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Controlled human infection models as a tool for malaria and schistosomiasis vaccine research

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Possibilities of
the controlled human
malaria infection model





Infectivity of *Plasmodium falciparum* sporozoites determines emerging parasitemia in infected volunteers.

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Abstract

Malaria sporozoites must first undergo intrahepatic development before a pathogenic blood-stage infection is established. The success of infection depends on host and parasite factors. In healthy human volunteers undergoing controlled human malaria infection (CHMI), we directly compared three clinical *Plasmodium falciparum* isolates for their ability to infect primary human hepatocytes *in vitro* and to drive the production of blood-stage parasites *in vivo*. Our data show a correlation between the efficiency of strain-specific sporozoite invasion of human hepatocytes and the dynamics of patent parasitemia in study subjects, highlighting intrinsic differences in infectivity among *P. falciparum* isolates from distinct geographical locales. The observed heterogeneity in infectivity among strains underscores the value of assessing the protective efficacy of candidate malaria vaccines against heterologous strains in the CHMI model.

Introduction

Malaria infections are initiated by blood-feeding female *Anopheline* mosquitoes injecting *Plasmodium* sporozoites into their mammalian host. Although most of the sporozoites get trapped in the dermis or draining lymph nodes, a small but crucial fraction succeed in penetrating dermal vasculature, whence they are carried into the bloodstream, including eventually the hepatic circulation (1). There they invade the liver parenchyma, traversing numerous cells before a few eventually succeed in invading a permissive hepatocyte where they undergo schizogony (2). Each liver-stage schizont releases an estimated $\geq 40,000$ merozoites (3), which invade erythrocytes, causing blood-stage disease. The respective steps in the preerythrocytic life cycle of the *Plasmodium* parasite initially form a substantial bottleneck and subsequently an enormous multiplication opportunity (4).

However, it is unclear how the success rate of these various preerythrocytic steps ties together to determine the total burden of parasites emerging from the liver and hence the course of pathogenic blood-stage infection. It is also unknown to what extent malaria isolates vary in infectivity with regard to preerythrocytic infection or, conversely, whether humans differ in their intrinsic permissiveness. Addressing the latter issue is central to the rational design of controlled human malaria infection (CHMI) studies for clinical evaluation of candidate malaria vaccines (5). Here, we investigated whether three clinical isolates of *Plasmodium falciparum* differed in their intrinsic ability to accomplish the sequential steps of preerythrocytic development in liver hepatocytes and how this affected the course of blood-stage infection in humans. We studied parasite dynamics both *in vivo* in malaria-naïve volunteers undergoing CHMI and in a recently optimized *in vitro* model using freshly isolated human hepatocytes (6, 7). *In vitro* experiments were performed with sporozoites harvested from exactly the same batches of infected mosquitoes used on the same day for the human infections. Alongside the longstanding laboratory strain NF54 (8) and the recently reported NF135.C10 clone (9), we described the development and use in humans of the *P. falciparum* clone NF166.C8. We showed that intrinsic differences in infectivity existed between *P. falciparum* isolates with regard to their ability to invade and multiply within human hepatocytes *in vitro*. The degree of liver-stage infection correlated directly with the magnitude of the first wave of blood-stage parasites to emerge from the liver *in vivo* and correlated inversely with the prepatent period in CHMI subjects.

Results

Dynamics of parasitemia during CHMI

In total, 23 subjects across two separate CHMI studies were each exposed to sets of five mosquitoes carrying the NF54, NF135.C10, or NF166.C8 isolates of *P. falciparum* (study flowcharts, study subject demographics, and laboratory characteristics of these *P. falciparum* isolates are provided in fig. S1 and tables S1 and S2, respectively). All subjects developed patent

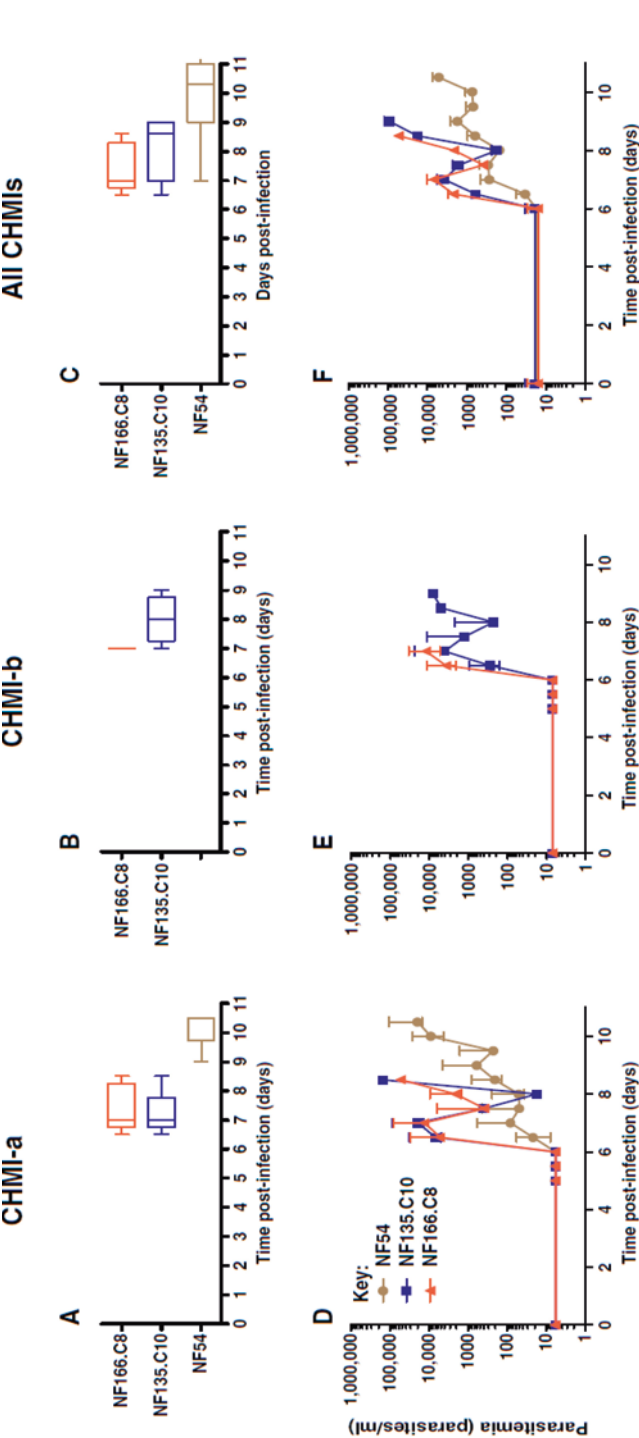


Figure 1. Prepatent period and kinetics of parasitemia in CHMI with three *P. falciparum* strains. (A) Prepatent periods in the CHMI-a study ($n = 5$ per group). (B) Prepatent periods in the CHMI-b study ($n = 4$ per group). All subjects infected with the NF166.C8 strain in the CHMI-b study had prepatent periods of exactly 7.0 days, which is shown as a single line in (B). (C) Combined data from all 10 comparable CHMI studies performed at our center using thick smear microscopy as an end point ($n = 56$ NF54, $n = 13$ NF135.C10, and $n = 5$ NF166.C8). Box-and-whisker plots represent median/interquartile range and range per strain. In the CHMI-a study (A) and all studies in (C), prepatent periods were determined by time to positive thick blood smear; in the CHMI-b study (B) and subsequent studies at our center, prepatent periods were determined by time to positive qPCR. (D to F) Development of submicroscopic parasitemia after infection, as determined retrospectively by qPCR in the CHMI-a study (D) ($n = 5$ per group), the CHMI-b study (E) ($n = 4$ per group), and combined data from all 18 CHMI studies at our center using either thick smear microscopy or qPCR as an end point (F) ($n = 90$ NF54, $n = 22$ NF135.C10, and $n = 14$ NF166.C8). Data represent geometric mean \pm 95% CI of all pretreatment samples per group per time point. All subjects in the CHMI-a and CHMI-b studies subsequently received a standard course of atovaquone-proguanil treatment and had their parasitemia cleared completely. Brown circles, NF54 strain; blue squares, NF135.C10 strain; red triangles, NF166.C8 strain.

parasitemia and were curatively treated with atovaquone-proguanil once thick smear–positive (first study, CHMI-a) or quantitative real-time fluorescence polymerase chain reaction (qPCR)–positive (second study, CHMI-b). In CHMI-a (Fig. 1A), the prepatent period as determined by thick smear was significantly shorter for subjects infected with either the NF135.C10 clone (mean, 7.2 days; range, 6.5 to 8.5 days) or the NF166.C8 clone (mean, 7.4 days; range, 6.5 to 8.5 days) than for those infected with the NF54 strain of *P. falciparum* (mean, 10.2 days; range, 9.0 to 10.5 days); mean differences were, respectively, 3.0 days [95% confidence interval (CI), 1.7 to 4.3] and 2.8 days (95% CI, 1.5 to 4.1) [$P < 0.001$ for both comparisons, one-way analysis of variance (ANOVA)/Tukey's post hoc test, $n = 5$ per group]. No significant difference existed between NF135.C10 and NF166.C8 (mean difference, 0.2 day; 95% CI, -1.1 to 1.5 ; $P > 0.05$). In CHMI-b (Fig. 1B), the prepatent period as determined by qPCR criteria did not differ significantly between NF135.C10 (mean, 8.0 days; range, 7.0 to 9.0 days) and NF166.C8 (7.0 days in all four subjects) *P. falciparum* strains (mean difference, 1.0 day; 95% CI, -0.3 to 2.3) ($P = 0.09$, one-sample t test, $n = 4$ per group). The prepatent period observed for NF54 in the CHMI-a study was similar to that of $n = 56$ malaria-naïve subjects in all 10 of our CHMI studies (mean, 10.3 days; range, 7.0 to 16.0 days), in which treatment was initiated upon positive standardized thick blood smear analysis (table S3). Likewise, the prepatent period for NF135.C10 in CHMI-a was similar to that of $n = 13$ subjects across all 3 of those 10 studies that also included infections with NF135.C10 strain parasites (mean, 8.0 days; range, 6.5 to 9.0 days; table S3). In a meta-analysis of all 10 studies, the prepatent period of NF54 remained significantly ($P < 0.001$) longer than that of either NF135.C10 or NF166.C8 (Fig. 1C).

As determined retrospectively by qPCR in CHMI-a subjects, submicroscopic parasitemia became detectable before thick smear positivity in all subjects. Parasitemia was evident 6.5 days after infection in all NF135.C10-infected and NF166.C8-infected subjects and by day 7.5 (6.5 to 8.5) in the NF54-infected group (mean difference, 1.0 day; 95% CI, 0.1 to 1.9 ; Fig. 1D and Table 1). The magnitude of the initial wave of parasitemia emerging from the livers of both NF135.C10-infected and NF166.C8-infected subjects was greater compared to that of NF54-infected subjects, although this magnitude did not itself differ significantly between NF135.C10-infected and NF166.C8-infected subjects (Fig. 1D and Table 1). Overall, peak parasitemia per subject before initiation of antimalarial treatment with atovaquone-proguanil did not differ between groups (Table 1).

In CHMI-b, submicroscopic parasitemia was first detected by qPCR on day 6.5 after infection in all four subjects in both the NF135.C10 group and the NF166.C8 group. The magnitude of the initial wave of parasitemia emerging from the liver did not differ between NF135.C10 and NF166.C8 (Fig. 1E and Table 1). Although the initial wave of parasitemia in CHMI-a was slightly lower for the NF54 strain ($P = 0.046$) and somewhat higher for the NF135.C10 strain ($P = 0.0045$), as compared to our other CHMI studies (table S3), the magnitude of the first wave of NF54 parasitemia was around 10-fold lower compared to either NF135.C10 or NF166.C8 parasitemias in a meta-analysis of all 18 of our CHMI studies (Fig. 1F and Table 1).

Table 1. Characteristics of *P. falciparum* parasitaemia in CHMI subjects *in vivo*.

	NF54 ¹	NF135.C10 ¹		NF166.C8 ¹		NF54 vs NF135.C10 ²	NF54 vs NF166.C8 ²	NF135.C10 vs NF166.C8 ²
CHMI-a								
No. of subjects per group	5	5	5	5				
First detection of parasitemia by qPCR (days after infection)	7.5 (6.5-8.5)	6.5 (6.5-6.5)	6.5 (6.5-6.5)	6.5 (6.5-6.5)		1.0 (0.1 to 1.9)*	1.0 (0.1 to 1.9)*	-
<i>Peak parasitemia (log₁₀ para-sites/mL)</i>								
First wave ¹	1.96 (1.23-3.52)	4.28 (3.41-4.79)	3.89 (2.83-4.79)			-2.32 (-3.69 to -0.96)**	-1.93 (-3.30 to -0.56)**	0.39 (-0.97 to 1.76)
Overall ²	4.43 (3.84-5.17)	4.64 (4.24-5.19)	4.22 (2.83-4.79)			-0.20 (-1.19 to 0.78)	0.21 (-0.77 to 1.20)	0.42 (-0.56 to 1.40)
CHMI-b								
No. of subjects per group	-	4	4					
First detection of parasitemia by qPCR (days after infection)	-	6.5 (6.5-6.5)	6.5 (6.5-6.5)			-	-	-
<i>Peak parasitemia (log₁₀ para-sites/mL)</i>								
First wave ¹	-	3.61 (2.59-4.19)	4.09 (3.77-4.34)			-	-	-0.48 (-1.63 to 0.66)
Overall ²	-	3.94 (3.82-4.19)	4.09 (3.77-4.34)			-	-	-0.15 (-0.62 to 0.32)
All CHMI studies								
No. of subjects per group ⁵	90	22	14					
First detection of parasitemia by qPCR (days post-infection) ⁵	7.2 (6.3-10.6)	6.7 (6.5-7.0)	6.5 (6.5-6.5)			0.52 (-0.004 to 1.0)	0.70 (0.070 to 1.3)*	0.18 (-0.57 to 0.93)

Table 1. Continued.

	NF54 ¹	NF135.C10 ¹		NF166.C8 ¹	NF54 vs NF135.C10 ²	NF54 vs NF166.C8 ²	NF135.C10 vs NF166.C8 ²
<i>Peak para-sitemia (log₁₀ parasites/mL)</i>							
First wave ^{1,5}	2.72 (1.00-4.67)	3.67 (2.59-4.79)	3.78 (2.78-4.79)		-0.95 (-1.41 to -0.49)***	-1.06 (-1.61 to -0.51)***	-0.11 (-0.79 to 0.58)
Overall ^{2,6}	4.28 (3.13-5.17)	4.72 (3.35-5.84)	4.22 (2.83-4.97)		-0.44 (-0.86 to -0.009)*	0.065 (-0.58 to 0.71)	0.50 (-0.23 to 1.23)

¹Data represent mean per group [range]. ²Data represent mean differences between groups [95% CI of difference]. Bold face indicates statistical significance (*p<0.05, **p<0.01 and ***p<0.001 by 1-way ANOVA/Tukey's post-hoc test). ³Peak height of parasitaemia during the first wave to emerge from the liver, defined as the highest parasite density measured by qPCR per subject between day 6.5-8.0 post-infection; data represent geometric mean [range] per group. ⁴Peak height of parasitaemia per subject at any time post-infection (generally at start of treatment); data represent geometric mean [range] per group. ⁵Data included from all 18 comparable CHMI studies performed at our centre using either thick smear microscopy or qPCR as an endpoint (table S3). ⁶Data included from only those 10 CHMI studies performed at our centre in which subjects were treated upon positive standardised thick blood smear (n=56 NF54-infected, n=13 NF135.C10-infected and n=5 NF166-infected subjects, respectively).

Clinical course and adverse events in subjects undergoing CHMI

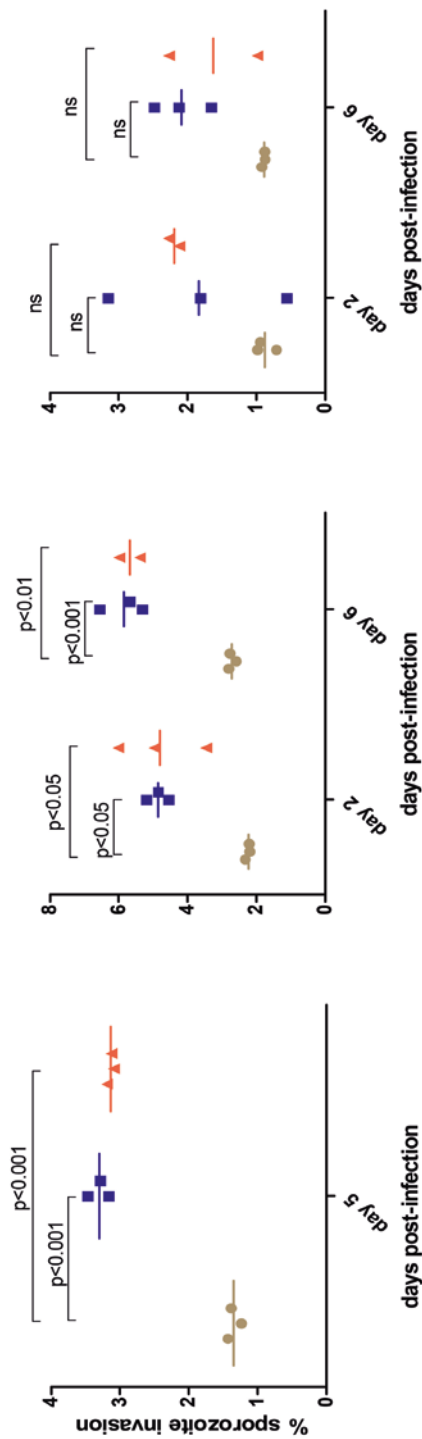
Mild-moderate adverse events were experienced by all CHMI-a subjects after infection (table S4A). Grade 3 adverse events occurred in five subjects, including general malaise/fatigue prohibiting daily activities in four subjects (across all three groups) and fever $>39.0^{\circ}\text{C}$ in two NF166.C8-infected subjects (table S4A). The duration of general fatigue/malaise was greater in NF54-infected subjects [5.0 (3.0 to 7.8) days] compared to NF135.C10-infected [2.7 (1.3 to 4.6) days] or NF166.C8-infected subjects [2.7 (1.6 to 4.0) days]; the mean difference for both comparisons was 2.6 days (95% CI, 0.0 to 5.1) ($P < 0.05$, one-way ANOVA/Tukey's post hoc test, $n = 5$ per group; table S4A). Overall, no other statistically significant difference in frequency, duration, or gradation of adverse events was observed between the three groups (table S4A). The clinical characteristics of the CHMI-b study did not differ markedly from those of the CHMI-a study, and no statistically significant differences were observed between the NF135.C10-infected and NF166.C8-infected groups (table S4B). No serious adverse events (SAEs) occurred in either study.

Genetic characterization of *P. falciparum* isolates

The genetic identity of the three *P. falciparum* isolates used in the CHMI-a and CHMI-b studies was assessed by PCR for the polymorphic repetitive RII region of Glutamate-Rich Protein (GLURP); the K1, MAD20, and R033 allelic variants of block 2 of Merozoite Surface Protein 1 (MSP1); and the IC1 and FC27 variants of block 3 of MSP2 (fig. S2). GLURP bands were observed corresponding to amplified DNA fragment lengths of about 950, 980, and 680 base pairs (bp) for NF54, NF166.C8, and NF135.C10 strains, respectively (fig. S2). MSP1 K1 bands corresponding to fragment lengths of 220 and 210 bp were observed for NF54 and NF166.C8 strains, but these isolates failed to produce an MSP1 MAD20 band (fig. S2). NF135.C10, in contrast, did produce an MSP1 MAD20 band with a fragment length of 180 bp (fig. S2). MSP2 IC1 bands corresponded to fragment lengths of 480, 580, and 560 bp for NF54, NF166.C8, and NF135.C10 strains, respectively (fig. S2). 18S bands were detectable as amplified DNA fragments of around 130 bp in all isolates (fig. S2).

Hepatocyte invasion and schizogony by sporozoites *in vitro*

In parallel, we assessed the infectivity of the three clinical *P. falciparum* isolates NF54, NF135.C10, and NF166.C8 for fresh human hepatocytes *in vitro*, using sporozoites dissected from the same batches of mosquitoes used to infect subjects in the CHMI-a study (experiment A) and derived from independent gametocyte cultures used in the CHMI-b study up to 2 years later (experiments B1 and B2) (Fig. 2). A higher proportion of NF135.C10 and NF166.C8 sporozoites established infection in human hepatocytes compared to the NF54 strain in these three experiments ($P < 0.001$ overall by repeated-measures ANOVA and $P < 0.01$ for NF54 versus NF135 and NF54 versus NF166 individually by Tukey's post hoc test; Fig. 2). In addition, mature NF166.C8 and NF135.C10 schizonts tended to be larger than NF54 schizonts, and concomitantly, the increase in the number of nuclei per schizont from day 2 to day 6 appeared to be greater for NF135.C10 and NF166.C8 compared to NF54 (Table 2). These combined data indicated that both sporozoite invasion and schizont development were more efficient in the NF135.C10 and NF166.C8 strains than in the NF54 strain.



Experiment A

Experiment B1

Experiment B2

Figure 2. Invasion of human hepatocytes *in vitro* by three *P. falciparum* strains. Freshly isolated human hepatocytes were co-incubated with freshly isolated NF54 sporozoites (brown circles), NF135.C10 sporozoites (blue squares), or NF166.C8 sporozoites (red triangles) *in vitro*. The number of infected hepatocytes per well was counted by immunofluorescence microscopy at the given time points after infection. Shown are the results of three independent experiments. Experiment A was performed with 40,000 sporozoites per well harvested from mosquito batches used to infect subjects in the CHMI-a study and assessed at 5 days after infection. Experiments B1 and B2 were performed with 50,000 sporozoites per well harvested from mosquitoes fed on gametocyte cultures used in the CHMI-b study and assessed at 2 and 6 days after infection. Experiment A was performed at a different institute to experiments B1 and B2, and each of the three experiments was performed with human hepatocytes from a different donor. Data represent the percentage of sporozoites achieving successful invasion in each of three triplicate wells per condition (some NF166.C8 strain wells only in duplicate); horizontal lines represent group means. *P* values by one-way ANOVA with Tukey's posttest. ns, not significant.

Table 2. Characteristics of *P. falciparum* infectivity of human hepatocytes *in vitro*.

			NF54	NF135.C10	NF166.C8
experiment B1	day 2	#schizonts/well ¹	1100 (1,091-1,158)	2420 (2,273-2,595)	2479 (1,727-3,005)
		schizont \varnothing (μm) ²	3.0 (2.3-4.1)	3.4 (0.9-4.5)	2.8 (1.8-3.6)
		#nuclei/well ³	18,533 (15,187-20,077)	20,090 (17,823-26,446)	17,980 (15,731-25,785)
		#nuclei/schizont ⁴	16.8	8.3	7.3
	day 6	#schizonts/well ¹	1,383 (1,289-1,398)	2,840 (2,656-3,276)	2,843 (2,692-2,993)
		schizont \varnothing (μm) ²	11.2 (9.0 – 13.5)	18.3 (12.5 – 25.0)	16.1 (8.9 – 26.8)
		#nuclei/well ³	56,181 (52,810-69,893)	216,449 (181,107-313,381)	248,961 (201,453-394,328)
		#nuclei/schizont ⁴	40.6	76.2	87.6
		multiplication rate ⁵	2.4	9.2	12.1
experiment B2	day 2	#schizonts/well ¹	373 (320-458)	902 (275-1575)	1,098 (1,059-1,137)
		schizont \varnothing (μm) ²	1.7 (0.9-3.6)	2.8 (1.8-5.4)	3.4 (1.8-7.1)
		#nuclei/well ³	8,858 (7,226-9,585)	16,726 (10,228-16,726)	21,968 (20,742-23,195)
		#nuclei/schizont ⁴	23.8	18.5	20.0
	day 6	#schizonts/well ¹	438 (436-461)	1,061 (826-124)	813 (492-1134)
		schizont \varnothing (μm) ²	11.4 (6.3-16.1)	18.9 (12.5-28.6)	12.5 (8.9-17.0)
		#nuclei/well ³	95,295 (94,347-11,184)	382,508 (364,489-451,483)	ND
		#nuclei/schizont ⁴	218	361	ND
		multiplication rate ⁵	11.5	19.4	ND

ND = not determined. ¹Number of liver-stage schizonts per well, counted by immunofluorescence microscopy following staining with anti-HSP70 MAbs, as in Fig 2. Data represent median [range] across triplicate wells. ²Schizont diameters, as measured by immunofluorescence microscopy and digital image analysis. Data represent mean [range] of $n=19$ to 49 schizonts per sample. NF135.C10 schizonts were larger than NF54 schizonts at day 2 ($P < 0.05$) and day 6 ($P < 0.001$) in both experiments and were also larger than NF166.C8 schizonts ($P < 0.05$ to $P < 0.001$ in all comparisons except experiment B2, day 2); NF166.C8 schizonts were larger than NF54 schizonts at day 2 in experiment B2 ($P < 0.001$) and at day 6 in experiment B1 ($P < 0.001$). P values by one-way ANOVA with Tukey's posttest. ³Number of parasite nuclei per well, as determined by qPCR; data represent median [range] of triplicate wells. ⁴Number of nuclei per schizont, as calculated by dividing the median #nuclei per well by the median #schizonts per well. ⁵Fold-increase in number of nuclei per infected human hepatocyte from day 2 to day 6 after infection, as calculated by dividing the median #nuclei per schizont at day 6 after infection by that at day 2.

Correlations between the *in vitro* human hepatocyte invasion assay and *in vivo* parasite dynamics

Given that identical sporozoite batches were used for both the human hepatocyte invasion assays and human infections in the CHMI-a study, we queried whether sporozoite infectivity *in vitro* might be predictive of blood-stage infection *in vivo*. The magnitude of the first wave of parasitemia emerging from the liver in study subjects inoculated with each strain (Table 1) appeared to correlate with the infectivity of sporozoites of that strain in the human hepatocyte invasion assay *in vitro* (Table 2) (Pearson's $R = 0.994$, $P = 0.068$, $n = 3$ strains). Furthermore, a strong negative association existed between sporozoite infectivity *in vitro* and the prepatent period of that strain *in vivo* ($R = -1.0$, $P = 0.005$).

2.

Discussion

Here, we systematically assessed the dynamics of the obligate liver stage of human malaria infection, from mosquito bite to the emergence of blood-stage parasites, by using three different clinical isolates of *P. falciparum* and directly linking data on sporozoite infectivity in an *in vitro* human hepatocyte invasion model with *in vivo* measurements of parasitemia in subjects undergoing CHMI.

Both *in vitro* and *in vivo* findings support the notion that a substantially greater initial burden of blood-stage parasites is released from the livers of subjects infected with the NF135.C10 or NF166.C8 *P. falciparum* strains than from those infected with the NF54 strain. This in turn has significant effects on the subsequent course of blood-stage infection, with total parasite numbers more quickly reaching levels detectable by thick smear microscopy, shortening the prepatent period by several days. The difference in parasite burden between strains *in vivo* was even greater than the differences in invasion and development observed *in vitro*, a phenomenon that may be partially explained by suboptimal conditions for sporozoite invasion and development *in vitro* as compared to *in vivo*. In support of this explanation, the diameter of the NF54 strain liver-stage schizonts in our human hepatocyte cultures in this study (Table 2), as well as in a previous study (10), was 1.5- to 5-fold smaller than those measured *in vivo* in two humanized mouse models [the fumarylacetoacetate hydrolase-deficient mouse (3) and the severe combined immunodeficient mouse homozygous for the urokinase-type plasminogen activator transgene under the albumin promoter (11)]. Measuring the size of liver-stage schizonts in these mouse models might reveal even bigger differences between *P. falciparum* isolates than those found here *in vitro*. Whereas more than a decade separates the original isolation date of each of these three strains of *P. falciparum* (table S2), the use of specific master cell banks (MCBs) for CHMI studies restricts the cumulative culture period for the generation of gametocytes of each strain. In our opinion, this is unlikely to explain the marked differences in phenotype of the strains *in vivo* with regard to liver-stage burden.

Both sporozoite infectivity (Fig. 2) and the size of mature liver-stage schizonts of strain NF135.C10 in particular (Table 2) appeared to be greater than those for the NF54 strain, suggesting that two distinct mechanisms may be contributing to the greater number of merozoites released *in vivo*. In contrast, the duration of liverstage development was markedly similar between isolates

(Fig. 1, D to F). Although the mean time to qPCR positivity was slightly longer for the NF54 strain, we regularly detected NF54 parasites in blood samples by day 6.5 after infection (Table 1) (12). The small magnitude of the first wave of NF54 parasitemia suggested that the earliest parasites to emerge from the liver may fall below the detection limit of the qPCR. In any case, this short delay in qPCR positivity (0.52 and 0.70 day relative to the NF135.C10 and NF166.C8 strains, respectively, across all studies, Table 1) would appear to form only a minor determinant of the overall delay in prepatent period (2.3 and 2.9 days, respectively; Fig. 1C).

Finally, the variability in both the timing and the magnitude of the first wave of parasitemia among subjects within each study group was relatively limited in comparison to the difference in that magnitude between groups, suggesting that innate host factors may have little influence on either the duration or efficiency of liver-stage development in primary *P. falciparum* infections in our study population.

Although no clear relationship has been found between salivary gland sporozoite load and the number of sporozoites inoculated by probing/feeding mosquitoes (13), higher salivary gland loads have been suggested to enhance the infectivity of sporozoites (14). However, NF54-infected mosquitoes used in the CHMI-a study actually contained higher salivary gland sporozoite loads than did NF166.C8-infected or NF135.C10-infected mosquitoes from the same colony (table S2), a finding that is consistent with batches of mosquitoes used for other CHMIs at our center (table S3). The advent of CHMIs using parenteral sporozoite inoculation allowing better control over the number of administered sporozoites may further help to disentangle this point.

As a direct consequence of their higher liver burden, the time frame between release of the first NF135.C10 and NF166.C8 parasites from the liver and the initiation of antimalarial treatment with atovaquone-proguanil (upon parasitemia attaining the threshold by thick smear microscopy) incorporated only a single erythrocytic cycle. This is insufficient to reliably determine their blood-stage multiplication rate (5).

A possible limitation of this study is that sporozoite infectivity and the development of parasitemia were only assessed in malaria-naïve volunteers from malaria nonendemic countries. The observed infectivity between the parasite strains may be different in endemic populations with variable degrees of semi-immunity. Therefore, it will be of interest to compare these clones in CHMI in endemic settings. A further limitation is that only three *P. falciparum* isolates from different geographical backgrounds were tested for comparison in human hepatocyte cultures and by CHMI. It remains to be seen to what extent the three strains are representative of the overall diversity among the global *P. falciparum* population (15). Increasing the portfolio of *P. falciparum* clones for CHMI studies may provide further support for our findings. Both the NF135.C10 (9) and NF166.C8 strains described here were developed and characterized with the specific intent to increase the currently limited portfolio of *P. falciparum* strains available for heterologous challenge CHMI studies. For NF54 or its derivative clone 3D7, the minimum infecting dose to guarantee a 100% infection rate in malaria-naïve subjects in such CHMI studies is generally held to be five mosquito bites (16), 3200 cryopreserved sporozoites inoculated intravenously (17), or 75,000 cryopreserved sporozoites inoculated intramuscularly (18). Given the higher observed infectivity of NF135.C10 and NF166.C8 sporozoites, the minimum dose to reliably achieve 100% infection must be explored in further studies. Whereas interventions that target hepatocyte invasion aim at one of the two most constrained bottlenecks in the parasite's life cycle (4), our data indicate that relatively limited but relevant heterogeneity does exist among strains with regard to sporozoite infectivity (Fig. 2), which should be taken into account when designing and evaluating such interventions.

A major advantage of CHMI studies is the relatively small number of subjects required for the evaluation of vaccine efficacy, due to the homogeneity of the profile of parasitemia within each group and between homologous groups, as illustrated here by our CHMI-a and CHMI-b studies. Given the clear-cut difference in parasite dynamics between *P. falciparum* isolates, however, homologous challenge studies of malaria vaccines will remain insufficiently representative, and CHMIs with heterologous strains are required to more accurately predict the efficacy of promising vaccine candidates.

Materials and Methods

2.

Study design

Two CHMIs were performed using the well-established *P. falciparum* research strain NF54 and the new clones NF135.C10 and NF166.C8. The primary objective of the first study (CHMI-a) was to compare parasite dynamics of these isolates during infection, and that of the second (CHMI-b) study was to determine the proportion of subjects in each group who developed patent parasitemia, as described below. Secondary objectives of both studies included clinical parameters and further measures of parasite kinetics.

Healthy malaria-naïve adult (age 18 to 35 years) Dutch volunteers were recruited at the Harbour Hospital, Rotterdam, after signing informed consent. Our screening procedures have been described previously (19), and a complete list of inclusion/exclusion criteria is provided in table S5. In the first CHMI study described here (CHMI-a), 15 subjects (9 men and 6 women; table S1) were randomly allocated (ratio 1:1:1) to three parallel groups of $n = 5$, to be infected by bites of five mosquitoes per subject carrying, respectively, the NF54 strain of *P. falciparum*, the NF135.C10 clone, or the new NF166.C8 clone. In the second study (CHMI-b), 24 subjects were randomly allocated (ratio 1:1:1:1:1:1) to six parallel groups of $n = 4$, to bites by one, two, or five mosquitoes carrying, respectively, either NF135.C10 or NF166.C8 strains. For comparison with CHMI-a, only the two CHMI-b groups exposed to the bites of five infectious mosquitoes are reported here (that is, eight subjects in total, of whom three were men, and five were women; table S1). Flow charts for both studies are shown in fig. S1. Sample sizes were chosen pragmatically, based on longstanding experience with NF54 and (limited) NF135.C10 data. The CHMI-a study was powered to detect a 0.9 log (7.9-fold) difference in parasite burden between strains.

At inclusion, subjects received a unique pseudonymized study code. Once the predetermined number of subjects per study was included, two departmental employees not otherwise involved in the study linked a random number generated in Excel to each study code and allocated the required number of study codes to each group, using simple ranking of the associated random numbers. This allocation list was provided only to insectary technicians, who prepared feeding cages containing the required strain and number of *P. falciparum*-infected mosquitoes and labeled these otherwise identical cages with only the respective study code. Thus, study subjects, clinical investigators, and laboratory personnel assessing study end points (thick smear microscopy and qPCR) remained blinded to group allocation throughout each study.

All subjects within each study underwent infection on the same day, and considering the extremely close monitoring of CHMI study subjects for safety reasons, all subjects were expected

to complete follow-up. Data from all subjects were included in the study, and there were no specific rules for data exclusion, outliers, or premature cessation of data collection (other than if required for safety reasons after consultation with the sponsor, safety monitor, and Data Safety and Monitoring Board).

Culture and characterization of clinical parasite isolates

P. falciparum blood-stage parasites were introduced into semi-automated culture from peripheral whole-blood samples of patients with clinical malaria and used to generate infective *Anopheles stephensi* mosquitoes, as described before (20). The origins of NF54 and NF135 strains of *P. falciparum* have been described previously (8, 9). The NF166 strain of *P. falciparum* originated in 2010 as a clinical isolate (parasitemia 0.5 to 1%) from a child who had recently visited Guinea (West Africa). The clone NF166.C8 was obtained by limiting dilution culturing and established as a stable producer of fertile gametocytes and subsequently oocysts in infected mosquitoes (table S2). To minimize a potential confounding effect, gametocytes used to infect mosquitoes for our CHMI studies are generated following a strict culture procedure. When a cryopreserved aliquot of any isolate from our MCB is thawed and brought into culture, new aliquots of the culture are frozen down again for future use after the first four rounds of subculture. The remaining culture is then maintained for the generation of gametocytes, generally between the 15th and 25th rounds of subculture. Because *in vitro* parasites lose their ability to generate gametocytes beyond ± 25 rounds of subculture, such cultures are discontinued. The MCB of NF54 was generated from the estimated 10th to 15th subculture since its original isolation in the 1970s. For NF135, the MCB was generated from the sixth subculture since isolation, and for NF166 from the fourth subculture.

The identities of NF54, NF135.C10, and NF166.C8 were defined by assessing the polymorphic regions of three *P. falciparum* antigenic genes by PCR in a method adapted from Snounou *et al.* (21). Briefly, parasite DNA was isolated using QIAamp DNA Blood Mini Kit (Qiagen) and amplified with GoTaq G2 Flexi Polymerase (Promega) using specific primers for the polymorphic repetitive RII region of *P. falciparum* GLURP (22); the K1, MAD20, and R033 allelic variants of block 2 of MSP1 (23); and the IC1 and FC27 variants of block 3 of

MSP2 (24) (all primers from Invitrogen); 18S ribosomal DNA was used as a loading control. The sensitivity of NF166.C8, NF135.C10, and NF54 to dihydroartemisinin (Sigma-Tau), chloroquine diphosphate salt (Sigma-Aldrich), mefloquine, proguanil (British Pharmacopoeia), atovaquone (GlaxoSmithKline), and lumefantrine (Novartis) was tested by the Malaria SYBR Green I-Based Fluorescence Assay in triplicate experiments (25). See also table S2 and fig. S2.

Controlled human malaria infections

Mosquitoes in small cages were allowed to feed for 10min on the forearms of study subjects. All blood-engorged mosquitoes were dissected to confirm sporozoite carriage. Where necessary, study subjects were exposed to additional mosquitoes until precisely the predetermined number of infective bites was achieved. From day 5 after infection, subjects were seen twice daily as outpatients. Solicited and unsolicited adverse events and vital parameters were recorded at each visit and venous whole blood was drawn for thick blood smear, qPCR (see below), safety parameters, and exploratory assays. Adverse events were graded as mild (not interfering with),

moderate (interfering with), or severe (prohibiting daily activities). Respective grading of fever was 37.5° to 38.0°C, 38.0° to 39.0°C, and > 39.0°C. Adverse events were recorded by clinical investigators as being probably, possibly, or unlikely related to study procedures.

In CHMI-a, subjects were treated with a standard regimen of atovaquone-proguanil (Malarone, 1000/400 mg daily for 3 days) upon their first positive thick blood smear (defined as ≥ 2 parasites per 225 high-powered fields, equating to 0.5 ml of whole blood examined). In CHMI-b, subjects were treated with the same regimen as soon as two consecutive whole-blood samples were positive by qPCR (defined as > 500 parasites/ml, calibrated against a daily standard curve of known parasite concentrations). The change in methodology between studies was proposed (12) and implemented to minimize potential safety risks upon a request by Netherlands' Central Committee on Human Research (CCMO), following concerns over a cardiac SAE possibly related to CHMI in an earlier study (26). Sequential daily thick smears/qPCRs were continued after treatment in each subject until complete clearance of blood-stage parasites. CHMI-a whole-blood samples were also retrospectively analyzed by qPCR. These studies were approved by Netherlands' CCMO (NL41004.078.12 and NL48704.000.14).

Quantitative real-time fluorescence polymerase chain reaction

P. falciparum parasitemia was quantified by qPCR as described before (27) with some modifications. Briefly, isolated DNA was resuspended in 100 µl of H₂O, using 5 µl as template and the TaqMan MGB probe AAC AAT TGG AGG GCA AG-FAM. Samples for post hoc analysis were stored at -80°C. For each sample (including standard curve dilutions), two qPCR replicates were performed; if these values differed by >5%, then the sample was remeasured a third time. In addition, all samples were spiked with heterologous DNA as an extraction control; if the qPCR value of the extraction control differed by > 5% from the running average of all other samples, then the extraction process for that sample was repeated. Quantification of *P. falciparum* nuclei in wells of the human hepatocyte infectivity assay was performed using the same qPCR.

Primary human hepatocyte infectivity assay

In vitro sporozoite infectivity assays in human hepatocytes were performed, as described previously (6), at two different laboratories. Briefly, fresh primary hepatocytes derived from patients undergoing elective (partial) hepatectomy were plated in 96-well plates (50,000 cells per well). Two to five days after plating the human hepatocytes, sporozoites were dissected from the salivary glands of 80 to 110 infected mosquitoes per *P. falciparum* strain and added to the human hepatocyte wells in triplicate. In the first *in vitro* experiment (experiment A), sporozoites were harvested from the exact same batches of mosquitoes used on the same day to infect human subjects in CHMI-a; 40,000 sporozoites were added per human hepatocyte well. In subsequent *in vitro* experiments (B1 and B2), sporozoites were harvested from mosquitoes fed on independent gametocyte cultures used to subsequently infect subjects in CHMI-b, and 50,000 sporozoites were added per well. After 2 to 6 days, the number of human hepatocytes in each well harboring viable parasites was assessed by intracellular staining with anti-*P. falciparum* HSP-70 rabbit polyclonal antibody (SPC-186C/D, StressMarq Biosciences) followed by fluorescence microscopy; no instances of >1 invaded sporozoite per human hepatocyte were

observed. Discrimination between sporozoite invasion of/adherence to human hepatocytes was achieved by staining with fluorescent monoclonal antibodies against circumsporozoite protein (CSP) before cell permeabilization (28); the proportion of adherents (that is, HSP-70⁺CSP⁺-double positive, noninvaded parasites) was generally <5% at day 2 after infection and consistently <0.5% at day 6 after infection. The diameter of $n = 19$ to 49 liver-stage schizonts per strain was measured at day 2 and day 6 after infection by fluorescence microscopy and digital image analysis (LeicaDMI6000B). Total numbers of parasite nuclei per well were determined by qPCR in separate triplicate wells after discarding the culture supernatant. It was technically infeasible to perform both microscopy and qPCR on the same wells. The median number of merozoites per liver-stage schizont was calculated for each strain as follows: $([\text{median \#parasite nuclei in triplicate wells}] - [\text{median \#adhered (noninvaded) parasites in triplicate wells}]) / ([\text{median \#infected human hepatocytes in triplicate wells}])$. Investigators carrying out the *in vitro* hepatocyte infectivity assay were blinded as to the strain of the sporozoites.

Statistical analysis

Differences in continuous variables between groups of study subjects were compared by one-way ANOVA/Tukey's post hoc test (CHMI-a, three groups), unpaired *t* test (CHMI-b, two groups), or one-sample *t* test (CHMI-b, two groups when all values for one group were identical). Differences in categorical variables between groups were compared by χ^2 /Fisher's exact test. Differences in *in vitro* invasive capacity between isolates were compared by repeated-measures ANOVA/Tukey's post hoc test (three groups). Correlations between *in vitro* and *in vivo* variables were assessed by Pearson's test on the mean value of each variable per strain; where appropriate, variables were first log-transformed. Two-sided $P < 0.05$ was considered statistically significant in all tests. Parasite densities below the lower quantification limit of the qPCR (35 parasites/ml in CHMI-a and 50 parasites/ml in CHMI-b) were plotted at 0.5 \times the log value of that limit. The magnitude of the initial wave of parasitemia emerging from the liver was defined as the highest parasite density measured by qPCR per subject between day 6.5 and 8.0 after infection.

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Supplementary Materials

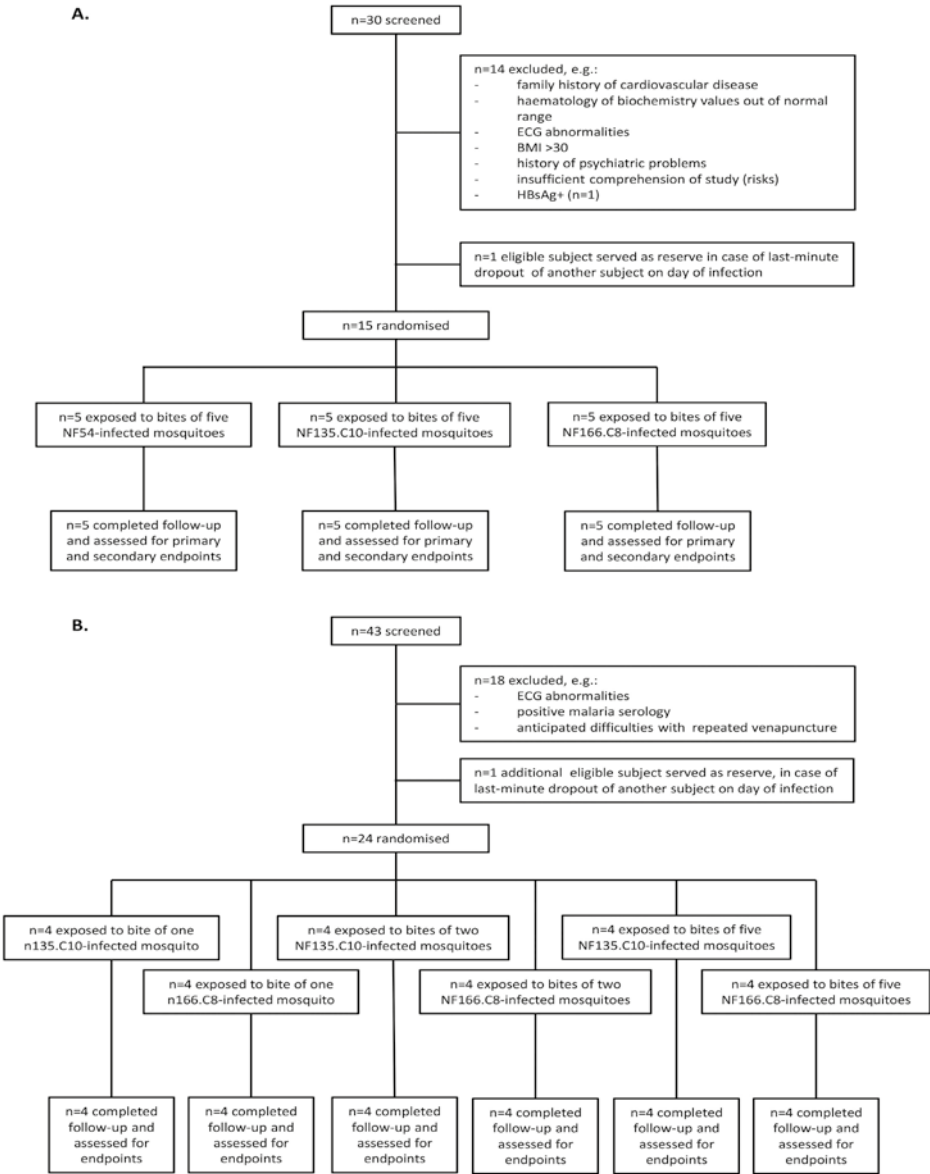


Figure S1. Study flow charts. A. CHMI-a (recruitment started August 2012, completed September 2012; day of infection 25-09-2012; final day of follow-up 30-10-2012); **B.** CHMI-b (recruitment started August 2014, completed October 2014; day of infection 07-10-2014; final day of follow-up 11-11-2014). **Note:** for comparison with CHMI-a, only the results of the two CHMI-b groups exposed to the bites of five infectious mosquitoes are presented in this paper.

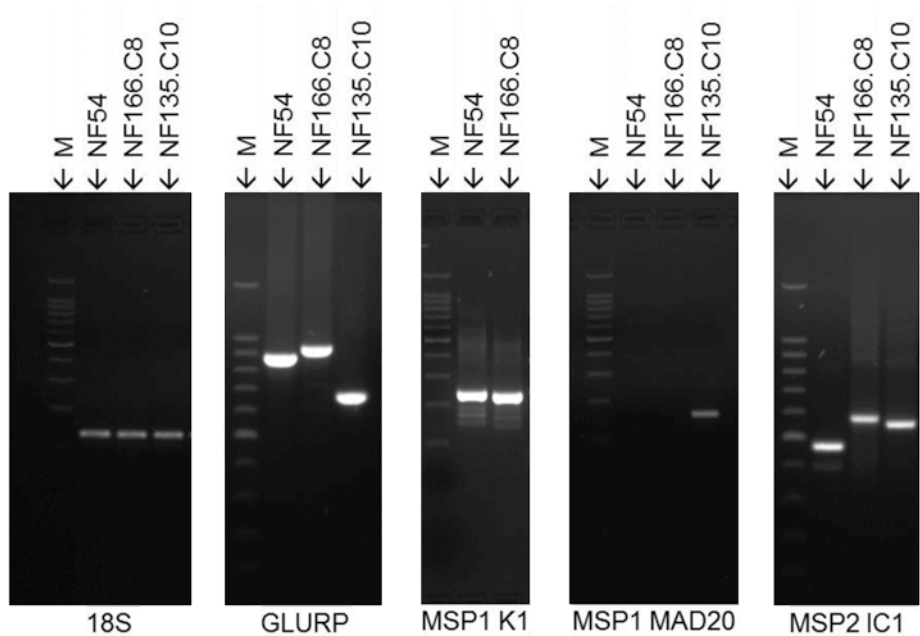


Figure S2. Genetic characterization of *P. falciparum* isolates. PCRs for 18S rDNA, GLURP, the K1, MAD20 & R033 allelic variants of MSP1 and the IC1 & FC27 variants of MSP2 were performed on the *P. falciparum* isolates NF54, NF166.C8 & NF135.C10. Note: NF54 and NF166.C8 failed to produce any MSP1 MAD20 band. M – 100bp marker.

Table S1. Study demographics.

	NF54	NF135.C10	NF166.C8
CHMI-a			
gender (male / total)	5 / 5	2 / 5	2 / 5
age (median [range])	19 [18-21]	22 [18-27]	24 [18-35]
CHMI-b¹			
gender (male / total)		0 / 4	3 / 4
age (median [range])		22.5 [19-27]	24 [19-25]

¹Data only shown for the two CHMI-b groups exposed to bites of five infectious mosquitoes.

Table S2. Characteristics of *P. falciparum* isolates used in CHMI studies.

	NF54		NF135.C10		NF166.C8	
Country of origin	Netherlands (West Africa)		Cambodia		Guinea	
Year of isolation	1979		1993		2010	
Drug sensitivity (IC₅₀):						
Chloroquine (nM)	8.8		96		9.9	
Mefloquine (nM)	19		37		11	
Atovaquone (nM)	1.2		0.94		0.37	
Proguanil (μM)	0.57		169		0.29	
Dihydroartemisinin (nM)	0.36		0.19		0.05	
Lumefantrine (nM)	94		122		49	
Parasite batches used in individual CHMIs:	CHMI-a	CHMI-b	CHMI-a	CHMI-b	CHMI-a	CHMI-b
No. sub-cultures since thawing of Master Cell Bank aliquot	23	-	17	21	18	17
proportion of oocyst-infected mosquitoes	10/10	-	10/10	10/10	10/10	10/10
mean oocyst count/ mosquito (n=10)	20.1	-	15.6	32.5	15.7	15.2
proportion of sporozoite-infectious mosquitoes	9/10	-	9/10	10/10	10/10	10/10
mean sporozoite count/ mosquito (n=10)	101x10 ³	-	69x10 ³	69x10 ³	40x10 ³	51x10 ³

Table S3. Overview of all comparable CHMI studies performed at our center.

Study# (ref#)	Year	Treatment threshold ¹	Number of subjects			Mosquito salivary gland sporozoite load		
			NF54	NF135.C10	NF166.C8	NF54	NF135.C10	NF166.C8
1 ² (31)	2000	TBS (NS)	5	-	-	N/A	N/A	N/A
2 ² (31)	2001	TBS (NS)	5	-	-	N/A	N/A	N/A
3 ² (31)	2002	TBS (NS)	5	-	-	N/A	N/A	N/A
4 ² (31)	2003	TBS (NS)	5	-	-	N/A	N/A	N/A
5 (32)	2007	TBS	5	-	-	31,500	-	-
6 ³	2008	TBS	18	-	-	72,800	-	-
7 (33)	2009	TBS	5	-	-	88,000	-	-
8 (9)	2010	TBS	4	3	-	69,000	12,500	-
9 (34)	2011	TBS	5	-	-	79,500	-	-
10 (19)	2011	TBS	5	-	-	100,000	-	-
11 (29)	2012	TBS	4	-	-	98,250	-	-
12 (CHMI-a)⁴	2012	TBS	5	5	5	101,250	69,000	40,000
13 (35)	2012	TBS	-	5	-	-	38,250	-
14 (36)	2012	TBS	5	-	-	75,800	-	-
15 (36)	2013	qPCR	4	-	-	98,000	-	-
16 (CHMI-b)⁴	2014	qPCR	-	4	4	-	69,000	51,000
17	2015	qPCR	5	-	-	74,000	-	-
18 ⁵	2015	qPCR	5	5	5	26,500/ 44,300	18,000/ 23,500	59,500/ 17,000
Total			90	22	14			

¹In initial studies at our centre, subjects were treated upon positive thick blood smear (TBS), whereas in more recent studies treatment was initiated upon positive quantitative PCR (qPCR). The lower detection threshold of the latter results in shorter pre-patent periods, lower parasitaemia at time of treatment and hence improved tolerability & safety for study subjects. In the very earliest studies, the methodology for performing thick blood smear analysis was not yet standardised (NS), which can result in subtly different detection thresholds between subjects and between studies. For meta-analysis of pre-patent periods and of highest parasite densities (usually at time of treatment), we have thus limited comparisons to those 10 studies (#5-14, including $n = 56$ NF54, $n = 13$ NF135.C10 and $n = 5$ NF166.C8 subjects) in which standardised thick blood smears were used to initiate treatment. For meta-analysis of time to first qPCR positivity and magnitude of the first wave of parasitaemia to emerge from the liver, all 18 studies are included.

²In the very earliest studies at our centre, subjects were exposed to the bites of between 4-7 *P. falciparum* NF54-infected mosquitoes; in all subsequent studies listed, all subjects were exposed to the bites of precisely 5 mosquitoes infected with the given isolate. No statistically significant difference was observed in the height of the first wave of parasitaemia in these first four studies compared to all later studies involving NF54.

³Subjects were immunised with a candidate malaria vaccine prior to CHMI, but were wholly unprotected against challenge.

⁴Studies for which directly-linked *in vitro* data on sporozoite invasion are available (boldface), as presented in this manuscript.

⁵Subjects were infected in two groups, for which two separate batches of *P. falciparum*-infected mosquitoes were used.

Table S4. Adverse events by severity grade during CHMI-a and CHMI-b studies.

table S4a. Adverse events by severity grade during CHMI-a

All adverse events	NF54 (n=5)			NF135.C10 (n=5)			NF166.C8 (n=5)		
	Subjects ¹	Duration ²	Grade ³	Subjects ¹	Duration ²	Grade ³	Subjects ¹	Duration ²	Grade ³
Fever ⁴	4	2.0 [0.2-2.8]	2 [2-2]	3	1.4 [0.4-3.2]	2 [1-2]	3	2.8 [0.6-6.8]	3 [1-3]
Headache	5	3.8 [0.3-7.8]	1 [1-2]	5	1.8 [0.1-5.3]	2 [1-2]	5	4.3 [1.6-7.3]	2 [1-2]
Malaise/fatigue ⁵	5	5.3 [3.0-7.8]	2 [1-3]	5	2.7 [1.3-4.6]	2 [1-3]	5	2.7 [1.6-4.0]	2 [1-3]
Myalgia	3	3.5 [1.6-5.9]	1 [1-2]	5	2.2 [1.0-4.0]	1 [1-2]	3	1.7 [0.8-3.3]	1 [1-2]
Arthralgia	-	-	-	1	4.1.	1.	-	-	-
Nausea ⁶	2	1.3 [1.0-1.1]	1.5 [1-2]	3	1.1 [0.0-3.0]	1 [1-2]	2	1.1 [0.1-2.2]	1.5 [1-2]
Chills/rigors	3	1.4 [0.7-2.6]	2 [1-2]	3	0.6 [0.1-1.1]	1 [1-1]	2	0.9 [0.2-1.7]	1.5 [1-2]
Diarrhoea ⁶	3	1.3 [0.0-3.9]	1 [1-1]	2	0.4 [0.0-0.8]	1 [1-1]	1	0.0.	2.
Abdominal pain ⁶	3	2.4 [0.2-4.9]	2 [1-2]	1	0.3.	1.	3	0.9 [0.1-1.5]	1 [1-2]
Unsolicited ⁷	4 (6)	3.0 [1.4-6.9]	1 [1-2]	2 (2)	2.0 [0.0-4.0]	1.5 [1-2]	5 (9)	4.8 [0.0-29]	1 [1-1]
Any ⁸	5 (35)	3.0 [0.0-7.8]	1 [1-3]	5 (30)	1.8 [0.0-5.3]	1 [1-3]	5 (33)	3.0 [0.0-29]	1 [1-3]
Grade 3 AEs									
Fever ⁴	-	-	-	-	-	-	2	3.9 [1.1-6.8]	-
Malaise/fatigue	2	6.0 [5.0-7.0]	-	1	3.0.	-	1	4.4.	-
Any ⁸	2 (2)	6.0 [5.0-7.0]	-	1 (1)	3.0.	-	3 (3)	4.0 [1.1-6.8]	-

table S4b. Adverse events by severity grade during CHMI-b^a

	NF135.C10 (n=4)			NF166.C8 (n=4)		
	Subjects ¹	Duration ²	Grade ³	Subjects ¹	Duration ²	Grade ³
All adverse events						
Fever	4	0.7 [0.2-1.9]	2 [1-3]	1	1.0 .	2 .
Headache	3	1.3 [0.0-3.6]	1 [1-2]	3	1.8 [0.3-2.9]	2 [1-3]
Malaise/fatigue	4	2.7 [1.4-3.6]	1 [1-2]	3	3.9 [0.7-8.3]	2 [1-2]
Myalgia	3	1.9 [1.0-3.3]	1 [1-2]	2	0.7 [0.6-0.7]	1 [1-1]
Arthralgia	1	1.0 .	1 .	-	-	-
Nausea ⁶	4	0.7 [0.0-1.6]	1 [1-2]	1	0.2 .	1 .
Chills/rigors	3	1.3 [0.8-2.0]	2 [1-2]	3	0.7 [0.1-1.3]	1 [1-2]
Diarrhoea	-	-	-	-	-	-
Abdominal pain ⁶	2	1.1 [0.1-2.1]	1 [1-1]	1	1.4 .	1 .
Unsolicited ⁷	2 (2)	4.0 .	1 [1-1]	2 (4)	2.4 [0.0-8.3]	1 [1-2]
Any ⁸	4 (27)	1.4 [0.0-4.0]	1 [1-3]	4 (18)	1.8 [0.0-8.3]	1 [1-3]
Grade 3 AEs						
Fever	1	1.9 .		1	2.9 .	
Any ⁸	1 (1)	1.9 .		1 (1)	2.9 .	

¹Number of subjects per group experiencing adverse event. Multiple similar events/episodes per subject were counted as one, with cumulative duration and maximum gradation recorded. For Unsolicited and Any adverse events, total numbers of events/episodes per group are shown in parentheses.

²In days; data represent mean [range] per group.

³AE gradation: 1=mild, 2=moderate, 3=severe; Fever gradation respectively: 37.5-38.0°C, 38.0-39.0°C, >39.0°C; data represent medians [range].

⁴Two NF135.C10 subjects and two NF166.C8 subjects (those with grade 3 symptoms) held persisting fever across the third and final day atovaquone-proguanil treatment, which subsequently resolved spontaneously.

⁵Mean difference in duration of malaise/fatigue: 2.6 [95% CI 0.0 to 5.1] days between NF54 and NF135.C10 ($P < 0.05$); 2.6 [95% CI 0.0 to 5.1] days between NF54 and NF166.C8 ($P < 0.05$); 0.0 [95% CI -2.6 to 2.5] days between NF135.C10 and NF166.C8 ($P > 0.05$); all comparisons by one-way ANOVA/Tukey's post-hoc test.

⁶Gastro-intestinal complaints, including nausea (but not vomiting), diarrhoea and abdominal pain were mainly experienced following intake of atovaquone-proguanil.

⁷Unsolicited adverse events in CHMI-a included 5 episodes of common cold, 2 of tender submandibular swelling, 1 of cold sore, 2 of anorexia, 1 of gastric reflux, 2 of syncope, 1 of hyperventilation syndrome, 1 of blurry vision, 1 of mild oedema orbitae and 1 of lumbago. In CHMI-b, there was 1 episode of atypical chest pain, 1 of dizziness, 1 of syncope, 1 of a minor transport accident, 1 of influenza-like illness (ongoing at the final day of follow-up) and 1 subject developed haematomas in both antecubital fossae.

⁸No other statistically significant difference in frequency, duration or gradation of any adverse events was observed between the three groups.

⁹Data only shown for the two CHMI-b groups exposed to bites of five infectious mosquitoes.

Table S5. Inclusion and exclusion criteria for CHMI studies.

CHMI-a	Inclusion criteria
1.	Males and females aged 18-35 years
2.	In general good health based on history, physical examination and basic haematology and biochemistry
3.	Negative pregnancy test for females
4.	Use of adequate contraception for females
5.	Signed informed consent, based on a thorough understanding of the concept and procedures of the study
6.	Volunteer agrees to allow informing his/her general practitioner about participation and agrees to sign a request for medical information from the GP concerning any contra-indications for participation in the study
7.	Willingness to undergo a Pf sporozoite challenge
8.	Agreement to stay in a hotel close to the trial center during part of the study (day 5 until three days post-treatment)
9.	Reachable (24/7) by mobile telephone during the whole study period
10.	Available to attend all study visits
11.	Agreement to refrain from blood donation to Sanquin or for other purposes, during the course of the study and thereafter following Sanquin guidelines.
12.	Willingness to undergo an HIV, HBV and HCV screening test
13.	Negative urine toxicology screening test at the screening visit and on the day before challenge
14.	Willingness to take a curative regimen of Malarone®
	Exclusion criteria
1.	History of malaria
2.	Plans to travel outside of the Netherlands during the study period
3.	Previous participation in any malaria vaccine study and/or positive serology for <i>P. falciparum</i>
4.	Symptoms, physical signs or laboratory values suggestive of systemic disorders, including but not limited to renal, hepatic, cardiovascular, pulmonary, skin, immunodeficiency, psychiatric and other conditions, which could compromise the health of the volunteer during the study or interfere with the interpretation of the study results
5.	History of diabetes mellitus or cancer (except basal cell carcinoma of the skin)
6.	Clinically significant ECG abnormalities at screening, or history of arrhythmia's or prolonged QT-interval
7.	Positive family history of cardiac disease in 1st or 2nd degree relatives < 50 years old
8.	An estimated ten year risk of fatal cardiovascular disease of ≥5%, as estimated by the Systematic Coronary Risk Evaluation (SCORE) system
9.	Body Mass Index (BMI) below 18 or above 30 kg/m ²
10.	Any clinically significant deviation from the normal range in haematological or biochemical blood tests or urine analysis

Table S5. Continued.

11.	Positive HIV, HBV or HCV screening tests
12.	Participation in any other clinical study within 30 days prior to the onset of the study or during the study period
13.	Pregnant or lactating women
14.	Volunteers unable to give written informed consent
15.	Volunteers unable to be closely followed during the study period for social, geographic or psychological reasons
16.	Previous history of drug or alcohol abuse interfering with normal social function in the period of one year prior to study onset
17.	A history of psychiatric disease or convulsions
18.	Known hypersensitivity to anti-malarial drugs
19.	History of severe reactions or allergy to mosquito bites
20.	The use of chronic immunosuppressive drugs, antibiotics, or other immune modifying drugs within three months prior to study onset (inhaled and topical corticosteroids are allowed) or during the study period
21.	Contra-indications for Malarone® use, including treatment taken by the volunteers that interferes with Malarone®
22.	Any confirmed or suspected immunosuppressive or immunodeficient condition, including asplenia
23.	Co-workers of the departments of Medical Microbiology of the UMC St Radboud, the department of Internal Medicine of the Havenziekenhuis or the department of Medical Microbiology & Infectious Diseases of the Erasmus MC
24.	A history of sickle cell, thalassaemia trait or G6PD deficiency
CHMI-b	Inclusion criteria
1.	Subject is aged ≥ 18 and ≤ 35 years and in good health.
2.	Subject has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
3.	Subject is able to communicate well with the investigator, is available to attend all study visits, lives in proximity to the trial centre (<10 km) or (if >10 km) is willing to stay in a hotel close to the trial centre during part of the study (day 5 post-infection until three days post-treatment). Furthermore the subject will remain within the Netherlands during the study period and is reachable (24/7) by mobile telephone throughout the entire study period.
4.	Subject agrees to inform his/her general practitioner and (if applicable) medical specialist about participation in the study and to sign a request to release by the GP any relevant medical information concerning possible contra-indications for participation in the study.
5.	Subject agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period and for a defined period thereafter according to current Sanquin guidelines.
6.	For female subjects: subject agrees to use adequate contraception and not to breastfeed for the duration of study.
7.	Subject has signed informed consent.

Table S5. Continued.

	Exclusion criteria
1.	Any history, or evidence at screening, of clinically significant symptoms, physical signs or abnormal laboratory values suggestive of systemic conditions, such as cardiovascular, pulmonary, renal, hepatic, neurological, dermatological, endocrine, malignant, haematological, infectious, immunodeficient, psychiatric and other disorders, which could compromise the health of the volunteer during the study or interfere with the interpretation of the study results. These include, but are not limited to, any of the following:
1.1	Body weight <50 kg or Body Mass Index (BMI) <18.0 or >30.0 kg/m ² at screening
1.2	A heightened risk of cardiovascular disease, defined as: an estimated ten year risk of fatal cardiovascular disease of ≥5% at screening, as determined by the Systematic Coronary Risk Evaluation (SCORE); history, or evidence at screening, of clinically significant arrhythmia's, prolonged QT-interval or other clinically relevant ECG abnormalities; or a positive family history of cardiac events in 1st or 2nd degree relatives <50 years old.
1.3	Functional asplenia, sickle cell trait/disease, thalassaemia trait/disease or G6PD deficiency.
1.4	History of epilepsy in the period of five years prior to study onset, even if no longer on medication.
1.5	Positive HIV, HBV or HCV screening tests.
1.6	Chronic use of i) immunosuppressive drugs, ii) antibiotics, iii) or other immune modifying drugs within three months prior to study onset (inhaled and topical corticosteroids and oral anti-histamines exempted) or expected use of such during the study period.
1.7	History of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past 5 years
1.8	Any history of treatment for severe psychiatric disease by a psychiatrist in the past year.
1.9	History of drug or alcohol abuse interfering with normal social function in the period of one year prior to study onset, or positive urine toxicology test for cocaine or amphetamines at screening or prior to infection.
2.	For female subjects: positive urine pregnancy test at screening or prior to infection.
3.	Any history of malaria, positive serology for <i>P. falciparum</i> , or previous participation in any malaria (vaccine) study.
4.	Known hypersensitivity to or contra-indications (including co-medication) for use of atovaquone-proguanil (Malarone®) or artemether-lumefantrine (Riamet®), or history of severe (allergic) reactions to mosquito bites.
5.	Receipt of any vaccinations in the 3 months prior to the start of the study or plans to receive any other vaccinations during the study period or up to 8 weeks thereafter.
6.	Participation in any other clinical study in the 30 days prior to the start of the study or during the study period.
7.	Being an employee or student of the department of Medical Microbiology of the Radboudumc, the department of Internal Medicine or Laboratory of the Havenziekenhuis or the department of Medical Microbiology & Infectious Diseases of the Erasmus MC.
8.	Any other condition or situation that would, in the opinion of the investigator, place the subject at an unacceptable risk of injury or render the subject unable to meet the requirements of the protocol.

