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RESEARCH ARTICLE

REVISED Population pharmacokinetics of artesunate and dihydroartemisinin in pregnant and non-pregnant women with uncomplicated *Plasmodium falciparum* malaria in Burkina Faso: an open label trial [version 2; peer review: 2 approved]

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Abstract

Background: Malaria during pregnancy is a major health risk for both the mother and the foetus. Pregnancy has been shown to influence the pharmacokinetics of a number of different antimalarial drugs. This might lead to an under-exposure in these patients which could increase the risk of treatment failure and the development of drug resistance. The study aim was to evaluate the pharmacokinetics of artesunate and dihydroartemisinin in pregnant and non-pregnant patients using a population modelling approach.

Methods: Twenty-four women in their second and third trimester of pregnancy and twenty-four paired non-pregnant women, all with uncomplicated *P. falciparum* malaria, were enrolled in this study. Treatment was a fixed-dose combination of oral artesunate and mefloquine once daily for three days. Frequent blood samples were collected and concentration-time data for artesunate and dihydroartemisinin were analysed simultaneously using nonlinear

mixed-effects modelling.

Results: Artesunate pharmacokinetics was best described by a transit-compartment absorption model followed by a one-compartment disposition model under the assumption of complete *in vivo* conversion of artesunate into dihydroartemisinin. Dihydroartemisinin pharmacokinetics was best described by a one-compartment disposition model with first-order elimination. Pregnant women had a 21% higher elimination clearance of dihydroartemisinin, compared to non-pregnant women, resulting in proportionally lower drug exposure. In addition, initial parasitaemia and liver enzyme levels (alanine aminotransferase) were found to affect the relative bioavailability of artesunate.

Conclusions: Results presented here show a substantially lower drug exposure to the antimalarial drug dihydroartemisinin during pregnancy after standard oral treatment of artesunate and mefloquine. This might result in an increased risk of treatment failure and drug resistance development, especially in low transmission settings where relative immunity is lower.

Trial registration: ClinicalTrials.gov NCT00701961 (19/06/2008)

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Any reports and responses or comments on the article can be found at the end of the article.

This article is included in the Mahidol Oxford Tropical Medicine Research Unit (MORU) gateway.

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Author roles: Birgersson S: Formal Analysis, Methodology, Software, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Valea I**: Conceptualization, Investigation, Writing – Review & Editing; **Tinto H**: Conceptualization, Investigation, Writing – Review & Editing; **Traore-Coulibaly M**: Conceptualization, Investigation, Writing – Review & Editing; **Toe LC**: Conceptualization, Investigation, Writing – Review & Editing; **Hoglund RM**: Formal Analysis, Methodology, Software, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Van Geertruyden JP**: Conceptualization, Investigation, Writing – Review & Editing; **Ward SA**: Conceptualization, Writing – Review & Editing; **D'Alessandro U**: Conceptualization, Funding Acquisition, Investigation, Resources, Writing – Review & Editing; **Abelö A**: Formal Analysis, Software, Writing – Review & Editing; **Tarning J**: Conceptualization, Formal Analysis, Funding Acquisition, Methodology, Software, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Introduction

Malaria infection during pregnancy has been associated with major adverse health outcomes for both the mother and the foetus. In 2007, there were an estimated 85 million pregnancies in areas of endemic *P. falciparum* malaria[1]. Pregnant women are more likely to get bitten by the vector, and to develop severe malaria[2,3]. Malaria during pregnancy has also adverse consequences for the foetus resulting in an increased risk of intrauterine growth retardation, low birth weight, stillbirth, and infant morbidity and mortality[4]. The World Health Organization (WHO) is today recommending artemisinin based combination therapy (ACT) as first line therapy of uncomplicated *P. falciparum* malaria. This recommendation includes pregnant women in their second and third trimester[5,6]. ACTs include an artemisinin derivative (i.e. artesunate, artemether, dihydroartemisinin) together with a longer acting drug (i.e. mefloquine, piperaquine, lumefantrine, amodiaquine)[7]. The artemisinins are highly effective, resulting in rapid parasite clearance during the first days of treatment[8]. The longer acting partner drugs are responsible for eliminating residual parasites to prevent recrudescent malaria.

Artesunate is rapidly converted by pre-systemic hydrolysis, systemic esterases and cytochrome P450 (CYP) 2A6 into its active metabolite dihydroartemisinin[9,10]. Dihydroartemisinin is metabolized into inactive metabolites by glucuronidation by UDP-glucuronosyltransferase (UGT) 1A9 and 2B7[11].

Pregnancy-related changes in the exposure to artesunate and dihydroartemisinin have been studied previously[12–14]. Morris *et al.* found a pregnancy-related increase in dihydroartemisinin clearance after oral artesunate treatment, resulting in approximately 42% lower drug exposure. Kloprogge *et al.* studied both intravenous and oral doses of artesunate in pregnant and non-pregnant women. Intravenous administration of artesunate showed similar disposition pharmacokinetics in both pregnant and non-pregnant women. However, a 23% decreased bioavailability of artesunate was observed after oral dosing which was explained by an increased pre-systemic activity during pregnancy.

A few other antimalarial drugs (e.g. chloroquine, lumefantrine and dihydroartemisinin) have shown a lower drug exposure in pregnant women compared to non-pregnant women, resulting in an increased risk for treatment failure and resistance development[15–21].

The main aim of the present study was to evaluate the population pharmacokinetics of artesunate and its active metabolite, dihydroartemisinin, in a comparative study in pregnant and non-pregnant patients with uncomplicated *P. falciparum* malaria in Burkina Faso.

Methods

Study design and ethical approval

The study was conducted at the Nanoro District Hospital, Nanoro in Burkina Faso from 7 September 2008 to 15 January 2009. Clinical details and results from a non-compartmental analysis has been published previously[22]. The investigation was a non-randomised parallel open label trial in pregnant and non-pregnant women with uncomplicated *P. falciparum* mono-infection. The study was approved by the National Health Ethics Committee in Burkina Faso (014-2008/CE-CM). The study was registered at www.clinicaltrials.gov (NCT00701961) on June 19th 2008.

Study subjects

Pregnant and non-pregnant women with uncomplicated *P. falciparum* malaria mono-infection (defined as <50 000 parasites/μL and with no danger signs of severe malaria) were identified in two health facilities, the Centre de Santé´ et de Promotion Sociale (CSPS) of Nazoanga and of Nanoro, Burkina Faso. Non-pregnant women were selected to match the recruited pregnant women by age (either less or more than 20 years old) provided that they were residing in the same village as the pregnant women. Inclusion criteria for the study were; gestational age of more than 12 weeks, *P. falciparum* infection with a parasite density of less than 50,000 parasites/μL, willingness to sign or thumb print the written consent form, willingness to stay in the hospital for three days and to return for regular follow-up visits until delivery for treatment and observation, willingness to deliver at the health facility. A woman was excluded from the study if; she had a history of drug sensitivity to the studied drug or recent treatment with antimalarials or drugs known to interact with the studied drug, presence of any danger signs, physical findings of severe illness/severe anaemia, inability to tolerate oral medicine, chronic medical conditions requiring special care which could not be met by the study. Study procedures and objectives were explained in the local language by the study physician before obtaining a signed informed consent.

Drug regimen and blood sampling

Treatment comprised a fixed-dose of artesunate/mefloquine-containing tablets (100 mg of artesunate and 220 mg of mefloquine) provided by Farmaguinhos, Rio de Janeiro, Brazil. A target daily dose of 8 mg/kg/day of mefloquine and 3.6 mg/kg/day of artesunate were given for three days to each patient. Number of tablets received was planned according to standard dosing according to body weight; women weighing <50 kg, 50–60 kg and >60 kg received a daily dose of 1.5, 2 and 2.5 tablets, respectively. Treatment was supervised and given after food.

Blood samples (2 mL) for pharmacokinetic analysis of artesunate and dihydroartemisinin were obtained by venous puncture or via a three-way tap attached to a catheter. Samples were collected before treatment and during the first day of treatment at time points: 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 hours after dose. All samples were placed on ice immediately after blood collection and processed within 15 minutes. Plasma samples were obtained after centrifugation at 1,500g for 10 minutes at 4°C and stored in liquid nitrogen until shipment on dry ice to the Department of Clinical Pharmacology, MORU, Bangkok, Thailand, for measurement of drug concentrations.

Drug analysis

Samples were extracted by solid phase extraction and quantified using a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method[23]. Briefly, artesunate and dihydroartemisinin standards and isotope-labelled internal standards were provided by the WorldWide Antimalarial Resistance Network (WWARN). Separation was performed using an Agilent 1200 system consisting of a binary LC pump, a vacuum degasser, a temperature-controlled micro wellplate autosampler set at 4°C and a column compartment set at 40°C (Agilent technologies, Santa Clara, USA). Quantification was performed using an API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, USA), with a TurboVTM ionization source (TIS) interface operated in the positive ion mode.

Data acquisition was performed using Analyst 1.5 (Applied Biosystems/MDS Sciex, Foster City, CA, USA). The lower limit of quantification (LLOQ) was 1.2 ng/mL and 2.0 ng/mL for artesunate and dihydroartemisinin, respectively. The total assay coefficient of variation was less than 7% for all quality control samples of artesunate (i.e. 2.90, 51.7 and 546 ng/mL, <3.5%) and dihydroartemisinin (i.e. 5.87, 117 and 1880 ng/mL, <6.4%) in this study. The laboratory participates in the WorldWide Antimalarial Resistance Network (WWARN) quality control and assurance proficiency testing program with satisfactory performance[24]. Pharmacokinetic results of mefloquine will be reported elsewhere.

Pharmacokinetic analysis

The population pharmacokinetic properties of artesunate and dihydroartemisinin were analysed using nonlinear mixed-effects modelling of the logarithmic plasma concentrations (NONMEM version 7.1.2; ICON Development Solutions, MD). Pearl-Speaks-NONMEM (PsN; version 3.4.2), Pirana (version 2.4.0) and Xpose (version 4.0) package in R (version 2.13.1; The R Foundation for Statistical Computing) were used for post processing of the results and for automation of the modelling process[25].

Artesunate and dihydroartemisinin were modelled simultaneously and complete conversion of artesunate into its metabolite, dihydroartemisinin, was assumed[9,26]. The first-order conditional estimation (FOCE) method with interactions was used in the model building process. Data below the LLOQ was modelled as missing data (M1), categorical data (M3) or fixed to half of the LLOQ (M5)[27]. When censored data were implemented with the M3-method a Laplacian estimation method was used.

Objective function value (OFV; proportional to minus twice the log likelihood of the observed data) and goodness-of-fit were used for model discrimination and graphical analysis, respectively. A drop in OFV of 3.84 was considered a significant (p=0.05) improvement in model fit when comparing two hierarchical models (one degree of freedom difference). One- two- and three-compartment models with first-order elimination from central compartment were fitted to the plasma concentration-time data. Several different absorption models were evaluated (zero-order, first-order, absorption lag-time, sequential absorption, and a flexible transit compartment model with 1–10 fixed transit compartments). Inter-individual variability was evaluated exponentially on all parameters in the model (Eq. 1).

$$\theta_i = \theta_{TV} \times \exp(\eta_i) \qquad \text{(Eq. 1)}$$

where θ_i is the individual parameter for the ith patient and θ_{TV} is the typical value for parameter θ. η_i is the inter-individual variability for parameter θ, assumed to be normally distributed with mean zero and variance ω^2. Relative bioavailability of artesunate was evaluated by fixing the population value to unity and estimating the inter-individual variability.

The residual random variability was modelled with two separate additive error models (i.e. artesunate and dihydroartemisinin) on the log-transformed drug concentrations, being essentially equivalent to an exponential residual error on an arithmetic scale.

Body weight was evaluated as an allometric function on all clearance and volume parameters. The allometrically scaled parameters were centered on the median body weight of the studied population and scaled to a power of 0.75 and 1 for clearance and volume parameters, respectively.

Stepwise forward inclusion (p<0.05) was used for all other continuous and categorical covariates followed by a stepwise backward exclusion (p<0.01). Parasite biomass, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin levels, and haemoglobin levels at enrolment were tested as continuous covariates while pregnancy was evaluated as both a continuous (estimated gestational age; 0–8 months) and a categorical covariate (pregnant vs non-pregnant).

A categorical pregnancy effect was evaluated using a full covariate approach (i.e. the pregnancy effect was added simultaneously on relative oral bioavailability, all clearance parameters and on mean transit time). The full covariate model was bootstrapped (n=500) and the 80% confidence interval of the covariate effect on each of the pharmacokinetic parameters visualized to investigate the clinical impact and the predicted variability of the covariate effect.

Basic graphical goodness-of-fit was evaluated by plotting observed artesunate and dihydroartemisinin concentrations against individually and population predicted concentrations and by plotting conditionally weighted residuals against population predicted concentrations and time. Eta and epsilon shrinkages were also evaluated to assess reliability of goodness-of-fit characteristics[28]. Prediction-correct visual predictive checks were performed using 2,000 simulations. Bootstrap diagnostics stratified on pregnancy, were performed using 1,000 re-sampled datasets to obtain standard errors for parameter estimates and non-parametric confidence intervals to evaluate parameter precision.

The final model was also used to simulate pregnant (n = 1,000) and non-pregnant (n = 1,000) women in order to investigate the differences in secondary exposure parameters (AUC and C_{MAX}) associated with pregnancy. Simulated pregnant and non-pregnant women were identical with respect to body weight and other co-variates, except pregnancy. All individual exposure parameters were divided on the average value for a non-pregnant women, and presented as a box-plot to illustrate trends and variabilities.

Results

48 women (24 pregnant and 24 non-pregnant patients) completed the pharmacokinetic study (one pregnant woman was lost to follow-up due to moving out of the study area). No adverse effects, related to study treatment, were observed and treatment efficacy was excellent in both groups with total parasite clearance by day three[22]. Consort checklist and flow-chart are available from figshare[29]). Parasites were detected in one pregnant woman at a revisit at day 49, classified as a new infection by genotyping. No recrudescent malaria was observed in any of the patients.

Demographic and clinical data are presented in Table 1. In both groups age, weight and height were comparable, but a higher parasite density and a significantly lower haemoglobin level were found in the pregnant women. The frequent sampling design resulted in full pharmacokinetic concentration-time profiles for artesunate and dihydroartemisinin in both groups. Artesunate was rapidly converted into dihydroartemisinin, and artesunate concentrations reached the LLOQ within six hours in all patients. 62%

of the artesunate samples, and 21% of the dihydroartemisinin samples were measured to be below the LLOQ.

Pharmacokinetic analysis

The pharmacokinetic properties of artesunate and dihydroartemisinin were described simultaneously in a drug-metabolite model, assuming complete *in vivo* conversion of artesunate into dihydroartemisinin (Figure 1). A transit-compartment (n = 3) absorption model for artesunate was superior to all other absorption models tested (ΔOFV > 242). Allowing for inter-individual variability in the relative bioavailability of artesunate improved the model fit substantially (ΔOFV = 27). Artesunate disposition was defined by a central disposition compartment with no additional benefit of adding a peripheral disposition compartment (p > 0.05). Dihydroartemisinin was best described by a one-compartment disposition model with first-order elimination. However, an additional peripheral disposition compartment for dihydroartemisinin improved the model fit significantly (ΔOFV = 21), but resulted in an unrealistic terminal elimination half-life of 9 hours and was therefore not carried forward[30–33].

Table 1. Admission demographics of study population.

Parameter	Pregnant women median (range)	Non-pregnant women median (range)
Number of patients	24	24
Age (year)	20.5 (18–39)	25 (18–48)
Weight (kg)	52 (46–70)	53 (45–70)
Height (cm)	160 (150–170)	160 (160–170)
Parasite density (count/μL)	810 (79–54,000)	240 (18–2,444)
Haemoglobin (g/dL)	9.10 (7.1–11)	12 (7.8–14)
AST (units/L)	20 (3.2–84)	27.9 (8.2–59.8)
ALT (units/L)	20.3 (3.2–63)	21.75 (9.9–61.2)
Bilirubin (mg/dL)	0.45 (0.2–1.9)	0.5 (0.2–2)
Gestational age (month)		
Second trimester, n=12	5 (4–5)	0
Third trimester, n=12	7 (6–8)	0

AST, aspartate aminotransferase; ALT, aspartate aminotransferase
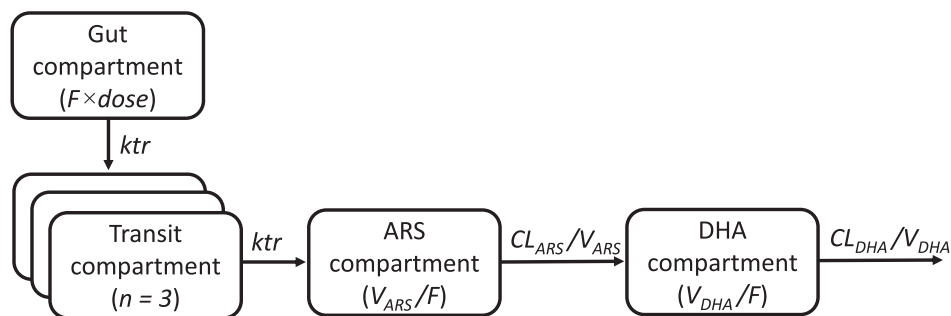
Figure 1. Structural representation of the final population pharmacokinetic model. *ARS* is artesunate; *DHA* is dihydroartemisinin; k_{TR} is the absorption rate constant; *CL/F* is the apparent elimination clearance; *V/F* is the apparent volume of distribution; *F* is the relative bioavailability of artesunate.

A third disposition compartment did not improve the model fit (p > 0.05). Ignoring data below the LLOQ (missing data) or imputing them as half the LLOQ resulted in model misspecifications due to the large fraction of censored data (results not shown). The best performing model was obtained when data below the LLOQ was modelled as categorical data (i.e. M3), thereby maximizing the probability that observations below the LLOQ are predicted to be below the LLOQ (Figure 2). Inter-individual variability was retained on artesunate elimination clearance, dihydroartemisinin elimination clearance, mean–transit-time,
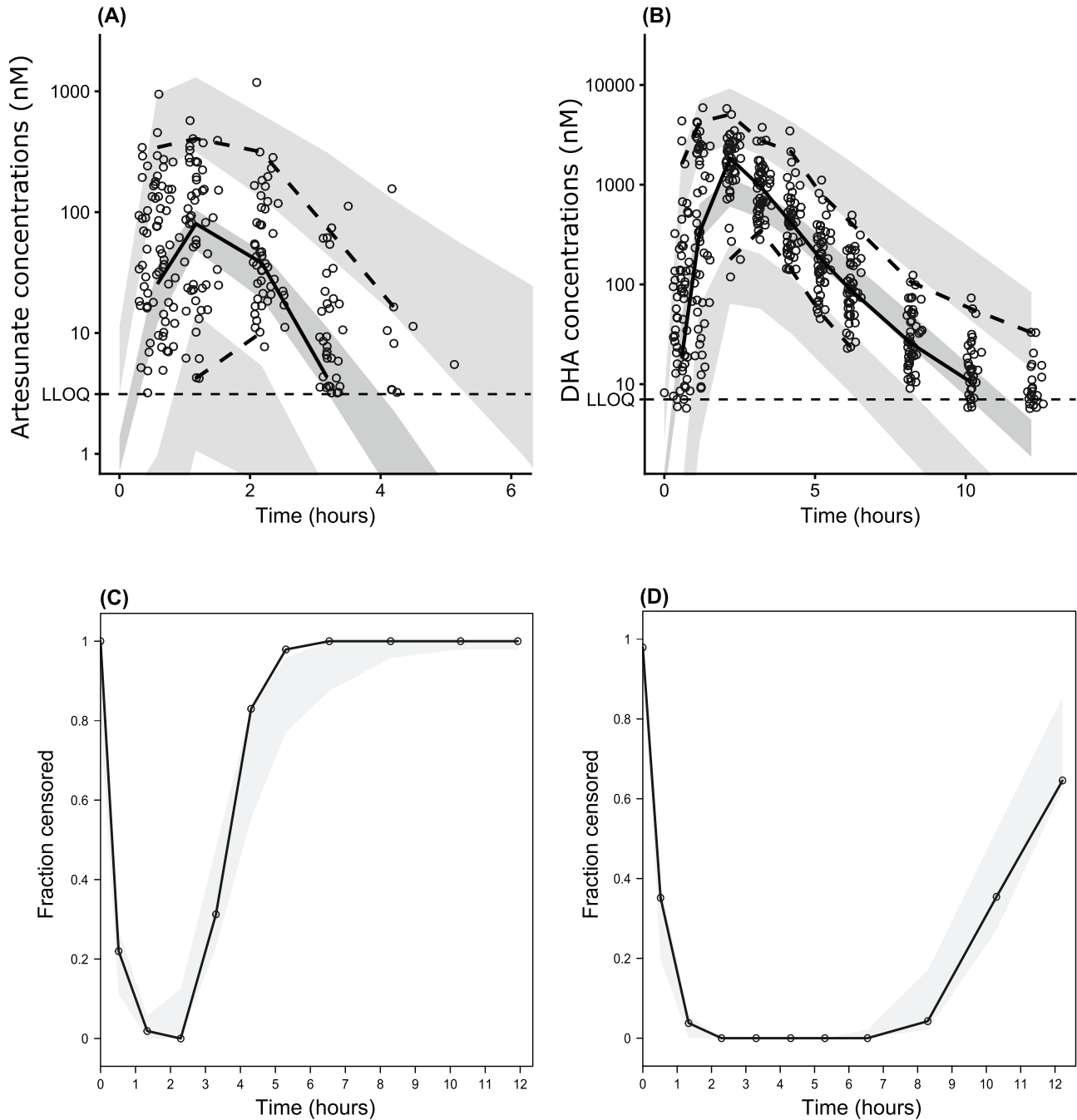
Figure 2. Prediction-corrected visual predictive check of the final population pharmacokinetic model. Predictive performance of the final population pharmacokinetic model of artesunate (**A**) and dihydroartemisinin (**B**) in pregnant and non-pregnant women. Open circles (upper panel) represent the observed concentrations and the solid lines represent the 5th, 50th and 95th percentiles of the observed concentrations. Shaded areas show the 95% confidence interval of the 5th, 50th and 95th percentiles of simulated concentrations. Solid black lines (lower panel) represent the observed fraction of data below the lower limit of quantification and the shaded area represents the 95% confidence intervals of simulated data below the lower limit of quantification for artesunate (**C**) and dihydroartemisinin (**D**).

and relative bioavailability. A separate additive error model for artesunate and dihydroartemisinin, respectively, described the random residual variability adequately.

Body weight implemented as an allometric function on clearance and volume parameters, improved the model fit (ΔOFV = 6.3) and was retained in the final model due to the strong biological prior of this covariate, and to remove the potential bias of systematically different body weights between pregnant and non-pregnant women. Pregnancy as a categorical covariate had a significant (ΔOFV = 20) impact on oral clearance of dihydroartemisinin, resulting in a 21% increased elimination clearance of dihydroartemisinin in pregnant women as compared to non-pregnant women. In addition, parasite biomass (ΔOFV = 10) and ALT (ΔOFV = 14) at enrolment were found to be significant covariates on the relative bioavailability; 14% increase in relative bioavailability per each increase in the natural logarithm of the parasite biomass, and 2.2% increase in relative bioavailability per each unit (IU) increase in ALT).

Final parameter estimates are summarized in Table 2. The full covariate approach investigating the impact of pregnancy confirmed the above covariate relationship, resulting in an increased dihydroartemisinin elimination clearance of 24% in pregnant women, causing a proportional decrease in total drug exposure to dihydroartemisinin (Figure 3).

The final model showed satisfactory goodness-of-fit diagnostics for both artesunate and dihydroartemisinin (Figure 4 and Figure 5) and predictive performance (Figure 2). The pharmacometric model code is available in Table 3. Calculated

epsilon-shrinkage was low (11.1% and 10.4% for artesunate and dihydroartemisinin, respectively) indicating that model diagnostics can be assessed reliably. However, the estimated eta shrinkage was relatively high for clearance parameters (i.e. artesunate clearance = 30%, dihydroartemisinin clearance = 40%, mean transit time = 5.7%, bioavailability = 25%) and empirical Bayes estimates should therefore be interpreted with caution[28]. Simulations of the exposure to artesunate and dihydroartemisinin in pregnant and non-pregnant women can be seen in Figure 6.

Discussion

In this study, a population pharmacokinetic model was developed for artesunate and dihydroartemisinin in pregnant and non-pregnant women in Burkina Faso, treated with a fixed-dose combination of artesunate and mefloquine. The final model was a one-compartment disposition model for both artesunate and its active metabolite, dihydroartemisinin. Artesunate absorption was described with a flexible transit compartment model allowing for inter-individual variability in the relative bioavailability. Pregnancy had a significant impact on the elimination clearance of dihydroartemisinin, resulting in a 21% reduced drug exposure compared to non-pregnant women.

Artesunate and dihydroartemisinin population pharmacokinetics have been described previously with one-compartment disposition models in pregnant and non-pregnant women after oral administration, supporting the present findings[12,31]. Artesunate absorption is known to be rapid and was best described by a transit compartment model (three transit compartments), which is in agreement with a previously published population pharmacokinetic model in pregnant women by *Kloprogge et al.*

Table 2. Final model parameters describing artesunate and dihydroartemisinin population pharmacokinetics in pregnant and non-pregnant women.

Parameters	Population estimate (RSE%)	CI. 95%	IIV CV% (RSE%)	CI. 95%
F (%)	100 *fixed*	-	30.5 (20.0)	16.8-38.7
Nr. of trans comp	3 *fixed*	-	-	-
MTT (h)	0.832 (8.56)	0.695-0.979	61.4 (12.8)	46.2-76.0
CL_{ARS}/F (L/h)	3,570 (9.22)	2,990-4,290	26.4 (27.5)	5.47-35.2
V_{ARS}/F (L)	1,700 (11.2)	1,370-2,110	-	-
CL_{DHA}/F (L/h)	190 (5.87)	168-213	9.00 (23.7)	3.63-11.6
V_{DHA}/F (L)	267 (6.49)	236-301	-	-
$PREG_{CL\_DHA}$ (%)	21.4 (16.3)	14.3-27.9	-	-
ALT_F (%)	2.15 (29.2)	1.10-3.57		
$Biomass_F$ (%)	13.8 (23.6)	7.32-20.0		
σ_{ARS}	0.892 (11.5)	0.707-1.10	-	-
σ_{DHA}	0.660 (9.84)	0.534-0.780	-	-

ARS, artesunate; DHA, dihydroartemisinin; CL/F, apparent elimination clearance; V/F, apparent volume of distribution; MTT, mean transit time of the absorption phase; F, relative oral bioavailability; Nr. trans comp, number of transit compartments in the absorption model; $PREG_{CL\_DHA}$, proportional increase in CL_{DHA}/F with pregnancy; ALT_F, linear increase in F with ALT; $Biomass_F$, linear increase in F with parasite biomass at enrolment; σ, additive residual error as variance.

RSE is the relative standard error calculated as 100x standard deviation/mean. CV% is the coefficient of variation calculated as $100 \times \sqrt{(e^{variance} - 1)}$ for inter-individual variability (IIV). Population parameter and IIV estimates are estimated directly by NONMEM. RSE% and 95% confidence intervals (CI. 95%) are based on 860 successful bootstrap runs (out of 1,000).
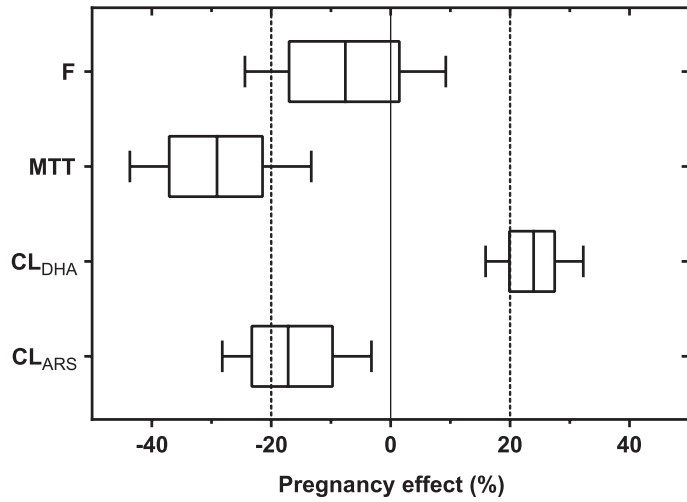
Figure 3. The impact of pregnancy on primary pharmacokinetic parameters. Box and whisker plot of the results from the full covariate model investigating pregnancy as a categorical covariate. Boxes represent the 25th to 75th percentiles and whiskers represent the 10th to 90th percentiles. The solid vertical line represents no covariate effect and the dashed vertical lines represent a covariate effect of ±20%, which is assumed to be associated with clinical significance. F is the relative oral bioavailability, CL_{ARS}/F is the apparent elimination clearance of artesunate, CL_{DHA}/F is the apparent elimination clearance of dihydroartemisinin, and MTT is the mean absorption transit time. The covariate was added as a categorical function and bootstrapped (n=500).
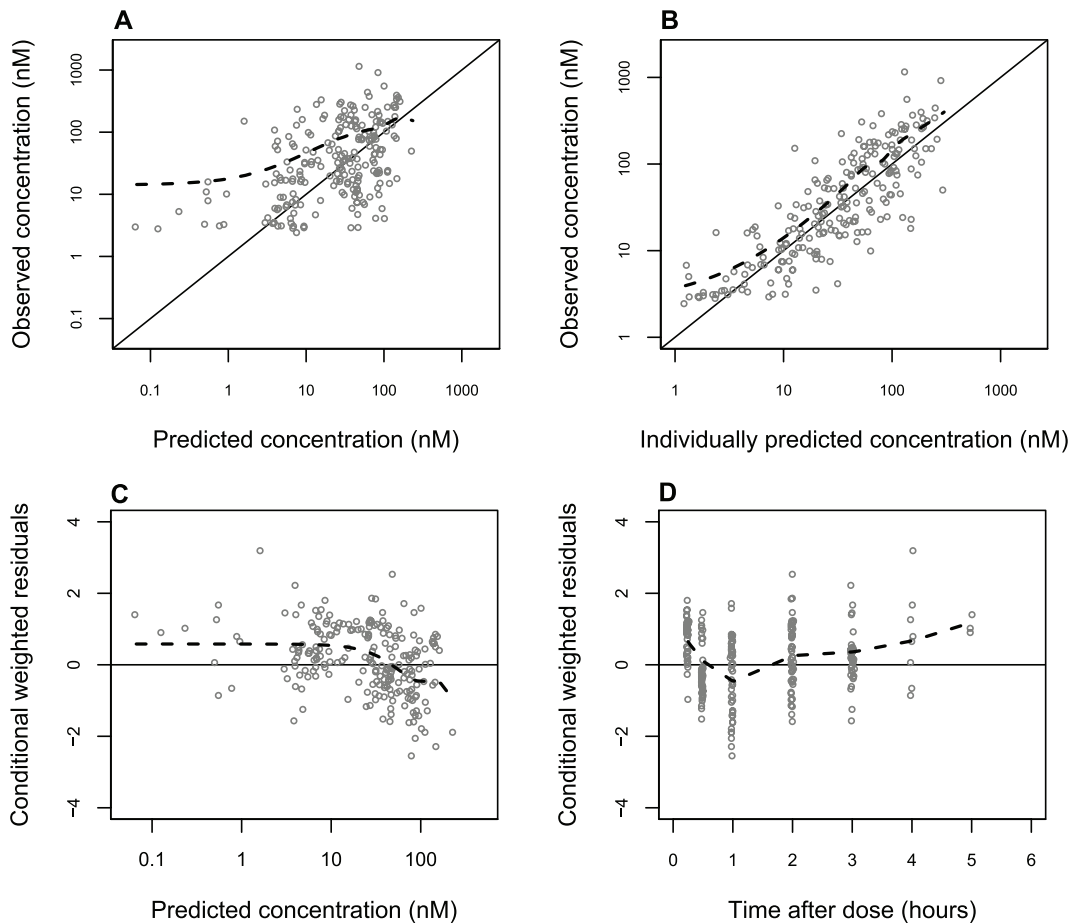
Figure 4. Goodness-of-fit diagnostics of the final population pharmacokinetic model for artesunate. Descriptive performance of the final population pharmacokinetic model in pregnant and non-pregnant women. Lines represent weighted least-squares regression (dashed) and lines of identity (solid).
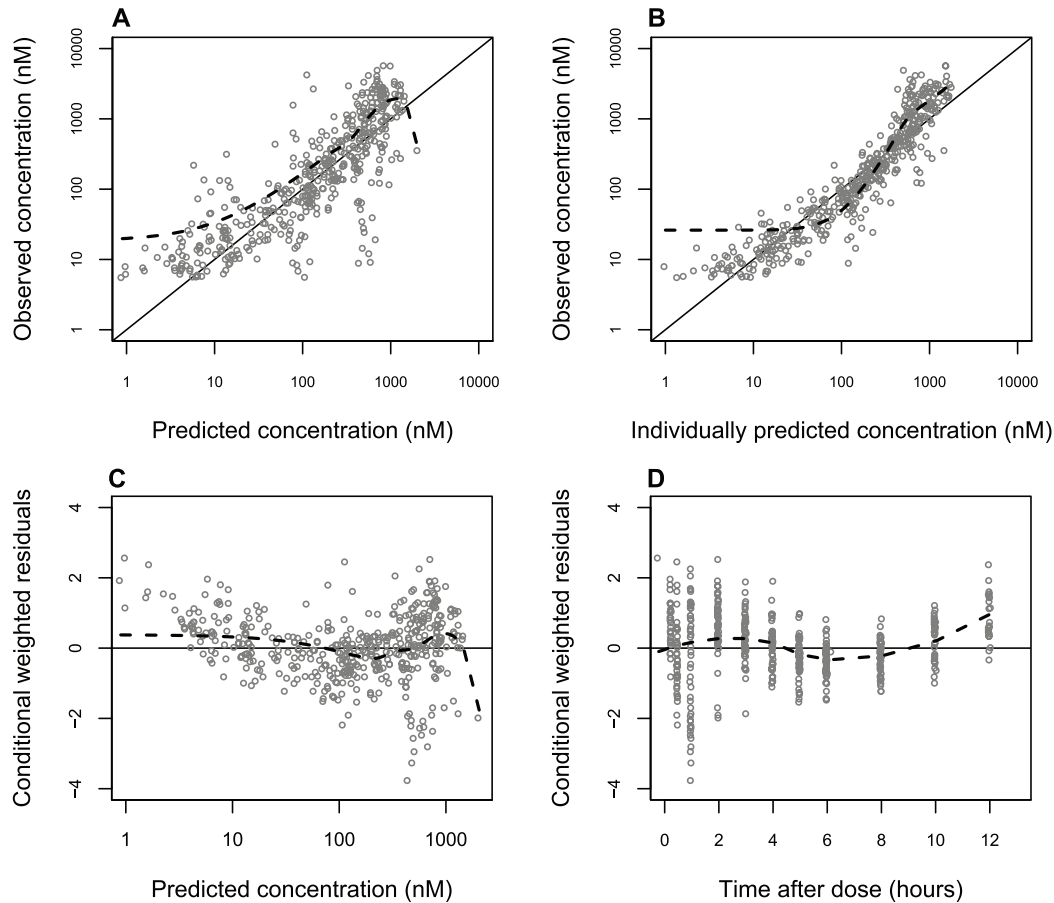
Figure 5. Goodness-of-fit diagnostics of the final population pharmacokinetic model for dihydroartemisinin. Descriptive performance of the final population pharmacokinetic model in pregnant and non-pregnant women. Lines represent weighted least-squares regression (dashed) and lines of identity (solid).

Furthermore, the transit absorption model offers a biologically plausible parameterization of drugs that show varying degrees of lag-time absorption. The pregnancy-related alterations on the pharmacokinetic properties of the artemisinins derivatives are also supported in literature. Morris *et al.* found a pregnancy-induced increase of 42% in the elimination clearance of dihydroartemisinin[12]. In the study by Tarning *et al.*[31], where oral dihydroartemisinin was administered alone, pregnancy was found to influence the bioavailability of dihydroartemisinin resulting in a 38% reduction in the exposure in pregnant compared to non-pregnant women. The present analysis also identified parasite biomass as a covariate on the relative bioavailability, resulting in an increased exposure to artesunate and dihydroartemisinin in patients with higher initial parasitaemia. This covariate effect has been identified also in another study[14]. This previously published clinical study showed opposite pharmacokinetic effects of malaria (87% increase) and pregnancy (23% decrease) on the absolute oral bioavailability of artesunate[13,14]. A sequential intravenous and oral dosing regimen in pregnant and post-partum women during the acute malaria phase and at recovery (i.e. healthy) enabled these covariate effects to be dissected and quantified. The results in the present study support these findings. In addition to the disease

effect, liver enzyme levels (ALT) was found to affect the relative bioavailability, resulting in an increased exposure with increasing ALT levels. This is likely to be explained by a decreased first-pass metabolism of artesunate.

The full covariate approach supported the step-wise covariate results, demonstrating a mean increased dihydroartemisinin clearance of 24% in pregnant women compared to non-pregnant women. There was also a trend towards a decreased mean transit absorption time in pregnant women. This suggests that an additional pregnancy effect in the absorption phase might be significant in a larger patient study. There was also a trend of decreasing artesunate clearance in pregnant women but it could not be substantiated by the step-wise covariate results.

The non-compartmental analysis of this clinical study by Valea *et al.*, demonstrated an increased exposure to artesunate in pregnant women, resulting from an unexplained decrease in artesunate elimination clearance[22]. This decrease was considered highly contradictive since elimination generally increases during pregnancy due to induced enzyme systems[34]. The increased artesunate exposure could not be explained by a model-independent analysis. Also, no significant differences were found in the

Table 3. Final population pharmacokinetic model of artesunate and dihydroartemisinin in pregnant and non-pregnant women with uncomplicated *falciparum* malaria.

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$INPUT
       ID                   ; Patient ID
       TIME                 ; Time of sample
       DV                   ; Dependent variable (natural logarithm of observed concentrations)
       WT                   ; Body weight (covariate)
       EVID                 ; Event ID record
       MDV                  ; Missing dependent variable (1=missing)
       AMT                  ; Dose amount
       CMT                  ; Compartment (1=dose, 2=artesunate, 3=dihydroartemisinin)
       BQL                  ; Below quantification limit (1=below BQL)
       PREG                 ; Pregnancy (covariate; 0=non-pregnant, 1=pregnant)
       LNPC                 ; Parasite count (covariate; natural logarithm of parasite count)
       HB                   ; Haemoglobin measurement (covariate)
       AST                  ; AST (covariate)
       ALT                  ; ALT (covariate)
       BIL                  ; Bilirubin (covariate)
       EGA                  ; Estimated gestational age (covariate)

$DATA
       dataset.csv IGNORE=#

$SUBROUTINE
       ADVAN5 TRANS1

$MODEL
       COMP = (1)      ; Dose
       COMP = (2)      ; Artesunate
       COMP = (3)      ; Dihydroartemisinin
       COMP = (4)      ; Transit compartment 1
       COMP = (5)      ; Transit compartment 2
       COMP = (6)      ; Transit compartment 3

$PK
;------------------------------Pregnancy covariate------------------------------------------------
       PREGNANCY = (1 + THETA(7) * PREG)       ; Linear covariate relationship for pregnancy
;------------------------------------------------------------------------------------------------

;------------------------------Liver enzyme level covariate--------------------------------------
       LIVER = (1 + THETA(8) * (ALT - 20.75))       ; Linear covariate relationship for liver enzyme
;------------------------------------------------------------------------------------------------

;------------------------------Parasite biomass covariate----------------------------------------
       PARASITE = (1 + THETA(9) * (LNPC - 5.88)) ; Linear covariate relationship for parasite biomass
;------------------------------------------------------------------------------------------------

       TVCLP  = THETA(1) * ((WT/52)**0.75)                ; Population artesunate clearance
       CLP    = TVCLP * EXP(ETA(1))                       ; Individual artesunate clearance

       TVV2   = THETA(2) * ((WT/52)**1)                   ; Population artesunate volume
       V2     = TVV2 * EXP(ETA(2))                        ; Individual artesunate volume

       TVCLM  = THETA(3) * ((WT/52)**0.75) * PREGNANCY    ; Population DHA clearance
       CLM    = TVCLM * EXP(ETA(3))                       ; Individual DHA clearance

       TVV3   = THETA(4) * ((WT/52)**1)                   ; Population DHA volume
       V3     = TVV3 * EXP(ETA(4))                        ; Individual DHA volume

       TVMT   = THETA(5)                                  ; Population mean transit time
       MT     = TVMT * EXP(ETA(5))                        ; Individual mean transit time

       TVF1   = THETA(6) * LIVER * PARASITE               ; Population relative bioavailability
       F1     = TVF1 * EXP(ETA(6))                        ; Individual relative bioavailability

       NN     = 3               ; Number of transit compartments
       KTR    = (NN + 1) / MT   ; Transit rate constant
       K14    = KTR             ; Transit rate between compartment 1 and 4
```

```
    K45     = KTR              ; Transit rate between compartment 4 and 5
    K56     = KTR              ; Transit rate between compartment 5 and 6
    K62     = KTR              ; Transit rate between compartment 6 and 2

    K23     = CLP / V2         ; Elimination of artesunate
    K30     = CLM / V3         ; Elimination of dihydroartemisinin
$ERROR
    IF(CMT.EQ.2) THEN
        IPRED = A(2) / V2       ; Predicted plasma concentration of artesunate
        W = SQRT(SIGMA(1,1))    ; Residual error artesunate
    ENDIF

    IF(CMT.EQ.3) THEN
        IPRED = A(3) / V3       ; Predicted plasma concentration of dihydroartemisinin
        W = SQRT(SIGMA(2,2))    ; Residual error dihydroartemisinin
    ENDIF

    IF(IPRED.GT.0)  IPRED = LOG(IPRED)  ; Natural logarithm of predictions


;----------------------------M3-method for incorporating concentratoins below LLOQ---------------------------
    DUM = (LLOQ-IPRED) / W
    CUMD = PHI(DUM)
;------------------------------------------------------------------------------------------------------------

; ----------------------------------------Prediction DV>=LLOQ-------------------------------------------------
    IRES = IPRED - DV
    IWRES = IRES / W
    IF(BQL.EQ.0.AND.CMT.EQ.2) THEN    ; Calculating Y for artesunate
      F_FLAG = 0
      Y = IPRED + ERR(1)
    ENDIF

    IF(BQL.EQ.0.AND.CMT.EQ.3) THEN    ; Calculating Y for dihydroartemisinin
      F_FLAG = 0
      Y = IPRED + ERR(2)
    ENDIF
;------------------------------------------------------------------------------------------------------------

;----------------------------------------- Likelihood DV<LLOQ-----------------------------------------------
    IF(BQL.EQ.1) THEN                 ; Calculating Y for data below LLOQ
      F_FLAG = 1
      Y = CUMD + 0.000001
    ENDIF
;------------------------------------------------------------------------------------------------------------

$THETA                                ; Initial estimates of theta
    (0, 3570)                         ; 1. Artesunate clearance
    (0, 1700)                         ; 2. Artesunate volume of distribution
    (0, 190)                          ; 3. Dihydroartemisinin clearance
    (0, 267)                          ; 4. Dihydroartemisinin volume of distribution
    (0, 0.832)                        ; 5. Mean transit time
    (1 FIX)                           ; 6. Relative bioavailability
    (-1, 0.214)                       ; 7. Pregnancy effect on dihydroartemisinin clearance
    (-0.024, 0.0215, 0.057)           ; 8. Liver enzyme effect on relative bioavailability
    (-0.199, 0.138, 0.334)            ; 9. Parasite biomass effect on relative bioavailability

$OMEGA                                ; Initial estimates for omega
    (0.0672)                          ; 1. IIV artesunate clearance
    (0 FIX)                           ; 2. IIV artesunate volume of distribution
    (0.00810)                         ; 3. IIV dihydroartemisinin clearance
    (0 FIX)                           ; 4. IIV dihydroartemisinin volume of distribution
    (0.320)                           ; 5. IIV mean transit time
    (0.0887)                          ; 6. IIV relative bioavailability

$SIGMA                                ; Initial estimates of sigma
    (0.892)                           ; Residual variability artesunate
    (0.660)                           ; Residual variability dihydroartemisinin
```
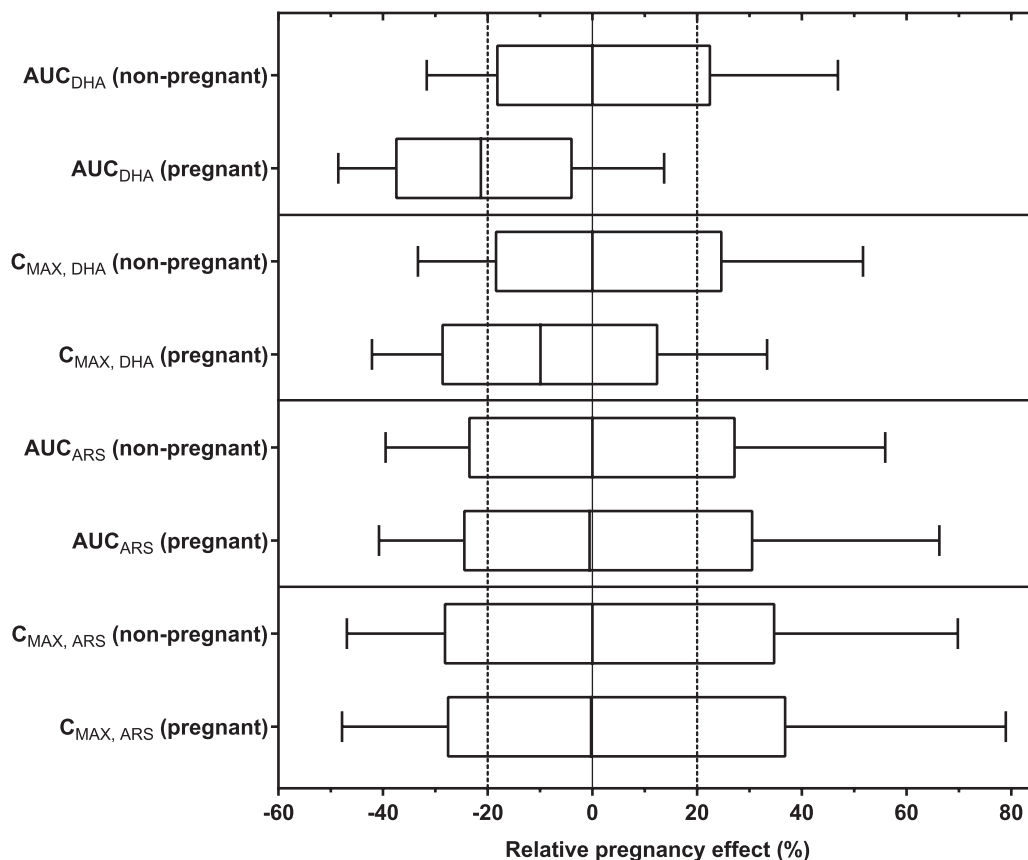
Figure 6. The impact of pregnancy on secondary pharmacokinetic parameters. Relative effect of pregnancy on simulated exposure (AUC and C_{MAX}) to artesunate and dihydroartemisinin. The final model was used to simulate 1,000 non-pregnant and pregnant woman after an oral single dose of artesunate. All individual exposure parameters were divided on the average value for a non-pregnant women, to generate relative pregnancy effects. Boxes represent the 25th to 75th percentiles and whiskers represent the 10th to 90th percentiles. The vertical black line represents no covariate effect and the dashed vertical lines represent a covariate effect of ±20%, which is assumed to be associated with clinical significance.

exposure to dihydroartemisinin in the model-independent analysis, but large variability was noted and thus limited power to detect differences. In the present paper, a model based approach was implemented for a more mechanistic understanding of the impact of pregnancy on the pharmacokinetics of artesunate and dihydroartemisinin. In contradiction to the NCA analysis, no pregnancy related effect was found on clearance for artesunate although a trend towards a decreased clearance was seen in the full covariate model.

It is well known that haemoglobin levels are decreased in pregnant women due to the increased need of iron for the mother and the foetus[35]. Indeed, haemoglobin levels were significantly lower in the pregnant group in this study. However, haemoglobin was not a significant covariate in the present study when pregnant and non-pregnant women were modelled simultaneously and separately.

In conclusion, a population pharmacokinetic model was developed for artesunate and dihydroartemisinin. A pregnancy

related increase in the elimination of dihydroartemisinin was found, resulting in a proportionally decreased exposure to dihydroartemisinin. This could result in an increased risk of failure and possibly a need of increased dosing of artesunate during pregnancy; especially in low-transmission settings where the acquired immunity is relatively lower. However, the clinical relevance of a lower exposure needs to be further evaluated in prospective studies.

Data availability
Underlying data
Due to ethical and security considerations, the data that supports the findings in this study can be accessed only through the Data Access Committee at Mahidol Oxford Tropical Medicine Research Unit (MORU). The application form and data sharing policy can be found here: http://www.tropmedres.ac/data-sharing.

The full NONMEM model code describing the final population pharmacokinetic model is available in Table 3, and

also in the open pharmacometric model repository hosted by DDMoRe.

NONMEM model code, Accession number DDMODEL00000297: http://repository.ddmore.foundation/model/DDMODEL00000297/

Reporting guidelines
CONSORT checklist and flow diagram for "Population pharmacokinetics of artesunate and dihydroartemisinin in pregnant and non-pregnant women with uncomplicated Plasmodium falciparum malaria in Burkina Faso" https://doi.org/10.6084/m9.figshare.7699835.v1[29].

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

Consent
The study was approved by the National Health Ethics Committee in Burkina Faso (014-2008/CE-CM). The study was registered at www.clinicaltrials.gov (NCT00701961). Study procedures and objectives were explained in the local language by the study physician before obtaining a signed informed consent.

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Open Peer Review

Current Peer Review Status: ✅ ✅

Version 2

Reviewer Report 21 January 2020

https://doi.org/10.21956/wellcomeopenres.17157.r37566

✅ **Lawrence Fleckenstein**
College of Pharmacy, University of Iowa, Iowa City, IA, USA

I have reviewed the revisions made by Professor Tarning and he has satisfactorily responded to all of the points I made in my review.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Clinical pharmacokinetics of antimalarial drugs.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 18 April 2019

https://doi.org/10.21956/wellcomeopenres.16178.r35023

✅ **Rada Savic**
Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco (UCSF), San Francisco, CA, USA

We congratulate the authors on using a well-designed modeling approach to improve our understanding of how exposure to dihydroartemisinin, a critical agent for malaria treatment, is

reduced in pregnancy due to increased dihydroartemisinin clearance. This is a particularly relevant contribution, as pregnant women are especially vulnerable to malaria and its consequences. In particular, we appreciate the author's clear description and thorough approach to the modeling methods.

We suggest a few clarifications.

Major Comments:
- Use of allometric scaling results in weight-based dosing recommendations for patients, which can complicate dosing regimens, and at the extreme could lead to lower drug coverage in low weight women and potential toxicity for larger women. We note a relatively small change in objective function value with addition of allometric scaling to the model. Was there a detectable negative correlation between weight and drug concentration in the observed data to suggest lower concentrations in women with greater weights?
- It is interesting that pregnant women had higher parasite biomass, and that pregnancy and parasite biomass had opposing covariate effects on CL/F. Specifically, higher dihydroartemisinin clearance was associated with pregnancy and higher relative bioavailability associated with higher parasite biomass. Can the authors comment on how they distinguished the covariate effects of pregnancy and parasite biomass during modeling (e.g. through evaluation of residual errors, OFV, VPCs, etc)? How did including parasite biomass impact the pregnancy covariate relationship?

Minor Comments:
- Showing the VPC for dihydroartemisinin stratified by pregnancy status may be helpful for the reader.
- Can the authors clarify which model was used for the final parameters listed in Table 2? One option would be to reverse the sentences in the third paragraph on page 7 of the results section as follows: "Final parameter estimates are summarized in Table 2. The full covariate approach investigating the impact of pregnancy confirmed the above covariate relationship, resulting in an increased dihydroartemisinin elimination clearance of 24% in pregnant women, causing a proportional decrease in total drug exposure to dihydroartemisinin (Figure 3)."
- Please include the change in OFV associated with adding the covariate parasite biomass in the text.
- Please reword the first sentence of the results section to improve clarity. As it reads now it could be thought you had 48 pregnant women not 48 women total.
- Please remove the extra ")" at the end of this sentence. "In addition, parasite biomass and ALT at enrolment were found to be significant covariates on the relative bioavailability; 14% increase in relative bioavailability per each increase in the natural logarithm of the parasite biomass, and 2.2% increase in relative bioavailability per each increase in ALT)."

In summary, we thank the authors for contributing to our understanding of artesunate and dihydroartemisinin pharmacokinetics during pregnancy. We appreciate their inclusion of NONMEM code to aid understanding and future efforts investigating this combination. We agree with their conclusions that more work is needed clarify how differences in pharmacokinetics impact treatment outcomes for pregnant women with malaria who are treated with ACTs, particularly when immunity in the population is low.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Using quantitative methods to optimize antimalarial treatment and prevention.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 20 Dec 2019
Joel Tarning, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Major Comments:

1. Use of allometric scaling results in weight-based dosing recommendations for patients, which can complicate dosing regimens, and at the extreme could lead to lower drug coverage in low weight women and potential toxicity for larger women. We note a relatively small change in objective function value with addition of allometric scaling to the model. Was there a detectable negative correlation between weight and drug concentration in the observed data to suggest lower concentrations in women with greater weights?

Reply: We agree with the point raised above by the reviewer, but adults are commonly treated with the same dose of antimalarial drugs while doses are scaled according to weight when treating children (the same dose of artesunate-mefloquine is recommended for all patients $\geq$30 kg). The main reason for using allometric scaling was to assure that potentially important body size differences were included in the model before evaluating the effect of pregnancy on pharmacokinetic parameters. We agree that the drop in OFV value was relatively small following addition of allometric scaling (ΔOFV = 6.3) but it was still an improved model with no additional degrees of freedom. Retaining allometric scaling to bodyweight was also strengthened by literature data of previous population pharmacokinetic modeling of artesunate and dihydroartemisinin in pregnant women (Kloprogge et al, 2015, Br J Clin

Pharmacol). However, there was no statistically significant trend when evaluating post-hoc exposure data vs body weight in the limited data presented here. Even so, no difference was seen in median bodyweights between pregnant and non-pregnant women recruited here, and its therefore very unlikely that the implementation of bodyweight as a covariate would generate biased results.

2. It is interesting that pregnant women had higher parasite biomass, and that pregnancy and parasite biomass had opposing covariate effects on CL/F. Specifically, higher dihydroartemisinin clearance was associated with pregnancy and higher relative bioavailability associated with higher parasite biomass. Can the authors comment on how they distinguished the covariate effects of pregnancy and parasite biomass during modeling (e.g. through evaluation of residual errors, OFV, VPCs, etc)? How did including parasite biomass impact the pregnancy covariate relationship?

Reply: Thank you for this insightful comment. We agree that it can be problematic to distinguish between correlated covariate effects. However, we observed a high degree of variability in parasites biomass within the pregnant and non-pregnant group, enabling us to discriminate between these two covariate effects. Both covariates were significant when evaluated in a stepwise manner. Pregnancy, as a covariate on CL/F, was included first and resulted in 18.9% difference between groups. This covariate relationship did not change substantially when including parasite biomass as a covariate on the relative bioavailability (21.4% difference between groups). To confirm these findings, patients were also divided in a pregnant and non-pregnant group, and parasite biomass evaluated as a covariate in each data set. This resulted in parasite biomass as a significant covariate in both of the separate data sets. This finding is also supported by previous literature, reporting opposite effects of pregnancy and parasite biomass (Kloprogge et al. 2015).

Minor Comments:

1. Showing the VPC for dihydroartemisinin stratified by pregnancy status may be helpful for the reader.

Reply: This is an excellent suggestion, but the relatively small number of patients (and data above the LLOQ) in each group makes a stratified VPC rather uninformative due to large simulated confidence intervals around the visualized percentiles. We therefore opted for pooling all the data when performing the simulation-based diagnostics.

2. Can the authors clarify which model was used for the final parameters listed in Table 2? One option would be to reverse the sentences in the third paragraph on page 7 of the results section as follows: "Final parameter estimates are summarized in Table 2. The full covariate approach investigating the impact of pregnancy confirmed the above covariate relationship, resulting in an increased dihydroartemisinin elimination clearance of 24% in pregnant women, causing a proportional decrease in total drug exposure to dihydroartemisinin (Figure 3)."

Reply: Thank you for this suggestion. Changed accordingly.

3. Please include the change in OFV associated with adding the covariate parasite biomass in the text.

Reply: Thank you for this suggestion. Changed accordingly.

4. Please reword the first sentence of the results section to improve clarity. As it reads now it could be thought you had 48 pregnant women not 48 women total.

Reply: The sentence has been revised for clarity and now reads.
"48 women (24 pregnant and 24 non-pregnant patients) completed the pharmacokinetic study (one pregnant woman was lost to follow-up due to moving out of the study area)."

5. Please remove the extra ")" at the end of this sentence. "In addition, parasite biomass and ALT at enrolment were found to be significant covariates on the relative bioavailability; 14% increase in relative bioavailability per each increase in the natural logarithm of the parasite biomass, and 2.2% increase in relative bioavailability per each increase in ALT)."

Reply: Thank you for this suggestion. Changed accordingly.

Competing Interests: No competing interests were disclosed.

Reviewer Report 16 April 2019

Lawrence Fleckenstein
College of Pharmacy, University of Iowa, Iowa City, IA, USA

This manuscript reports on population pharmacokinetic modeling of artesunate and dihydroartemisinin in pregnant and non-pregnant women with uncomplicated *Plasmodium falciparum* malaria in Burkina Faso. Women in their second and third trimester of pregnancy and non-pregnant women, all with uncomplicated *P. falciparum* malaria, were studied. Treatment was a fixed-dose combination of oral artesunate and mefloquine once daily for three days. Frequent blood samples were collected and concentration-time data for artesunate and dihydroartemisinin were analysed simultaneously using nonlinear mixed-effects modelling.The principal finding of this manuscript reveals a 21% higher clearance of dihydroartemisinin in pregnant women compared to non-pregnant controls, resulting in a proportionately lower drug exposure.

Comments:
1. The authors note in the Study Design section that the clinical details and results from a non-compartmental pharmacokinetic analysis were previously published (Reference

22). Comparing the current manuscript with reference 22 (Both ClinicalTrials.gov NCT00701961) show an apparent difference in the values for the demographics of the study population. Both studies report 24 subjects studied, yet the values for age, weight and height appear to differ (Table 1, Valea vs. Table 1, Birgersson). Please clarify.

2. The values given for baseline characteristics of the study population (haemoglobin, parasite density) do not appear to be the same for the two studies (Table 1, Valea vs. Table 1, Birgersson). Parasite biomass was found by Birgersson to be a significant covariate on relative bioavailability. Please clarify that parasite biomass numbers are correct, and confirm whether it remains a significant covariate.

3. The laboratory parameters for study participants also appear to differ (Table 2, Valea vs. Table 1, Birgersson). This is particularly relevant because the ALT values reported by Valea appear to fall within the normal range, while the maximum values reported by Birgersson appear to be slightly elevated. In the PK analysis by Birgersson, ALT at enrolment was found to be a significant covariate on relative bioavailability. Can the authors confirm this covariate relationship is correct? The last line of paragraph 2 on page 7 of the Birgersson manuscript is unclear, stating: "…2.2% increase in relative bioavailability per each increase in ALT." Please clarify.

4. To confirm a disease effect with regard to liver status (ALT) as referred on page 9 in the first paragraph; it would seem to require data from individuals with liver disease. It is not clear that evidence of liver disease is present in the study population. The values reported for ALT appear only slightly elevated for some individuals (perhaps isolated and transient values). AST and bilirubin values appear normal and there is no clinical description of liver disease. Please clarify the discussion with respect to the relationship between ALT and liver disease.

5. It would be useful for the authors to comment further in the discussion section on how the transit-compartment absorption model reconciles with the known mechanism of artesunate absorption.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Clinical pharmacokinetics of antimalarial drugs.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 20 Dec 2019

Joel Tarning, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

1. The authors note in the Study Design section that the clinical details and results from a non-compartmental pharmacokinetic analysis were previously published (Reference 22). Comparing the current manuscript with reference 22 (Both ClinicalTrials.gov NCT00701961) show an apparent difference in the values for the demographics of the study population. Both studies report 24 subjects studied, yet the values for age, weight and height appear to differ (Table 1, Valea vs. Table 1, Birgersson). Please clarify.

Reply: Thank you for this very observant comment. The presentation of demographic data is based on the same original data in both papers (Valea et al and Birgersson et al). However, data are presented as median and absolute range in the current manuscript, while data were presented as mean and standard deviation in the paper by Valea et al.

2. The values given for baseline characteristics of the study population (haemoglobin, parasite density) do not appear to be the same for the two studies (Table 1, Valea vs. Table 1, Birgersson). Parasite biomass was found by Birgersson to be a significant covariate on relative bioavailability. Please clarify that parasite biomass numbers are correct, and confirm whether it remains a significant covariate.

Reply: The representation of demographic data is based on the same original data in both papers, but presented differently (see comment 1 above). We can confirm that the parasite biomass numbers are correct, and that this was a significant covariate in the model.

3. The laboratory parameters for study participants also appear to differ (Table 2, Valea vs. Table 1, Birgersson). This is particularly relevant because the ALT values reported by Valea appear to fall within the normal range, while the maximum values reported by Birgersson appear to be slightly elevated. In the PK analysis by Birgersson, ALT at enrolment was found to be a significant covariate on relative bioavailability. Can the authors confirm this covariate relationship is correct? The last line of paragraph 2 on page 7 of the Birgersson manuscript is unclear, stating: "…2.2% increase in relative bioavailability per each increase in ALT." Please clarify.

Reply: Similar to the above comments, also the laboratory data is based on the same original data in both papers, but presented differently (i.e. median and range vs mean and standard deviation). We can confirm that the demographic and laboratory data is presented according to the original database in the current manuscript. ALT was a significant covariate on the relative bioavailability. We agree that the sentence mentioned above is unclear and we have re-written it to clarify the results.

"…2.2% increase in relative bioavailability per each unit (IU) increase in ALT."

4. To confirm a disease effect with regard to liver status (ALT) as referred on page 9 in the first paragraph; it would seem to require data from individuals with liver disease. It is not clear that evidence of liver disease is present in the study population. The values reported for ALT appear only slightly elevated for some individuals (perhaps isolated and transient values). AST and bilirubin values appear normal and there is no clinical description of liver disease. Please clarify the discussion with respect to the relationship between ALT and liver disease.

Reply: This is a very important comment raised by the reviewer. Both artesunate and dihydroartemisinin are metabolized via liver enzymes; cytochrome P450-family and UGT-enzymes, respectively. It was therefore of interest to evaluate ALT and AST as covariates in the population pharmacokinetic analysis to describe the potential impact of these on different parameters related to metabolism. It was not an objective to evaluate liver disease in this population, but the intention was to characterize the pharmacokinetic data and explain the observed variability. Thus, we do not claim to have characterized the impact of liver disease, but we could still identify a relationship between observed values of ALT in different patients and their estimated relative bioavailability. We agree that this covariate description can be misunderstood on account of using "liver status", and we have reworded the section to clearly state that there was a relationship found between observed values of ALT and the relative bioavailability.

5. It would be useful for the authors to comment further in the discussion section on how the transit-compartment absorption model reconciles with the known mechanism of artesunate absorption.

Reply: Thank you for this comment. Artesunate is known to have a rapid absorption with short lag-time in the absorption, resulting in detectable concentrations within 15 min of drug administration in healthy volunteers and patients (summarized in Morris et al, 2011). In this paper several different absorption models were evaluated; zero-order, first-order, absorption lag-time, sequential absorption, and a flexible transit compartment model with 1–10 transit compartments. The transit compartment model was superior to all other models tested with an estimated mean transit absorption time of 0.8h. This is in agreement with a previously published population pharmacokinetic model of artesunate in pregnant women (Kloprogge et al 2015). Furthermore, the transit absorption model offers a biologically plausible parameterization of drugs that show varying degrees of lag-time absorption. We have expanded the discussion slightly to clarify this.

Competing Interests: No competing interests were disclosed.