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MAJOR ARTICLE

Absence of Malaria-Associated Coagulopathy in Asymptomatic *Plasmodium falciparum* Infection: Results From a Cross-sectional Study in the Ashanti Region, Ghana

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Background. Coagulopathy is common in acute symptomatic *Plasmodium falciparum* malaria, and the degree of coagulation abnormality correlates with parasitemia and disease severity. Chronic asymptomatic malaria has been associated with increased morbidity. However, the role of coagulation activation in asymptomatic, semi-immune individuals remains unclear. This study investigates the potential effect of asymptomatic *P falciparum* infection on coagulation activation in semi-immune Ghanaian adults.

Methods. Blood from asymptomatic Ghanaian adults with *P falciparum* blood stage infection detectable by polymerase chain reaction (PCR) or by both PCR and rapid diagnostic test and from noninfected individuals, was investigated. Markers of coagulation activation including global coagulation tests, D-dimer, antithrombin III, fibrinogen, and von Willebrand factor antigen were tested. Furthermore, blood count, inflammation markers, and liver and kidney function tests were assessed.

Results. Acquired coagulopathy was not found in asymptomatic *P falciparum* infection. Asymptomatic malaria was associated with significantly lower platelet counts. Systemic inflammation markers and liver and kidney function tests were not altered compared to noninfected controls.

Conclusions. There is no laboratory evidence for acquired coagulopathy in adults with asymptomatic *P falciparum* malaria in highly endemic regions. Lack of laboratory evidence for systemic inflammation and liver and kidney dysfunction indicates that asymptomatic malaria may not be associated with significant morbidity.

Keywords. asymptomatic malaria; coagulopathy; Ghana; *Plasmodium falciparum*.

Plasmodium falciparum is the deadliest malaria parasite globally and the most prevalent in Africa. According to the World Health Organization, there were an estimated 241 million malaria cases and 627 000 related deaths worldwide in 2020, whereby the African region accounted for 95% of cases and 96% of deaths [1]. Alterations in coagulation markers are frequently observed in acute malaria [2, 3]. Thrombocytopenia predicts an increased risk of death in patients with *P falciparum* infection [4] and is more profound in severe malaria [5]. Similarly, the degree of coagulation activation is associated with disease severity and parasitemia levels in acute symptomatic malaria [2, 6].

The pathophysiology of asymptomatic malaria infection in semi-immune individuals vastly differs from that of acute symptomatic malaria and remains poorly understood. Recent evidence suggests that chronic, asymptomatic malaria induces dysregulated immune responses and tissue damage with unknown, unforeseen complications and should be considered as potentially harmful [7]. Intriguingly, inflammation has been shown to promote a procoagulant milieu, and thus enhances the risk of thromboembolic adverse events [8, 9].

While coagulopathy has been extensively reported in symptomatic malaria cases, its potential role and severity in asymptomatic infected, semi-immune persons are not known.

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To fill this knowledge gap, the aim of our study was to investigate the effect of *P falciparum* infection on coagulation activation in the absence of clinical symptoms.

METHODS

Patients

Asymptomatic adult volunteers were recruited in September 2018 in the 4 villages Afrisere, Ananekrom, Dukusen, and Serebuoso in the Asante Akim North district, Ghana, as reported previously [10]. Exclusion criteria comprised clinical signs of malaria (axillary temperature >37.5°C, headache, chills, myalgia, dizziness, nausea, and/or diarrhea), clinical signs of venous thromboembolism (unilateral leg swelling >3 cm, unilateral pain and/or warmth of the lower extremities), pregnancy, puerperium, risk factors for increased clotting activity (trauma and/or surgery in the last 6 weeks), and anticoagulation. The exclusion criteria were ruled out by using a questionnaire and by measurement of the axillary temperature by the study staff after written informed consent. Since coagulation disorder is consistently detectable only in *P falciparum* and not in *Plasmodium vivax* malaria [6], participants with non-falciparum or mixed malaria were not included.

Blood Sampling and Processing

Blood samples were collected by antecubital venepuncture. Methods for malaria screening and species identification have previously been described [10]. In short, a rapid diagnostic test (RDT) was immediately performed on venous blood (BinaxNOW Malaria Test, Binax, Scarborough, Maine). This RDT targets the histidine-rich protein 2 specific for *P falciparum* and the pan-malarial antigen aldolase present in all *Plasmodium* species that can cause malaria in humans [11].

To measure coagulation function and clinical chemistry, 3.2% sodium citrate– and heparin-anticoagulated whole blood (Sarstedt) was immediately transported to the laboratory in the Presbyterian Hospital, Agogo, and processed within 8 hours after blood collection. Following centrifugation (4000 rpm, 15 minutes) to obtain platelet-poor plasma, the samples were stored and shipped at −80°C to the University Medical Center Hamburg-Eppendorf, Hamburg, Germany. Samples were analyzed in the Institute of Clinical Chemistry and Laboratory Medicine using Siemens Healthcare Atellica analyzers and reagents, respectively (Erlangen, Germany).

Coagulation tests (prothrombin time [PT], activated partial thromboplastin time [aPTT], thrombin time [TT]), fibrinogen, antithrombin III (ATIII), D-dimer, and von Willebrand factor antigen (VWF:Ag) were measured using an COAG 360 analyzer. Creatinine, C-reactive protein (CRP), lactate dehydrogenase (LDH), total bilirubin, alkaline phosphatase (AP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured by the Solution CH930 system.

For blood counts and polymerase chain reaction (PCR), venous blood was collected in ethylenediaminetetraacetic acid (EDTA)–anticoagulated blood tubes (Sarstedt). Blood counts were performed within 4 hours of collection using Sysmex XP-300 automated hematology analyzer (Sysmex, Kobe, Japan) in the laboratory of the Presbyterian Hospital, Agogo. For PCR analyses, EDTA-anticoagulated blood was centrifuged (4000 rpm, 15 minutes) and the remaining pellet was stored and shipped at −80°C to the National Reference Centre for Tropical Pathogens, Bernhard Nocht Institute of Tropical Medicine, Hamburg, Germany.

DNA was extracted from 200 µL of frozen red blood pellet using the QIAamp DNA Blood Mini-Kit (Qiagen, Hilden, Germany). Screening was performed by genus-specific real-time PCR for *Plasmodium* species (RealStar Malaria PCR kit 1.0, Altona Diagnostics, Hamburg, Germany). Species-specific real-time PCR (Altona Diagnostics) and, in case the latter was negative, real-time in-house PCR were performed to detect *P falciparum* as previously described [10].

Study Design

This study is a nested case-control study of a parasitological survey published previously [10], which included 391 participants, of whom 107 were negative for *Plasmodium* infection and 218 had *P falciparum* monoinfection. Of these, 91 were positive by RDT and 127 by PCR only. Participants with PCR only had a higher cycle threshold value corresponding to lower parasite load than those with RDT positivity.

Since the extent of coagulopathy appears to correlate with parasitemia in acute symptomatic malaria [2], participants were divided into 3 subgroups based on diagnosis of *P falciparum* infection: participants with *P falciparum* monoinfection detectable only by PCR, participants with *P falciparum* monoinfection detectable by both PCR and RDT, and participants who tested negative for *P falciparum*. Participants with infections due to *Plasmodium* species other than *P falciparum* or coinfections were excluded. To investigate markers for coagulation activation within the 3 subgroups, 150 samples (50 samples per subgroup) were randomly selected (using the dplyr package in R Studio 3.6.2) and assessed for coagulation studies and clinical chemistry.

Statistical Analysis

Descriptive statistics are reported using either median and interquartile range (IQR) or counts and percentages. The distribution of continuous variables was assessed using histograms and the Kolmogorov-Smirnov test. Nonparametric tests (Wilcoxon rank-sum tests and Kruskal-Wallis test) and χ^2 test with Yates correction were used to test for differences between groups as appropriate. Data analyses were conducted using R software (R Foundation for Statistical Computing, version 3.6.2).

Ethics and Patient Consent Statement

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and consistent with Good Clinical Practice. The study was approved by the Committee on Human Research, Publication, and Ethics at the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana (CHRPE/AP/455/18). Written informed consent was obtained from all patients.

RESULTS

The median age of the 150 participants was 32 years (IQR, 23–46 years) and 78 (52%) were females. Age and gender were similarly distributed between the 3 subgroups ($P = .21$ and $P = .79$, respectively). As expected [10], infected participants with both positive PCR and positive RDT had significantly lower cycle threshold values in genus-specific real-time PCR ($P < .001$) than participants who tested positive by PCR but negative by RDT, corresponding to higher parasitemia levels in RDT-positive participants [12]. Hemoglobin, white blood count, and neutrophils were similar among the 3 subgroups (Supplementary Table 1). Confirming previous findings [10], platelet counts differed significantly between uninfected controls and asymptomatic *P falciparum*–positive participants ($P = .03$), with higher values in uninfected participants compared to those with asymptomatic *P falciparum* infection (Supplementary Figure 1). Importantly, when discriminating between the 2 *P falciparum*–positive subgroups, platelet counts were lowest among only PCR-positive participants (Figure 1).

Enhanced coagulation activation has been described in chronic inflammatory and infectious diseases [13, 14] and contributes to morbidity and mortality in severe cases of *P falciparum* malaria [15]. We therefore aimed to assess if coagulation activation is detectable in *P falciparum*–positive participants in the absence of clinical symptoms. Global coagulation tests comprising aPTT, PT, and TT (Figure 2*A*–*C*) and plasma levels of D-dimer, ATIII, and fibrinogen (Figure 3*A*–*C*) did not significantly differ between participants diagnosed by only PCR versus individuals who were positive in both PCR and RDT, suggesting that the level of parasitemia in asymptomatic *P falciparum* malaria is not associated with coagulation biomarkers. Accordingly, we did not find any difference in coagulation parameters between uninfected versus *P falciparum*–positive participants (Supplementary Figures 2*A*–*C* and 3*A*–*C*). Thus, *P falciparum* infection is not associated with significant coagulation activation in patients in the absence of clinical symptoms.

Von Willebrand factor is a large plasma glycoprotein released by activated endothelial cells upon inflammatory stimuli [16]. It plays a crucial role in primary hemostasis where it mediates platelet adhesion onto exposed collagen at sites of vascular injury. Importantly, elevated levels of circulating VWF have been reported in patients with *P falciparum* malaria [17]. Analogous to our findings on coagulation markers, we did not detect any changes in plasma levels of circulating VWF:Ag within the *P falciparum*–positive subgroups (Figure 3*D*). Similarly, VWF:Ag plasma levels did not differ when comparing uninfected versus *P falciparum*–positive participants (Supplementary Figure 3*D*).

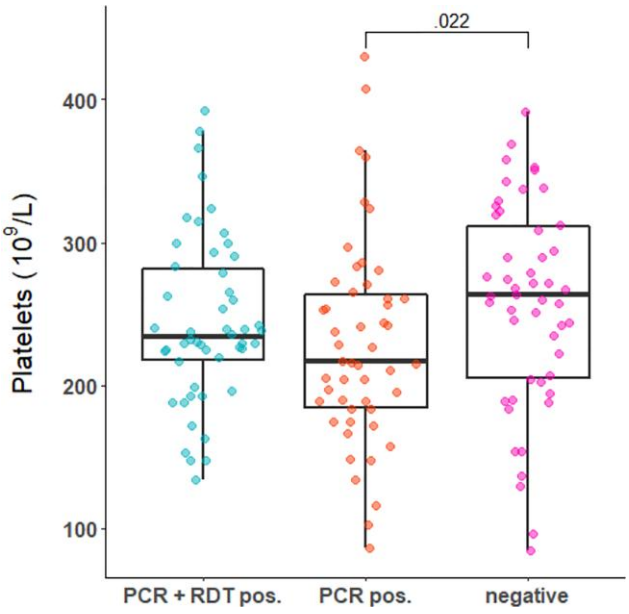

Figure 1. Lower platelet counts in asymptomatic *Plasmodium falciparum* malaria. Platelet counts in peripheral blood samples derived from asymptomatic *P falciparum*–positive individuals diagnosed by polymerase chain reaction (PCR) and rapid diagnostic test (RDT) (blue dots) or only by PCR (orange dots), and from uninfected controls (purple dots). *P* values determined by Wilcoxon rank-sum test only shown when significant.

Markers for systemic inflammation in malaria (CRP and LDH) and liver and renal function were not different between uninfected and *P falciparum*-infected participants (Supplementary Table 1).

In summary, our data show that *P falciparum* infection in the absence of clinical symptoms is not associated with enhanced coagulation activation.

DISCUSSION

We show that enhanced coagulation activation is not present in asymptomatic *P falciparum* infection. We did not find differences in global coagulation tests such as aPTT, PT, and TT or in D-dimer levels when compared to uninfected controls. In correspondence, fibrinogen and VWF:Ag plasma levels were not higher in *P falciparum*–infected individuals. Confirmation of malaria only by PCR or by additional detection in RDT, as surrogate markers of parasite densities, had no impact on coagulation markers. Furthermore, systemic

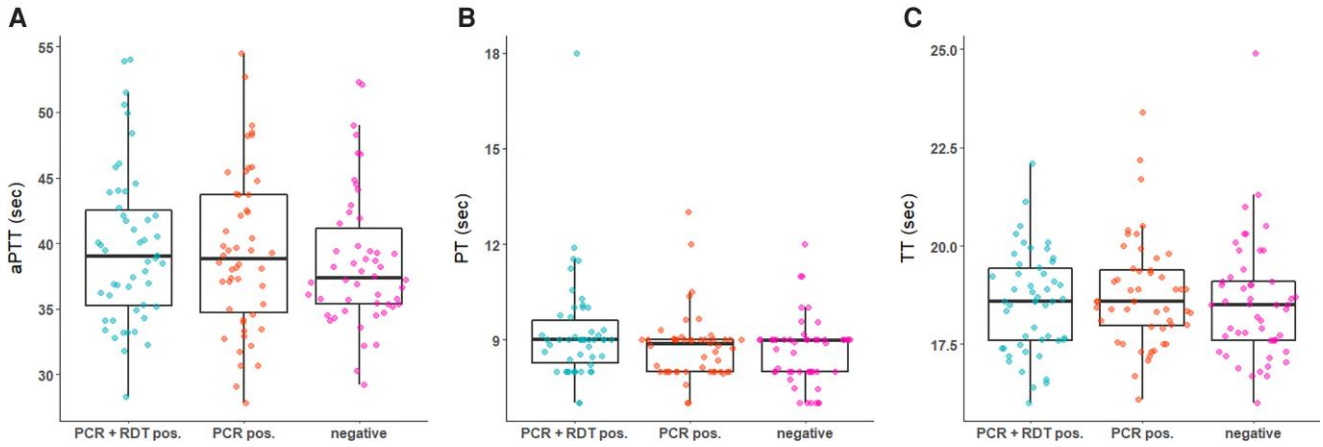

Figure 2. No alterations of global coagulation tests in asymptomatic *Plasmodium falciparum* malaria. Plasma levels of activated partial thromboplastin time (aPTT) (*A*), prothrombin time (PT) (*B*), and thrombin time (TT) (*C*) in samples of asymptomatic *P falciparum*–positive individuals diagnosed by polymerase chain reaction (PCR) and rapid diagnostic test (RDT) (blue dots) or only by PCR (orange dots), and in uninfected controls (purple dots). *P* values determined by Wilcoxon rank-sum test only shown when significant.

inflammation markers and liver and kidney function tests were not increased in asymptomatically infected participants.

Acquired coagulopathy in acute malaria infection is frequent and—when developing into disseminated intravascular coagulation (DIC)—a potentially life-threatening clinical condition [6]. Findings from previous studies suggest that a hypercoagulable state may already be induced very early on during controlled human malaria infection in malaria-naive volunteers, even if the *P falciparum* density is still low [18]. In a study by Riedl et al, first detection of parasites was associated with enhanced in vitro thrombin generation. However, parasitemia was not associated with changes in other coagulation markers including D-dimer and VWF [18]. In line with this, we did not find any laboratory differences in global coagulation tests between both *P falciparum*–infected groups, nor between infected and uninfected individuals. Similarly, global coagulation parameters such as aPTT or fibrinogen levels did not change in the presence of *P falciparum* in another experimental human malaria study by de Mast et al [19].

In our study, lower platelet count was the only significant laboratory finding in asymptomatic *P falciparum* malaria when compared to uninfected individuals. Corresponding to our findings, platelet counts were lower in children with asymptomatic *Plasmodium* infection when compared to uninfected controls [20, 21]. In an experimental study on controlled malaria infection, platelet levels dropped as soon as *P falciparum* parasites were microscopically detectable in thick blood smears [22]. Taken together, these findings underline that decline in platelet counts can be an early laboratory sign of malaria infection in the absence of clinical symptoms.Several pathomechanisms causing thrombocytopenia in malaria have been postulated including immune-mediated lysis [23] or increased platelet clearance [24]. However, in acute symptomatic *P falciparum* malaria, decrease in platelet counts has mostly been attributed to activation of the coagulation cascade [25, 26]. Our laboratory analyses did not include measurement of additional prothrombotic markers that are produced upon thrombin generation, such as thrombin-antithrombin complexes and prothrombin fragments (F1 + 2), or that are indicative of (compensatory) fibrinolysis, such as plasmin-antiplasmin complexes. These markers are elevated early on during coagulation activation, for example during non-overt DIC, in which global hemostatic parameters such as D-dimer or coagulation function tests can still be within the normal range [27]. Also, since platelet numbers were lower than in uninfected controls but still within the normal range in the majority of cases in our study, this finding can be indicative of very early, subtle alterations in hemostasis that were not yet visible in the global coagulation tests used in our study.

Oftentimes, thrombocytopenia in malaria is accompanied by elevated VWF plasma levels. Both endothelial cells and platelets store and release VWF upon inflammatory stimuli [28]. In malaria, increase in VWF plasma levels has predominantly been linked to endothelial cell activation; for example, in a study from Indonesia, children but not adults with asymptomatic *P falciparum*, *P vivax*, or mixed malaria infection had increased VWF:Ag concentrations, indicating mild endothelial activation [29]. In a controlled study setting of healthy volunteers infected with *P falciparum*, very early increase in plasma VWF:Ag occurred at infected erythrocyte levels <0.01% [22]. In contrast, VWF:Ag were not elevated in asymptomatic *P falciparum* monoinfection in our study. We did not analyze the amount of high or ultra-large molecular weight VWF multimers, which promote platelet-dependent coagulation activation, resulting in

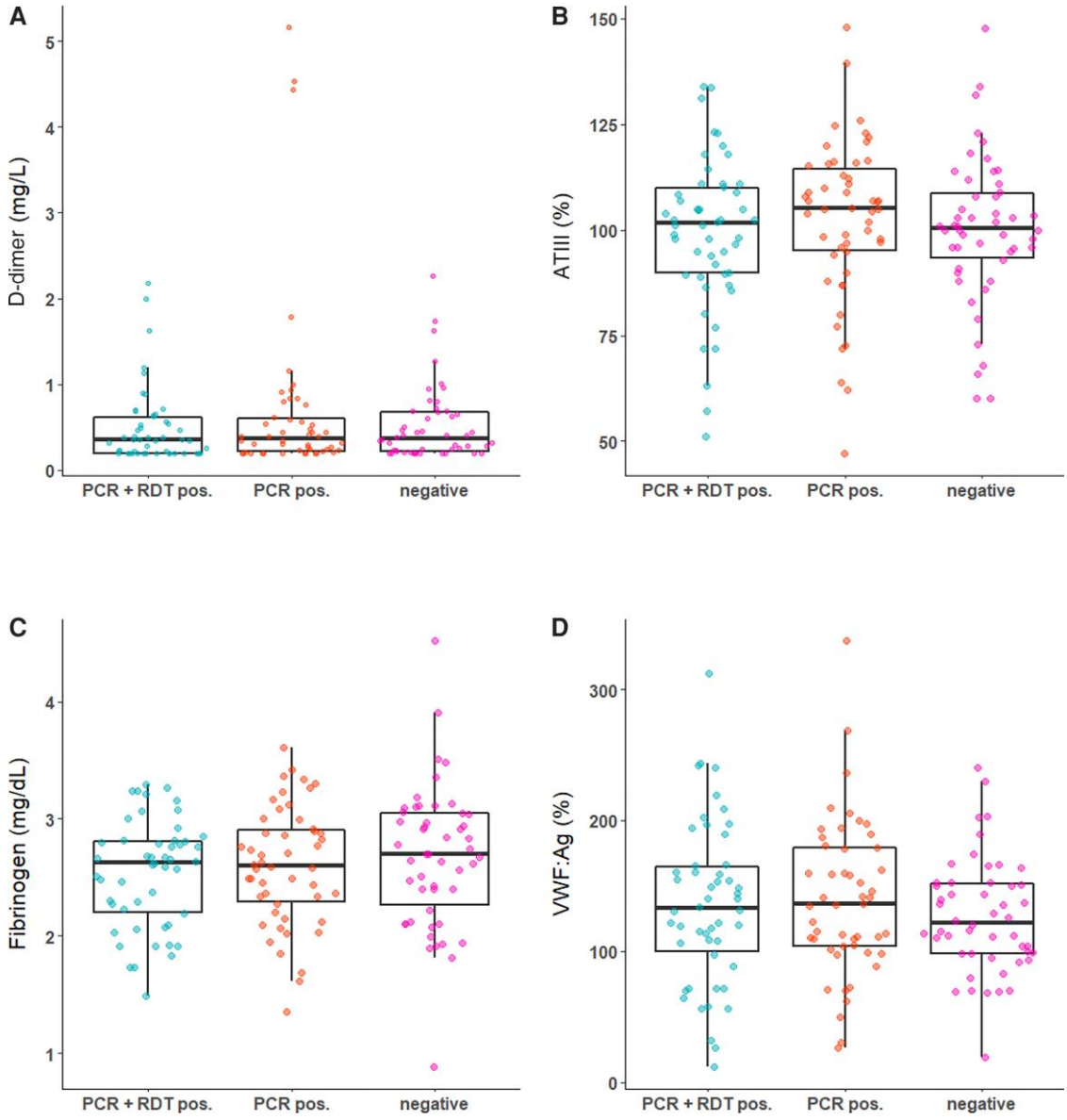

Figure 3. No alterations of coagulation markers in asymptomatic *Plasmodium falciparum* malaria. Plasma levels of D-dimer (*A*), antithrombin III (ATIII) (*B*), fibrinogen (*C*), and von Willebrand factor antigen (VWF:Ag) (*D*) in samples of asymptomatic *P falciparum*–positive individuals diagnosed by polymerase chain reaction (PCR) and rapid diagnostic test (RDT) (blue dots) or only by PCR (orange dots), and in uninfected controls (purple dots). *P* values determined by Wilcoxon rank-sum test only shown when significant.

platelet consumption [30], and which have been detected at elevated levels in severe *P falciparum* malaria [31]. Intriguingly, a recent study from Brazil on patients with *P vivax* suggested that total parasite biomass rather than peripheral parasitemia predicts endothelial cell activation in malaria [32].

In conclusion, asymptomatic *P falciparum* malaria is not necessarily associated with acquired coagulopathy. Absent laboratory evidence for systemic inflammation and liver and kidney dysfunction indicates that asymptomatic malaria may not be associated with significant morbidity in Ghanaian adults. Future prospective studies are needed to investigate potential risk factors for coagulation activation in malaria infection. Identification of markers for malaria-associated coagulopathy will allow early intervention in patients at risk and thus reduce morbidity and mortality long-term.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. C. C. R., T. Ro., and M. H. designed the study. R. O. P., W. T., K. M. A., J. K. A. P., S. O.-M., B. O.-W., C. D. V., L. H., and M. H. collected the samples. C. C. R., R. O. P., F. L., P. F., P. K., S. K., T. Re., E. T., and M. H. conducted laboratory testing. C. C. R., T. Ro., and M. H. analyzed the data. C. C. R. and M. H. drafted the manuscript, and all authors revised the manuscript.

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Data availability. Data are not publically available.

Disclaimer. The funding body had no influence on the design of the study; collection, analysis, and interpretation of data; or writing of the manuscript.

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Potential conflicts of interest. All authors: T. Ro is currently an employee of BioNTech SE.

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