HIV-positive Nigerian adults harbour significantly higher serum lumefantrine levels than HIV-negative individuals seven days after treatment for *Plasmodium falciparum* infection

Running title: lumefantrine-nevirapine interaction in HIV patients

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ABSTRACT

Management of co-infection with malaria and HIV is a major challenge to public health in developing countries and yet potential drug-drug interactions between antimalarial and antiviral regimens have not been adequately investigated in people with both infections. Each of the constituent components of artemether-lumefantrine, the first-line regimen for malaria treatment in Nigeria, and nevirapine, a major component of highly active antiretroviral therapy, are drugs metabolised by the cytochrome P450 3A4 isoenzyme system, which is also known to be induced by nevirapine. We examined potential interactions between lumefantrine and nevirapine in 68 HIV-positive adults, all of whom were diagnosed with asymptomatic *Plasmodium falciparum* infections by microscopy. *Post hoc* PCR analysis confirmed the presence of *P. falciparum* in only a minority of participants. Day 7 capillary blood levels of lumefantrine were significantly higher in HIV positive participants than in 99 HIV negative controls (P=0.0011). Associations between day 7 levels of lumefantrine and risk of persistent parasitaemia could not be evaluated due to inadequate power. Further investigations of the impact of nevirapine on *in vivo* malaria treatment outcomes in HIV-infected patients are thus needed.

Introduction

Malaria and HIV are two of the most important health problems facing developing countries and are among the most common infections in sub-Saharan Africa. HIV co-infection is thought to contribute to 3 million additional malaria cases, higher malaria parasite densities in immunosupressed children and a 5% greater mortality rate (1, 2). HIV also increases the risk of *Plasmodium falciparum* infection progressing to clinical malaria in adults, especially in those with advanced immunosuppression, by eroding the efficacy of acquired immunity (3). The choice of antimalarial drug for the treatment of HIV patients therefore is of utmost importance considering the dangers of comorbidity, but sufficient pharmacokinetic and parasitological evidence to make this choice is currently lacking.

Combination therapies in current use for malaria in Africa comprise a derivative of the artemisinin family of drugs combined with at least one non-artemisinin partner drug. The most widely used such combination is artemether plus lumefantrine (co-artemether, AL). Artemether is metabolized in the liver by the isoenzyme CYP3A4, to its active metabolite dihydroartemisinin (DHA) with peak plasma concentration being reached around 2-3 hours after oral administration (4); elimination half life is estimated at approximately 1 hour. There is thus only limited opportunity for DHA to participate in drug-drug interactions. Lumefantrine is partially metabolised to desbutyl-lumefantrine, predominantly through CYP3A4, reaching peak plasma levels approximately 10 hours after oral administration and is then cleared slowly, showing a terminal half life of 4-6 days in *P. falciparum* malaria cases (5-9). Oral bioavailabilty of lumefantrine is variable and highly dependent on administration with fatty foods (5, 9, 10).

The anti-retroviral drug nevirapine (NVP) is a non-nucleoside reverse-transcriptase inhibitor that is well absorbed after oral administration with >90% bioavailabilty, generally achieved about 4 hours after oral dosing and has a long half-life (11). NVP is extensively metabolised by the same CYP3A4 isoform as artemether and lumefantrine, and is also known to upregulate the isoenzyme (12, 13). Thus NVP autoinduces its own metabolism, and potentially that of any other drugs metabolised through this route. This raises the possibility of significant drug-drug interactions of NVP with lumefantrine and other anti-malarials (1). Kredo and colleagues (6) initiated a pharmacokinetic study in 18 South African volunteers that were HIV-infected and receiving NVP therapy, compared to 18 naïve controls, each of whom took a full adult course of AL; none of the study subjects were

infected with *Plasmodium sp.* This study found differences between NVP recipients and controls in several pharmacokinetic parameters for lumefantrine, the most important of which was a significantly higher day 7 lumefantrine concentration in the NVP group. These authors concluded that further studies of drug-drug interactions between NVP and lumefantrine were urgently needed in malaria-infected subjects.

Artemether-lumefantrine (AL), which is currently the recommended therapy for malaria treatment, was introduced in Nigeria in 2005 as the first line regimen for uncomplicated malaria. Rivers State, in the Niger Delta area of southern Nigeria, has a high prevalence of HIV infection (7.4% of the population) and is hyperendemic for malaria transmission. The study was designed to address the lack of data regarding the pharmacokinetics of AL among HIV-positive subjects in this setting, where asymptomatic parasite carriage is common. We hypothesised that following treatment with AL for concomitant *P. falciparum* infections, day 7 blood concentrations of lumefantrine in HIV-positive individuals on NVP therapy would differ from those in HIV-negative individuals. Any such difference may also have a measurable impact on parasite clearance in treated asymptomatic individuals, as day 7 lumefantrine concentration is known to be an important determinant of antimalarial efficacy in individuals with symptomatic malaria (4).

Materials and methods

Study area

The study was carried out at the University of Port Harcourt Teaching Hospital and the Braithwaite Memorial Specialist Hospital Port Harcourt, Nigeria, from September 2010 to May 2011. Port Harcourt is the capital of Rivers State in the Niger Delta, rich in the nation's oil resources. The region is dotted with oil and gas activities which attract an international workforce, and commercial sex workers follow the camp (14). These socio-economic conditions contribute to a high estimated population prevalence of HIV infection of 7.4% (15). Malaria is holoendemic in Nigeria with transmission all the year round, but malaria cases are most common during the rainy season from April to September, with peak of the rains and intense transmission between May and July. Annual rainfall averages more than 3,550 millimeters in the region.

Patients and samples

This paper describes an exploratory pharmacokinetic study with a simple unmatched case-control design, ancillary to a study designed to track molecular markers of drug resistance in HIV-infected individuals, using active detection of *P. falciparum* infection followed by treatment with AL as the regimen recommended by the University of Port Harcourt Teaching Hospital guidelines for uncomplicated malaria in adults. The work was conducted from September 2010 to May 2011. The main endpoints of the current analysis were day 7 peripheral blood lumefantrine levels, and parasite carriage at day 3 and day 28 post-treatment. The primary endpoint for which the study was designed and powered was carriage of parasite genetic markers of antimalarial resistance. This analysis is ongoing and will be reported elsewhere.

Participants were recruited if they met the following eligibility criteria: age 16-65 years, willingness to have HIV status confirmed from clinical records or by a point-of-care test, *P. falciparum* positive by microscopic examination of a blood film and provision of a signed informed consent form. HIV-positive patients were recruited from the HIV adult clinic of both hospitals. HIV-negative participants were recruited from the hospital communities including staff and students. HIV-negative patients were screened and confirmed virus-negative with the use of the HIV Determine point-of-care test (Alere Medical Co. Ltd Matshuhidai-shi, Chiba, Japan). Each was then screened for malaria by standard microscopy. Permission for the study was obtained from the Research

Ethics Committees of the University of Port Harcourt Teaching Hospital, the Braithwaite Memorial Specialist Hospital and the London School of Hygiene and Tropical Medicine, London.

Enrolled patients were treated with AL ('Coartem', Novartis Pharma, Nigeria) according to manufacturer's dosing regimen: 4 tablets twice daily for 3 days for persons with weight>35kg. Patients were advised to eat before taking the tablets. Most of the patients took their first dose at the site having been pre-informed to eat before coming. Patients were followed up till day 28. On day 7, capillary blood samples were taken from a finger prick.

For drug measurements, 100µl of blood were measured using a pipette and dropped on a filter paper (Glass microfibers paper, Fisherbrand FB59431) pre-treated with 0.75M tartaric acid (Fisher Scientific). The papers were allowed to air-dry and then stored in individual pouches with a silica desiccant to absorb moisture. The preserved papers were transferred to the London School of Hygiene and Tropical Medicine. Filter paper adsorbed blood samples were analysed for lumefantrine using liquid chromatography-mass spectrometry (LCMS; Thermo Finnigan LCQ instrument) following a modified protocol based on previously published methods (16). Briefly bloodspots were extracted in methanol / water (4:1; 350 µl), and the extracts were filtered through a cotton wool plug. Each sample (20 µl) was separated on a Dionex Acclaim® 120 3µm C18 (4.6 x 150 mm, with 120 Å pore size, fitted with a guard column) eluting with mobile phase MeOH: 20 mM formate buffer, pH 2.7 (85:15) isocratically at a flow rate of 500µl/min. The column temperature was maintained at 35°C. The ESI source was operated in positive mode with the capillary temperature set to 350°C and sheath and auxiliary gas (nitrogen) flow rates of 60 and 20 arbitrary units respectively. Peak identity was confirmed by using blood spiked with lumefantrine standards (0-30 µg/ml), adsorbed onto filter paper and extracted in the same manner as the patient samples. Quantitation was performed using selective ion monitoring for the transitions m/z 530 to 512. LLOD was determined to be 0.1 µg/ml, LLOQ 1.0 µg/ml and ULOQ 20.0 µg/ml.

Plasmodium falciparum DNA was prepared from dried spots (10–20 µl) on Whatman paper as previously described (17) and codons 24 to 201 of the *pfmdr1* locus amplified by nested PCR (18). Relative quantitfication of parasite DNA was performed by an established qPCR method as previously described (19).

158 Data were entered into spreadsheets and analysed in STATA 11 (Stata Corp, Madison WI).
159 Continuous data were compared between groups using Wilcoxon's rank sum test, while
160 categorical comparisons in 2 x 2 format were performed using the χ^2 distribution.

Results

Out of 80 attendees at the two HIV clinics who agreed to have a malaria film read, 68 were identified as positive for *P. falciparum* and returned for day 7 follow-up (85%). None of these individuals reported concurrent symptoms suggestive of clinical malaria. 126 individuals agreed to have a rapid HIV test performed, of which 99 were found to be negative for HIV-specific antibodies, were identified as infected with *P. falciparum* and attended for day 7 follow-up (79%); none of these individuals were symptomatic. These 167 participants were treated with a full adult course of AL, and followed up on day 3, day 7 and day 28 for repeat blood sampling.

To confirm microscopic diagnosis of *P. falciparum* parasitaemia at enrolment, nested PCR amplification of the amino-terminal fragment of the *pfmdr1* gene was carried out on DNA extracted from the first blood sample taken from each participant. Nested PCR was also performed on DNA extracted from all day 3 and day 28 filter paper blood samples. Unexpectedly, a high proportion of enrolees (78.1%) were found to be aparasitaemic by nested PCR, suggesting poor specificity of the original microscopic diagnosis (Table 1). There was a strong association between PCR positivity at day 0 and day 3 (O.R. 5.56, 95% C.I. 1.76 - 17.32; P = 0.0004), suggesting good reproducibility of parasite detection for the PCR method, in contrast to results obtained with microscopy.

Using the PCR data as a more reliable test for parasite carriage, we found weak evidence that HIV positive people were more likely to be parasitaemic at day 0 (OR 2.05, 95% C.I. 0.917 - 4.60; P = 0.054), which may reflect slightly higher parasite densities in this group, and thus a greater likelihood of parasites being correctly identified by the screening microscopists. HIV-positive subjects were not significantly more likely to be PCR positive for *P. falciparum* at day 3 and/or day 28 after AL treatment than were HIV negative individuals (OR 1.75, 95% C.I. 0.776 - 3.95; P = 0.141).

Both HIV status and lumefantrine concentration at day 7 were recorded for all 167 individuals. We examined the distribution of lumefantrine concentration at day 7 in all study participants, and found highly significant departure from normality ($z=7.581$, $P<0.0001$), which remained after (natural) logarithmic transformation ($z=5.372$, $P<0.0001$). In an exploratory analysis following the methods of Kredo *et al.*, (6), we removed as “outliers” 5 samples with extremely low measured lumefantrine concentrations (0, 0, 0.01, 0.08, 0.08μM respectively, all in the HIV neg group) and

log-transformed. Departure from the normal distribution was then marginally non-significant (z=1.594, P = 0.054). After consideration of these findings, we decided to take the conservative approach of using only non-parametric tests for testing statistical significance of comparisons, and retained all data in the analysis.

HIV status, and thus nevirapine use, was found to have a significant effect on the concentration of lumefantrine 7 days after treatment (Wilcoxon ranksum test z = -3.270, P=0.0011), with a median concentration in the HIV negative group of 2.75µM (IQR 1.03 – 4.31), and in the HIV positive group of 3.55µM (IQR 2.07 – 5.37) (Figure 1). However, the 5 individuals with extremely low lumefantrine readings (identified in the previous paragraph) were all in the HIV-negative group, so to test for possible bias caused by this group, we performed the comparison with these 5 measurements removed. In this exploratory analysis, a significant association remained between HIV status and lumefantrine concentration at 7 days post AL treatment (z = -2.830, P=0.0046).

As many of our subjects were shown to be parasite negative by PCR, we tested for any effect of parasitaemia on lumefantrine concentrations at day 7. Overall, in all 166 evaluable individuals, PCR positive parasitaemia at day 0 was not associated with any difference in day 7 lumefantrine concentration in our sample set (37 positive vs 129 negative individuals). There was a weak association between day 3 PCR-detected parasitaemia and higher lumefantrine concentration (z = -2.305, P = 0.021), suggesting that greater lumefantrine bioavailability among NVP recipients was not improving AL treatment outcomes. This effect was not strong enough to confer a statistically significant deficit in parasite clearance for HIV-positive individuals as a group; considering only those participants with follow-up data from both day 3 and day 28 (N = 140) 33.9% of HIV-positive individuals had PCR-detectable parasitaemia on either or both days 3 and 28, compared to 22.7% of HIV negative individuals (O.R. 1.75; 95% C.I. 0 .776 - 3.95; P = 0.141). Assessment of day 0 parasitaemia using qPCR was performed for 8 individuals (including 5 HIV+) who were subsequently PCR-positive on day 3, and 15 who had cleared parasites by day 3 (including 9 HIV+). This exploratory analysis did not provide any evidence that higher starting parasitaemia increased the likelihood of an individual remaining PCR-posiive for *P. falciparum* on day 3 (Wilcoxon ranksum test: z = -0.904, P = 0.37).

Discussion

The co-formulated combination of artemether, a sesquiterpene lactone derived from the natural compound artemisinin, with the aryl amino-alcohol lumefantrine, as a systemic racemic flourene mixture, has become the most widely distributed and available ACT throughout Africa. As anti-retroviral chemotherapies have also become more widely available for treatment of HIV patients in health systems in Africa, detailed understanding of any interactions between these two chemotherapies is urgently needed. In this study we show that HIV positive adults taking regular NVP who were treated with AL for microscopically apparent *P. falciparum* infection, had significantly higher day 7 plasma concentrations of lumefantrine compared to treated adults who were HIV test-negative and not receiving NVP. However, we found no evidence that sub-microscopic parasite persistence at day 3 after AL treatment was prevented in individuals with higher day 7 plasma levels of lumefantrine, in fact HIV-positive individuals were slightly more likely to have PCR-detectable parasitaemia on day 3 or day 28 than were HIV-negative participants, although this was not significant.

Our findings are consistent with those of Kredo *et al.* (6) and confirm that drug-drug interactions between AL and NVP are potentially important. However, NVP-stimulation of the CYP3A4 isoenzyme would be expected, *a priori*, to lower peripheral lumefantrine levels, due to an increase in the amount of lumefantrine metabolised to desbutyl-lumefantrine, a potent derivative that is normally found at a concentration between 0.5% and 5% of that of the parent compound at day 7 in the few studies available (8, 20). Food intake also alters lumefantrine metabolism; we were not able to supervise the food intake of our participants while they were taking AL, but all were informed of the need to accompany their medication with fatty food. The apparently increased bioavailability of lumefantrine in NVP recipients produced no measurable parasitological benefit in our patients; on the contrary, one third of HIV-positive (and thus NVP-receiving) participants were found to have persisting PCR-detectable *P. falciparum* parasitaemia at day 3 and/or day 28, compared to less than a quarter of the control group. This difference, which suggests perturbation of the immune system in HIV infection remains a significant factor in these dual-treated patients, was not statistically significant. The case-control design used here may be prone to selection bias, and this could affect parasitological outcomes. However univariate analysis of post-treatment parasitaemia versus age, weight, gender and educational attainment found no evidence of

confounding by any of these parameters (data not shown). The recent observation that co-administration of NVP with AL leads to reduced maximal concentration of both artemether and DHA (21) suggests an alternate explanation for reduced parasite clearance at day 3 in patients receiving both regimens. Nevertheless, further studies of the parasitological impact of antiretroviral-antimalarial drug-drug interactions in adequately powered studies are urgently needed, not least because of the important role of the host immune system in clearing drug-treated malaria parasites (3, 22). In our study, all HIV-infected participants were identified through attendance at a weekly clinic in which all received NVP (except for a single patient on efavirenz; when this patient was excluded from the analysis the relationship between NVP use and lumefantrine concentration at day 7 remained significant). Compliance with antiretroviral treatment was not evaluated directly. Future studies with HIV patients not receiving NVP may permit discrimination between drug-drug interactions, and the impact of retroviral disease *per se* on lumefantrine bioavailability.

A major weakness of our study was the poor quality of enrolment microscopic diagnosis, such that the majority of participants had in fact failed a major inclusion criterion. This had two main impacts. Firstly, the study was greatly under-powered to evaluate any parasitological outcomes, as so many participants were actually uninfected (with *P. falciparum*). Secondly, we were not able to analyse parasite densities with any confidence, and thus were left with the binary variable of PCR positivity as the remaining reliable measure of malaria infection. Further, by this method we cannot rule out the possibility that some of our positive PCR reactions on post-treatment blood samples were detecting gametocytes of *P. falciparum* only. These sexual stage parasite forms are infective to *Anopheles sp.* mosquitoes, but do not contribute to clinical malaria symptoms and cannot divide. Gametocytes are well known to survive in a minority of AL-treated patients after clearance of the actively dividing asexual parasite stages (23, 24). Nevertheless, we have recently described persistence of asexual parasites in asymptomatic Ghanaian school children treated with ACT, suggesting that sub-clinical parasitaemia may be more difficult to clear than previously thought (25).

In conclusion, this is the second study to find evidence that NVP-recipient HIV patients harbour a significantly higher peripheral blood concentration of lumefantrine than do HIV-negative controls, 7 days after receiving a full treatment course of AL. Our findings corroborate the findings of Kredo *et al.* (6) in a larger group of AL-treated individuals, some of whom were infected with *P.*

falciparum. Insufficient parasitological data were available to determine whether this difference in lumefantrine concentration provides any parasitological benefit to NVP-treated HIV patients with malaria infections.

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TABLES

TABLE 1. **Parasite carriage by microscopy and PCR in 68 HIV positive and 99 HIV negative individuals.**

All 167 individuals were reported as positive for *P. falciparum* parasites on microscopic examination of thick blood films.

Numbers PCR positive for *P. falciparum*	Day 0	Day 3	Day 28
HIV pos N = 68*	17 (29.9%)	8	12
HIV neg N = 99	20 (17.2%)	12	12

*67 of these individuals were receiving daily nevirapine anti-retroviral therapy; one received efavirenz; all HIV positive patients also received the nucleoside reverse-transcriptase inhibitors lamivudine and zidovudine.

386
387 Figure Legends
388
389 **Figure 1. Day 7 lumefantrine concentration in AL-treated participants.**
390 Mid-line of each box-plot is the median, with the edges of the box representing the
391 inter-quartile interval. Whiskers delineate the 5^{th} and 95^{th} percentile. Lumefantrine
392 concentration was below the normal limits of detection in five individuals, all in the
393 HIV negative group (see text).
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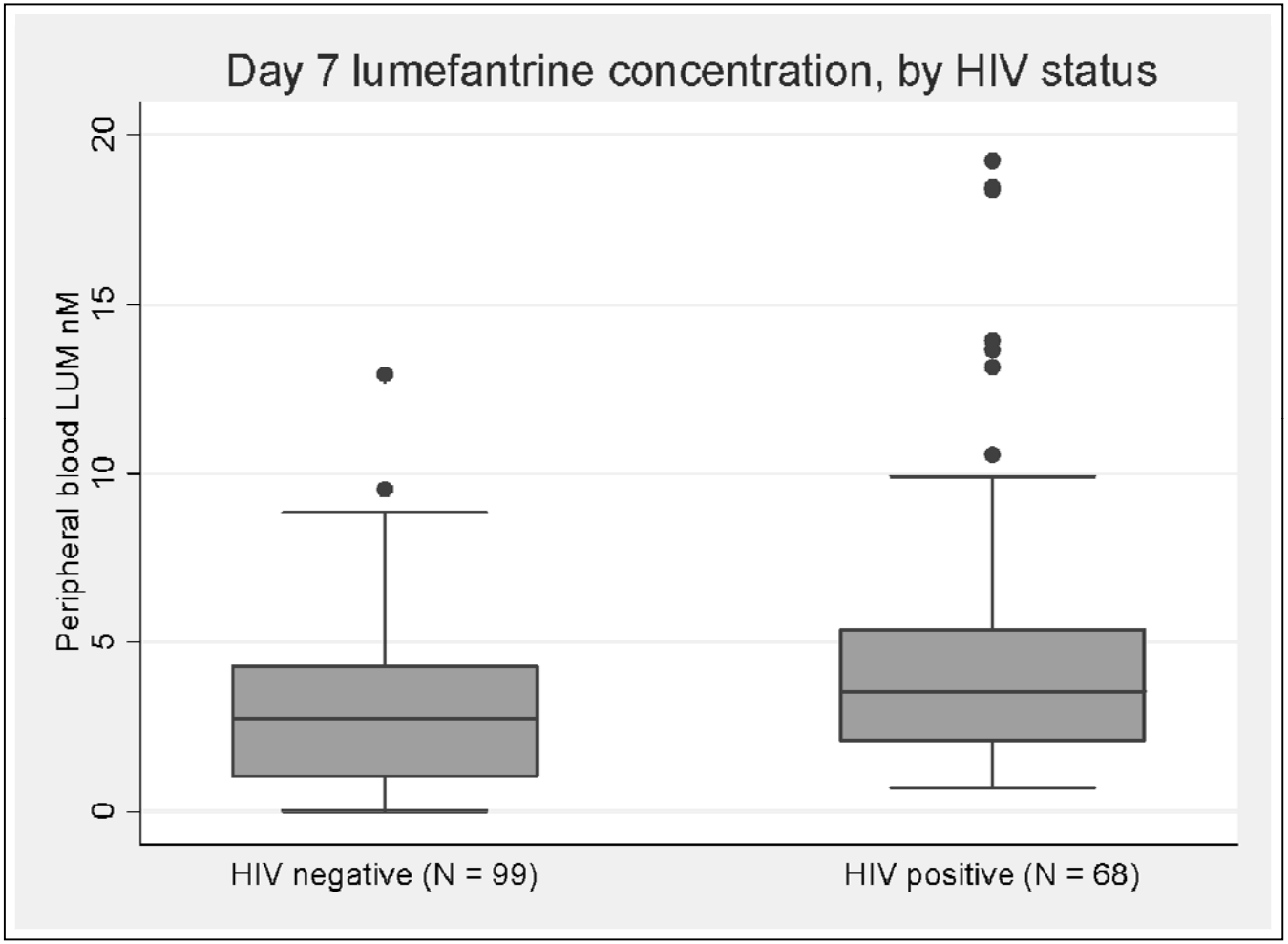
Day 7 lumefantrine concentration, by HIV status
Peripheral blood LUM nM
0
5
10
15
20
HIV negative (N = 99)
HIV positive (N = 68)