blood cells (% white blood cells)	13.2 [10.0-21.0]	7.0** [1.3-10.0]	12.3 [7.8-21.3]	10.7 [5.3-16.0]				

Footnotes:

All values are median [interquartile range] unless otherwise indicated.

Kruskal-Wallis with Dunn's multiple comparisons test, significantly different to controls (***p<0.0005, **p<0.005, *p<0.05).

Wilcoxon matched-pairs signed rank test, significantly different to day of discharge ($^{###}p$<0.0005, $^{\#}p$<0.05).

Abbreviations: CD62P, P-selectin; TRAP, thrombin receptor activating peptide; PF4, platelet factor-4.

Table S4. Association between platelet activation and platelet-RBC complexes, $TUNEL^{+}PF4^{+}$ iRBC and indicators of disease severity in the Papua cohort**.**

Infecting species	**Variables correlated**	**Platelet-iRBC%**			**Platelet-uRBC%**			**$TUNEL^{+}PF4^{+}$ iRBC%**			**Hemoglobin (g/dL)**			**Parasitemia (per µL)**		
		r	*p*	*n*	r	*p*	*n*	r	*p*	*n*	r	*p*	*n*	r	*p*	*n*
P. falciparum	Platelet-monocytes%	0.38	0.102	20	**0.67**	**0.001**	**20**	0.36	0.026	38	**-0.45**	**0.003**	**43**	-0.25	0.106	43
	Platelet-neutrophils%	0.44	0.056	20	**0.79**	**<0.0001**	**20**	0.31	0.060	38	-0.31	0.041	43	-0.33	0.032	43
	Soluble PF4 ng/mL	0.26	0.235	23	0.51	0.012	23	0.09	0.534	50	0.03	0.816	55	0.10	0.461	55
P. vivax	Platelet-monocytes%	-0.05	0.815	26	0.24	0.230	26	0.07	0.709	28	-0.15	0.387	34	-0.20	0.269	34
	Platelet-neutrophils%	0.22	0.290	26	0.39	0.046	26	0.19	0.325	28	-0.01	0.956	34	-0.31	0.071	34
	Soluble PF4 ng/mL	0.19	0.343	26	0.24	0.237	26	0.35	0.052	32	-0.01	0.947	38	-0.27	0.096	38
P. malariae	Platelet-monocytes%	0.17	0.703	8	0.48	0.243	8	0.25	0.521	9	-0.18	0.576	12	-0.52	0.089	12
	Platelet-neutrophils%	0.10	0.840	8	0.48	0.243	8	0.47	0.213	9	0.03	0.943	12	-0.66	0.022	12
	Soluble PF4 ng/mL	-0.38	0.313	9	-0.47	0.213	9	0.25	0.455	11	-0.02	0.936	14	-0.28	0.341	14
Mixed infection	Platelet-monocytes%	0.31	0.564	6	0.54	0.297	6	0.30	0.683	5	-0.47	0.179	10	0.09	0.811	10
	Platelet-neutrophils%	0.03	1.000	6	0.49	0.356	6	1.00	0.017	5	**-0.86**	**0.003**	**10**	-0.24	0.513	10
	Soluble PF4 ng/mL	0.18	0.713	7	0.21	0.662	7	0.09	0.919	6	-0.03	0.939	12	-0.15	0.651	12
Controls	Platelet-monocytes%	-	-	-	0.06	0.823	15	-	-	-	-0.04	0.851	21	-	-	-
	Platelet-neutrophils%	-	-	-	0.21	0.442	15	-	-	-	-0.17	0.460	21	-	-	-
	Soluble PF4 ng/mL	-	-	-	-0.12	0.811	17	-	-	-	0.07	0.737	24	-	-	-

Footnotes:

Non-parametric Spearman correlations shown (significant correlations shown in bold font; $p<0.01$ after Bonferroni correction).

Abbreviations: PF4, platelet factor-4; iRBC, infected RBC; uRBC, uninfected RBC.