INTRODUCTION

In 2016, *Plasmodium* parasites caused 219 million cases and 435,000 malaria-attributable deaths. The standard approach for the detection of these burdensome parasites has long been through microscopic examination of thick and thin Giemsa-stained blood smears, now often supplemented with rapid diagnostic tests. Thick blood smears (called “blood smears” hereafter) have also served as the gold standard for controlled human malaria infection (CHMI) studies. Controlled human malaria infection studies are clinical trials where experimental infection with sporozoite-stage parasites are conducted to test early stage vaccine and drug candidates. Over the past 20 years, sensitive nucleic acid tests (NATs) that afford earlier detection at lower parasite densities have been developed (reviewed in Refs. 5 and 6). Methods have included single-step and nested PCRs with electrophoresis gel–based detection, DNA dye- or probe-based real-time quantitative PCR (qPCR), nucleic acid–based sequence amplification (NASBA), and real-time quantitative reverse transcription–PCR (qRT-PCR) with probe-based detection.

The most common NAT targets of Plasmodium are its conserved 18S rRNA-coding genes (rDNA) by qPCR or the expressed 18S rRNAs themselves by qRT-PCR or NASBA. *rRNA expression is relatively stage specific,*2–4 with asexual (A)-type 18S rRNAs more highly expressed in erythrocyte-stage parasites and sexual (S)-type 18S rRNAs more abundant in mosquito stages. Each *Plasmodium* parasite expresses thousands of 18S rRNAs from a few coding genes,10,12–12 making it even possible to detect single parasites in a 0.05–1 mL blood sample by qRT-PCR or NASBA.10,12,13 Because of the difference in *rRNA versus rRNA* copy number per parasite, extractions for DNA qPCR generally require larger volumes of blood than those for qRT-PCR or NASBA to achieve the same parasite limit of detection. Initially, malaria NATs were used retrospectively in trials, but as the techniques matured, such methods have been increasingly used as primary endpoint assays in CHMI trials in non-endemic sites.14–16

Beyond Blood Smears: Qualification of *Plasmodium* 18S rRNA as a Biomarker for Controlled Human Malaria Infections

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Abstract. 18S rRNA is a biomarker that provides an alternative to thick blood smears in controlled human malaria infection (CHMI) trials. We reviewed data from CHMI trials at non-endemic sites that used blood smears and *Plasmodium* 18S rRNA/rDNA biomarker nucleic acid tests (NATs) for time to positivity. We validated a multiplex quantitative reverse transcription–polymerase chain reaction (qRTPCR) for *Plasmodium* 18S rRNA, prospectively compared blood smears and qRT-PCR for three trials, and modeled treatment effects at different biomarker-defined parasite densities to assess the impact on infection detection, symptom reduction, and measured intervention efficacy. Literature review demonstrated accelerated NAT-based infection detection compared with blood smears (mean acceleration: 3.2–3.6 days). For prospectively tested trials, the validated *Plasmodium* 18S rRNA qRT-PCR positivity was earlier (7.6 days; 95% CI: 7.1–8.1 days) than blood smears (11.0 days; 95% CI: 10.3–11.8 days) and significantly preceded the onset of grade 2 malaria-related symptoms (12.2 days; 95% CI: 10.6–13.3 days). Discrepant analysis showed that the risk of a blood smear–positive, biomarker-negative result was negligible. Data modeling predicted that treatment triggered by specific biomarker-defined thresholds can differentiate complete, partial, and non-protective outcomes and eliminate many grade 3 malaria-related symptoms post-CHMI. *Plasmodium* 18S rRNA is a sensitive and specific biomarker that can justifiably replace blood smears for infection detection in CHMI trials in non-endemic settings. This study led to biomarker qualification through the U.S. Food and Drug Administration for use in CHMI studies at non-endemic sites, which will facilitate biomarker use for the qualified context of use in drug and vaccine trials.
Following CHMI, immunologically naive volunteers usually become blood smear positive 10–12 days later. However, in such persons, the blood stage begins approximately 6 days post-CHMI, as determined by in vitro culture of clinical samples\textsuperscript{17,18} and by NATs.\textsuperscript{13} Traditionally, CHMI participants were monitored by once- or twice-daily blood smears and received treatment on becoming patent. Dangerously, high-density or prolonged infections do not occur with this approach—to our knowledge, there have been no severe malaria cases or deaths in any CHMI trial to date. Whereas some CHMI participants at non-endemic sites are asymptomatic on blood smear patency,\textsuperscript{13,19} malaria-related symptoms (e.g., headache, myalgia, fever, chills, sweats, nausea, vomiting, and diarrhea) up to and including grade 3 symptoms often commence days earlier.\textsuperscript{10,20–23} Although well-controlled, sensitive assays for 18S rRNA/rDNA have been used in the context of CHMI trials, regulatory qualification of this biomarker\textsuperscript{23} has not been previously achieved. Here, analytical/clinical validation and regulatory qualification of the Plasmodium 18S rRNA/rDNA biomarker for non-endemic site CHMI studies are reported.

**MATERIALS AND METHODS**

**Literature review.** Peer-reviewed publications of Pf CHMI that used *Plasmodium* 18S rRNA and/or rDNA assays and blood smears were identified in PubMed. Publicly available data (main manuscripts and Supplemental Information) were evaluated. Subject-level data were not evaluated unless publicly available.

**Plasmodium 18S rRNA qRT-PCR assay.** Extraction and amplification for the third-generation biomarker assay were entirely performed on the Abbott m2000sp and m2000rt instruments, respectively (Abbott Molecular, Niles, IL). Ethylenediamine tetraacetic acid (EDTA) venous whole blood was collected from trial participants, and 50 μL was mixed with 2 mL of NucliSENS lysis buffer (bioMérieux, Durham, NC). One milliliter of lysate was processed by the m2000sp using mSample RNA preparation kit (Abbott Molecular), and the m2000 whole blood RNA extraction protocol was followed by automated pipetting of mastermix and template into 96-well plates.

Our multiplex approach simultaneously evaluated a pan-*Plasmodium* 18S rRNA target, a *Plasmodium falciparum*-specific 18S rRNA target, and a human housekeeping mRNA target. The triplex qRT-PCR reaction was performed using 35 μL SensiFAST\textsuperscript{TM} Probe Lo-ROX One-Step Kit (Bioline, Taunton, MA) and 15 μL of extracted eluate. Primers/probes were as follows for the pan-*Plasmodium* 18S rRNA segment (Forward PanDDT1043F19: 5′-AAAGTTAAGGGGTAGGAA-3′; Reverse PanDDT1197R22: 5′-AAGACCTTGAATGGTCT-3′; Probe: 5′-CAL Fluor Orange 560-ACCGTCG TAATCTTTACATAACTA[T(Black Hole Quencher-1)] GCCGACTAG-3′[Spacer C3], a Pf-specific 18S rRNA sequence (Forward PanDDT1043F19: 5′-GGAGTACACTA TATTTCTAT-3′; Reverse PanDDT1197R22: 5′-ATTATAGTGA AACAGGGAAA-3′; Probe: 5′-[6-FAM]-ATTATACGTA AATTTACAG-3′[Black Hole Quencher-1], and the human TATA-Binding Protein (TBP) mRNA (Forward: 5′-GATAAGA GAGGCCAACGCCAC-3′; Reverse: 5′-CGAGGACCACTG GAAAGTC-3′; Probe: 5′-[Quasar 670]-CACAGGGACACG GAGGGAACAGT-3′[Black Hole Quencher-2]). Probes were dual high performance liquid chromatography-purified (LCG BioSearch Technologies, Novato, CA). All probes and human TBP primers were at 0.1 μM final, Pf-specific primers 0.4 μM, and pan-*Plasmodium* primers 0.2 μM. Cycling conditions were reverse transcription (10 minutes) at 48°C, denaturation (2 minutes) at 95°C, and 45 PCR cycles of 95°C (5 seconds) and 50°C (35 seconds).

**Pf culture.** Control samples and some validation samples were generated from Pf 3D7 strain parasites cultured using the methods of Trager and Jensen as previously reported.\textsuperscript{10} ABO blood group-matched whole blood was used for diluting erythrocyte cultures to prevent rosetting.

**Calibration and reporting.** A custom lot of quantified Armored RNA encoding full-length Pf 18S rRNA (Asuragen, Austin, TX) was used as an absolute 18S rRNA calibrator by addition to lysed Pf-negative whole blood before extraction. Copies/mL of whole blood were converted to estimated parasites/mL of whole blood by dividing by the per-parasite copy number conversion factor (see Results). Quantitative results were reported for estimated parasite densities ≥ 20 parasites/mL, qualitative “low-positive” results for results equivalent to 10 to < 20 estimated parasites/mL, and “not detected” for lower and undetectable results.

**Quality assurance.** High (4 × 10\textsuperscript{2} parasites/mL), low (8 × 10\textsuperscript{2} parasites/mL), and negative controls were tested in each assay run and monitored using 30-run Levey-Jennings plots. Westgard 13s (run rejected if control > 3 SDs from expected value), 2s\textsubscript{r} (run rejected if two consecutive controls are > 2 SDs from expected value), and 12r rule (run rejected if 12 consecutive control measurements are on one side of the mean).\textsuperscript{25} Human TBP mRNA was monitored as an endogenous internal control. The laboratory exchanged samples with outside laboratories for external quality assurance (EQA) and enrolled in the WHO EQA scheme for malaria nucleic acid amplification testing (http://www.who.int/malaria/publications/atoz/NAAT-EQA-manual/en/).

**Discrepant analysis.** Discrepant analyses were performed following Food and Drug Administration (FDA) guidelines.\textsuperscript{26} Agreement and CIs were calculated following the Clinical and Laboratory Standards Institute (CLSI) standards.\textsuperscript{27} Tests used to assess the primary result (pan-*Plasmodium* channel of biomarker assay) included the Pf-specific channel of the current assay, the first generation of the University of Washington (UW) Pf 18S rRNA qRT-PCRs,\textsuperscript{10} and assays performed by outside laboratories, including the University of Maryland 18S rRNA biomarker qRT-PCR (University of Maryland),\textsuperscript{28} the Laboratory for Malaria Immunology and Vaccinology 18S rDNA qPCR (unpublished. J. Neuel, personal communication), and the NIH Clinical Center 18S rDNA qPCR (NIH).\textsuperscript{29}

**Human clinical samples.** Leftover clinical whole blood specimens (50 μL) from local hospitals were used under a protocol approved by the UW Institutional Review Board (IRB) (Protocol 47026, S. Murphy). Samples were also obtained from the following completed IRB-approved clinical trials. The MC-001 Demo Trial (IND 14224; ClinicalTrials.gov NCT01500980) was a single-center, open-label phase 1 trial to demonstrate the ability to conduct CHMI trials under an investigational new drug (IND) application and obtain immunological endpoints after one exposure.\textsuperscript{10,21} The MC-003 trial (IND 14752; ClinicalTrials.gov NCT01500980) was a phase 1, randomized,
partial double-blind, placebo-controlled study at the same site by Infection-Treatment-Vaccination using chloroquine and primaquine. The PfSPZ-CVac pyrimethamine (PYR) study (NIAID Protocol Number 15-I-0169; IND 16650; ClinicalTrials.gov NCT03083847) was a phase 1 study conducted at the NIH Clinical Center of Sanaria® PfSPZ-CVac (P. falciparum sporozoite-chemoprophylaxis vaccination30) using chloroquine and PYR as the prophylactic drugs. The CHMIs for the MC-001 and MC-003 studies were given by five infected mosquito bites per participant, whereas CHMI for the NIH PfSPZ-CVac PYR study was by direct venous injection (DVI) of $3.2 \times 10^3$ aseptic, purified, cryopreserved Pf sporozoites (Sanaria PfSPZ Challenge).20,31 In all studies, participants provided informed consent; additional details are available in the Supplemental Information and at ClinicalTrials.gov. Studies were evaluated for onset of blood smear positivity, 18S rRNA positivity, and malaria-related symptom onset and severity. Solicited malaria-related adverse events (AEs) included temperature $\geq 38^\circ$C, fever, malaise, myalgia, headache, nausea, vomiting, chills, lower back pain, diarrhea, abdominal pain, arthralgia, and chest pain. Temperature grading was grade 1 (38.0–38.4°C), 2 (38.5–38.9°C), 3 (39.0–40°C), and 4 (> 40°C). Symptom grading used comparable scales as defined in individual trial protocols.

Data management and statistics. Clinical and laboratory data for included trials were managed by DF/Net Research (Seattle, WA). For most comparisons, paired or unpaired Student’s t-tests were used, depending on the nature of the data. Food and Drug Administration and CLSI guidance were followed for calculating CIs of assay data as indicated. Statistical significance was considered at $P < 0.05$.

RESULTS

Literature review: Plasmodium 18S rRNA/rDNA biomarker versus blood smears in CHMI trials at non-endemic sites. Twenty-two CHMI studies at non-endemic sites were reviewed on 488 volunteers (290 naive, 198 vaccinated), where both blood smears and biomarker NATs were performed10,16,20–23,29,30,32–45 (Figure 1, Supplemental Table 1). Data on participants administrated intramuscular or intradermal PfSPZ Challenge were not included. Controlled human malaria infection was by either three to seven infectious mosquito bites or DVI of $3.2 \times 10^3$ PfSPZ Challenge (Sanaria, Rockville, MD). Across all studies and groups, the biomarker time to positivity (TTP) was shorter than that for blood smears (Figure 1), with biomarker assays becoming positive before blood smears (mean difference: 3.4 days; 95% CI: 2.8–4.0). The reported

![Figure 1](image-url)
Biomarker TTPs were not significantly different for CHMI by mosquito bite versus PfSPZ DVI. Positive blood smears occurred 3.2 days earlier (range: 2–5.2 days) than blood smears (mean: 10.9 days; range: 7–16 days). For the DVI CHMIs, biomarker positivity (mean: 8.0 days; range: 7–11 days) occurred 3.2 days earlier (range: 2.4–11 days) than blood smears (mean: 11.2 days; range: 10–17.9 days). Blood smear and biomarker TTPs were considered separately. For vaccines deemed effective for this analysis, 34/48 participants showed biomarker positivity (mean: 7.4 days; range: 6.3–8.0 days) than blood smears (mean: 11.6 days; range: 9–20 days). Compared with naive controls, blood smears and 18S rRNA biomarker TTPs for the effective vaccine group were significantly shorter than those from naive participants (P = 0.001 and P < 0.001, respectively), whereas there were no significant differences between the ineffective vaccine group and the naive controls for either endpoint.

**Analytical validation of the Plasmodium 18S rRNA multiplex qRT-PCR.** A multiplex Plasmodium 18S rRNA qRT-PCR was analytically validated in accordance with CLSI guidelines. Key characteristics and assay performance are summarized in the following paragraphs. The assay detects and quantifies pan-Plasmodium and Pf-specific regions of the 18S rRNA and a human TBP endogenous control mRNA. Pan-Plasmodium primers (PanDDT1043F19/PanDDT1197R22) and a probe are 100% conserved in all human-infecting plasmodia (Supplemental Figure 1, Supplemental Table 2); in rodent-infecting species *Plasmodium yoelii*, *Plasmodium chabaudi*, and *Plasmodium berghei*; and in primate-infecting species *Plasmodium cynomolgi* and *Plasmodium reichenowi*. The pan-Plasmodium amplicon contains nucleotide variation that permits species identification by amplicon sequencing. The Pf target used novel Pf-specific primers (PFDDT1451F21/ PFDDT1562R21) and a published Pf probe that are 100% matched to A-type 18S rRNA genes MAL5_18S/PF3D7_0531600 and MAL7_18S/PF3D7_0725600 and 10 full- or partial-length PfA-type 18S rRNA sequences in GenBank (not shown). Pf reagents have mismatches to Pf S-type genes and are absent from non-Pf 18S rRNA genes (Supplemental Figure 2). These Pf primers also detected additional Pf infections in field studies that were missed by earlier generations of the assay, demonstrating improved coverage of variant Pf strains (S. Das, G. Domingo, S. Murphy, personal communication).

The method was tested for accuracy, precision, analytical sensitivity, sample stability and analytical specificity (interferences), reportable range, and carryover using whole blood samples. Briefly, standard curves demonstrated an efficient reaction and stable, reproducible performance (pan-Plasmodium slope −3.38 cycles/log_{10} copies/mL lysate, 95% CI: −3.17 to −3.60).
to -3.59; intercept 41.9 cycles; 95% CI: 39.4–44.3 cycles; \( r^2: 0.998 \). The Armored RNA calibrator was used to generate a conversion factor for 18S rRNA copies per ring–stage parasite of \( 7.4 \times 10^5 \) 18S rRNA copies/parasite (mean and median; 95% CI: 6.17 \times 10^5 \text{ to } 8.71 \times 10^5; n = 22 samples) following our previously published approach\(^{10,11}\) using EQA samples extensively characterized by qPCR and qRT-PCR\(^{12}\); the conversion factor was within 2-fold of the values determined for the earlier generations of the assay.\(^{10,11}\)

Among 106 samples of known density (range: \( 1 \times 10^2 \) to \( 10^5 \) parasites/mL), the average \( \log_{10} \) difference (bias) between measured and expected results was \( +0.11 \log_{10} \) parasites/mL (95% CI: 0.23 to 0.44 \( \log_{10} \) parasites/mL) in the pan-
Plasmodium channel; with no evidence of concentration-dependent differences in recovery (Supplemental Figure 3). Within-run and between-run precision\(^{48}\) were acceptable (Supplemental Table 3). For low-density samples, the pan-
Plasmodium channel detected 100% (10/10), 95% (19/20), and 95% (19/20) of samples at nominal concentrations of 100, 50, and 20 parasites/mL, respectively (Supplemental Table 4). To further evaluate assay sensitivity, Armored RNA was added to whole blood samples to create samples equivalent to low parasite densities. Positive Armored RNA results included 21/21 samples at a copy number equivalent to one parasite per 50 \( \mu \)L of whole blood (1.48 \times 10^6 copies/mL of blood or 20 parasites/mL and 20/20 at a copy number equivalent to one-third of a parasite per 50 \( \mu \)L of whole blood (5.3 \times 10^5 copies/mL of blood or ~7 parasites/mL). The limit of quantification determined by CLSI methods\(^{49}\) was 20 parasites/mL using Armored RNA added to lysed malaria-negative whole blood (1.48 \times 10^6 copies/mL whole blood) (Supplemental Table 5), for a reportable range of 20 to \( 1 \times 10^5 \) parasites/mL. No carryover was noted (44 known negative samples processed immediately after paired high positives). The assay detects asexual-stage Plasmodium parasites as well as gametocytes and sporozoites. There were no interferences from hemolysis (to 10 times upper limit of normal [ULN] for free hemoglobin), lipemia (to 6.5-fold ULN), bilirubinemia (to 8-fold ULN), heparin (to 40 U.S. Pharmacopeia heparin units/mL), Candida albicans (to 4 \times 10^3 cfu/mL), Cytomegalovirus (5 \times 10^4 IU/mL), Epstein–Barr virus (5 \times 10^4 IU/mL), HIV-1 (viral load 4.76 \log_{10} \) RNA copies/mL, HIV-2 (viral load 3.24 \log_{10} \) RNA copies/mL), or Trypanosoma brucei spp. (1 \times 10^5 parasites/mL). Leukocytosis up to 25 \times 10^9 cells/L did not interfere, but more severe leukocytosis led to biomarker underestimation (−0.34 \log_{10} \) estimated parasites/mL less than controls). Weak cross-reactivity for high-density Babesia microti samples (5% parasitemia) occurred in the pan-Plasmodium channel (not the Pf channel); a Babesia-specific assay is available to discriminate.\(^{50}\)

Stability studies showed that the Plasmodium 18S rRNA is stable in parasite-containing EDTA whole blood samples (2 \times 10^6 estimated parasites/mL) for 96 hours at room temperature or 72 hours at 4°C before lysis buffer addition (Supplemental Table 6). Whole blood could also be frozen and thawed one time with subsequent processing into lysis buffer on thawing without significant biomarker degradation (\( P = 0.21, n = 9 \) paired samples), whereas repeated freeze-thawing resulted in more significant degradation (\( P = 0.003, n = 9 \) paired samples) (Supplemental Figure 4). Finally, correlation studies showed close agreement with earlier assay generations\(^{10,11}\) (first- and third-generation assays: \( n = 68 \) samples, slope: 1.06, intercept: 0.03 \log_{10} \) parasites/mL, \( r^2: 0.91 \); second- and third-generation assays: \( n = 98 \) samples, slope: 1.00, intercept: 0.10 \log_{10} \) parasites/mL, \( r^2: 0.99 \).**

**Clinical validation of the 18S rRNA biomarker.** We performed a clinical validation of the Plasmodium 18S rRNA to support biomarker qualification through the FDA.\(^{51}\) We tested samples from three CHMI studies from non-endemic sites (MC-001, MC-003, and PSPZ-Cvac PYR; Table 2), in which blood smears and second- or third-generation Plasmodium 18S rRNA qRT-PCR were performed. Quantitative reverse transcription–PCR and blood smear results (one sample per day for each protocol-defined testing day) and malaria-related clinical data were included. Discrepant analyses were performed for two trials. Data for each study are described and evaluated in Supplemental Information and Supplemental Tables 7–11. Summary data are below.
Combined analysis of *Plasmodium 18S* rRNA infection detection across all studies. In the included trials, blood smear–positive participants showed the presence of the biomarker in peripheral blood on or before the time of blood smear positivity (n = 37). Across all such volunteers, the mean TTP for any level of biomarker positivity (including low positives) was 7.6 days (range: 6–15 days; n = 37), with correspondingly longer times needed to reach higher qRT-PCR–estimated densities (Table 3 and Figure 2A). By contrast, for blood smear–positive participants, mean blood smear TTP was 11.0 days (range: 7–18 days; n = 37). On average, biomarker positivity began 3.4 days (95% CI: 3.0–3.8 days) earlier than blood smear positivity. When modeled against different qRT-PCR–determined parasite densities, there were density-dependent accelerations compared with blood smears (Figure 2B, Table 4). For instance, the mean TTP difference between biomarker equivalent to ≥250 est. parasites/mL and blood smears was 2.2 days (95% CI: 1.9–2.6 days), whereas the difference was greater if a more sensitive biomarker-based threshold of 20 parasites/mL was used (3.3 days; 95% CI: 2.9–3.7 days).

For the included studies, the mean time for first malaria-related symptom of any grade was 9.7 days (range: 6–14 days; n = 37) and for first grade 2 malaria-related symptom was 12.2 days (range: 6–15 days; n = 28) (Table 5). The mean difference between any biomarker positivity and malaria-related symptom onset was 2.2 days (95% CI: 1.6–2.7 days). As for blood smears, modeling of different treatment thresholds indicates that treatment at lower densities is likely to mitigate symptom onset seen at higher densities up to and including blood smears (Figure 2C–D, Table 4).

The mean parasite density on becoming qRT-PCR positive was 950 estimated parasites/mL (range: 19–10,904; n = 37) by qRT-PCR (Supplemental Table 12). At blood smear positivity, the mean parasite density by blood smear was 19,668 estimated parasites/mL (range: 1,700–82,000; n = 37) and 41,979 estimated parasites/mL by qRT-PCR (range: 1,687–162,152; n = 37) (Supplemental Table 12). Quantification by blood smears and qRT-PCR was moderately correlated (r²: 0.45; n = 37 samples), with qRT-PCR estimates slightly higher than those by blood smears (bias +0.28 log₁₀ parasites/mL, 95% CI: −0.53 to +1.10 log₁₀ parasites/mL; data not shown).

Discrepant analyses support accuracy of prepatent detection of *Plasmodium 18S* rRNA. *Plasmodium 18S* rRNA/PYR biomarker assays have lower limits of detection and are therefore more analytically sensitive than blood smears. Thus, a common result in low-density infections is "biomarker positive, blood smear negative." Volunteers in these studies who were initially qRT-PCR positive progressed to higher density infections and eventually became blood smear positive. To further assess the ability to reliably detect biomarker at sub-patent densities, all positive and negative samples from two of the studies (MC-001 and PISPZ-CVac PYR) were subjected to discrepant analyses using published assays conducted in our laboratory and at collaborating centers (Supplemental Figure 5). There was 100% positive percent agreement between blood smears and the described pan-*Plasmodium 18S* rRNA qRT-PCR in both studies (Supplemental Table 13). As expected, negative percent agreement (NPA) between blood smears and pan-*Plasmodium* qRT-PCR was < 100% in all cases. As the limit of detection of comparator molecular assays approached
that of the pan-*Plasmodium* 18S rRNA qRT-PCR, NPA between the pan-*Plasmodium* qRT-PCR result and the comparator test approached 100%. Discrepancy analyses showed that the risk of a blood smear–positive/qRT-PCR–negative result is close to zero. These data show that qRT-PCR reliably detects *Plasmodium* 18S rRNA at sub-microscopic densities.

**Evaluation of biomarker-defined treatment thresholds for CHMI studies.** Clinical trials with NAT endpoints can be designed to initiate treatment based on qualitative NAT positivity or on the basis of a quantitative NAT-defined threshold corresponding to a specific copy number or estimated parasite density. Waiting longer to treat post-CHMI may result in more symptoms, but allow better resolution of partially protected phenotypes between groups. To evaluate such trade-offs, correlations between biomarker, blood smears, and symptoms were evaluated for the three included trials. As described, lower biomarker levels were associated with progressively earlier infection detection compared with onset of blood smear patency and symptom onset (Table 4). The greatest acceleration was achieved using the most sensitive cutoff at the 20 parasite/mL assay limit of detection—as described earlier, blood smears and symptoms would be expected to trail 2–3 days behind this level of biomarker positivity. Of all symptoms that occur in CHMI studies, it is especially desirable to limit the frequency of grade 3 symptoms. Initiation of treatment based on the most sensitive biomarker threshold would be expected to eliminate nearly all grade 3 symptoms (Table 6).

Because not all assays achieve a limit of detection of 20 parasites/mL, and because sampling bias may affect the limit of detection at such low densities, we also evaluated a cutoff of 250 estimated parasites/mL. This cutoff accelerated infection detection by > 2 days compared with blood smears and preceded malaria-related symptoms (any grade by 1.0 day and grade 2 by 2.1 days) (Table 4). The threshold of 250 parasites/mL would also be expected to precede nearly all grade 3 symptoms (Table 6).

To evaluate the ability of different biomarker levels to differentiate degrees of partial protection, we also evaluated biomarker time to first positive, time to 250 estimated parasites/mL, and estimated parasite density at first positive against CHMI results stratified by day of blood smear patency. Subject-level data were binned into groups of no delay (blood smear–positive days 7–9, n = 13 participants), low delay (blood smear–positive days 10–11; n = 13 participants), moderate delay (blood smear–positive days 12–13; n = 5 participants), large delay (blood smear–positive days 14; n = 6 participants), or complete protection (n = 9 participants). Although time to first positive biomarker result could significantly differentiate the group with patency on days 7–9 from those on days 12–13 or ≥ 14, it could not do so for the day 10–11 group (Figure 3A). By contrast, time to ≥ 250 estimated parasites/mL by qRT-PCR closely mirrored the blood smear groupings and differentiated between most groups (Figure 3B). Early positive blood smears (days 7–9) were also accompanied by higher biomarker-based parasite densities than participants patent on day 10 or later (Figure 3C). Thus, these data and those of others support a *Plasmodium* 18S RNA–based treatment threshold–based approach for differentiating outcomes of complete, partial, and zero protective efficacy.

**DISCUSSION**

Controlled human malaria infection trials at non-endemic sites are increasingly used to test malaria drug and vaccine

### Table 4

Comparisons of *Plasmodium* 18S rRNA versus blood smears and symptoms for all studies

| Biomarker threshold (est. parasites/mL) | Mean days from biomarker to TBS positivity* | Mean days from biomarker to any related symptom† | Mean days from biomarker to any grade 2 related symptom‡ |
|----------------------------------------|--------------------------------------------|-----------------------------------------------|--------------------------------------------------|
| Any (+) (incl. low positives)          | 3.4 (3.0–3.8) ****                         | 2.2 (1.6–2.7) ****                           | 3.3 (2.7–4.0) ****                               |
| ≥ 20                                   | 3.3 (2.9–3.7) ****                         | 2.0 (1.5–2.6) ****                           | 3.2 (2.6–3.8) ****                               |
| ≥ 100                                  | 2.8 (2.4–3.2) ****                         | 1.6 (1.0–2.1) ****                           | 2.7 (2.1–3.3) ****                               |
| ≥ 250                                  | 2.2 (1.9–2.6) ****                         | 1.0 (0.5–1.4) ****                           | 2.1 (1.6–2.6) ****                               |
| ≥ 500                                  | 1.9 (1.6–2.3) ****                         | 0.7 (0.2–1.2) ****                           | 1.8 (1.3–2.2) ****                               |
| ≥ 1,000                                | 1.5 (1.2–1.7) ****                         | 0.2 (–0.3 to 0.7) 0.25                      | 1.2 (0.7–1.8) ****                               |
| ≥ 10,000                               | 0.2 (–0.1 to 0.4) 0.12                     | –0.9 (–1.5 to –0.3) 0.99                    | 0.1 (–0.5 to 0.8) 0.37                           |
| Positive TBS                           | NA                                         | –1.1 (–1.7 to –0.6) 0.99                    | –0.2 (–0.8 to 0.4) 0.70                           |

NA = not applicable; TBS = thick blood smears. *P < 0.05; **P < 0.001; ***P < 0.0001. †Tests performed among participants who eventually tested positive by TBS.

### Table 5

Summary of post-CHMI malaria-related symptoms in blood smear–positive participants

| Days from CHMI to | Statistic | MC-003 infectivity and drug controls (N = 11) | MC-003 vaccinated (N = 14) | PRSPZ-Cvac PYR (N = 6) | All studies (N = 37) |
|------------------|-----------|-----------------------------------------------|---------------------------|------------------------|---------------------|
| First symptom (any grade) | N = 6 | 11 | 14 | 4 | 35 |
| Mean (days) | 9.7 | 8.2 | 9.8 | 12.8 | 9.7 |
| 95% CI (days) | 6.4–12.2 | 7.2–9.1 | 8.5–11.1 | 10.4–14.2 | 8.9–10.4 |
| First grade 2 symptom | N = 5 | 10 | 9 | 4 | 28 |
| Mean (days) | 12.2 | 8.8 | 10.0 | 13.8 | 10.7 |
| 95% CI (days) | 10.6–13.3 | 7.9–9.6 | 8.2–11.8 | 12.2–14.7 | 9.7–11.6 |

CHMI = controlled human malaria infection; PYR = pyrimethamine.
candidates and assess innate and naturally acquired resistance to malaria. In studies conducted at non-endemic sites using blood smear endpoints, many volunteers who develop parasitemia experience malaria-related AEs by the time parasites are microscopically detected. In these studies, delayed blood smear patency is used as an indicator of partial protection. Here, we provide analytical and clinical validation that, compared with blood smears, the *Plasmodium* 18S rRNA/rDNA biomarker can accelerate infection detection and reduce AEs while preserving the ability to discern complete, partial, and lack of protection post-CHMI. Review of the literature demonstrated consistently accelerated infection detection for the biomarker compared with blood smears in 22 published CHMI studies. Testing of archival samples from three CHMI trials using the analytically validated biomarker assay further confirmed these trends and showed that the biomarker is consistently detected approximately 7 days post-CHMI in immunologically naive persons, which is ∼2–4 days earlier than blood smear patency and symptom onset. The use of biomarker testing as the primary treatment endpoint is notably expected to eliminate most grade 3 symptoms and grade 3 AEs in CHMI studies at non-endemic sites. With an appropriately sensitive biomarker assay, discrepant analyses showed that the risk of a blood smear–positive/biomarker-negative result was close to zero. Thus, these data support the use of the *Plasmodium* 18S rRNA/rDNA biomarker as an alternative for blood smears in CHMI studies at non-endemic study sites. Not surprisingly, several recently published studies have used this biomarker as the primary endpoint for some or all drug and vaccine CHMI cohorts.

These data were submitted to the FDA through the Center for Drug Evaluation and Research (CDER) Drug Development Tool Biomarker Qualification program to be considered for qualified use in non-endemic CHMI trials. Through a 2014–2018 process, the FDA recently qualified the biomarker for the following agency-wide context of use (COU): *Plasmodium* 18S rRNA/rDNA biomarker (reported in copies/mL blood) can be tested by a NAT for...
monitoring to inform initiation of treatment ≥ 6 days post-
CHMI in *P. falciparum* studies at non-endemic sites. Nota-
ably, CHMI trial participants need to be biomarker negative at
the start of a study and meet general CHMI inclusion/
exclusion criteria. Potential interferences from extreme
levels of leukocytosis and *Babesia* (as noted previously)
were also noted by the FDA. These interferences are unlikely
to be problematic in CHMI studies—individuals with leuko-
cytosis > 25 × 10⁹/L would not be eligible for most studies at
screening and *Babesia* cross-reactivity would only occur
with high-density, clinically apparent infections. Such par-
ticipants would be disqualified at screening by symptoms
and by any reactive biomarker testing performed pre-CHMI.
The Pf channel described in this multiplex assay does not
cross-react with *Babesia*, and true Pf-positive results are
expected to be pan- and Pf-channel positive. The FDA re-
quests that biomarker data be reported in copies/mL—as
such, we issue results in copies/mL as well as in estimated
parasites/mL using a Pu-specific conversion factor. The
FDA’s qualified COU considers any positive cycle threshold
to be a qualitative positive biomarker result, but directs trial
protocols to specify a biomarker threshold where treatment
would be initiated—this threshold could be at the limit of
detection or higher. Our study assessed a variety of
thresholds and found that a density equivalent to 250 esti-
imated parasites/mL is likely to reduce symptoms and allow
differentiation between complete, partial, and lack of pro-
tection, which could be obscured if a much lower threshold
was used. Another study found that a comparable threshold
of 100 estimated parasites/mL behaved similarly.⁵² The FDA
recommends that the WHO 18S rRNA calibrators be de-
veloped and that laboratories participate in EQA. Additional
recommendations by the FDA include that the WHO 18S rRNA
mRNA be used, ¹⁴,⁵⁶ recommended COU for other settings,
such as endemic site CHMI and/or field studies.

This study has several limitations. First, this study did not
address posttreatment clearance of the 18S rRNA bio-
maker, although these data are being compiled. Sensitive
NATs can sometimes lead to prolonged positivity following
adequate treatment. Second, human samples were limited to
non-endemic CHMI trials reliant on NF54 strain Pf paras-
ite. Other CHMI strains such as 7G8, NF135.C10, and
NF166.C8 are in earlier stages of development and use, ¹⁴,⁵⁶–⁵⁹
and biomarker agreement with NF54 and/or
3D7-based studies may need to be evaluated because
prepatent periods can differ.⁶⁰ Biomarker use in endemic
sites will likely add other complexities such as preexisting
infections, parasite strain and species variation, and higher
rates of partial immunity. Third, this report describes studies
where treatment was initiated by blood smear positivity and
TTP modeling was based on sample collection times. If
biomarker-positive participants are not treated until the
day after becoming treatment eligible, the projected re-
duction in symptoms may not be fully realized. However, we
and others have since conducted numerous clinical tri-
als using the biomarker to initiate treatment including the
DSM265 drug trial,¹⁵ published PfSPZ vaccine trials,¹⁴,¹⁶ an
as-yet-unpublished multi-cohort drug trial (J. Koblin/
S. Murphy, personal communication), an unpublished
Sanaria PfSPZ-Cvac trial, and an ongoing study of geneti-
cally attenuated sporozoites administered by mosquito
bites (L. Jackson, personal communication). In these stud-
ies, the biomarker has led to elimination of the domiciled
hotel phase in the study designs. When these trials are
published, the collective experience is likely to demonstrate
accelerated infection detection and reduction in symptoms.
Thus, the biomarker-based approach can safely accelerate
infection detection in CHMI studies and provide nuanced pro-
tection data for evaluating early stage drugs and vaccines.

Received January 30, 2019. Accepted for publication March 21, 2019.
Published online April 22, 2019.

Note: Supplemental information, tables, and figures appear at
www.ajtmh.org.

Acknowledgments: We thank the clinical trial staff at the Fred
Hutchinson Cancer Research Center Prevention Center, the Center
for Infectious Disease Research (now at Seattle Children’s Research
Institute), the Seattle Malaria Clinical Trials Center, the Medicines for
Malaria Venture, the Center for Vaccine Development and Global
Health at the University of Maryland School of Medicine, and LMIV,
and the NIH Clinical Center for assistance with clinical trials. We thank
Jane Mills (Center for Infectious Disease Research) for assistance with
data access. We thank the UW Center for AIDS Research for labora-
tory assistance, FDA CDER Biomarker Qualification panel members,
and Sophie Allaunen and the late Alan Magill at the Bill & Melinda
Gates Foundation (BMGF) for guidance and support.

Financial support: Funding was from the BMGF ( OPP1133622 to
S. M.), and the biomarker laboratory was supported in part by the NIH
( AI-38858; P30 AI027757); the MC-001 study was supported by the
Seattle Biomedical Research Institute; the MC-003 study was sup-
ported by the BMGF, which was involved in development and design
of the study; and the NIH PfSPZ-Cvac PYR study was supported by the
National Institute of Allergy and Infectious Diseases and NIH,
(U01AI097970 to Sanaria).

Ethics, Consent, and Permissions: The MC-001 and MC-003 studies
were reviewed and approved by the Western IRB (WRB), in collabo-
rution with UW IRB. The NIH PfSPZ-Cvac PYR study was reviewed and
approved by the NIAID IRB. Volunteers were enrolled after
obtaining informed consent using IRB-approved documents. The
IRB-approved informed consent for all studies included the consent
to publish.

Availability of data and material: Additional Biomarker Qualification
data reviews are available on the FDA website.⁵¹ All sequence data
were from publicly available sources through plasmodb.org.

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Biomarker Qualification of Plasmodium 18S rRNA in ChMI Studies

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REFERENCES

1. WHO. 2018. World Malaria Report 2018. Geneva, Switzerland: World Health Organization.
2. Laurens MB et al.; Consensus Group on Design of Clinical Trials of Controlled Human Malaria Infection, 2012. A consultation on the optimization of controlled human malaria infection by mosquito bite for evaluation of candidate malaria vaccines. Vaccine 30: 5302–5304.
3. Bruce-Chwatt LJ, 1984. DNA probes for malaria diagnosis. Lancet 1: 795.
4. Payne D. 1988. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. Bull World Health Organ 66: 621–626.
5. Murphy SC, Shott JP, Parikh S, Etter P, Prescott WR, Stewart BA, 2013. Malaria diagnostics in clinical trials. Am J Trop Med Hyg 89: 824–839.
6. Roth JM, Korevaar DA, Leeffang MM, Mens PF, 2016. Molecular malaria diagnostics: a systematic review and meta-analysis. Crit Rev Clin Lab Sci 53: 87–105.
7. Gunderson JH, Sogin ML, Wollett G, Hollingdale M, de la Cruz VF, 1987. Structurally distinct, stage-specific ribosomes occur in Plasmodium. Science 238: 933–937.
8. Waters AP, Slyn C, McCutchan TF, 1989. Developmental regulation of stage-specific ribosomes in Plasmodium. Nature 342: 438–440.
9. Li J, McConkey GA, Rogers MJ, Waters AP, McCutchan TR, 1994. Plasmodium: the developmentally regulated ribosome. Exp Parasitol 78: 437–441.
10. Murphy SC et al., 2012. Real-time quantitative reverse transcription PCR for monitoring of blood-stage Plasmodium falciparum infections in malaria human challenge trials. Am J Trop Med Hyg 86: 383–394.
11. Murphy SC, Daza G, Chang M, Coombs R, 2012. Laser cutting eliminates nucleic acid cross-contamination in dried-blood-spot processing. J Clin Microbiol 47: 4126–4130.
12. Schneider P, Wolters L, Schoone G, Schallig H, Sillekens P, Hermens R, Sauerwein R, 2005. Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of Plasmodium falciparum. J Clin Microbiol 43: 402–405.
13. Hodgson SH et al., 2015. Increased sample volume and use of quantitative reverse-transcription PCR can improve prediction of liver-to-blood inoculum size in controlled human malaria infection studies. Malar J 14: 33.
14. Lyke KE et al., 2017. Attenuated PSpZ vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection. Proc Natl Acad Sci USA 114: 2711–2716.
15. Murphy SC et al., 2018. A randomized trial evaluating the prophylactic activity of DSM265 against preerythrocytic Plasmodium falciparum infection during controlled human malaria infection by mosquito bite and direct venous inoculation. J Infect Dis 217: 693–702.
16. Ishizuka AS et al., 2016. Protection against malaria at 1 year and immune correlates following PSpZS vaccine. Nat Med 22: 614–623.
17. Murphy Jr, Baqar S, Davis JR, Herrington DA, Clyde DF, 1989. Evidence for a 6.5-day minimum exoerythrocytic cycle for Plasmodium falciparum in humans and confirmation that immunization with a synthetic peptide representative of a region of the circumsporozoite protein retards infection. J Clin Microbiol 27: 1434–1437.
18. Fairley NH, 1947. Sidelights on malaria in man obtained by sub-inoculation experiments. Trans R Soc Trop Med Hyg 40: 621–676.
19. Hodgson SH et al., 2014. Evaluating controlled human malaria infection in Kenyan adults with varying degrees of prior exposure to Plasmodium falciparum using sporozoites administered by intramuscular injection. Front Microbiol 5: 686.
20. Mordmuller B et al., 2015. Direct venous inoculation of Plasmodium falciparum sporozoites for controlled human malaria infection: a dose-finding trial in two centres. Malar J 14: 117.
21. Talley AK et al., 2014. Safety and comparability of controlled human Plasmodium falciparum infection by mosquito bite in malaria-naive subjects at a new facility for sporozoite challenge. PLoS One 9: e109654.
22. Sheehy SH et al., 2012. Chad83-MVA-vectorized blood-stage malaria vaccines targeting MSP1 and AMA1: assessment of efficacy against mosquito bite challenge in humans. Mol Ther 20: 2355–2368.
23. Lyke KE et al., 2010. Plasmodium falciparum malaria challenge by the bite of aseptic Anopheles stephensi mosquitoes: results of a randomized infectivity trial. PLoS One 5: e13490.
24. Leptak C et al., 2017. What evidence do we need for biomarker qualification? Sci Transl Med 9: eaal5698.
25. Westgard JO, 2007. Basic Quality Control Practices, 4th edition. Westgard Quality Corporation.
26. FDA. 2007. Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests. FDA Center for Devices and Radiological Health.
27. CLSI. 2009. User Protocol for Evaluation of Qualitative Test Performance, EP12-A2, 2nd edition.
28. Adams M et al., 2015. An ultrasensitive reverse transcription polymerase chain reaction assay to detect asymptomatic low-density Plasmodium falciparum and Plasmodium vivax infections in small volume blood samples. Malar J 14: 520.
29. Seder RA et al.; VRC 312 Study Team, 2013. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science 341: 1359–1365.
30. Mordmuller B et al., 2017. Sterile protection against human malaria by chemoattenuated PSpZ vaccine. Nature 542: 445–449.
31. Roestenberg M et al., 2013. Controlled human malaria infections by intradermal injection of cryopreserved Plasmodium falciparum sporozoites. Am J Trop Med Hyg 88: 5–13.
32. Verhage DF, Teltg DS, Bousema JT, Hermens CC, van Gernet GJ, van der Meer JW, Sauerwein RW, 2005. Clinical outcome of experimental human malaria induced by Plasmodium falciparum-infected mosquitoes. Neth J Med 63: 52–58.
33. Thompson FM et al., 2008. Evidence of blood stage efficacy with a virosomal malaria vaccine in a phase iia clinical trial. PLoS One 3: e1493.
34. Spring MD et al., 2009. Phase 1/2a study of the malaria vaccine candidate apical membrane antigen-1 (AMA-1) administered in adjuvant system AS01B or AS02A. PLoS One 4: e6254.
35. Roestenberg M et al., 2009. Protection against a malaria challenge by sporozoite inoculation. N Engl J Med 361: 468–477.
36. Roestenberg M et al., 2011. Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. Lancet 377: 1770–1776.
37. Epstein JE et al., 2011. Live attenuated malaria vaccine designed with controlled human malaria infection. PLOS ONE 7: e38434.
38. Chuang I et al., 2013. DNA prime/adenovirus boost malaria vaccine encoding P. falciparum CSP and AMA1 induces sterile protection associated with cell-mediated immunity. PLOs One 8: e55571.
39. Kamau E, Alemayehu S, Feghali KC, Komisar J, Regules J, Cowden J, Ockenhouse CF, 2014. Measurement of parasitological data by quantitative real-time PCR from controlled human malaria infection trials. Malar J 13: e38434.
40. LaVange L, Zineh I, Buckman-Garner S, Woodcock J, Edelman R, Adams M, Strauss KA, Shrestha B, Levine MM, Edelman R, Lyke KE, 2019. The controlled human malaria infection experience at the University of Maryland. Am J Trop Med Hyg 29 (Suppl 1): S23–S26.
41. FDA, 2018. Qualified Biomarkers and Supporting Information (CDER Biomarker Qualification Program). Available at: https://www.fda.gov/Drugs/DevelopmentApprovalProcess/ DrugDevelopmentToolsQualificationProgram/BiomarkerQualificationProgram/ucm535383.htm. Accessed March 19, 2019.
42. Coffeng LE, Hermansen CC, Sauerwein RW, de Vlas SJ, 2017. The power of malaria vaccine trials using controlled human malaria infection. PLOs Comput Biol 13: e1005255.