Circulating neutrophil extracellular traps and neutrophil activation are increased in proportion to disease severity in human malaria

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40-word summary: Neutrophil extracellular traps (NETs) may inhibit parasite growth in asymptomatic *Plasmodium falciparum* parasitemia. Conversely, in symptomatic infection,

circulating NETs and neutrophil activation increase in proportion to malaria disease severity and parasite biomass, and may contribute to malaria pathogenesis.

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Abstract

Background: Neutrophil activation results in *Plasmodium* parasite killing in vitro, but neutrophil products including neutrophil extracellular traps (NETs) mediate host organ damage and may contribute to severe malaria. The role of NETs in the pathogenesis of severe malaria has not been examined.

Methods: In Papua, Indonesia, we enrolled adults with symptomatic *Plasmodium falciparum* (n=47 uncomplicated, n=8 severe), *P. vivax* (n=37) or *P. malariae* (n=14) malaria; asymptomatic *P. falciparum* (n=19) or *P. vivax* (n=21) parasitemia; and healthy adults (n=23) without parasitemia. Neutrophil activation and NETs were quantified by immunoassays and microscopy, and correlated with parasite biomass and disease severity.

Results: In patients with symptomatic malaria, neutrophil activation and NET counts were increased in all three *Plasmodium* species. In falciparum malaria, neutrophil activation and NET counts positively correlated with parasite biomass (Spearman rho=0.41, p=0.005 and r^2=0.26, p=0.002, respectively) and were significantly increased in severe disease. Conversely, NETs were inversely associated with parasitemia in adults with asymptomatic *P. falciparum* infection (r^2=0.24, p=0.031), but not asymptomatic *P. vivax* infection.

Conclusion: Whilst NETs may inhibit parasite growth in asymptomatic *P. falciparum* infection, neutrophil activation and NET release may contribute to pathogenesis in severe falciparum malaria. Agents with potential to attenuate these processes should be evaluated.

Keywords: neutrophil, neutrophil extracellular traps, *Plasmodium*, malaria, neutrophil activation, severe, human, innate

Introduction

Malaria remains a major health burden with more than 200 million symptomatic cases and 400,000 deaths annually [1]. *Plasmodium falciparum* accounts for most fatalities, though all *Plasmodium* species can cause severe disease [2-4]. Neutrophils are innate immune cells with antimicrobial functions and regulate the effector functions of other immune cells [5]. Multiple studies demonstrate that neutrophils can kill *P. falciparum* parasites in vitro [6-8].

As well as antiparasitic effects, neutrophil activation in malaria has been linked to pathogenesis. Neutrophils are activated in vivax [9] and severe falciparum malaria [10-12], with activation associated with endothelial damage in both uncomplicated [13] and severe falciparum malaria [14], and hepatotoxicity in vivax malaria [15]. Severe disease is also associated with high expression of neutrophil granule-related genes linked to the innate immune response to high parasite load in pediatric falciparum malaria [16]. Microscopic detection of malaria pigment in neutrophils is a prognostic marker in falciparum malaria and is predictive of fatal outcomes [17]. Neutrophil activation is also associated with pathogenesis in murine models of malaria [18, 19].

A key mechanism underlying both the pathogen-killing and pathogenic effects of neutrophils in other human infections is the release of neutrophil extracellular traps (NETs). Activated neutrophils form NETs by releasing extracellular DNA, chromatin and granule proteins capable of capturing and destroying pathogens [20], however, they can also exacerbate angiopoietin-mediated endothelial activation [21] and tissue damage [22, 23]. *P. falciparum*-infected red blood cells (RBCs) stimulate human neutrophils to release NETs in vitro [19]. *Plasmodium* species produce a DNase that degrades NETs, and

73 experimental knockout of this DNase results in lower parasitemia in mice [8]. While NETs
74 may reduce parasite biomass *in vivo*, other murine studies show that host NETosis is
75 linked to tissue damage [22, 23], including malaria-associated acute lung injury [19].

76 Key pathogenic processes in severe falciparum malaria include parasite sequestration in
77 the microvasculature and endothelial dysfunction, resulting in impaired tissue perfusion
78 and organ dysfunction [2]. NETs have been hypothesized to exacerbate these processes
79 and contribute to malaria pathogenesis [24]. A previous study, without neutrophil-specific
80 markers, reported the presence of NETs in children with uncomplicated falciparum
81 malaria with parasites trapped within [25]. However, the role of NETs in controlling
82 parasitemia and contributing to malaria pathogenesis are not known for any human
83 *Plasmodium* species. To determine the role of NETs in human *Plasmodium* infection, we
84 measured NETs and neutrophil activation in patients with and without infection with *P.*
85 *falciparum, P. vivax* or *P. malariae*, and determined relationships with disease severity
86 and parasite biomass. We also determined if hemolysis and platelet activation, both
87 known to trigger NET release in other diseases [26, 27], were associated with NET
88 formation in malaria.

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Methods

Study site and participants

The study was conducted between 2014 and 2017 in the Mimika district of southern Papua, Indonesia, a region with unstable transmission of *P. falciparum*, *P. vivax* and *P. malariae* [28, 29]. Individuals in the following groups were enrolled at Rumah Sakit Mitra Masyarakat (RSMM) Hospital: i) uncomplicated malaria (UM), defined as a blood smear positive by microscopy for *P. falciparum*, *P. vivax* or *P. malariae*, fever or history of fever in the last 48 hours, no clinical evidence of concurrent infection or major comorbidity, and no prior antimalarial therapy in the preceding 24 hours. ii) Severe malaria (SM), defined according to the WHO 2014 research criteria [2]. iii) Controls, defined as visitors or relatives of malaria patients, with no fever or history of fever in the preceding 14 days and a blood smear negative for malaria parasites. Exclusion criteria included the following: i) pregnant or lactating, ii) aged <16 or >60 years, iii) a hemoglobin level ≤7 g/dL. Malaria patients at RSMM were treated as described previously [30]. Demographic and clinical information were collected using a standardized data collection form. Peripheral venous blood collection and hematology methods are described in Supplementary Methods. Longitudinal blood samples were available for a subset of patients requiring hospital admission and had blood recollected before discharge.

As an additional comparator group to those with symptomatic malaria, adults with asymptomatic *P. falciparum* or *P. vivax* parasitemia by microscopy, enrolled in a 2013 community household survey of the Mimika district [28], had NETs quantitated on Giemsa slides. Slides were included if asymptomatic individuals were aged >16 years, not

pregnant, had hematology results available, and were afebrile at recruitment (<37.5 °C) and in the preceding 24 hours.

The studies were approved by the Human Research Ethics Committees of Gadjah Mada University, Indonesia, and Menzies School of Health Research, Australia. Written informed consent was obtained from all participants.

Neutrophil activation and parasite biomass (PfHRP2)

Neutrophil elastase (NE) and proteinase-3 (PR3) concentrations were determined in platelet-free plasma using the Human Polymorphonuclear NE Enzyme Linked Immunosorbent Assay (ELISA) kit (Abcam, Cambridge, UK) and the PR3 Human ELISA kit (HycultBiotech, Uden, Netherlands), respectively. Myeloperoxidase (MPO) concentrations were measured in heparin plasma using the Human MPO ELISA kit (Abcam, Cambridge, UK). All assays were performed according to manufacturer's instructions. Sample dilutions were adjusted for each assay. Plasma *P. falciparum* histidine-rich protein-2 (PfHRP2), a measure of total parasite biomass, was measured by ELISA as previously described [31].

Neutrophil extracellular traps

NETs were quantified in a subset of blood samples by conventional Giemsa microscopy and immunofluorescent staining. In Giemsa-stained thin smears, NETs were identified at

1000x magnification as extracellular structures with staining of decondensed chromatin associated with fragmented neutrophil-like cells or small granules.

In the immunofluorescent assay, thin smears were prepared from CTAD-anticoagulated blood on glass slides on the day of venipuncture, dried and then stored at -80°C in enclosed slide holders. Frozen slides were thawed and rapidly fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes at room temperature (RT), then blocked with 5% (w/v) bovine serum albumin plus 0.005% (w/v) saponin in PBS for 20 minutes at RT. Samples were stained with rabbit anti-human NE (Merck, Darmstadt, Germany) and mouse anti-human histone H1 (Abcam, Cambridge, UK) primary antibodies for 1 hour at RT, followed by secondary labeling for 1 hour with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate and sheep anti-mouse IgG conjugated to cyanine-3 at RT (both from Sigma Aldrich, Missouri, US). Slides were mounted with SlowFade™ Gold Antifade Mountant with DAPI (Invitrogen, Massachusetts, US) and kept in the dark at 4°C until ready to read. An Axio Scope A1 fluorescent microscope coupled to an Axiocam ICm-1 CCD camera (all from Carl Zeiss, Germany) was used to count NETs at 630x magnification, identified as NE-positive cells with extracellular string-like structures of DNA positive for histone-H1 and DAPI.

Giemsa and fluorescent slides were read by two research microscopists (LL and SK) blinded to each other's results. For both methods, the number of NETs was counted as a percentage of total neutrophils; slides with <50 neutrophils counted were excluded. The number of NETs per µL blood was calculated from automated neutrophil counts in whole blood. Parasitized and uninfected RBCs within NETs were quantified from Giemsa slides.

Factors potentially triggering NET release

Platelet binding to neutrophils and cell-free heme are known triggers of NET release [26, 27]. Fresh whole blood flow cytometry was used to quantify circulating platelet-bound neutrophils as previously published [32]. Cell-free hemoglobin (CFHb) concentrations were measured on twice-centrifuged CTAD-anticoagulated plasma using the Human Hemoglobin ELISA Quantitation Set (Bethyl Laboratories, Montgomery, US).

Statistics

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA) or Stata 14 (StataCorp, Texas, US). The Kruskal-Wallis test followed by Dunn's multiple comparisons was used for comparisons between controls with *P. falciparum* groups, and between controls with *P. vivax* and *P. malariae*. The Mann-Whitney test was used for comparisons between severe and non-severe *P. falciparum*. The Wilcoxon matched-pairs signed rank test was applied to paired datasets. Microscopy images were processed with ZEN-2 software (Carl Zeiss, Germany) and flow cytometric data were analyzed using FlowJo (TreeStar, Ashland, OR). Associations between two variables were assessed using linear regression with robust standard errors, or Spearman correlation. Individuals with a NET count or HRP2 concentration of zero were given a value of half of the lower limit of detection for each assay (0.5 and 0.3, respectively). Correlations were adjusted for potential confounders using multivariable linear regression with robust standard errors. A two-sided value of $P<0.05$ was considered significant.

Results

Participant characteristics and neutrophils

In total, 129 adults were enrolled from RSMM hospital, comprising 8 with severe falciparum malaria, 98 with UM (47 falciparum, 37 vivax and 14 malariae malaria) and 23 with no infection (controls). From the household survey, asymptomatic individuals microscopy-positive with *P. falciparum* (n=19) and *P. vivax* (n=21) were included in the NETs analysis. Baseline characteristics of participants, including parasite biomass, hematology results and criteria for SM cases, are listed in **Table 1**. Clinical details of SM patients are described in **Table S1.** Median neutrophil count per µL blood was higher in SM (p=0.010) and lower in UM with *P. malariae* (p=0.0009) compared to controls (**Table 1** and **Figure S1**).

Paired samples collected on admission and discharge from hospital were available for 9 patients with uncomplicated *P. falciparum* and 4 with uncomplicated *P. vivax* malaria. Median neutrophil counts were lower on discharge from hospital in all patients and this was statistically significant for falciparum malaria (4,400 vs 2,200 per µL blood [p=0.012], Wilcoxon test).

Neutrophil activation is increased in human malaria

NE and PR3 concentrations were elevated in UM from all species compared to controls (p<0.0005), and in falciparum malaria were proportional to disease severity (p<0.0005); **Figure 1A** and **1B**, and **Table S2**. The third marker, MPO, was significantly higher in SM relative to UM and controls (both p<0.0001), but was not elevated in any group of UM

patients; **Figure 1C** and **Table S2**. Differences in activation markers between groups remained significant after adjusting for neutrophil counts (all $p<0.0005$, except MPO $p<0.05$). Longitudinally, NE and PR3 concentrations had fallen by hospital discharge in falciparum ($p=0.004$ and $p=0.008$, respectively) and vivax malaria patients; **Figure 2A** and **2B**.

Circulating neutrophil extracellular traps are elevated in human malaria

NETs were visualized using two methods – Giemsa staining and immunofluorescence; **Figure 3**. Images of normal neutrophils not undergoing NETosis are illustrated in **Figure 3A*i*** and **3B*i*** (lower cell), with NETosis seen in **Figures 3A*ii-ix*** and **3B*ii-x***. When quantified by Giemsa staining, median number of NETs per µL blood were higher in UM than controls for all species; **Figure 3C** and **Table S2**. In falciparum malaria, the number of NETs was increased in proportion to disease severity; **Figure 3C** and **Table S2**.

In the immunofluorescent assay, circulating NETs were similarly elevated in UM relative to controls in all species, and in the falciparum patients higher in SM compared to UM ($p<0.005$); **Figure 3D** and **Table S2**. In patients with paired samples collected on admission and hospital discharge, circulating NET counts in patients with falciparum and vivax malaria were higher on admission compared to discharge, although this only reached significance for *P. falciparum* ($p=0.016$); **Figure 4A** and **4B**.

In total 80 samples had NETs quantified by both immunofluorescence and Giemsa staining. Bland-Altman results indicated that Giemsa stains generally returned higher NET counts than immunofluorescence, but the difference in NET counts were still within

the 95% confidence interval; **Figure S2A**. The correlation rho-squared between the two methods was 0.133; (p=0.001); **Figure S2B**.

The size, shape and frequency of NETs varied between samples; **Figure 3A** and **3B**. In patients with malaria, parasitized RBCs were observed on Giemsa staining to be in direct contact with at least one NET in 29% (10/35) of patients with *P. falciparum*; **Figure 3A*ii-iv***. NET contact with parasitized RBCs was apparent in 38% (6/16) of patients with *P. vivax* and 8% (1/12) with *P. malariae*; **Figure 3A*v,vi*** and **3A*ix***, respectively. In patients with NET-bound parasites, the median percentage of NETs that contained parasites was higher in *P. falciparum* than *P. vivax* (**Figure 4C)**, with NETs containing 1-2 parasites in *P. falciparum* (all rings) and a maximum of one parasite in *P. vivax* (either a ring or trophozoite). Among patients with malaria, the majority of NETs contained between 1 and 9 uninfected RBC; **Figure 3A**. While the immunofluorescent assay confirmed the presence of NETs in malaria and could identify parasitized RBCs in close proximity to NETs (**Figure 3B*i,iv,viii***), parasites were not visualized inside NETs due to overlap in the staining of parasite and host nuclear material.

NETs were also present in asymptomatic parasitemia, with median numbers per µL blood significantly elevated relative to controls in both asymptomatic *P. falciparum* and *P. vivax* infections; **Figure 3C**. Ring and trophozoite-stage parasites were present within NETs in 10.5% (2/19) of individuals with asymptomatic *P. falciparum* infection, but none in those with *P. vivax*.

NETs and neutrophil activation correlate with parasite biomass in malaria

In UM with *P. falciparum*, parasite biomass correlated with neutrophil counts, NE and MPO concentrations, and NET counts; **Figure 5A**. The correlation between parasite biomass and NET counts remained significant after adjusting for neutrophil counts and NE or MPO concentrations (p<0.02). In patients with *P. vivax*, peripheral parasitemia correlated with neutrophil counts, NE, PR3 and MPO concentrations, and NET counts; **Figure 5B**. The associations between *P. vivax* parasitemia and neutrophil activation markers remained significant after adjusting for neutrophil counts (NE p=0.0004, PR3 p<0.0001, MPO p=0.001). There was no correlation between peripheral parasitemia and neutrophil parameters in 14 patients with UM due to *P. malariae*. In SM, there were strong associations between parasite biomass and neutrophil activation markers (NE Spearman r=0.96, p=0.003; PR3 r=0.76, p=0.048, both n=7); **Figure 5A**.

In contrast to symptomatic malaria, NET counts were inversely correlated with parasitemia in asymptomatic *P. falciparum* infection (p=0.031; **Figure 6**) and this remained significant after adjusting for neutrophil counts (p=0.028). There was no association between NET counts and asymptomatic *P. vivax* parasitemia; **Figure 6**.

NETs, platelet-bound neutrophils and hemolysis

Median platelet-bound neutrophils were significantly lower in malaria from each *Plasmodium* species relative to controls; **Table 1**. The number of platelet-bound neutrophils correlated with NET counts in UM patients with *P. falciparum* (linear regression with robust standard errors, r^2=0.17, p=0.049, n=27) and *P. malariae* (Spearman r=0.66, p=0.031, n=11), which remained significant after adjusting for *P.*

falciparum biomass and platelet counts (*p*=0.012). Median CFHb concentrations were significantly higher in vivax and severe falciparum malaria relative to controls; **Table 1**. CFHb correlated with parasite biomass and NE concentrations in UM with *P. falciparum* (Spearman r=0.32, p=0.029 and r=0.30, p=0.047, respectively, both n=46), and a trend with MPO in SM (Spearman r=0.75, p=0.066, n=7). There was no correlation between CFHb and NET counts in any of the patient groups.

Discussion

In adults with uncomplicated falciparum, vivax and malariae malaria, circulating NETs and neutrophil activation were increased compared to aparasitemic controls. The presence of parasites inside NETs and the inverse relationship between NETs and parasitemia in asymptomatic *P. falciparum* infection are consistent with antiparasitic effects of NETs. However, the direct association between NETs and neutrophil activation with parasite biomass in falciparum and vivax malaria, suggests only a limited antiparasitic effect in clinical disease. In symptomatic falciparum malaria, these markers were elevated in proportion to disease severity, suggesting that both neutrophil activation and NET formation may contribute to malaria pathogenesis.

Our findings support previous studies investigating neutrophil activation in falciparum and vivax malaria [9-15], and provide evidence of neutrophil activation in *P. malariae* patients. NE and PR3 were increased in malaria from each *Plasmodium* species and across disease severity, and are known mediators of acute lung injury in murine malaria models [19] and vascular damage in vitro [33]. MPO, which was elevated only in severe falciparum malaria, has been linked to impaired parasite clearance [34]. Uncontrolled malarial neutrophil activation impairs function [35-37], reduces chemotaxis [9], compromises endothelial integrity [13, 38, 39], and has been associated with cerebral vasculopathy in children with cerebral malaria [14] and liver damage in vivax malaria [15].

Specific neutrophil immunofluorescent markers confirm that extracellular traps previously seen in children with falciparum malaria [25] may indeed be NETs, and this is now confirmed in adult patients with falciparum, vivax and malariae malaria. Further studies quantifying NETs in children with malaria and the association with disease severity are

warranted. NE and MPO are known to promote chromatin decondensation and NET formation [40], and are thus likely to stimulate NETosis in malaria for each parasite species, particularly in severe disease.

Platelet binding to neutrophils is an important mechanism for NET formation in sepsis [26], and platelet-neutrophil complexes have greater adhesive properties and capacities to undergo oxidative bursts [41], a key trigger for NETosis. In malaria patients, circulating platelet-bound neutrophils were reduced, hence these complexes are either lost, migrate to tissues, or form NETs. The positive correlations between platelet-bound neutrophils and NETs in malaria patients suggest that platelet-neutrophil interactions contribute to NET formation in human malaria. Hemolysis is associated with endothelial and organ dysfunction in SM [42, 43] and stimulates NET formation in sickle cell disease [27]. In the present study, plasma CFHb was associated with neutrophil activation, but not NETs, suggesting that hemolysis may promote SM through mechanisms other than NET formation.

Parasites trapped within NETs and the inverse relationship between NETs and asymptomatic *P. falciparum* parasitemia highlight a potential antiparasitic function. Despite the paucity of asymptomatic individuals with parasites visible inside NETs, it is plausible that NET-mediated protection contributes to control of *P. falciparum* replication in low parasitemia infections, as seen in asymptomatic parasitemia, where the neutrophil dysfunction that occurs in clinical disease [35-37] is not apparent. A greater anti-parasitic effect on *P. falciparum* was also supported indirectly by the higher frequency of NETs containing *P. falciparum* than *P. vivax* in clinical disease.

Associations between NE and parasite biomass have been reported previously in falciparum malaria [11], but not for other activation markers or in vivax malaria. We show that direct (NETs) and indirect markers (NE, PR3 and MPO) of neutrophil activation are each associated with *P. falciparum* and *P. vivax* parasite density, key determinants of disease severity from each species [31, 44]. The correlation between NETs and parasite biomass in clinical disease is consistent with the direct stimulation of NETs by *P. falciparum* seen in vitro [19], and also an inability of NETs to control parasite biomass in clinical disease. This inability may be related to the significant dysfunction of neutrophils that is found in symptomatic falciparum malaria [35-37]. After antimalarial treatment and recovery, elevated neutrophil activation and circulating NET counts in discharged patients returned to control levels, consistent with the rapid and early resolution of parasite-induced neutrophil activation and NET release following clearance of parasitemia.

The association between NETs and disease severity in clinical disease suggests a contribution from neutrophil activation and NETs to malaria pathogenesis [24]. NETs have been identified in the blood and lungs of mice suffering from malaria-associated lung injury, where depletion of neutrophils reduced tissue damage and increased mouse survival [19]. Accumulation of neutrophils in the inter-alveolar spaces of *P. vivax* patients dying from respiratory complications [45, 46] has also been reported, however, the presence of NETs has not been examined. It was notable that the single severe falciparum malaria patient with acute lung injury had markedly elevated circulating NETs and neutrophil activation markers, however the patient also had multiple other severity criteria, including coma. Marked NETosis was also seen in others with multiorgan failure, including severe acute kidney injury. In pediatric cerebral malaria, a lack of neutrophils

and NETs in brain autopsies has been described [14], suggesting local NET formation may not be a contributing factor to coma. Studies examining NETs in organs outside the brain in SM are warranted.

Multiple mechanisms have been proposed for how NETs may promote SM [24]. NET products such as metalloproteinases, PR3 and NE are known to breakdown endothelial glycocalyx [47], a carbohydrate-rich layer important in maintaining endothelial integrity. Glycocalyx loss is considered a key event in SM pathogenesis [48]. Angiopoietin-induced NET release may also contribute to angiopoietin-mediated endothelial activation [21] and accentuate the microvascular dysfunction due to parasite sequestration [49]. Further, the increase in NETs may exacerbate intravascular thrombosis [22] and promote ischemia and reduced tissue perfusion [24].

The mechanisms leading to malarial anemia are not well understood. It was notable that the majority of circulating NETs in our malaria patients contained uninfected RBCs. Accumulation of RBCs within NETs has been described in models of thrombosis [22], and uninfected RBC phagocytosis by neutrophils also occurs *ex vivo* in the presence of *P. vivax* [15]. These findings suggest that neutrophil-mediated loss of bystander RBCs may be a possible contributor to anemia from both falciparum and non-falciparum malaria.

Giemsa stains generally returned higher NET counts than neutrophil-specific immunofluorescent staining. Overestimation of NETs in the Giemsa stains was likely due to the difficulty in differentiating extracellular traps originating from neutrophils versus other white blood cells [50]. However, both methods were able to show comparable differences between malaria patients and controls, suggesting that both methods are appropriate for quantifying NETs.

In conclusion, our study indicates that neutrophils play an important role in malaria. Neutrophil activation and circulating NET formation were increased in clinical illness with *P. falciparum*, *P. vivax* and *P. malariae*, and were highest in patients with severe disease. In asymptomatic *P. falciparum* infection, where parasitemias are low and neutrophil dysfunction is not apparent, NETs may inhibit parasite growth. However, the associations of neutrophil activation and NETs with parasite biomass and disease severity support the notion that neutrophil activation and NETs contribute to malaria pathogenesis. While it is not known whether interventions targeting these processes would be timely in those presenting with severe malaria, agents which attenuate neutrophil activation and NET release should be further evaluated as potential adjunctive agents for severe malaria.

Footnotes

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Conflict of interest

All the authors declare that no conflicts of interest exist.

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531

Figure legends

Figure 1. Neutrophil activation in human malaria. Markers for neutrophil activation were quantified by ELISA in frozen plasma from malaria patients and controls. Markers included neutrophil elastase **(A)** and proteinase-3 **(B)**, determined in 23 controls, 8 patients with severe *P. falciparum* (Pf), 46 uncomplicated malaria (UM) patients with Pf, 13 UM with *P. malariae* (Pm) and 36 UM with *P. vivax* (Pv). A third marker, myeloperoxidase **(C)**, was measured in 23 controls, 7 patients with severe Pf, 47 with UM Pf, 14 with UM Pm and 37 with UM Pv. Plots show median and individual data points in each group. The Kruskal-Wallis with Dunn's multiple comparisons test was used for statistical comparisons to controls. Significant differences in all plots are signified by asterisks (***p<0.0005, ****p<0.0001). The Mann-Whitney test was used for comparison between UM and severe Pf ($^{\#}$p<0.0005, $^{\#\#}$p<0.0001). Data presented in **Table S2**.

Figure 2. Neutrophil activation in malaria inpatients on admission and discharge from hospital. Paired data (connected dots) were available for 9 *P. falciparum* (Pf) and 3 *P. vivax* (Pv) patients whom were admitted and had neutrophil elastase **(A)** and proteinase-3 **(B)** concentrations tested again before being discharged from hospital. The Wilcoxon test was used for statistical comparisons. Significant differences are signified by asterisks (*p<0.05, **p<0.005).

553 **Figure 3. Circulating NETs in human malaria.** Giemsa **(A)** and 3-color
554 immunofluorescent staining (IF) **(B)** were used to visualize NETs in blood smears from
555 controls and malaria patients. These were identified as extracellular web-like structures
556 of decondensed chromatin released by neutrophils. NETs were positive for DNA, histones
557 and neutrophil elastase by IF. A small proportion of circulating NETs were found to contain
558 parasitized RBCs (iRBCs) with *P. falciparum* (Pf) **(*Aii-iv*)**, *P. vivax* (Pv) **(A*v,vi*)** and *P.*
559 *malariae* (Pm) **(A*ix*)**, though the majority of NETs contained uninfected RBCs **(A*vii,viii*)**.
560 Parasitized RBCs nearby NETs were more frequent **(B*i,iv,viii*)**. The number of NETs per
561 µL blood was quantified by Giemsa **(C)** and IF staining **(D)** in controls (n=12), severe
562 patients with Pf (Giemsa n=6 and IF n=8), and uncomplicated malaria (UM) patients with
563 Pf (Giemsa n=29 and IF n=28), Pm (Giemsa n=12 and IF n=13) and Pv (n=16). Additional
564 Giemsa stains from asymptomatic *Plasmodium* carriers with Pf (n=19) and Pv (n=21)
565 were analyzed for NETs **(C)**. Plots show median and individual data points in each group.
566 The Kruskal-Wallis with Dunn's multiple comparisons test was used for statistical
567 comparisons to controls. Significant differences in all plots are signified by asterisks
568 (*p<0.05, **p<0.005, ****p<0.0001). The Mann-Whitney test was used for comparison
569 between UM and severe Pf ($^{\#}$p<0.005, $^{\#\#}$p<0.0005). Data presented in **Table S2**.

570

571 **Figure 4. Circulating NETs in malaria inpatients on admission and discharge from**
572 **hospital, and percentage of NETs containing parasites in falciparum and vivax**
573 **malaria.** Paired NET counts (connected dots) were available for 7 *P. falciparum* (Pf) **(A)**
574 and 4 *P. vivax* (Pv) patients **(B)** whom were admitted and had NETs quantified again by
575 immunofluorescence before being discharged from hospital. The Wilcoxon test was used

for statistical comparison. In patients with NET-bound parasites, the percentage of NETs containing parasites in each sample was compared between Pf and Pv **(C)**. Plots show median and individual data points in each group. The Mann-Whitney test was used for statistical comparisons. Significant differences in all plots are signified by asterisks (*p<0.05).

Figure 5. Parasite biomass correlations with neutrophil parameters. Parasite biomass was correlated with neutrophil counts, neutrophil activation markers and circulating NET counts in uncomplicated malaria (UM) patients with *P. falciparum* **(A)** and *P. vivax* **(B)**. HRP2 was used as a measure for parasite biomass in *P. falciparum* patients, and parasitemia was used for *P. vivax.* All data were log transformed. Linear regression (with robust standard errors) (LR) or Spearman correlation was used. Lines of best fit were determined by linear regression. In *P. falciparum* plots, data for UM (outlined circles, continuous line) and severe malaria patients (filled circles, dotted line) are shown. The number of samples correlated, rho or rho-squared, and *p*-value are presented in each plot and correspond to correlations in UM patients. A *p*-value of less than 0.05 was considered significant.

Figure 6. Correlation between parasitemia and NETs in asymptomatic *Plasmodium* carriers. Linear regression (with robust standard errors) between parasitemia and circulating NET counts in asymptomatic individuals with *P. falciparum* (n=19) or *P. vivax* infection (n=21). Parasitemia values were log transformed.

598 **Table 1. Baseline characteristics of n=169 participants from Papua, Indonesia**

	Controls *n*=23	*P. falciparum*			*P*-value [b]	Asymptomatic *P. vivax* *n*=21	UM *P. vivax* *n*=37	UM *P. malariae* *n*=14	*P*-value [c]
		Asymptomatic *n*=19	UM *n*=47	Severe [a] *n*=8					
Age, years	28 [26-34]	36 [26-49]	27 [19-36]	33 [18-53]	0.078	36 [31-41]	23 [19-31]	34 [26-47]	<0.0001
Male, *n* (%)	9 (39)	11 (58)	22 (47)	7 (88)	-	8 (38)	23 (62)	4 (29)	-
Ethnicity, *n* of highland/ lowland/non-Papuan	12/10/1	1/10/8	42/2/3	8/0/0	-	1/6/14	32/3/2	14/0/0	-
Parasitemia (parasites/µL)	Not detected	360 [236-3,330]	10,800 [3,400-44,000]###	283,000 [94,200-745,000]	-	161 [67-1,040]	5,750 [2,120-10,900]	1,230 [820-2,540]	-
***P. falciparum* HRP2 [a] (ng/mL)**	-	-	113 [8-322]####	2,120 [1,220-5,020]	-	-	-	-	-
Hemoglobin (g/dL)	12.0 [11.4-14.7]	11.6 [10.6-13.6]	12.4 [10.2-13.3]	12.8 [9.7-13.5]	0.539	13.2 [12.3-14.5]	12.0 [10.6-13.1]	9.6 [8.6-10.6]***	<0.0001
Cell-free hemoglobin (ng/µL)	33.1 [23.9-44.8]	-	37.8 [27.9-80.7]	73.9 [37.4-261.4]*	0.039	-	48.5 [32.9-71.6]*	39.2 [23.6-77.4]	0.060
White blood cell count (x1000/µL)	7.4 [6.0-8.1]	5.9 [4.9-7.9]	4.8 [3.6-6.1]****/###	7.9 [6.3-12.4]	<0.0001	6.8 [5.6-8.9]	5.6 [5.0-7.6]	4.2 [3.2-5.1]****	<0.0001

Neutrophils (% white blood cells)	45.2 [41.4-53.2]	50.0 [44.4-53.2]	62.0 [51.4-73.3]***/#	77.0 [70.3-80.7]****	<0.0001	44.9 [36.2-54.6]	61.0 [55.4-70.0]****	48.6 [35.0-51.7]	<0.0001
Neutrophils (x1000/µL)	3.4 [2.4-4.3]	2.9 [2.4-3.9]	2.7 [1.9-4.3]####	5.7 [5.1-8.6]**	0.0006	2.9 [2.4-4.5]	3.8 [2.8-4.3]	1.6 [1.5-2.4]***	0.0002
Platelet count (x1000/µL)	198 [170-228]	116 [90-192]	74 [50-102]****/####	19 [13-37]****	<0.0001	115 [85-171]**	81 [54-118]****	80 [56-101]****	<0.0001
Platelet-bound neutrophils (% neutrophils) [d]	7.7 [6.7-9.9]	-	3.6 [2.4-5.1]****/##	1.1 [1.0-1.5]***	<0.0001	-	3.9 [2.3-5.5]****	4.9 [3.3-6.1]**	<0.0001
Platelet-bound neutrophils (per µL) [d]	249 [172-395]	-	115 [85-164]****	51 [46-83]**	<0.0001	-	132 [100-209]**	87 [57-111]****	<0.0001

599

600 Footnotes:

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

602 [a] Severe malaria criteria encountered: cerebral malaria (Glasgow Coma Score ≤10 for >30 minutes) (n=1), jaundice
603 (visible jaundice OR creatinine >1.5 mg/dL OR bilirubin >3 mg/dL AND >100,000 parasites/µL) (n=5), acute renal failure
604 (creatinine >3 mg/dL +/- urine output <400 mL/day OR urea > 20 mM) (n=2), hypoglycemia (plasma glucose <40 mg/dL)

(n=1), hyperparasitemia (asexual parasitemia >10%) (n=4), and respiratory distress (respiratory rate >30/minute AND O_2 saturation <92%) (n=1). See Table S1 for clinical features of severe malaria patients.

[b] Kruskal-Wallis with Dunn's multiple comparisons test between controls, and asymptomatic, uncomplicated and severe *P. falciparum*.

[c] Kruskal-Wallis with Dunn's multiple comparisons test between controls, asymptomatic and uncomplicated *P. vivax*, and uncomplicated *P. malariae*.

[d] Previously published data [32].

Significantly different to control: ****p<0.0001, *** p<0.0005, ** p<0.005, *p<0.05.

Significantly different to severe *P. falciparum*: ####p<0.0001, ###p<0.0005, ##p<0.005, #p<0.05 (Mann-Whitney test).

Abbreviations= UM, uncomplicated malaria; HRP2, histidine-rich protein-2.

615 **Supplementary List**

616

617 **Supplementary Methods**

618 Blood collection and hematology

619

620 **Supplementary Materials**

621 Table S1. Clinical features of severe malaria patients

622 Table S2. Neutrophil activation and NETs in malaria patients

623 Figure S1. Distribution of neutrophils between infection groups

624 Figure S2. Comparison of Giemsa and immunofluorescence to count NETs