Use of artemether-lumefantrine in the treatment of asymptomatic-malaria infection in HIV-positive and HIV-negative Nigerian adults.

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Declaration

I, Ifeyinwa Nwogo Chijioke-Nwauche, declare that the work presented in this thesis is my own original work, and confirm that to the best of my knowledge I have acknowledged every information, quotation or results derived from other sources.

Signed

Ifeyinwa Nwogo Chijioke-Nwauche **Date**

Dedication

To

Jokes

Mon Cheri

For believing in me

And my lovely girls

Amaka my lady

Zomii my virtous woman

Ozi my flower girl

and

Ruby my sweet baby

Acknowledgements

I must first of all acknowledge the help and grace of my LORD Jesus, for granting me this golden opportunity to come to LSHTM and for the strength and fortitude to come up to this stage of my programme.

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May the Lord bless and reward you all greatly.

Abstract

Malaria /HIV co-infection 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 HIV and *Plasmodium falciparum* infections. Earlier studies on the use of artemether-lumefantrine (AL) in Nigeria have neither addressed its use in HIV-positive subjects nor in asymptomatic-malaria infection.

The present study investigated associations between drug resistant *P. falciparum* and the use of medication for HIV management, drug-drug interactions between artemether-lumefantrine and antiretroviral drugs (ARV) and the molecular markers of artemether-lumefantrine and other antimalarial drugs.

Results of the study revealed an elevated day 7 lumefantrine concentrations in HIV subjects on nevirapine treatment compared to their HIV-negative counterparts. Associations between elevated day 7 levels of lumefantrine and the persistent parasitaemia could not be evaluated due to inadequate power. Genetic analysis by DNA sequence of *P. falciparum* isolates revealed strong selection for the *pfmdr1*codon86N allele among all treated individuals. This polymorphism is a strong indicator of AL treatment failure or slow clearance *in vivo*. There was a 72.6% prevalence of the *pfcrt*76T mutations in the population and this was observed to be higher in the HIV-positive subjects. Three new mutations F73S, S97L and G165R were detected on the *pfmdr1* gene and the first case S436F mutation on the *pfdhps* gene to be reported in Nigeria. The *dhps*K540E and *dhfr*I164L mutations, associated with high-level resistance to sulfadoxine-pyrimethamine (SP) were not observed in our small sample size.

The study also revealed that HIV-positive subjects were more likely to harbour parasites, at a higher density, before and after treatment. Improvement of the immune status of HIV-infected patients was suggested by the increase of CD4 cell count level in about 68% of the HIV-positive patients.

This is a preliminary study and first of its kind to investigate drug-drug interactions between ARVs and the antimalarial drug AL in HIV-positive patients co-infected with *P. falciparum* in relation to parasite clearance. The findings of the study are very important but more work is urgently needed with a larger sample size to confirm these findings.

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List of abbreviations

3TC	Lamivudine
°C	degrees celsius
µg	microgram
µl	microliter
A	Alanine
ABC	Abacavir
ACTs	Artemisinin combination therapies
AIDS	Acquired immune deficiency syndrome
AL	Artemether-lumefantrine
AQ	Amodiaquine
ARVs	Antiretroviral drugs
AS	Artesunate
AZT	Azidothymidine
BMH	Braithwaite MemorialHospital
C	Cysteine
CCR5	Chemokine receptor antagonist
CD4	Cluster of differentiation
CDC	Centre Disease Control
CI	Confidence Interval
CQ	Chloroquine
CQR	Chloroquine resistant
CQS	Chloroquine sensitive
C_T	Cycle threshold
d4T	Stavudine
DHA	Dihydroartemisinin
DNA	Deoxyribonucleic
dNTP	deoxynucleotide
D	Aspartic
DV	Digestive vacuole
E	Glutamic acid

EDTA	Ethylene diamine tetra acetic acid
EFV	Efavirenz
F	Phenylalanine
FMOH	Federal Ministry of Health
FRN	Federal Republic of Nigeria
FSW	Female sex workers
G	Glutamine
GARPR	Global AIDS Response Progress Report
HAART	Highly active antiretroviral therapy
Hb	Haemaglobin
HIV	Human immunodeficiency virus
HPA	Health Protection Agency
HPLC	High Performance Liquid chromatography
HR	Hazard ratio
I	Isoleucine
IDV	Indinavir
INFγ	Interferon gamma
IPT	Intermittent prevention of malaria
IPTi	Intermittent prevention of malaria in infants
IPTc	Intermittent prevention of malaria in children
IPTp	Intermittent prevention of malaria in pregnancy
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Nets
IVM	Integrated vector Management
K	Lysine
Kb	Kilo base
Kcl	Potassium Chloride
L	Leucine
LAMP	Loop mediated amplification kit
LCMS	Liquid chromatography mass spectrometer
LLINs	Long-Lasting Insectidal Nets
LLOD	Lower limit of detection

LLOQ	Lower limit of quantification
M	Molar
M	Methione
Mdr	Multidrug resistance
MFQ	Mefloquine
$MgCl_2$	Magnesium chloride
Min	Minute
Ml	millilitre
MSM	Men having sex with men
Msp	Merozoite surface protein
N	Asparagine
NACA	National Agency for the Control of AIDS
NMCP	National Malaria Control Programme
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NPC	National Population Control
NRTIs	Nucleoside reverse transcriptase inhibitors
NtRTIs	Nucleotide reverse transcriptase inhibitors
NVP	Nevirapine
OR	Odds ratio
PB	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDA	Photo Diode Array
pfatpase6	*P. falciparum* atpase6 gene
pfcrt	*Plasmodium falciparum* chloroquine resistance transporter gene
Pfcrt	*Plasmodium falciparum* chloroquine resistance transporter protein
Pfdhps	*Plasmodium falciparum* dihyropterate synthetase
pfdhfr	*Plasmodium falciparum* dihydrofolate reductase
pfmdr	*Plasmodium falciparum* multidrug resistance gene
pgh-1	*Plasmodium falciparum* glycoprotein homologue 1
PIs	Protease Inhibitors
PMI	President's Malaria initiative
PQ	Piperaquine

PYR	Pyronaridine
Q	Glutamine
qPCR	Real time Polymerase Chain Reaction
R	Arginine
RNA	Riboxy nucleic acid
rpm	Revolutions per minute
RTV	Ritonavir
S	Serine
SIV	Simian immunodeficiency virus
SMC	Seasonal Malaria Chemoprevention
SNP	Single nucleotide polymorphism
SP	Sulphadoxine-pyrimethamine
SQV	Saquinavir
T	Threonine
TNF	Tumor necrosis factor
TS	Trimethoprim-sulphamethoxazole
T-20	Enfuvirtide
ULOQ	Upper limit of quantification
UNAIDS	United Nations Programme on HIV/AIDS
UNGASS	United Nations General Assembly Special Session
UNICEF	United Nations International Children Emergency Fund
UPTH	University of Port Harcourt Teaching Hospital
V	Valine
WBC	White blood cells
WHO	World Health Organisation
Y	Tyrosine
ZDV	Zidovudine

Chapter One

General Introduction

1.0 Introduction

Malaria and HIV are two of the most common and important health problems facing developing countries and the most common infections in sub-Saharan Africa (UNAIDS, 2005). They constitute the major public health threats responsible for morbidity and mortality in these areas.

Half of the world population is at risk of malaria and an estimated 243 million cases occurred in 2008. Of these, there was an estimated 863,000 malaria deaths, 767,000 (89%) of which occurred in Africa where malaria is the leading cause of mortality in children under 5 years. (WHO 2004a; WHO 2009a). However the World Malaria Report, 2011 indicates that over a period of two years, the malaria cases have reduced from 243 million cases in 2008 to 216 million in 2010 and the number of deaths have reduced to 655, 000 (WHO, 2011). Despite this reduction, the global burden of malaria has remained very high especially in the tropics. A recent report (UNAIDS, 2011) shows that by 2010, about 34million people were living with HIV/AIDS of which 2.7million are new infections and the greatest burden is in sub-Saharan Africa where an estimated population of 22.9million (68%) of HIV-infected people live. The report further states that the total number of new HIV infections in this area dropped by more than 26% since 1997 when HIV infection epidemic was at the peak.

Malaria and HIV/AIDS are both diseases of poverty, they cause poverty and are commonly found among the poor. Together, the two diseases cause over 4 million deaths a year. Both diseases are highly endemic, and there is a wide geographic overlap in sub-Saharan Africa (WHO, 2005). HIV co-infection is thought to contribute to 3 million additional malaria cases, higher malaria parasite densities in immunosuppressed children and a 5% greater mortality. (UNAIDS, 2005; (Korenromp et al., 2005).

1.1 Malaria

Malaria is a protozoan infection in humans caused by the parasite Plasmodium which until recently was thought to be classified into four species *P. falciparum, P. ovale, P. malariae* and *P. vivax*. However current study (Sutherland et al., 2010) has shown that there are two non-recombining species of *ovale* (*ovale curtisi* and *ovale wallikeri*) which are non-sympatric in nature (Oguike et al., 2011). Of all these, *Plasmodium falciparum* is the agent of the most malignant form of malaria, usually presenting with severity mostly in children in sub-Saharan Africa (Urdaneta et al., 2001). It is the most dangerous form of malaria with the highest rates of complications. It is also the commonest species in virtually all parts of Africa accounting for up to 98% of the confirmed cases in Nigeria and is associated with significant morbidity and mortality. *Plasmodium falciparum* is responsible for virtually all the features of severe malaria. *P. malariae* usually occurs as a mixed infection with *P. falciparum.* (NPC & NMCP, 2012). The main vector of malaria in Nigeria is *Anopheles. gambiae* but *A. funestus* and *A. arabiensis* are also commonly encountered. *A. melas* is found in the coastal areas. (NPC & NMCP, 2012).

1.2 Malaria biology

1.2.1 Life cycle of the malaria parasite-*P. falciparum*

The life cycle of *Plasmodium falciparum* begins with the bite of an infected female mosquito. The mosquito while taking a blood meal, releases sporozoites into the blood stream which invade the liver cells within 30 minutes of the release. Inside the hepatic cells of the liver, the parasites rapidly differentiate and undergo asexual multiplication resulting in the release of merozoites which invade other liver cells. These merozoites from the hepatocytes burst into the host's blood stream where they invade the erythrocytes. Further multiplication takes place inside the erythrocytes enlarging into ring trophozoites which divide asexually producing schizonts. The schizonts divide further causing a release of merozoites when the erythrocytes are ruptured. The released merozoites in the blood stream are responsible for the clinical manifestation in malaria illness such as fever, joint and muscle pains and chills. This stage called the erythrocytic stage is the asexual life cycle of the parasite and usually lasts for 48hours.

Some of the schizonts divide into sexual forms resulting in male and female gametocytes. The gametocytes are taken up by the female anopheles mosquito during another blood meal from

a host. The male gametocytes undergo a rapid nuclear division inside the midgut of the mosquito resulting to microgametes which fertilise the female microgametes producing ookinete. The ookinete later crosses the gut wall as oocyst which subsequently ruptures releasing merozoites into the body cavity of the mosquito from where they eventually migrate to the salivary gland of the mosquito. With another blood meal the mosquito transmits the merozoites into another victim and continues the life cycle.

(Adapted from http://www.cdc.gov/malaria/about/biology/).

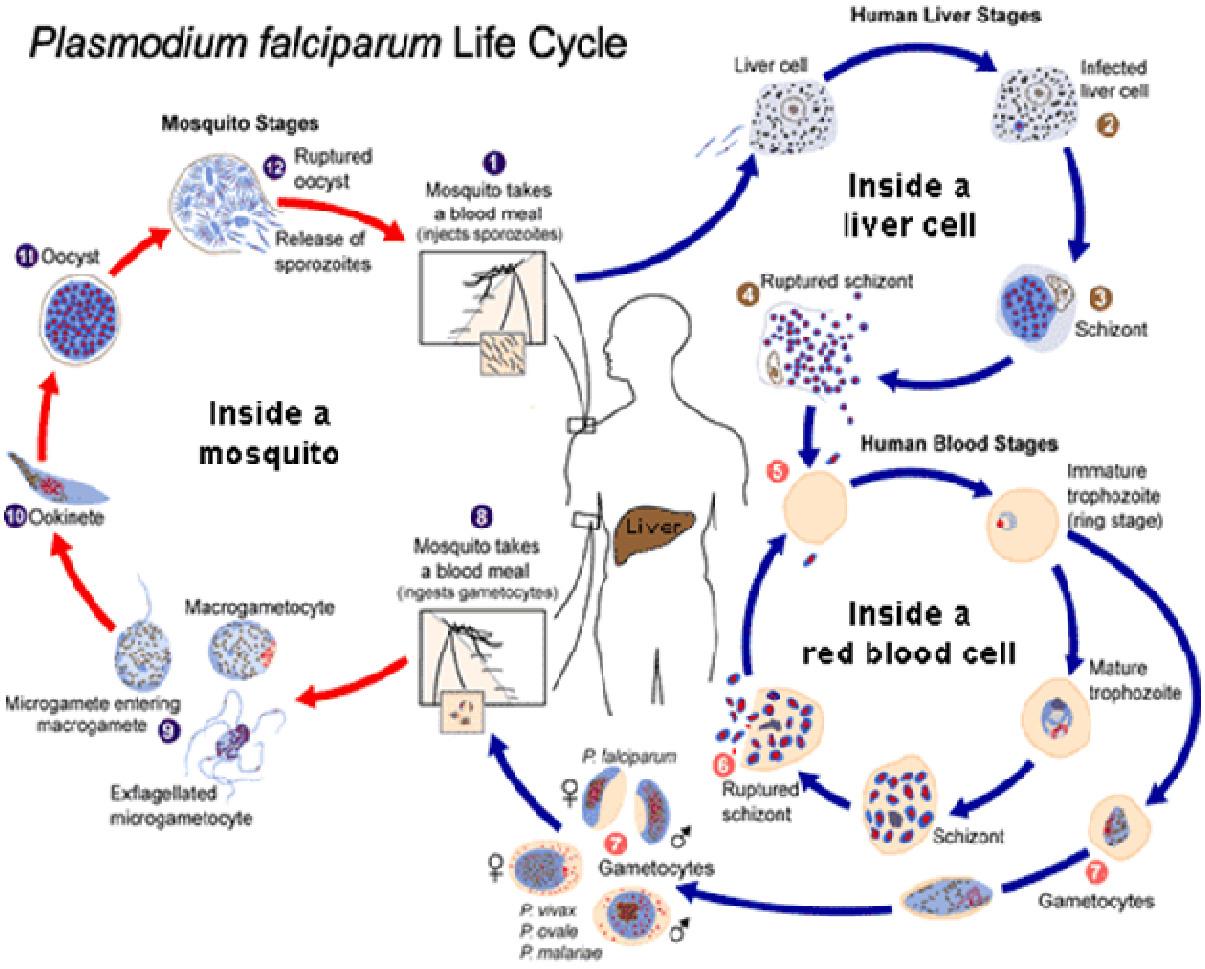


Figure 1.1 Plasmodium life cycle

Source: http://www.parasitaesinhumans.org/plasmodium-falciparum-malaria.html

1.3 Endemicity of malaria.

Malaria is essentially a disease of the tropics and subtropics particularly the sub-Saharan African region although it can be found in some people in temperate areas due to migration from the tropics. Malaria tends to affect mainly children and pregnant women.

The level of malaria endemicity in Africa varies between different countries and sometimes from one part to another within the same country. Some of the factors responsible for the variations in endemicity include: rainfall pattern, altitude and temperature. High rainfall pattern is associated with high malaria transmission while places with high altitudes and low temperatures tend to be associated with lower rates of transmission (WHO, 2004a). In areas of stable transmission, there is often an increased incidence coinciding with increased mosquito breeding during rainy season.

1.4 The burden of malaria in Nigeria.

Nigeria bears up to 25% of the malarial disease burden in Africa, hence contributing significantly to the one million lives lost per year in the region (PMI, 2011).

Malaria has proven to be a colossal and very difficult human disease amongst the health problems confronting countries in sub-Saharan Africa thereby hampering their development with a high proportion of its wealth being drained by the disease which accounts for over 60% of outpatients visit in Nigeria. It is the commonest cause of hospital attendance in all age groups in all parts of Nigeria. It is also one of the four commonest causes of childhood mortality in the country. It is estimated that 50% of the population has at least one episode of malaria each year while children under five have on the average of 2-4 attacks in a year (FMOH, 2005a). Treatment of malaria illnesses accounted for 40% of the curative health care cost incurred by households.

National Malaria Control Programme (NPC & NMC), 2012, reports that the disease is responsible for 30% childhood mortality, 25% of infant mortality and 11% maternal death and 300,000 Nigerians die yearly from malaria related deaths. The direct financial loss due to malaria according to the report is estimated to be about 132 billion Naira (about 8.8million US dollars) in the form of treatment costs, prevention and loss of person-hours annually.

Treatment of malaria illnesses accounted for 40% of the curative health care cost incurred by households (FMOH, 2005a). The indirect cost of malaria cannot be quantified, but malaria has been shown to have a long term effect on the cognitive function and educational attainment in children (Jukes et al., 2006, Clarke et al., 2008). However this observation has not been reported in Nigeria. Malaria is the commonest cause of fever and death especially in young children (WHO, 2000). This is made worse by the situation of multi-drug resistance as a result of self- medication, sub-optimal doses of antimalarial drug, improper diagnosis and improper treatment due to resource limitations as is the case in Nigeria.

Malaria is holoendemic in Nigeria with transmission all the year round. Nigeria has two main seasons in the year, the dry season which is from October to about March and the rainy season which is from April to September with peak of the rains between May and July when malaria transmission is very intense. Rainfall pattern in Nigeria varies with the South having more rains than the North. Annual rainfall decreases northward; rainfall ranges from about 2,000 millimeters in the coastal zone (averaging more than 3,550 millimeters in the Niger Delta) to 500–750 millimeters in the north. The far south is defined by its tropical rainforest climate, where annual rainfall is 60 to 80 inches (1,524 to 2,032 mm) a year.

With an estimated population of 162 million and being the most populous African country and the eighth most populous country in the world (PRB, 2009; UN, 2013), the burden of malaria in Nigeria is conversely a world burden especially with the level of migration of Nigerians particularly to the western world. The UK Health Protection Agency (HPA, 2011), states that about 1,614 malaria cases were reported in the UK yearly between 2005 and 2010, and 22% of these cases in 2010 were from Nigeria.

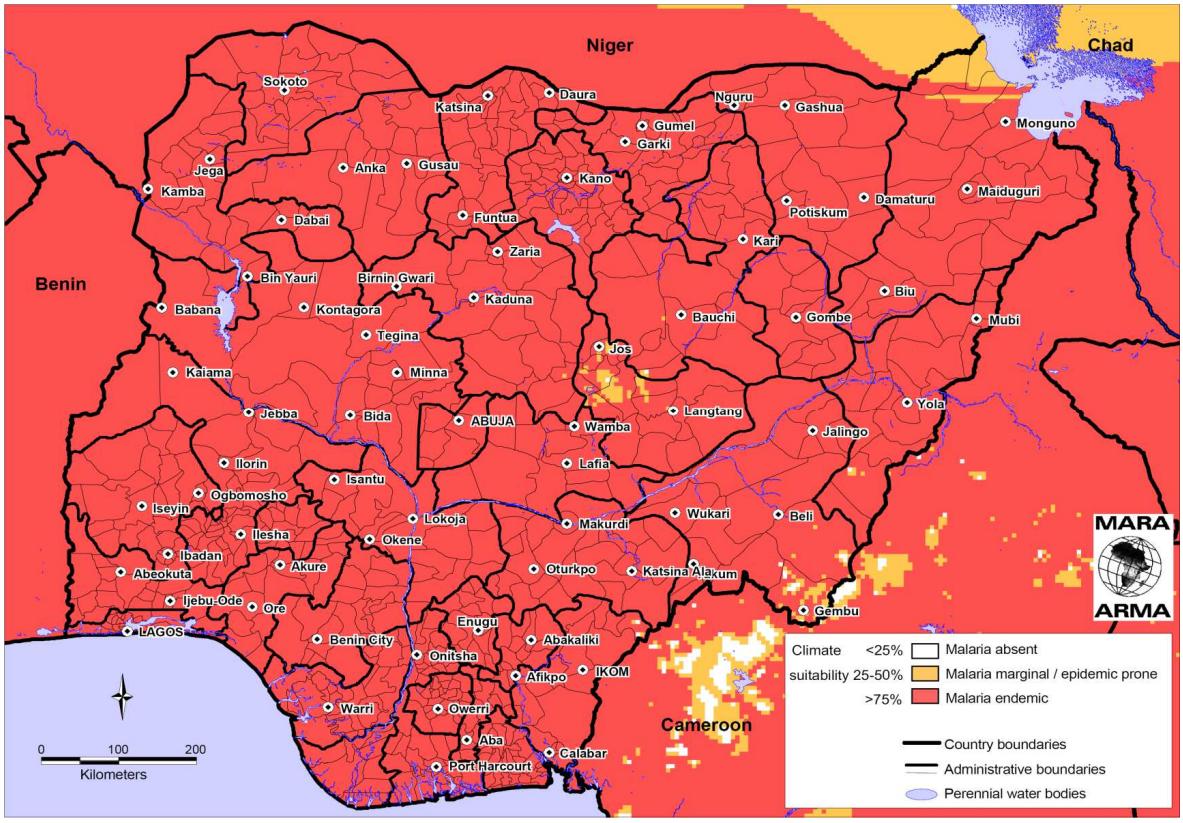


Figure 1.2: Nigeria Distribution of Endemic Malaria

Source: (http://www.mara.org.za**) July 2005**

Red Colour= >75% malaria endemic

1.5 Malaria and Immunity

The immune system of man plays a very important role of defense against the infections that attack the body and malarial infection is not exempt in this. The human immune system is comprised of innate or natural and adaptive immunity. As the name implies, innate immunity is that which the individual is born with**;** it is non-specific and does not depend on any previous infection. It is usually genetic hence it is passed down to the offspring. It is the first line of

defense which recruits the second line of defense into action in the face of pathogenic invasion of the body. This second line of defense is the adaptive or the acquired immune system.

Adaptive/acquired immunity is antigen-specific developing after an infection and provides long lasting protection depending on the host, the type and number of infections. Its role is essentially to destroy invading pathogens and any toxic molecules they produce. Adaptive immune responses are carried out by white blood cells (WBCs) which are made up of the lymphocytes, monocytes, granulocytes, basophils, neutrophils and eosinophils. The T-lymphocytes are the most important of the lymphocytes involved in malaria infection and are activated by antigens that are presented to it by the dendritic cells.

Upon activation, the T-lymphocytes proliferate and mature into effector cells which can be either T-helper cells or cytotoxic cells. T-helper cells play a very important role in the acquisition and regulation of malaria immunity. The predominant T cells are the CD4 cells and are the major cells that control blood stage malaria infection (Perlmann and Troye-Blomberg, 2000) while on the other hand cytotoxic T- cells kill infected cells. The activation of CD4 T-helper cells leads to the stimulation of certain responses which manifest in the release of macrophages and cytokines (IL-10) through the secretion of IFN-γ to prevent the pathology of the disease. IFN-γ which is also secreted by CD8 T cells is known to be associated with protective immune responses (Schofield et al., 1987, Moreno et al., 1991, Seguin et al., 1994, Doolan and Hoffman, 2000). Studies (Lyke et al., 2004) have illustrated that elevated levels of cytokines such as interleukins IL-6, IL-12, IL-1 and IL-10 influence the severity of the malaria disease while TNF and INF-γ stimulate neutrophils which increase parasite destruction (Kumaratilake et al., 1992, Lyke et al., 2004). Although the TNF is protective against parasite by increasing the parasite destruction, in very high serum concentrations it is associated with greater severity and death (Kwiatkowski, 1990).

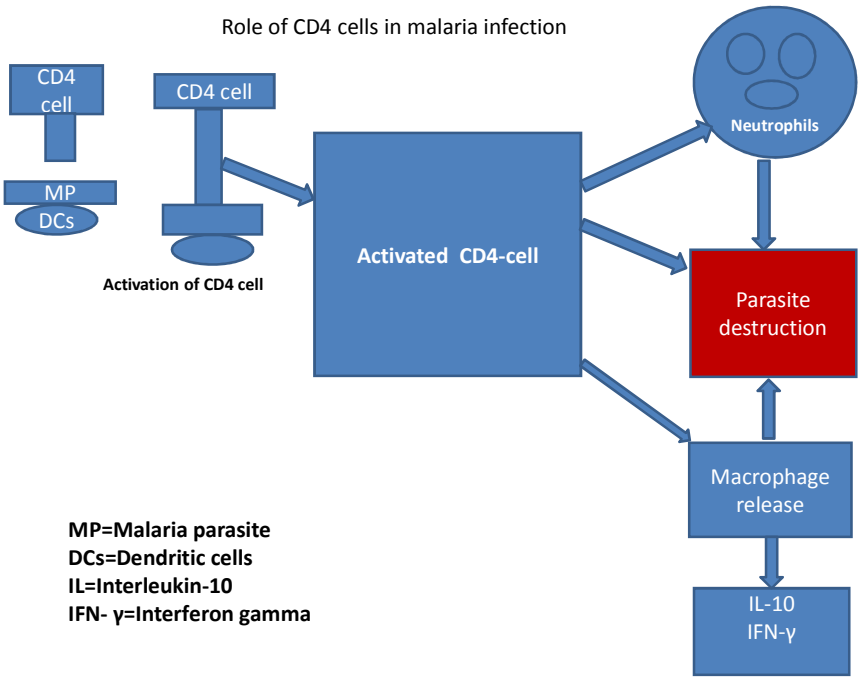


Figure 1.3: Role of CD4 cells in malaria infection.

Sketch based on studies: (Schofield et al., 1987, Moreno et al., 1991, Seguin et al., 1994, Doolan and Hoffman, 2000, Kumaratilake et al., 1992, Lyke et al., 2004).

CD8 T cell responses are suppressed during blood stage malaria and this maybe the explanation for the increased or higher viral load in HIV-positive individuals with *Plasmodium falciparum* infection (Hoffman et al., 1999). The CD4 cells are therefore seen as the bedrock of the body immune system because upon exposure to malaria infection, they are primed for protection by initiating the recruitment and release of the other cells and chemicals that prevent the pathology of the disease. The severity of the malaria disease is dependent on the speed of the response of the immune system.

Individuals without pre-existing malaria immunity are at risk of malaria infection whereas in malaria endemic areas where there is moderate or intense transmission, immunity that is acquired by virtue of several exposures plays a very important role in protecting the individuals from developing the clinical and severe form of the disease. (Bates et al., 2004, Cohen et al.,

2005). Therefore over time, individuals infected with malaria acquire partial protection and therefore have reduced risk of severe malaria hence many adults are not at risk of malaria-caused deaths. In immunosuppression such as is obtained in HIV-positive persons, the situation is not the same. Studies have shown that vulnerability and frequency of malaria infections appear to be increased in HIV-positive adults particularly those with low CD4 cell counts (French et al., 2001, Whitworth et al., 2000; Ladner et al., 2002).

1.6 Clinical features of malaria.

The first symptoms of malaria are non-specific and similar to the symptoms of minor systemic viral illness. The characteristic presentation of uncomplicated malaria includes fever, headache, joint pains, weakness, muscle pains, chills and rigors. Nausea, vomiting, orthostatic hypotension and abdominal pain may occur and in children there may be loss of appetite. In addition to the above, there is also general malaise and fatigue (**http://www.cdc.gov/malaria/about/disease.html, last reviewed 2010)**.

The severity of clinical malaria depends very much on the acquired immunity and intensity of malaria transmission in the area of residence. In areas of stable transmission which prevail in much of sub-Saharan Africa, partial immunity to clinical malaria has been acquired in early childhood therefore clinical diseases is almost always confined to young children who suffer high parasite densities and acute clinical disease (WHO. 2006a).

1.7 Control of malaria

Malaria control is multifaceted and consists of vector control, chemotherapy or the treatment of malaria with drugs and chemoprophylaxis which includes seasonal malaria chemoprevention (SMC). Malaria vector control is the reduction of transmission of malaria through the eradication and control of vectors involved in transmission. The various methods include indoor residual spraying (IRS), insecticide treated nets (ITNs) and long-lasting insecticidal nets (LLINs), larviciding and integrated vector management (IVM). In sub-Saharan Africa, the primary interventions for preventing malaria are the ITNs and the LLINs (Lengeler, 2004, Hill et al., 2006). Recent studies have suggested that the combination of LLIN and IRS provides additive protective benefit by preventing mosquito blood feeding (Okumu et al., 2013).

Chemoprophylaxis on the other hand is the prevention of the development of malaria through the regular use of anti-malarial drugs. SMC is the intermittent administration of full treatment courses of an antimalarial medicine during the malarial season with the objective of therapeutic antimalarial drug concentrations in the blood throughout the period of greatest malarial risk (WHO, 2012a) and was previously referred as Intermittent preventive treatment of malaria in children (IPTc). Study of SMC in Senegal showed a lower prevalence of molecular markers of resistance associated with SP or AQ in SMC areas than in control areas, reflecting the lower prevalence of parasitaemia in areas where SMC was delivered (Lo et al., 2013). Malaria prophylaxis is generally not necessary in persons living in malaria endemic regions. It is however needed in non-immune people, persons with sickle cell anaemia, children and infants and in pregnant women because of the risk of severe disease.

Intermittent preventive treatment in infants (IPTi) which is closely related to SMC is the administration of a full course of an effective anti-malarial treatment at specified time points to infants at risk of malaria, regardless of whether or not they are infected with malaria, with the objective of reducing the infant malaria burden (WHO, 2009a). The administration of this prophylaxis is usually included during the course of the immunization of the child and reduces the incidence of clinical malaria in under-ones by about 30% (Aponte et al., 2009). This intermittent preventive prophylaxis is similar to the treatment of asymptomatic-malaria infection which has been proposed as a method to reduce malaria–associated morbidity (O'Meara et al., 2006).

Intermittent preventive treatment in pregnancy (IPTp) is the administration of full treatment of an antimalarial drug to pregnant women at specific intervals. In Africa**,** Sulfadoxine /Pyrimethamine (SP) has been in use for several years and has been shown to be effective. Trimethoprim-Sulphamethoxazole (TS) has also been reported to be more effective than SP-IPTp in reducing malarial infections and anemia and a combination of both drugs provided further reduction in malaria parasitaemia. However the toxicity of combining the two was not assessed in the study (Kapito-Tembo et al., 2011). However pregnant women who are HIV positive and are on Trimethoprim-Sulphamethoxazole (TS) chemoprophylaxis are not to be placed on IPTp with SP because of the possible increased risk of adverse effects of sulphonamides (Peters et al,2007). The efficacy of antimalarial drugs for use in prophylaxis and treatment is greatly affected by antimalarial drug resistance. This is particularly of importance

in situations of immunosuppression as in HIV-positive patients who are regularly placed on TS and in pregnant women who are placed on SP for prophylaxis against malaria. The focus of this study is on chemotherapy or treatment of malaria.

1.7.1 Treatment of malaria.

Treatment of malaria with drugs is the most common and one of the most important measures for the control of malaria. The goals of treatment of uncomplicated malaria are: to provide rapid and long lasting cure, to reduce morbidity including malaria-related anaemia, to prevent the progression of uncomplicated malaria to severe and potentially fatal diseases and to minimize the likelihood and rate of development of drug resistance in addition to reducing transmission (Sutherland et al., 2005, Sawa et al., 2013).

Antimalarial drugs can be classified into related groups based on the chemical structure of the compounds as follows

- 8-Amino quinolines (Mefloquine, primaquine)
- 4-Amino quinolines (Chloroquine, amodiaquine, piperaquine)
- Antifolates (Sulfonamides, sulfones)
- Aryl amino alcohols (Quinine, Lumefantrine, Halofantrine)
- Artemisinis (Artemisinin, Dihydroartemisinin, artesunate, artemether, arteether)
- Biguanides (proguanil and chlorproguanil)
- Unclassified quinolones (Pyronaridine)
- Antibiotics (tetracyclines, azithromycine)
- Naphthoquinones (Atovaquone)

They can also be classified based on their mode of action (Warhust, 2001a) as blood schizonticides, antifolates and antimitochondrials.

1.7.2 Blood Schizonticides: The blood schizonticides are the drugs that are used for clinical and suppressive cure of malaria. They act only on the haemoglobin-digesting cycle in the RBC (the asexual erythrocytic stage of the parasite). These include the 4-aminoquinolines such as chloroquine and amodiaquine, the aryl aminoalcohols which include quinine (a natural aryl aminoalcohol), lumefantrine, halofantrine and mefloquine (synthetic aryl aminoalcohols), quinine and their related derivatives such as mefloquine and also artemisinin and its derivatives like artemether, arteether, artesunate and dihydroartemisinin and other antimalarial endoperoxides.

Artemisinin and its derivatives possess an endoperoxide bridge in their structure which is crucial to its antimalarial activity (Brossi et al., 1988). The endoperoxide bridge is very unstable in the presence of heme iron and malaria parasites are rich in heme. This bridge is broken down upon interaction with intraparasitic heme in the digestive vacuole leading to a release of free radicals. These free radicals are toxic to the parasite and damage specific intracellular targets possibly via alkylation (Meshnick, 2002). Another suggested mode of action of artemisinins is the inhibition of sarcoplasmic-endoplasmic reticulum Ca2+-ATPase ortholog SERCA known as PfATP6 (Eckstein-Ludwig et al., 2003, Valderramos et al., 2010).

Due to the mortality and morbidity rate of malaria especially in children, the choice of antimalarial drug to use is of utmost importance particularly in the immunocompromised. The choice of antimalarial drug is dependent on a number of factors such as efficacy and safety, the cost and dose regimen of the drug. The World Health Organization (WHO) has established an antimalarial treatment policy which is a set of recommendations and regulations concerning the availability and rational use of antimalarial drugs in a country. The primary purpose of antimalarial treatment policy is to select and make accessible to the population at risk of malaria safe, effective, good quality and affordable antimalarial drugs so that malaria disease can be promptly, effectively and safely treated.

For several decades, the gold standard for the treatment of malaria was chloroquine, a 4-aminoquioline that was previously characterized by its efficacy, low toxicity and affordability (Fidock et al., 2004). However the efficacy of the drug has deteriorated worldwide due to chloroquine-resistant strains of *P. falciparum.* The resistant foci of chloroquine were first detected at the Thailand-Cambodia border in the late 1950s (Payne, 1987) and has spread to

many malaria-endemic areas thereby making the drug increasingly ineffective. The spread of these resistant strains extended from South America, Southeast Asia and India in the 1960s and 1970s and to Africa in the late 1970s when resistance was detected in Kenya and Tanzania (Wellems and Plowe, 2001). However, chloroquine resistance was not reported in West Africa until early 1980s in Burkina Faso, Gabon and Senegal (Nuwaha, 2001, Trape et al., 1989) and by the 1990s, there was increased mortality rate in children due to chloroquine resistance in West Africa (Trape et al., 1998).

Another equally cheap drug in use in sub-Saharan Africa which has also come under serious resistance is sulphadoxine-pyrimethamine (SP). These two drugs were the first and second line drugs for use in treatment of uncomplicated malaria in Nigeria. However in the six geopolitical zones of the country, resistance to these drugs has been reported to occur in 23-96% of cases hence the need for a change in policy (FMOH, 2005a).

1.7.3 Treatment failure

One of the challenges in the chemotherapy of malaria is treatment failure which is the inability of a drug to produce the desired therapeutic effect. Treatment failure is caused by various factors which include drug resistance, poor bioavailability, fake or substandard drug quality, poor patient compliance, suboptimal dose, wrong diagnosis, antagonistic interactions with other medications and the immune status of patient (Bloland, 2001). On the other hand, the ability of a drug to produce the desired therapeutic effect is described as the efficacy of the drug. Of paramount importance to the efficacy of the drug is the host immunity and this may be ineffectual in HIV-infected subjects who are immunosuppressed (Kamya et al, 2001; Birku et al, 2002). Where the administration of the drug has met the appropriate standards of quality, patient compliance, optimal dose, the other factor exacting immense influence on efficacy is the bioavailability of orally administered drugs, i.e. the concentration of therapeutically active drug or drug metabolite that reaches the blood and is available at the site of action. Day 7 blood/plasma concentration has been established as a simple measure of assessment of the bioavailability of antimalarial drugs (Ezzet et al., 2000).

1.7.4 Combination therapy

Antimalarial combination treatment is the simultaneous use of two or more blood schizonticidal drugs with independent modes of action and different biochemical targets in the parasites (WHO, 2001).Combination therapy is based on the synergistic or additive potential of two or more drugs to improve treatment efficacy and retard the development of resistance to individual components of the combination. Therefore combination therapy offers better advantages over monotherapies. The combination therapy particularly advocated is the artemisinin based combination (ACT) because artemisinin produces rapid and sustained reduction of the parasite biomass and rapid resolution of clinical symptoms (White, 1997, White et al., 1999a).

1.7.5 Artemisinin and its derivatives

Artemisinin and its derivatives is currently the mainstay for the treatment of malaria. Artemisinin is the active component developed from the leaves of the old Chinese herbal medicine *Artemisia annua*-sweet wormwood or qinghao which has been used for many centuries by the Chinese for the treatment of fever. The commonly used derivatives of artemisinin are artesunate, artemether and dihydroartemisinin. Other derivatives which are less often used include the oil-based lipid soluble arteether (artemotil) and artenilic acid. Every form of artemisinin is converted to the active metabolite dihydroartemisinin (DHA).

The artemisinins and their derivatives are the most rapid acting and efficacious antimalarial drugs currently available (Chavchich et al., 2010). Artemisinins are very effective and rapid in clearing parasites by killing young, circulating ring-stage parasites and preventing further maturation and sequestration of these parasites (ter Kuile et al., 1993, White, 2008). However**,** this is short-lived when used as a monotherapy because of its very short half life. Consequently**,** this results in high rate of recrudescence**;** therefore for effective treatment of malaria, artemisinin is combined with a long acting blood schizonticide which will clear remaining parasites, thereby reducing the risk of developing resistance.

Among the drugs used in combination with artemisinin (AS) or its derivatives are lumefantrine (L), mefloquine (MFQ), piperaquine (PQ), sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ), pyronaridine (PYR). ACTs come in various combinations: AL; AS+AQ; AS+SP; AS+MFQ,

DHA+PQ and each of the combinations have been proved to be effective in the management of uncomplicated malaria (Yeka and Harris, 2010, Croft et al., 2012).

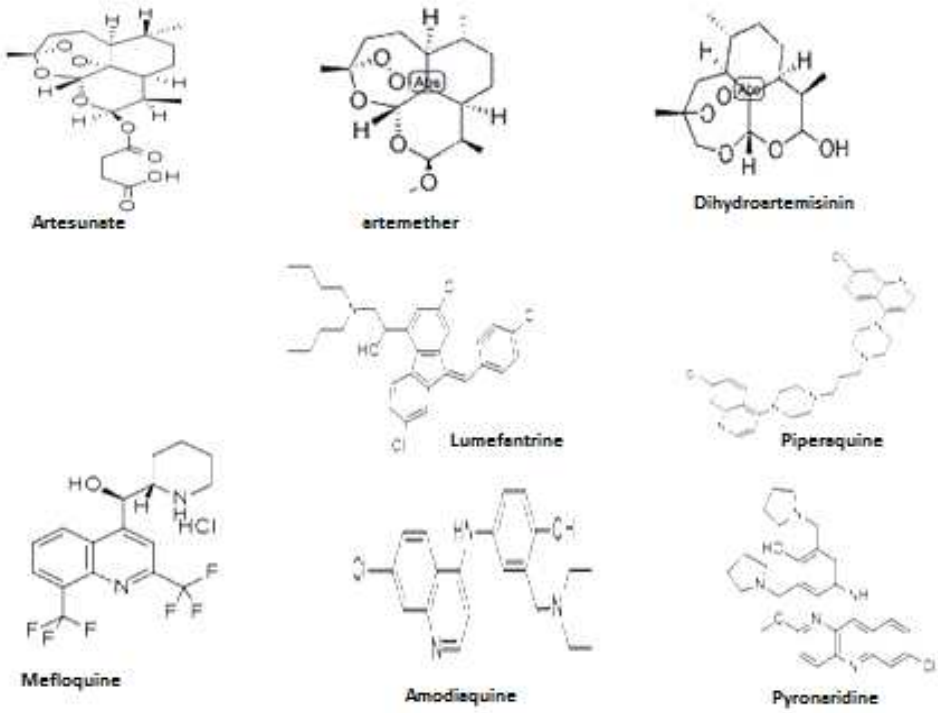
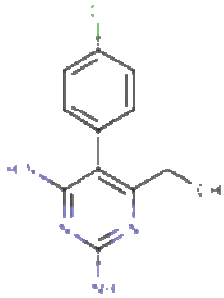


Figure 1.4 Chemical structures of the artemisinins and partner drugs

Source: http://www.chemicalbook.com

Sulphadoxine



Pyrimethamine

Figure 1.5 Chemical structures of sulphadoxine and pyrimethamine

Source: http://www.drugbank.ca/drugs/DB00205

1.7.6 Artemether-lumefantrine

Artemether-lumefantrine was introduced in Nigeria in 2005 as the first line antimalarial drug for the treatment of uncomplicated malaria. The drug artemether-lumefantrine is a fixed combination of artemether-lumefantrine in the ratio 1(20mg, artemether): 6(120mg, lumefantrine). It is indicated for the treatment of acute, uncomplicated infections due to *Plasmodium falciparum* or mixed infection including *P. falciparum* and strains from multi-drug resistant areas for infants, children and adults. Artemether is a sesquiterpene lactone derived from the naturally occurring compound artemisinin. It is the methyl ether of dihydroartemisinin while lumefantrine is a racemic mixture of a synthetic flourene derivative of the aryl amino-alcohol family. Lumefantrine has a similar mechanism of action to halofantrine probably due to similarities in structure and pharmacokinetic properties, but unlike halofantrine, lumefantrine is very safe as it does not prolong the electrocardiographic QT interval (van Vugt et al., 1999). Lumefantrine was first synthesized and registered in China and is now commercially available only in co-formulated product with artemether as coartemether (Annerberg et al., 2005) Both drugs act as blood schizonticides (Lefevre et al., 2001).

1.7.6.1 Pharmacology: The site of antiparasitic action of artemether and lumefantrine is the digestive food vacuole of the malaria parasite, where they are thought to interfere with the conversion of heme, a toxic intermediate produced during haemoglobin breakdown to the non-toxic haemozoin malaria pigment (del Pilar Crespo et al., 2008).

Artemether being a derivative of artemisinin is metabolized to DHA and posesses an endoperoxide bridge which is very crucial to its antimalarial activity (Brossi et al., 1988). The endoperoxide bridge which is very unstable in the presence of heme iron and upon interaction between the two is broken down to antiparasitic free radicals in blood stage malarial parasites. These free radicals are toxic to the parasite and damage specific intracellular targets possibly via alkylation as previously noted above. Lumefantrine interferes with the polymerization process. Both artemether and lumefantrine have a secondary action involving inhibition of nucleic acid and protein synthesis within the malaria parasite.

Pharmacodynamics reports (van Vugt et al., 2000, Lefevre et al., 2001) of artemether-lumefantrine indicate high parasitological cure rates (>95%) in three Thailand studies. It eliminates parasites and symptoms significantly faster than most current antimalarials (van Agtmael et al., 1999, Kshirsagar et al., 2000) and is rapidly gametocytocidal helping to reduce transmission (Sutherland et al., 2005, Sawa et al., 2013). It is also effective in multi-drug resistant areas.

The two drugs complement each other in their pharmacokinetic activities. They are both metabolised by hepatic cytochrome P450 enzyme, CYP3A4 and CYP2B6. Artemether is metabolised to its more active metabolite dihydrartemisinin (DHA). Both artemether and DHA offer potent antimalarial properties causing significant reduction in asexual parasite mass of approximately 10,000-fold per reproductive cycle with prompt resolution of symptoms (Djimde and Lefevre, 2009). Lumefantrine is metabolized by N-debutylation to desbutyl-lumefantrine which has a 5-8-fold higher antiparasitic effect (White et al., 1999b, Khoo et al., 2005).

Peak plasma concentrations of artemether occur around 0.5-2 hours after oral administration (Ezzet et al., 1998) and elimination half-life is approximately 2 hours. Lumefantrine on the other hand reaches peak plasma levels approximately 10 hours after oral administration (WHO, 2006a) but is cleared more slowly, showing a terminal half-life of 4-6 days with *P. falciparum* malaria (Lefevre and Thomson, 1999). Oral bioavailabilty is variable and is highly dependent on

administration with fatty foods (Ezzet et al., 1998, White et al., 1999b). A meal with only a small amount of fat (1.6g) is considered sufficient to achieve adequate exposure to lumefantrine (Djimdé and Lefèvre, 2009).

The manufacturer recommends that Coartem be instituted at the onset of symptoms as a six-dose regimen with 1-4 tablets per dose depending on body weight and should be administered over the course of 3 days. The six-dose regimen was first tested in sub-Saharan Africa in 2002 and showed both high efficacy and transmission reduction (Sutherland et al., 2005).

1.7.7 Antimalarial Drug Resistance

Antimalarial drug resistance is defined as the ability of a parasite strain to survive and multiply despite the proper administration and absorption of an antimalarial drug in the dose normally recommended (WHO, 2006). It is a shift to the right of the dose-response curve, thus requiring higher drug concentrations to achieve the same parasite clearance (White, 2004). In many cases even the higher concentration results in treatment failure. However not every treatment failure is as result of drug resistance. Assessment of antimalarial drug resistance can be done through many ways and this includes: *in vivo* assays which is usually regarded as the gold standard, *in vitro* tests which involves drug assays and assessment of molecular markers of resistance present in the study area of interest.

Many factors influence antimalarial drug resistance and this includes the nature of the parasite, pharmacological properties of the drug, host factors of which the primary one is human immune responses, (Travassos and Laufer, 2009), and the ability to clear a resistant infection is closely related to age (Djimde et al., 2003).

Individuals with lower immunity such as HIV-positive patients, children and pregnant women are more vulnerable to antimalarial drug resistance. Reduced immunity allows the survival of a residuum of parasites thereby potentially intensifying the development, intensification and spread of resistance (Byakika-Kibwika et al., 2010). Additionally, delayed cure rate and higher rate of recrudescence which occur in HIV-positive individuals accelerate the spread of resistant parasites and increase the parasite biomass in both symptomatic and asymptomatic carriers (Birku et al., 2002, Shah et al., 2006, Van Geertruyden et al., 2006).

Resistance to antimalarial drugs by *Plasmodium falciparum* parasite has become a major health problem since the first resistance to Chloroquine was recorded in Thailand and Cambodia in the late 1950s (WHO, 2006). This has spread to other malaria endemic regions and against other antimalarial drugs like sulphadoxine-pyrimethamine (SP) and mefloquine. Studies (Noedl et al., 2008) reporting artesunate-resistant malaria in western Cambodia and reduced *in vivo* susceptibility of artesunate (Dondorp et al., 2009) and *in vitro* resistance to its derivative artemether (Jambou et al., 2005) indicate a great threat to the management of malaria since artemisinin and its derivatives are currently the mainstay for the treatment of uncomplicated malaria. Dokomajilar et al., (2006) also reported parasite tolerance to AL in some parts of Africa shortly after its introduction.

1.7.7.1 Mechanisms of drug resistance

Antimalarial drug resistance can result either from changes in drug accumulation or efflux or reduced affinity of the drug target resulting from point mutations in the respective genes encoding the target (White et al., 1999b). The efflux of the drug from the DV of the malaria parasite via the transporters involved in the carriage of the antimalarial drug have been associated with drug resistance such as that obtained in transporters of chloroquine or the artemisinins (Sanchez et al, 2005., Duraisingh and Cowman, 2005). Genetic polymorphisms in one or more genes that do not actually encode the drug target itself but affect drug efflux can lead to reduced drug concentrations within the parasites such as in chloroquine, amodiaquine, quinine, mefloquine and halofantrine thereby resulting in drug resistance (Valderramos and Fidock, 2006). Another mechanism of resistance is through altered affinity for the drug target caused by single or multiple point mutations in genes that encode the drug target as in pyrimethamine, cycloguanil, sulphonamide and atovaquone (Wang et al., 1997). Resistance can also be due to expression of higher levels of the gene through amplification thereby resulting in increased copy number. This has been reported in the treatment with AL in Sudan where increased copy number of *pfmdr1* gene was associated with clinical failure (Gadalla et al., 2011) and decreased parasite susceptibility to artelinic acid and artemisinin *in vitro* was linked to increased copy number of *pfmdr1* gene (Chavchich et al., 2010). Antimalarial drug resistance can also occur where there has been no prior resistance in the parent drug because of inadequately treated biomass infections (White and Pongtavornpinyo, 2003) or reduced sensitivity to a given drug or class of drugs which occurs as a result of spontaneous mutations

(Bloland, 2001) and subsequent spread as a result of survival and multiplication. Where a large population of parasites are exposed to drug pressure, resistance is reported to develop more quickly (Farooq & Mahajan, 2004). Some malaria parasites, especially in West Africa exhibit an innate resistance to new drugs of diverse chemical classes such as mefloquine, halofantrine or even artemisinin (Oduola et al., 1987, Oduola et al., 1992).

1.7.8 Molecular markers of resistance

Molecular markers of drug resistant malaria are based on genetic changes that confer parasite clearance to drugs used to treat and prevent malaria (Plowe et al., 2007). Molecular markers have been proven to be tools for surveillance of resistance (Djimde et al., 2001a, Djimde et al., 2001b), provide additional data that compliment clinical observations of the *in vivo* efficacy of a drug and has been instrumental to policy making with regards to control of malaria epidemic (Mugittu et al., 2004). They have also served as monitoring tools in parasite drug susceptibility following change in treatment policy (Kublin et al., 2003, Laufer et al., 2010). Since resistance results from mutations in genes or changes in the copy number of the genes relating to the drug's target of the parasite gene, investigating molecular markers of resistance is therefore a very important way of assessing resistance to a drug. Certain molecular markers have been associated with certain group of drugs as evidence of resistance. Table 1.1 lists molecular markers related to this study.

Table 1.1 Molecular markers related to this study (Genes of interest)

GENE	GENE ID	CODONS OF INTEREST	DRUGS	REFERENCES
Pfcrt	PF3D7_0709000	72-76	CQ, AQ	Fidock et al, 2000, Sidhu et al, 2002, Warhust, 2003; Folarin et al, 2008; Beshir et, al 2010
Pfmdr1	PF3D7_0523000	86,184,1034, 1042,1246	CQ,AQ, AL	Sutherland et al, 2002; Dokomajilar et al, 2006; Humphreys et al, 2007; Holmgren et al, 2007; Happi et al, 2009; Malmberg et al, 2013.
Pfdhps	PF3D7_0810800	431,436,437,540,581, 613	SP, TS	Pearce et al, 2003; Roper et al, 2003; Happi et al, 2005; Sutherland et al, 2009; Naidoo et al, 2010
pfdhfr	PF3D7_0417200	50,51,59,108,164	SP,TS	Pearce et al, 2005; Happi et al, 2005; McCollum et al, 2007; Malamba et al, 2010.

1.7.8.1 Plasmodium falciparum multidrug resistance gene1 *(Pfmdr1)*

A similarity in decreased drug accumulation found in the antitumour drugs and antimalarial drug resistance led to the discovery of the *Plasmodium falciparum* multi drug resistance (*pfmdr1*) gene (Krogstad et al., 1988). *Pfmdr1* is a 4251 base pair gene on chromosome 5 that encodes a protein PfMDR1 majorly localised on the digestive vacuole (DV) of the erythrocyte stage of the parasite, very little is present at the parasite plasma membrane (Cowman et al., 1991). Changes in the transmembrane protein *pfmdr1* which encodes the P-glycoprotein homologue 1 (PGH-1), results in resistance to the artemisinins. PfMDR1 has been demonstrated to be a transporter of various chemicals such as chloroquine, quinine, halofantrine into the DV of the parasites and the polymorphisms on this protein define the substrate specificity of the antimalarial drug (Sanchez et al., 2010). This has been evidenced by its regulation in the *in vitro* and *in vivo* response to mefloquine, halofantrine and quinine (Price et al., 1999b, Price et al., 2004, Nelson et al., 2005, Duraisingh and Cowman, 2005).

Point mutations in the *pfmdr1* gene and increased copy number result in decreased accumulation of the drugs because of the increased efflux from the DV with subsequent resistance or decreased sensitivity (Duraisingh et al., 2000b). Five major mutations (N86Y, Y184F, S1034C, N1042D and D1246Y) on the *pfmdr1* gene have been implicated in multidrug resistance of antimalarial drugs. Studies by (Dokomajilar et al., 2006, Sisowath et al., 2007), have indicated that polymorphisms at 86N, 184F and 1246D are involved in the AL resistance while a selection of 86Y, 184Y and 1246Y leads to AQ resistance (Humphreys et al., 2007). Mutations on points N86Y and D1246Y have also been associated with contributors to chloroquine resistance (Plowe, 2003). Happi et al, (2009) in a study of AL for the treatment of Nigerian children with uncomplicated falciparum malaria observed that the NFD haplotype of the *pfmdr1* gene was significantly associated with treatment failure. Additionally, (Gadalla et al., 2011) established that amplification of *pfmdr1* locus increased copy number may contribute to recurrent parasitaemia following AL therapy just as decreasing the copy number was demonstrated to heighten susceptibility to lumefantrine, halofantrine, mefloquine, quinine and artemisinin (Sidhu et al., 2006).

1.7.8.2 Plasmodium falciparum dihyropterate synthetase (*Pfdhps*) and *Plasmodium falciparum* dihydrofolate reductase *(pfdhfr)*

Resistance to the antifolate and sulpha drugs Sulphadoxine-pyrimethamine (SP) and Trimethoprim-sulphamethoxazole (TS) is mediated by mutations on the *Pfdhps* and the *pfdhfr* genes. SP is used for both treatment and intermittent prevention of malaria (IPT) while the closely related drug TS which is primarily used for prophylaxis of opportunistic infections in HIV-infected persons has also been confirmed to have antimalarial activity (Walker et al., 2010, Manyando et al., 2013).

The Plasmodium parasite decreases the affinity of binding of the antifolates pyrimethamine, trimethoprim, cycloguanil, proguanil to the enzyme target dihydrofolate reductase *(dhfr)* thereby leading to resistance to the drug. Sulpha drugs are inhibitors of the enzyme dihdropteroate synthetase (*dhps)* the target enzyme in the folate biosynthesis of the plasmodium parasite. A combination of the two classes usually given as sulphadoxine-pyrimethamine (SP) therefore provides sequential inhibition of folate biosynthesis and show a marked synergy in antimalarial activity (White et al., 1999b).

Mutations in the genes of these enzymes lead to altered drug binding and the resultant effect is resistance. Polymorphisms at certain loci on the genes encoding these enzymes are responsible for resistance to these drugs (Kublin et al., 2002, Karema et al., 2010). On the *dhfr* gene, point mutations at positions 51, 59, 108 and 164 have long been associated with pyrimethamine resistance (Cowman et al., 1988) while on the *dhps* gene mutations at positions 437G and 540E are linked to SP resistance (Kublin et al., 2002, Bwijo et al., 2003).

The presence of a combination of the five mutations (*dhfr* N51I, C59R, S108N and the *dhps* A437G and K540E on both genes which is generally referred to as the 'quintuple mutant' have been statistically associated with SP treatment failure *in vivo* (Nzila et al., 2000, Kublin et al., 2002). A Nigerian study, (Happi et al., 2005) established an independent association of SP treatment failure with the *dhfr* triple mutant N51I, C59R, S108N and the double mutant *dhps* A437G and K540E in children less than 5years. The study further stated that the genotype strongly associated with the failure is the quintuple mutant.

Another locus of interest in the *dhps* gene is substitution of valine for isoleucine at codon I431V (Sutherland et al., 2009). The significance of this novel *dhps* to antifolate resistance has not yet been established however it is has been detected in occurrence together with mutations at 581 and 613 and was first reported in samples of UK infections from Nigerian isolates. Preliminary data analysis from the study shows 431V was in occurrence with 437G, 581G and 613S. Mutations at codons 581 and 613 have long been associated with SP resistance (Plowe et al., 1997).

1.7.8.3 Plasmodium falciparum chloroquine resistance transporter gene (*Pfcrt)*

The emergence of Chloroquine resistant (CQR) *P. falciparum* parasites in the late 1950s (Payne, 1987) in South-east Asia and South America many years after the first use of CQ as the mainstay for treatment and prophylaxis of malaria and its subsequent spread to Africa in the late 70s (Wootton et al., 2002) has resulted in a great challenge in malaria control. The mechanisms of this resistance are not completely understood however many studies have established the role and implication of the gene *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt)* in CQR. *Pfcrt* is a gene on chromosome 7 and encodes a putative transporter PfCRT which is a 48kDa protein containing 424 amino acids and is localized in the digestive vacuole (DV) of the parasite during the eryhthrocytic stage (Fidock et al., 2000).

PfCRT has been shown to function as a transporter that directly mediates the efflux of CQ from the DV of the parasite (Warhurst, 2003, Bray et al., 2005). Polymorphisms with multiple mutations in this protein resulting in changes in pH on the DV have been closely correlated with CQR and this is evidenced by the differences observed in the accumulation of chloroquine in the DV of CQR and CQS parasites. Furthermore, the reduced accumulation can be reversed in the presence of verapamil and thereby increase the sensitivity (Wellems et al., 1990, Bray et al., 2005, Fidock et al., 2000, Sidhu et al., 2002). Point mutations on this gene particularly change in the amino acid at position 76 from lysine K to threonine T (K76T) has been shown to confer resistance to CQ by reducing accumulation of the drug in the DV.

Although there are many point mutations on this gene resulting in different alleles, the most critical is the change at codon 76 from lysine K to threonine T (K76T) and has been shown to confer resistance to CQ in every geographical setting just like the CQS strains in every geographic setting maintain the wild type K76. This was emphasized in the report (Warhurst, 2003) stating that in the context of other mutations, CQR results from the change of the positively-charged lysine-76 to neutral threonine which could facilitate drug efflux.

The Plasmodium parasite digests haemoglobin from the host red cell which is degraded to heme and amino acids in the DV. Being a weak base, CQ accumulates inside the acidic DV, of the Plasmodium parasite. CQ-sensitive parasites accumulate high levels of CQ partly because of the weak base trapping and binding to haem (Yayon et al., 1985) producing a complex which is toxic to the parasite (Orjih et al., 1994, Warhurst, 2003). In the CQS form of PfCRT the positively charged side chain of lysine 76 may repel the positively charged CQ molecule reducing its efflux through the channel (Warhurst, 2003). In CQ-resistant strains however, there is less accumulation of the drug due to the drug efflux (Sanchez et al., 2003). The pH of the DV may also have an influence in the reduction of the accumulation of CQ (Ginsburg and Stein, 1991).

Various mutations of CQR on the PfCRT present in different haplotypes depending on the location however all the alleles maintain the 76T mutation. In Africa and Southeast Asia the common haplotype seen is the CVIET (change at positions 72-76), while in South America the common allele found is SVMNT (Fidock et al, 2000). Additionally SVMNT was also reported in South-East Asia (Dittrich et al., 2005), in Tanzania (Alifrangis et al., 2006) and a high prevalence of >50% in Angola (Sa and Twu, 2010). Another less common haplotype is the CVMNT as

reported in Ecuador and Brazil (Vieira et al., 2004) and South-Western Nigeria (Gbotosho et al., 2012).

Transfection studies have also proven the role of PfCRT not just on CQR but also on resistance to quinoline antimalarial drugs (Bray et al., 2005). This has been evidenced by its involvement in AQ failure presenting as SVMNT haplotype (Beshir et al., 2010a, Sa and Twu, 2010). Its expanded role in influencing parasite susceptibility to other structurally diverse antimalarials was established in its role in conferring hypersensitivity to MFQ and Halofantrine (Johnson et al., 2004).

The implications of antimalarial drug resistance are such that there is continued transmission of drug-resistant parasites thereby limiting the efforts to control malaria. This is because drug resistance can also lead to increased gametocyte carriage, thereby facilitating the spread of resistance through the population (Price et al., 1999a). The continued use of a drug with prevalence of resistance in a locality confers a selective advantage to parasites carrying resistant genes and leads to higher rates of transmission of drug resistant parasites (Handunnetti et al., 1996, Sutherland et al., 2002).

Antimalarial drug resistance is the predominant factor maintaining the global burden of malaria particularly in sub-Saharan Africa (Barnes and White, 2005). This has therefore made the management of malaria very difficult and warranted the recommendation of combination of antimalarial drugs for the treatment of malaria by the World Health Organisation (WHO).

1.8 HIV/AIDS.

The acquired immune deficiency syndrome (AIDS) is defined as the state of profound immunosuppression produced by chronic infection with human immunodeficiency virus (HIV). Many theories have been projected as the origin of HIV. It has however been accepted as a descendant of the African Green monkey virus, the Simian immunodeficiency virus (SIV) virus because of the similarities between the two viruses (Cohen, 1999).

HIV is a retrovirus that belongs to the family of Lentiviruses from the retroviridae. The retroviruses possess the enzyme reverse transcriptase which they use to transcribe their RNA genome into DNA. After transcription to DNA, the viral DNA then gains access to the DNA of the

cell of the host and there replicates itself. There are two types of HIV -types 1 and 2. Both HIV-1 and HIV-2 replicate in CD4 T cells and have been documented as causative agents of AIDS. HIV-1 is more virulent than HIV-2, more easily transmitted and is the cause of the vast majority of infections globally (Reeves and Doms, 2002).

1.8.1 Epidemiology of HIV/AIDS

The first case of AIDS was reported in the USA amongst homosexual men (Gottlieb et al., 1981). It later became apparent that other population groups were affected including males who have sex with men and intravenous drug users (CDC, 1991). Later reports based on retrospective studies have suggested that the oldest HIV infection was that found in a sailor from Manchester who died from AIDS-like illness in 1959 and also from African plasma sample that was previously found to be seropositive thereby predating the infection before the 1981 case (Zhu et al., 1998). The African sample is believed to be the first known case of AIDS and was collected from a man who died in the Democratic Republic of Congo in 1959 (Pickrell, 2006).

An estimated 34-47 million people were infected with HIV/AIDS in 2006, with approximately 4.3 million of these being newly diagnosed infections (UNAIDS/WHO, 2006). Over 40million people are HIV-positive and over half of this number are in Africa (WHO, 2004a). HIV/AIDS accounted for the deaths of 2 million people in 2007 and 2.7 million were new infections of which 1.9 million occurred in sub-Saharan Africa(Quinn, 2008).

In Africa, reports in 2003 show that AIDS claimed the lives of an estimated 2.4million people and over 600,000 children were newly affected with the virus (WHO, 2004a). HIV/AIDS increasingly accounts for a large proportion of mortality among children below five years in the heavily affected countries and also affect mainly adults in their most productive years of life (15-49years) (UNAIDS /WHO, 2001). A more recent report (UNAIDS, 2011) shows that by 2010, about 34million people were living with HIV/AIDS of which 2.7million are new infections and the greatest burden is in sub-Saharan Africa with an estimated population of 22.9million (68%) of infected people.

1.8.1.2 HIV/AIDS in Nigeria

Nigeria is ranked second in the number of people living with HIV/AIDS after South Africa and accounts for about 9% of the global HIV burden (UNGASS, 2010). AIDS was first reported in Nigeria in 1986. The first two cases of HIV and AIDS in Nigeria were identified in 1985 and were reported at an international AIDS conference in 1986. (FMOH, 2005b, Nasdi and Harry et al, 2006). Consequently the number of people living with HIV/AIDS steadily increased and the epidemic became established with an increase of HIV-seroprevalence from 1.8% in 1991 to 5.8% in 2001 and 5.0% in 2003. This meant that Nigeria had 3.5million infected persons, the third highest in the world (FMOH, 2005b). Currently an estimated 3.1 million people are living with HIV/AIDS and national prevalence is 4.1% (NACA, 2011). Nigeria has the second highest number of new infections yearly (UNGASS, 2010).

In Nigeria, about 80% of HIV transmission is through the heterosexual route (UNGASS, 2010) while blood transfusion with a 10% rate accounts for the second largest source of HIV infection (FMOH, 2009). In a study of transfusion-transmissible infections among blood donors in Port Harcourt, Nigeria, Ejele et al, (2005a) observed that out of the 146 donors positive for HIV, 138 (95.5%) had HIV-1 and 8 (5.5%) had HIV-2 with sub-type A and G of HIV-1 being the predominant one in circulation (Peeters et al, 2004).

Another report established an overall prevalence of 1% among blood donors in Niger Delta region of Nigeria with the highest occurring among the age group 20-29 years (Ejele et al, 2005b). Another major route of transmission is the mother to child accounting for about 360,000 children with HIV. Mode of transmission model studies show that high risk groups men that have sex with men (MSM 1%) and female sex workers (FSW 23%) significantly contribute to new HIV infection (Nwauche et al, 2006b; UNGASS, 2010).

Prevalence of HIV/AIDS is highest in the age group 25-29years (5.6%) and lowest in 40-44years (2.9%) from the 2008 ANC survey (UNGASS, 2010). In settings as obtained in Nigeria, where heterosexual transmission is common, women are more likely to be affected in the epidemic thereby increasing the burden of mother to child transmission. The high prevalence of HIV in women of reproductive age has led to a growing population of HIV–infected and affected children. It is estimated that one HIV- positive child is born every five minutes, in Nigeria (Eneh, 2007). In 2010, the HIV prevalence was highest in urban areas and among the 30-34 years age

group. The prevalence of HIV in the 36 states of Nigeria shows Benue state to be on top of the ladder while Kebbi was on the lowest pedals. River state which was our study area is on the 10^{th} position (Figure 1.6).

The impact of HIV/AIDS in Nigeria is profound and multifaceted ranging from healthcare, political and social instability but the greatest impact is on the economy because HIV/AIDS attacks people mostly in their productive years and in resource-limited countries it is usually fatal. HIV fosters and promotes poverty, destabilizes the family structure and takes a great toll on the nation's economy. The major effects on the economy are seen on the increased costs and reduction in labour supply. This is particularly evident in agriculture which is the primary source of activity of the Nigerian population occupying about 45% of the employed labour force (Bolinger, et al, 1999). The indirect costs of HIV/AIDS include lost time due to illness, recruitment and training and cost to replace workers and care of orphans. Approximately 54% of annual income of an affected household is estimated to be spent on HIV and this involves income losses due to sickness and out–of-pocket expenses on healthcare (Mahal et al, 2008). Personal cost is estimated to be over 200% of household expenditure and include expenses on antiretroviral drugs (ARVs), other drugs (e.g for opportunistic infections), laboratory tests and investigations (Onwujekwe et al., 2009).

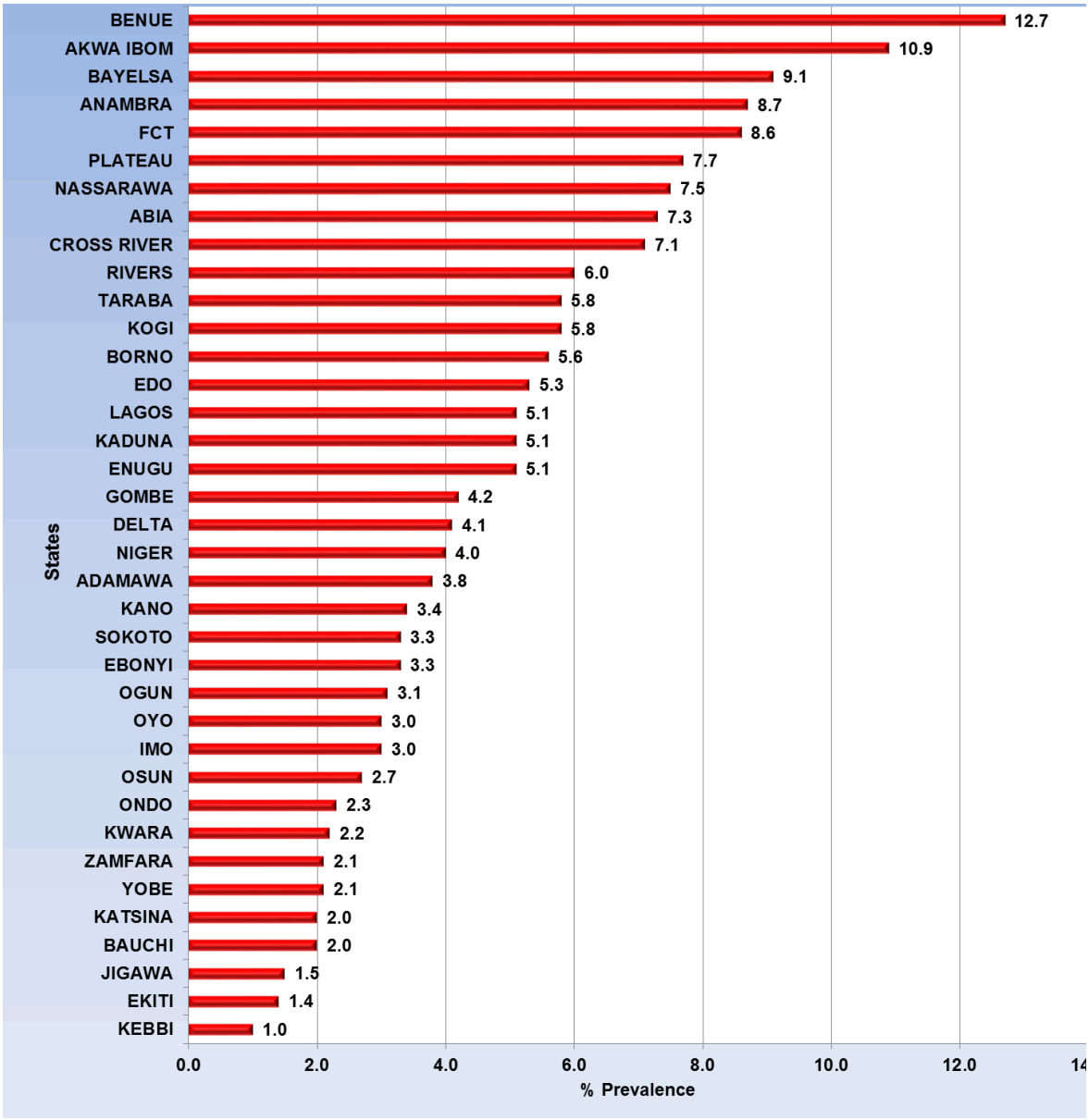


Figure 1.6 HIV prevalence by States, 2012

Source : Federal Republic of Nigeria Global AIDS Response Progress Report, 2012 (GARPR)

1.8.1.3 Pathogenesis of HIV/AIDS.

HIV possesses the enzyme reverse transcriptase which enables it to transcribe its genetic material from ribonucleic acid (RNA) to deoxyribonucleic acid (DNA). Docking glycoprotein gp 120 and transmembrane glycoprotein gp41 found on the HIV virion wall have a strong affinity

for the CD4 receptor protein found predominantly on the T helper/inducer lymphocytes. It makes use of CD4 molecules to gain entry into the T helper cells of the host since CD4 is its primary receptor. It makes use of CD4 molecules to gain entry into the T helper cells of the host since CD4 is its primary receptor. Reports have shown that even though CD4 is the primary receptor for viral entry into the host cell, there are additional co-receptors required for HIV to gain entry into the lymphcyte. The HIV glycoprotein 120 has been found to interact with both CD4 as well as CXCR4, a receptor expressed on many T-cells for adherence to the cell in order to effect the required conformational changes in the gp120/gp41 complex which then allows its fusion to the host cell membrane (Feng et al, 1996). Another co-receptor involved in HIV-membrane infusion to the host cell is the CCR5 expressed on the macrophages and on some populations of T-cells (Deng et al, 1996; Idemyor, 2005). However, the binding of the HIV gp120 to CCR5 is CD4-dependant. This is evidenced by reports that individuals with certain mutations on the CCR5 are resistant to HIV infection (Liu et al, 1996; Samson et al, 1996). The fusion of the HIV cell to the host surface and subsequent entry into the host cell results in the formation of DNA through the use of its reverse transcriptase enzyme.

The newly formed DNA is integrated into the host cell and begins to replicate into several billion cells daily. The multiplicity of the virus leads to the destruction and depletion of the CD4 cells ultimately overwhelming the immune system therefore the individual becomes immunocompromised and susceptible to other opportunistic infections and tumours. When the CD4 cell count of an individual infected with HIV gets depleted to a very low level (less than 200 cells/mm^3, the person is diagnosed with AIDS. HIV also invades other cells such as macrophages, monocytes and glial cells of the brain (NIH. 2012). Due to their role in the immune system, the T-cells are therefore used as prognostic markers for monitoring the progress of immunosuppression such as HIV infection (Abdulazeez, 2012).

1.8.1.4 Prophylaxis of HIV/AIDS

Despite the decline in global incidence of new HIV infections from 3.1 million in 2001 to 2.7 million in 2010 (UNAIDS, 2011), HIV still claims many lives in sub-Saharan Africa where about 68% of people leaving with HIV reside. Awareness campaigns, HIV testing, prevention of mother to child transmission (PMTCT), lifestyle changes etc. have contributed to a great extent to the decline in new infections of HIV (Coates et al., 2008), however there is still need for more

efforts to further prevent new infections and reduce the incidence. Like the old adage goes 'prevention is better than cure' there is need to devise means of reducing HIV epidemic or preventing new infections. Prophylaxis has proven to be effective in the control of many diseases, such as malaria and opportunistic infections in HIV/AIDS patients. This approach is currently being advocated as an additional tool for the control of HIV. HIV prophylaxis can be administered either before (pre-exposure) or after (post exposure) to the infection.

Post exposure prophylaxis (PEP) is short-term antiretroviral treatment to reduce the likelihood of HIV infection after potential exposure, either occupationally or through sexual intercourse (WHO, 2007a). The administration of antiretroviral medications 72 hours after exposure of the percutaneous and mucous membrane to HIV either through needle-stick injury or unanticipated sexual intercourse has been shown to prevent infection (Olshen & Samples, 2003, Smith et al, 2005). Post exposure prophylaxis is not suitable for individuals with repeated high-risk of infection who are unwilling to adhere to risk reduction practices (Omrani and Freedman, 2005).

Pre-exposure prophylaxis (PrEP) on the other hand is the administration of antiretroviral drugs to uninfected HIV individuals who are at high risk of infection. It works on the principle of supplying a therapeutic level of ARV drugs in the blood stream before exposure to the virus so it is crucial that adequate level of drug are present in the system (Morin, 2012). This therefore calls for long-term adherence among uninfected persons considered to be at high risk of HIV infection who are placed on prophylaxis.

There have been trials on the use of microbicides and topical applications of antiretroviorals (Cohen et al, 2007; Abdool Qarim et al, 2010) for use in PrEP. The US Food and Drug Administration (FDA) approved the use of combination of two medications, tenofovir disoproxil fumarate and emtricitabine (TDF-FTC), for daily use as PrEP for people at high risk of HIV infection through sexual exposure. Studies have reported a 44% reduction in HIV acquisition when PrEP is administered to HIV-negative men or trans-gender women who have sex with men (among adherent users) (Grant et al, 2010). Many more trials are being carried on the target audience which include people who inject drugs, serodiscordant couples, heterosexual men and women, women at high risk of HIV exposure and men and transgender women who have sex with men (WHO, 2012b).

1.8.1.5 Treatment pattern for HIV infection.

In order to improve the quality and duration of life of HIV-positive subjects, the goals of therapy are

1. Reduce viral load
2. Prevent deterioration of immune function and/or restore immune status
3. Treat and prevent opportunistic infections
4. Relieve symptoms.

The time for initiating antiretroviral treatment is determined by the clinical stage of the HIV infection as indicated by symptoms, and where available the CD4 cell count. The optimum time for initiation of treatment depends primarily on the CD4 cell count, the plasma viral load and the clinical symptoms of the patient. In the United States of America it is strongly advocated that treatment be initiated when the CD4 cell counts fall below 350 cells/mm^3 (AIDSINFO, 2012) while in low income countries, treatment is initiated in patients with CD4 cell counts between 200-350 cells/mm^3 (Stover et al, 2010). Studies in Nigeria have shown that there is no short-term immunological advantage in starting HAART at a baseline CD4 count of > 350 cells/ul rather than at 200-350 or < 200 cells/ul (Ejele et al, 2005c). However in conditions of pregnancy, co-infection with hepatitis B virus, HIV-associated nephropathy and history of an AIDS-defining illness, initiation of treatment is advocated in HIV-positive individuals regardless of CD4 cell count (AIDSINFO, 2012).

The drug treatment for HIV disease is tripartite comprising antiretroviral therapy, management of opportunistic infections or malignancies and symptom control. The aim of the first of these, antiretroviral therapy is reducing the HIV viral load as much as possible, for as long as possible and restoring immune function. Opportunistic infections in HIV infections are managed by the administration of trimethoprim/sulphamethoxazole (TS). The introduction of antiretroviral drugs changed the whole course of HIV/AIDS disease and management so that many infected people now live for extended period.

1.8.2 Classes of antiretroviral drugs.

Following the deployment of Azidothymidine (AZT) as the first drug (Fischl et al., 1987) for treatment of HIV/AIDS, the whole course of the disease prognosis and management has

changed such that with the administration of ARVs, there is decreased morbidity and mortality (Mahungu et al., 2009b) and many infected persons now live extended periods of time (Palella et al., 1998). Several drugs have been developed for the treatment of HIV/AIDS and currently there are seven classes of antiretroviral drugs based on the site and mechanism of action (Arts and Hazuda, 2012).

1. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), which inhibit viral replication by binding directly onto the reverse transcriptase enzyme thus preventing the transcription of viral RNA to DNA. Examples are Nevrapine (NVP), etravirine and efavirenz (EFV).

2. Nucleoside reverse transcriptase inhibitors (NRTIs), which incorporate themselves into the DNA of the virus, thereby stopping the building process. The resulting DNA is incomplete and cannot encode a new virus. Examples are Lamivudine (3TC), stavudine (d4T) zidovudine (ZDV) and abacavir (ABC).

3. Nucleotide reverse transcriptase inhibitors (NtRTIs), which act at the same stage of the viral life cycle as the NRTIs, but do not require to be phosphorylated for effective antiretroviral activity. Examples are Tenofovir Disoproxil Fumarate.

4. Protease inhibitors (PIs), which work at the last stage of the virus reproduction cycle. They prevent HIV from being successfully assembled and released from the infected CD4 cell. Examples are Saquinavir (SQV), Ritonavir (RTV) and Indinavir (IDV).

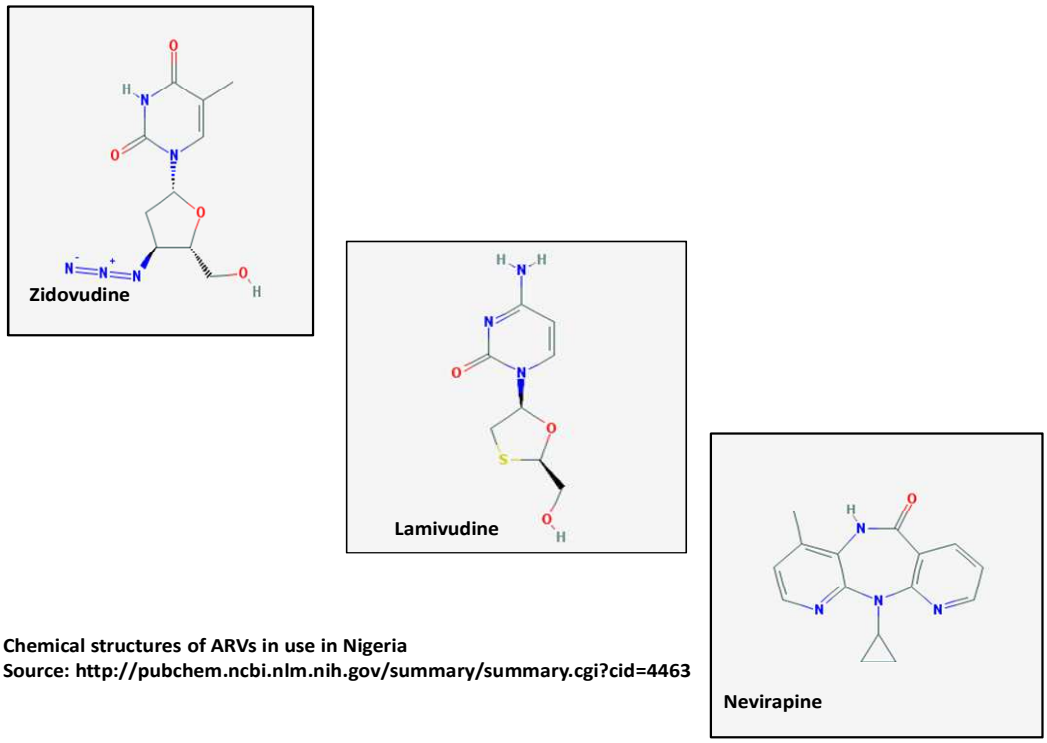
5. Fusion inhibitors (Entry inhibitors), which prevent the HIV particle from infecting the CD4 cell. An example is Enfuvirtide (T-20).

6. Integrase inhibitors, which interfere with the ability of the HIV DNA to insert itself into the host DNA and thereby copying itself. An example of this class is Raltegravir

7. Chemokine receptor antagonist (CCR5 antagonist) which are CCR5 antagonists. These drugs bind to the CCR5 receptors thereby blocking its binding to gp120. An example of this class is Maraviroc.

In order to prevent resistance and increase efficacy, the combination of three or more drugs from at least two different classes acting on at least two different points in the viral replication

cycle is recommended for therapy. This combination is called highly active antiretroviral therapy (HAART). Currently the first line combination in use in the treatment of HIV/AIDS in Nigeria comprises of 2NRTI+1NNRTI. A typical combination in use in Nigeria is Stavudine+Lamivudine+Nevirapine or Zidovudine+Lamivudine+Nevirapine (FMOH, 2005b; Nwauche et al, 2006a).



Chemical structures of ARVs in use in Nigeria
Source: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=4463

Figure 1.7 Chemical structures of the ARVs in use in Nigeria.

1.9 Malaria/HIV interactions.

Malaria has been associated with a rise in viral load (Kublin et al, 2005, Hoffmann 1999) and a fall in CD_4 cell count (Mermin et al, 2006) potentially worsening the clinical course of people with HIV infection. An important study from Malawi by Kublin et al, (2005) showed that HIV-1 plasma viral loads were significantly higher in patients with malaria infection than in those

without and these levels remained higher for up to 10 weeks after treatment. The increases in viral load were greatest in those with clinical malaria and high levels of parasitaemia. This is supported by another Malawian study which revealed that HIV-1 viral burden is higher in patients with *Plasmodium falciparum* than in controls and thus viral burden can in some patients be partly reduced with antimalarial therapy (Hoffman et al., 1999).

1.9.1 The Nigerian situation

Eni et al, (2005) in a study in Ahmadu Bello University Teaching Hospital, Kaduna, Nigeria observed that 70% of HIV-1 patients have concurrent malaria parasitaemia compared to HIV-negative adults and children which are 22.5% and 57.5% respectively.
In another study of Seroprevalence of malaria infection among human immunodeficiency virus patients in Ondo state, Western Nigeria by Onifade and Ogudare (2006), it was observed that 33.5% of HIV patients examined was positive for malaria and the highest prevalence of malaria infection in the patients was 66.7% for age range 0-9 years. In a study on the prevalence of malaria as a co-infection in HIV infected individuals in South Eastern Nigeria, (Onyenekwe et al., 2007) showed 3X higher prevalence of *Plasmodium falciparum* malaria in symptomative HIV seropositive group compared to seronegative group. Another study in Nnewi a sub-urban town in South-Eastern Nigeria reports 46.7% prevalence in asymptomatic-malaria in HIV-positive patients compared to 26.9% in HIV-negative group (Ukibe et al, 2010). A more recent study in Jos, North Central Nigeria, established a 24% prevalence of malaria parasitaemia in HIV-positive adults compared to 9% in HIV-negative controlled group (Iroezindu et al., 2012).

HIV infection impairs T-cell immunity, which is of crucial importance for antimalarial responses (Graziosi et al., 1998). On the other hand, malaria infection activates T cells, potentially promoting HIV replication (Xiao et al., 1998, Froebel et al., 2004). Since increased HIV RNA levels are associated with accelerated disease progression, malaria could potentially facilitate faster progression to AIDS and death (Tkachuk et al., 2001). In areas of stable malaria transmission, (areas where transmission of malaria is intense and continuous, though seasonal variations may occur) malaria infection rates and the frequency of clinical illnesses (fever and other findings) appear to be increased in HIV infected adults particularly those with immunosuppression as measured by low CD4 T cell counts. A study involving Ugandan adults, observed that the odds of parasitemia, and risk of malarial fever increased with decreasing

CD4 cell count, such that individuals with CD4 cell counts<200cells/µl were more than twice as likely to suffer malarial fever as individuals with >500 cells/µl (Whitworth et al., 2000, French et al., 2001). Increased prevalence of severe malaria has also been reported in HIV-infected adults in South Africa (Cohen et al, 2005).

Malaria/HIV interactions have been clearly demonstrated in young children in whom malaria induced anaemia leads to blood transfusions which may transmit HIV (Kublin et al., 2005, Mermin et al., 2006). In pregnant women also HIV contributes to higher malaria infection rates, higher parasite density, more clinical illness, more anaemia and diminished response to treatment (ter Kuile et al., 2004). A more recent study on malaria in HIV observed that HIV-1 infected malaria patients had lower haemoglobin (Hb) compared to HIV-1 uninfected patients (Van Geertruyden et al., 2009). It is thought that malaria/HIV interaction is a contributing factor to the reversal of the gradual declines in malaria mortality in the 1990s, the period during which HIV was at its peak (UNICEF, 2003).

The control of malaria parasitaemia in untreated individuals is immune mediated, and this prevents most malarial infections from becoming clinically apparent in semi-immune adults in endemic areas (Whitworth, 2006). This in addition with the reports cited suggests that HIV-associated immunosuppression interferes with parasite control. The above report also stated that HIV infection may reduce the efficacy of immunity which protects persons with parasitemia from clinical disease and may reduce the effectiveness of the acquired protective immune response that prevents parasitemic persons from developing clinical illness (Hewitt et al, 2006).

With the above reports and records it can be predicted that the efficacy of antimalarial therapy will be lower in immunosuppressed individuals co-infected with HIV and malaria living in regions of stable transmission. Increasing parasite burdens and reduced host immunity, both of which occur with HIV infection, are associated with increased treatment failure rates (WHO, 2006a). A Ugandan study (Kamya et al., 2006) observed that HIV-1 infection was associated with a >3-fold hazard ratio [HR] increased risk of clinical treatment failure of adults treated with three different antimalarial regimen including an ACT. In a study of delayed clearance of *Plasmodium falciparum* in patients with human immunodeficiency virus co-infection, Birku et al, (2002) demonstrated a prolongation in fever and parasite clearance time in adults following

Artemisinin treatment for uncomplicated malaria. Further study (Kamya et al., 2001) established clinical failure to chloroquine therapy in HIV-positive children in comparison with HIV- negative children. This decrease in response to antimalarial therapy in immunosuppressed individuals could be attributed to the fact that HIV infection impairs T- cell immunity, which is of crucial importance for antimalarial responses (Graziosi et al., 1998). It could also be because of increased susceptibility to malaria re-infection or because of recrudescence of infection since antimalarial therapy is most effective in individuals who already have some acquired immunity (Hewitt et al., 2006, Diallo et al., 2007).

1.9.2 Influence of HIV on the emergence of antimalarial drug resistance.

An important factor that influences drug resistance is host immunity and in HIV-infected people there is reduced immunity. Other factors contributory to drug resistance are frequency at which resistance mutation occurs in a population and the number of parasites exposed to drugs. In HIV-positive individuals, there is increased probability of malaria infection progressing to symptomatic illness and to higher parasite densities, thereby increasing the probabilities of treatment being required and thus of contact between parasites and the drug (Whitworth et al., 2000, French et al., 2001, Patnaik et al., 2005). The resulting effect is increased drug exposure and thus selective drug pressure. Prolongation in fever and parasite clearance time as established by (Birku et al., 2002), higher risk of treatment failure (Kamya et al., 2001) and higher rates of recrudescence (Van Geertruyden et al., 2006, Shah et al., 2006) accelerate the spread of resistant parasite biomass in both symptomatic patients and asymptomatic HIV-infected people.

The WHO Technical consultation Geneva, Switzerland advocates that research be carried out to investigate the treatment of malaria among people living with HIV to determine whether a reduced response to antimalarial treatment among HIV-infected people increases malaria case fatality rate. (WHO, 2004a).

Management of co-infection with malaria and HIV is a major challenge to public health yet potential drug-drug interactions between antimalarial and antiviral regimen have not been adequately investigated in people with both infections. Djimde and Lefevre (2009) in their study on the pharmacokinetics of artemether-lumefantrine (Coartem) stressed the need for additional data on the pharmacokinetics of artemether-lumefantrine in patients undergoing

HIV/AIDS chemotherapy. The mortality and morbidity of the two diseases is already high, and if the interaction of the treatment undermines the efficacy of the antimalarial drug, it will increase the outcome risks and may require the need to modify and optimize therapy and may even worsen the problem of drug resistance, or could result in the elevation of the concentration of the antimalarial leading to a possible increased toxicity. On the other hand, the elevation may be an advantage in enhancing the antimalarial effect especially with antiretrovirals that possess antimalarial activity (Parikh et al., 2005, Soyinka et al., 2010, Nsanzabana and Rosenthal, 2011). The choice of antimalarial drug for the treatment of HIV-positive patients therefore is of utmost importance considering the dangers of co-morbidity but sufficient pharmacokinetic and parasitological evidence to make this choice is currently lacking. Dosages of ACTs currently used for the treatment of malaria are based on trials among HIV- negative people.

As well as drug-drug interaction between antimalarials and antiretroviral drugs, there is no clear understanding of the importance of resistance to antimalarials in HIV-infected individuals. The influence of host factor on resistance particularly immune status cannot be over-emphasised. (Diallo et al., 2007, Travassos and Laufer, 2009). Since studies have shown that drug resistance is usually genetically related because of polymorphic changes, and is influenced by host immune status, therefore there is need to design studies to address relationship between immune status and drug resistance.

The *pfmdr1* allele NFD at codons 86, 184 and 1246 which has been linked to AL selection, tolerance and treatment failure in several studies even in Nigeria (Humphreys et al, 2007; Happi et al, 2009) was found in about 20% of the few Nigerian samples obtained from the Malaria reference laboratory. There is little or no information of the *mdr*86N selection and the prevalence of the NFD haplotype in the Niger Delta particularly among HIV-positive people. A study on Nigerian children by (Happi et al., 2009) established the selection of *Plasmodium falciparum* multidrug resistance gene 1 allelles in asexual stages and gametocytes by artemether-lumefantrine. None of these studies involved HIV-positive individuals.

Also widespread use of trimethoprim-sulphadoxine (TS) as prophylaxis among HIV-positive patients may be placing sulfonamide selective pressure for alleles at the *dhps* locus that are not directly related to antimalarial use (Sutherland et al., 2009). Any impact of *dhps* mutations

on AL treatment outcome is expected to be indirect only since AL action is independent of the folate pathway in the parasite. Sutherland et al, (2009) identified a novel haplotype *dhps* I431V found only in Nigerian isolates. This study will involve further investigation of this haplotype to establish its presence and evaluate the prevalence in Nigerian subjects in the Niger Delta region.

Results of the studies above were obtained from HIV-negative individuals. As earlier stated, host factors especially immunity play a major role in the influence of drug resistance (Diallo et al., 2007, Travassos and Laufer, 2009). There is therefore need for such studies to be carried out in HIV-positive individuals because of their immunosuppression. The paucity of data regarding the use of this drug combination in HIV-positive subjects calls for investigation which this research is designed to address.

1.10 Justification.

Given the high prevalence of these two diseases in Nigeria and in consideration of the reducing effects of this interaction on the response to therapy, it is imperative to design studies that will investigate and analyse the cohorts of HIV/malaria infected patients and their treatment. There seems to be a paucity of data on the use of antimalarial drugs on HIV-positive patients on antiretroviral therapy particularly with the regards to drug-drug interactions between ARVs and antimalarial drugs in addition to the influence of the HIV status on the drug response with reference to resistant markers. The study (Brentlinger et al., 2007) on the challenges in the prevention, diagnosis and treatment of malaria in HIV-infected adults in sub-Saharan Africa advocated for a study on description of longitudinal response of CD4 cell count and HIV viral load to malaria in persons taking highly active antiretroviral therapy. Therefore there is need to assess the effect of different CD4 cell count levels on the efficacy of the drug in HIV-infected individuals in order to establish the success of therapy to the level of infection. Our study involved the evaluation of CD4 cell count levels of the HIV subjects before and after treatment of the asymptomatic-malaria with AL.

1.11 Rationale for study:

Earlier studies on AL use have not focused on its use in HIV subjects and there is an urgent need for this study in HIV-positive patients with malaria. Recent studies (Kredo et al., 2011, Byakika-

Kibwika et al., 2012, Huang et al., 2012) on HIV-positive volunteers who were not infected with malaria support the view that drug-drug interactions are important in the management of patients with co-infection. This study therefore is being carried to analyse/evaluate the antimalarial effect of artemether-lumefantrine in HIV-positive and HIV-negative adult patients in this region.

Since the deployment of AL as first-line in 2005, studies have established its high cure rates in the treatment of uncomplicated malaria in Nigeria (Meremikwu et al., 2006, Sowunmi et al., 2007). Resistance studies (Falade et al., 2005, Falade et al., 2008, Happi et al., 2009) have established an association of either treatment failure or slow clearance with polymorphisms in the *pfmdr1* gene. As at date and time of our study, none of these studies involved the inclusion of HIV-subjects. There is paucity of data on AL malaria treatment of HIV-positive patients in Nigeria especially in Rivers state, Niger Delta where there is a high prevalence (6.0%) of HIV compared to the national prevalence of 4.1% (FRN, 2012). The study was designed to address this paucity and establish a current status of markers to resistance of AL in this very important region of the country. Therefore monitoring of the resistance markers related to the drugs is of vital importance in order to sustain their use in Nigeria.

Summarily, information is lacking in the areas of *in vivo* drug-drug interactions between antimalarials and ARVs, the impact of ARVs on malaria parasites, interaction between resistant parasites and HIV and the impact of asymptomatic-malaria on HIV infection as well as its significance to the spread and transmission of resistant parasites. The study is therefore designed to address some of these gaps and possibly generate more questions for further studies.

1.12 Research Questions

- Are there any associations at enrolment between drug resistant *P. falciparum* and the use of medication for either HIV treatment or prevention of opportunistic infections with particular reference to the use of TS prophylaxis?

- Are there any drug-drug interactions between the ARVs and the antimalarials and if there is, does it have any impact on parasitaemia?

- Are there any differences between the HIV-positive and HIV-negative people in the selection pattern of mutations on the *mdr1* and *crt* genes?

- Is there any evidence of persistent parasitaemia being higher in HIV-positive individuals than in the control group at day 3 or day 28?

- Does AL treatment of asymptomatic malaria infected adults with HIV provide any benefit on the immunity of the HIV-positive people in terms of CD4 count?

1.13 Objectives

- To measure and compare the prevalence of different *dhps* haplotypes in association with 431V in the two different groups and test for possible associations with recent use of TS.

- To compare the pharmacokinetics of AL based on the day 7 blood concentrations and bioavailablity of lumefantrine in HIV-positive and HIV-negative adults. Studies have shown that the D7 plasma level concentration is an important predictor of the efficacy or treatment failure of AL (Ezzet at al, 2000).

- To investigate the prevalence in both HIV-positive and HIV-negative adults of parasites carrying molecular markers of resistance known to be selected by AL treatment.

- To evaluate CD4 cell counts as an indicator of impact of AL on HIV chemotherapy.

Chapter Two

Materials and Methods

2.1. Study design

A 28-day study was designed to assess the use of artemether-lumefantrine in the treatment of asymptomatic malaria infection in HIV-positive and HIV-negative adult Nigerians residing in Port Harcourt, Niger Delta Region. The study was patterned after the classic WHO, 2003 however treatment outcome was based only on parasitologic and not therapeutic outcome definitions since the participants are asymptomatic (Kublin et al., 2003).

2.1.1. Nigeria Geography

Nigeria consists of a Federal Capital Territory and 36 states which are further divided into 774 local government areas. It is divided into six geo-political regions: North Central, North East, North-West, South-East, South-South and South. Nigeria is made up of 374 ethnic groups (NPC, 2012). Nigeria was a federation of three regions (Northern, Western and Eastern) before it gained its independence from the Colonial British government in October 1960.

Nigeria is in the West African sub-region, lying between latitudes 4˚16' and 13˚53' north and longitudes 2˚40' and 14˚41' east. It is bordered in the north by Niger Republic, in the east by the Republic of Chad and Cameroun, in the west by the Republic of Benin and in the south by the Atlantic Ocean. It has a total surface area of approximately 923,768 square kilometers and 800km of coast line. Nigeria has two main seasons, the dry seasons which occur from October to March and the wet or rainy season occurring from April to September with peak of the rains between May and July when malaria transmission is very intense. Transmission is throughout the country and 97% of the population is at risk. The main vector for transmission is *Anopheles gambiae* and *A. funestus, A. arabiens* are also commonly encountered predominantly in the north while *A. melas* is found in the mangrove coastal zone (PMI, 2011).

2.1.2. Study Area

The study was carried out in two sites; the University of Port Harcourt Teaching Hospital (UPTH) and the Braithwaite Memorial Specialist Hospital (BMH) both in Port Harcourt, Nigeria. The study was conducted from September 2010 to September 2011. Port Harcourt is the capital of Rivers State. It lies along the Bonny River in the Niger Delta 41 miles upstream from the Gulf of Guinea, rich in the nation's oil resources and it is the TREASURE BASE of the nation. The region

is dotted with oil and gas activities which attract many foreigners and migrant workers and commercial sex workers follow the camp (Nwauche and Akani, 2006). These socio-economic conditions contribute to a high seroprevalence of HIV infection (6.0%) which is higher than the national prevalence (FRN, 2012).

Port Harcourt exhibits lengthy and heavy rain seasons and very short dry seasons with average temperatures between 25˚C-28˚C. Due to this vulnerability, there is an increased interaction with malaria which is already endemic in Nigeria especially in the Niger Delta as a result of its mangrove swamp forest vegetation where transmission is year round (PMI, 2011). Annual rainfall in this area averages more than 3,550 millimeters in the Niger Delta. The epidemiology of malaria/ HIV co-infection and interaction has not been studied in this area.

University of Port Harcourt Teaching Hospital shares a wall with the University Park of the University of Port Harcourt; it is sandwiched between the Port Harcourt International Airport, the New Calabar River and the East- West Express Road. The hospital has 30 departments and has a bed compliment of about 800, the largest referral hospital in Niger Delta. The hospital runs HIV adult clinic on two days of the week (Wednesdays and Thursdays). The Braithwaite Memorial Hospital is the state specialist hospital right in the city metropolis and has a 350 bed compliment. Its HIV clinic days are Tuesdays and Thursdays.

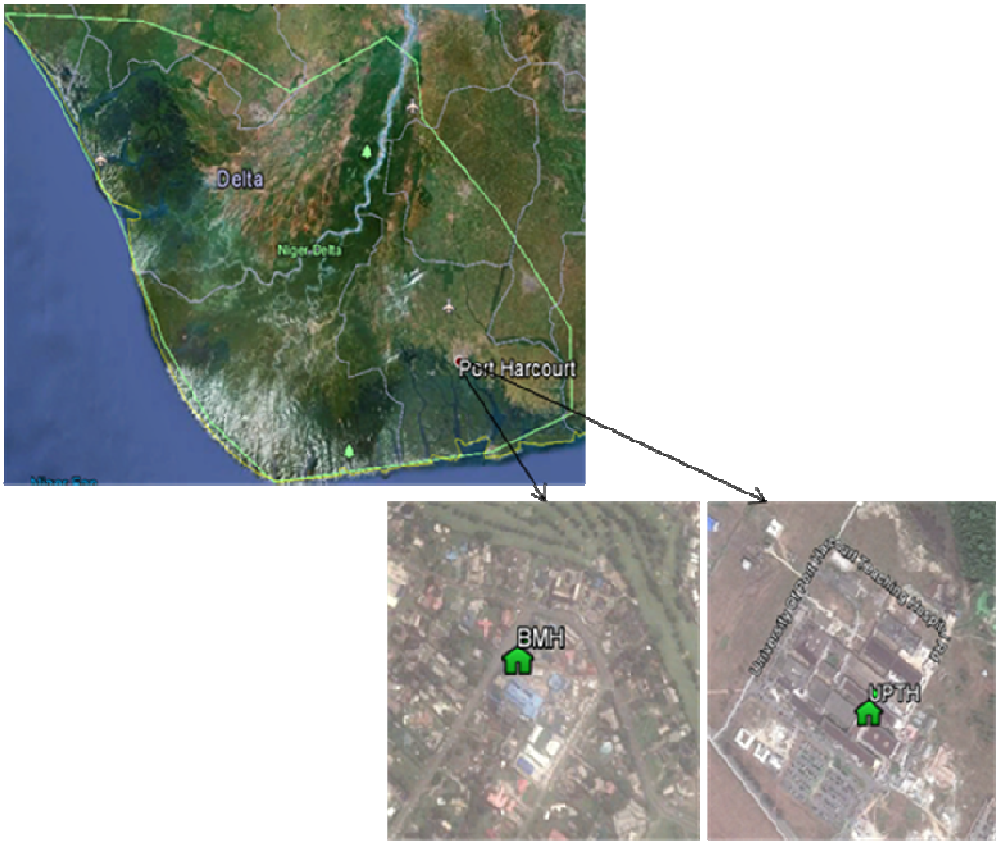


Figure 2.1 Geographic location of study

BMH: Braithwaite Memorial Hospital
UPTH: University of Port Harcourt Teaching Hospital Teaching

2.1.3. Recruitment of Participants and sample collection

A temporary clinic was established in an office in the hospital to allow some privacy for the study. Patients who visited both hospitals came from all over the Niger Delta region since both are the two largest hospitals in the state. Information sheet about the study was given to the participants and the purpose of the study was thoroughly explained to them. Self-administered questionnaires were given to obtain family and social history or any clinical history of the participants that may be of importance to the study (Annexe 6 & 7)

2.1.4. Inclusion criteria

Participants were recruited if they met the following eligibility criteria:

- Aged between 16-65 years,
- Willingness to have HIV status confirmed from clinical records or by a point-of-care test.
- Positive for *P. falciparum* by microscopic examination of a blood film
- Provision of a signed informed consent form.

2.1.5. Exclusion criteria

Excluded from the study were

- People aged 15 years and below
- People with complicated malaria that is evidenced by cerebral involvement like convulsions, vomitting etc,
- Pregnancy and severe underlying disease requiring specific therapeutic intervention.

The cohort of our study participants were asymptomatic malaria parasite carriers recruited from HIV-positive participants and the HIV-negative group. HIV- positive participants 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 participants 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 Ethics Committee of the University of Port Harcourt Teaching Hospital (UPTH) Port Harcourt, the Braithwaite Memorial Specialist Hospital (BMH), Port Harcourt and also the London School of Hygiene and Tropical Medicine, London.

2.1.6. Primary analysis

The study was designed to look at the association of the polymorphism at the *dhps* I431V with TS use, specifically

- Associations at enrolment between drug resistant *falciparum* and the use of medication for HIV treatment and prevention of opportunistic infection.
- Evidence of persistent parasitaemia at day 3 or day 28 being more common in HIV participants than in the HIV-negative control group.

2.1.7. Secondary analysis

- Any drug-drug interactions between ARVs and AL and if so does it have any impact on parasitaemia
- Any benefit of AL treatment of malaria-infected adults with HIV in terms of improved immunity based on CD4 cell count level

2.1.8. Sample size calculation

The study was designed based on carriage of parasite genetic markers of antimalarial drug resistance of the *dhps* gene. Sample size was calculated to be 229 of which 143 should be HIV-negative arm and 86 will be HIV-positive arm respectively. The calculation was based on the prevalence of the *dhps* I431V mutant found from the survey of clinical cases in Lagos, Nigerian (Sutherland, unpublished data). Based on the hypothesis that there will probably be an increased number of these mutations among HIV-positive people because of the use of TS for prophylaxis, and using a power of 80% to extrapolate with a 95% confidence interval, the estimated sample size was 86 for the HIV-positive group and 143 for the HIV-negative group. Our enrolment figure of 370 based on microscopy was higher than the calculated sample size

however the PCR confirmed number fell far below this number. Sample size calculation was not possible to calculate for the secondary analysis because there is no idea of parasitological impact of the drug in HIV-positive persons (or how effective the parasitological impact is in relationship to lumefantrine levels) since the study is exploratory to generate a first estimate of sample size which can be used to design other studies.

2.2.0 Materials for field work

2.2.1 Sample collection

- Cotton wool
- Methylated spirit
- Syringe and needles
- Lancet
- Glass slides
- EDTA Bottles
- Hand gloves
- RDT kit (First Response, PREMIER Medical Corporation Limited, Kachigam, Nani Daman (UT) 396 215. India
- RDT kit (Paracheck, Orchid Biomedical Systems, M46/47, Phase 111 B, Verna Industries Estate Verna, Goa 403 722, India
- Microscope
- Filter paper for PCR: Printed filtermat A Glass fibre filter size 102x258mm. WALLAC Oy. Turku Finland
- HIV Determine point-of-care test (Alere Medical Co. Ltd Matshuhidai-shi, Chiba, Japan).
- 10% w/v Giemsa stain prepared in buffered distilled water at pH 7
- Coartem (Novartis Pharma, Nigeria)

2.2.2. CD4 tests

- CD4 mAb PE (monoclonal antibody to human CD4)
- EDTA bottle

- No Iyse buffer
- Partech Cyflo Counter FCM System (Partech GmbH. Otto-Hahn-StraBe 32. D-4816 Munster. Germany).

2.2.3. WBC/PCV analysis

- Full automatic blood counter (PCE-201(N) ERMA INC. Tokyo)
- Haematology Autoanalyzer Model: MicroS 60 CS/CT 8 (Horiba ABX Diagnostics, France).

2.2.4. Lumefantrine concentration

- Liquid chromatography mass spectrometry (LCMS) Thermo Finnigan LCQ instrument
- 0.75M Tartaric acid (Fisher Scientific, UK)
- Filter paper (Glass microfibers paper, Fisherbrand FB59431, Fisher Scientific, UK)
- 20mM MeOH formate buffer, pH 2.7 (85:15 Fisher Scientific, UK)
- Dionex Acclaim® 120 3µm C18 (4.6 x 150 mm, with 120 Å pore size, fitted with a Nitrogen gas

2.3. Field work methods

Enrolled patients were treated with AL containing 20mg artemether and 129mg lumefantrine in a fixed dose combination ('Coartem ®'Novartis Pharma, Nigeria) according to manufacturer's dosing regimen of 4 tablets twice daily for 3days for persons with weight>35kg. Patients were advised to eat before taking the tablets. Most of the patients took their first dose under supervision at the site having been pre-informed to eat before coming. Subsequent doses were self-administered by participants with instructions to take the drug with food and also how to take the drug, the second dose 8 hours after, and the rest at 12hr interval for the next two days. A medication chart (Table 2.2) of how to take the drug was given to each participant on the day of enrolment. This was used to check for compliance on the follow up days. Compliance was found to be >95% of the participants. Patients were followed up till day 28 and were contacted by phone to remind them of coming for the sampling the following day in addition to ensuring they have taken their medication. Finger prick blood was collected on days 1,2,3,7 and 28 for blood spots on filter paper for PCR (Printed Filtermat Glass fibre filter size 102x258mm. WALLAC

Oy. Turku Finland), thick smears on days 1, 2, 3, and 28 and venous blood additionally on day 28 for CD4.

Table 2.1 Sampling table for each patient

	D0	D1	D2	D3	D7	D28
RDT	+					
Thick film	+	+	+	+	+	+
CD4	+					+
WBC	+			+		
Lume. Conc.					+	
Filter paper	+	+	+	+	+	+

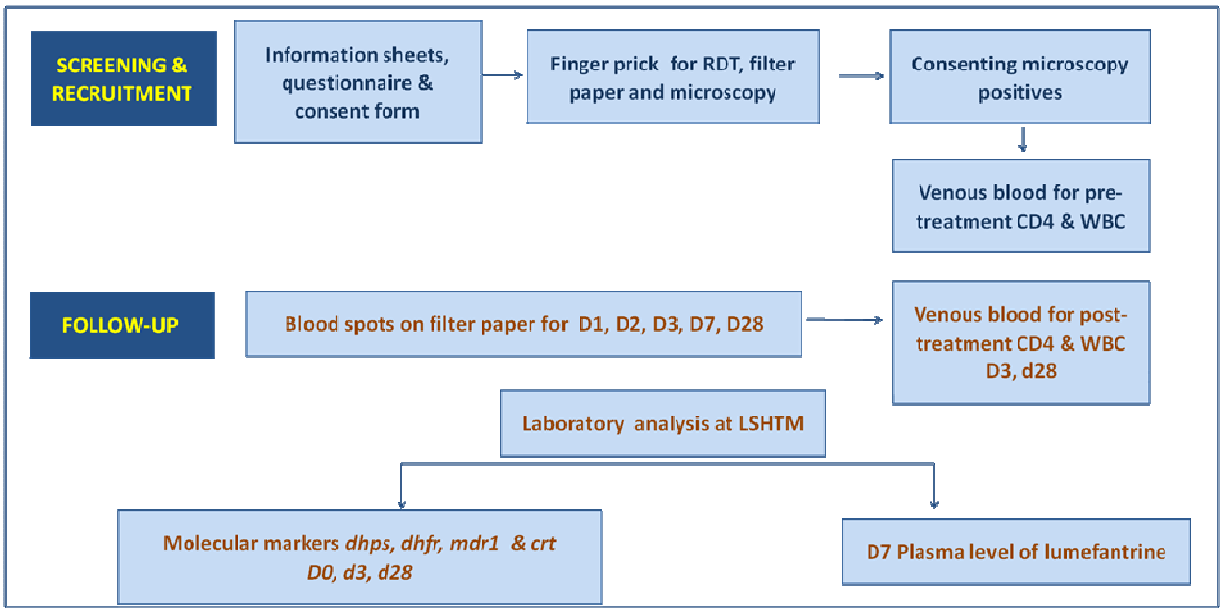


Fig.2.2. Flow chart of sampling method

Table 2.2 Patient's record of drugs taken

Days	Morning	Evening
D0		
D1		
D2		

2.3.1. Diagnosis of malaria (Microscopy)

The definitive diagnosis of malaria was based on the demonstration of plasmodium parasites in the blood. Immediately after collecting finger-prick blood samples, thick blood smears were prepared on two glass slides fixed in methanol and stained with 10% w/v Giemsa for 30minutes while patients were waiting. Initial microscopy was carried out by microscopists on the thick blood smear since low parasitaemia can be more readily identified by thick film. The stained slides were washed under slow running water, air dried on a slide rack and examined in oil immersion under light microscope at x100 magnification. Asexual parasites were counted against 200 white blood cells (WBCs). Parasite density was calculated as number of parasites per microlitre of blood by multiplying the number of parasites with 8000/µl (the assumed number of WBCs per microlitre of blood) and then dividing by 200 (Greenwood and Armstrong, 1991).

2.3.2. Diagnosis of malaria (RDT)

About 5µl of whole blood was added into the well of the RDT kit and two drops of the assay buffer was added into the well. Result was interpreted within 20 minutes based on the indication of the bands presented.

2.3.3. Determination of CD4 levels

CD4 cell count tests were carried out in Port Harcourt on venous blood collected from HIV-positive patients using the Partech Cyflo Counter FCM System (Partech GmbH. Otto-Hahn-StraBe 32. D-4816 Munster. Germany). Using a dry sterile syringe, 5mls of venous blood was collected from participants into EDTA (coagulant) bottles and mixed. Briefly, 20µl of whole blood were collected from the EDTA bottle and mixed with 20µl of CD4 mAb PE (Monoclonal antibody to human CD4) in a Partec test tube and mixed gently, then incubated at room temperature in the dark for 15 minutes. 800µl of no Iyse CD4 buffer was added and shaken gently. Blood sample was analysed using a Partec cyflow counter.

2.3.4. Evaluation of WBCs and PCV

The WBC count and PCV of the blood samples in the EDTA bottles were analysed in Port Harcourt using Haematology Autoanalyzer Model: MicroS 60 CS/CT 8 (Horiba ABX Diagnostics, France). Briefly, 10µl of blood was aspirated and delivered into the WBC chamber for first dilution. 28.3µl of diluted blood was delivered into the RBC chamber for the RBC/PLT measurement.

2.3.5. Evaluation of lumefantrine levels using Liquid chromatography mass spectrometry (LCMS)

Capillary blood samples were taken on day 7 from finger prick of participants. 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 (Blessborn et al., 2007). Briefly bloodspots were extracted in methanol / water (4:1; 350 µl), and the extracts were filtered through a cotton wool plug. Each sample (20µl) was separated on a Dionex Acclaim® 120 3µm

C18 (4.6 x 150 mm, with 120 Å pore size, fitted with a guard column) and eluted with ammonium formate (20mM, pH 2.7 and methanol (v/v; 85:15) isocratically at a flow rate of 500µl/min. The column temperature was maintained at 35°C. The electron spray ionisation (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. Lower limit of detection (LLOD) was determined to be 0.1µg/ml, lower limit of quantification (LLOQ) 1.0 µg/ml and upper limit of quantification (ULOQ) 20.0 µg/ml (Blessborn et al, 2007).

2.3.6 Assaying of Trimethoprim-Sulphamethoxazole (TS) using High Performance Liquid Chromatography (HPLC)

Filter paper adsorbed blood samples were analysed for trimethoprim and sulphamethoxazole using high performance liquid chromatography (HPLC) with photodiode array (PDA) detection. Separation and quantification of components of the formulations was achieved using a Dionex UltiMate 3000 HPLC system (Thermofisher, UK) with its built in software, Hertfordshire, UK. Separations were carried out utilising a GENESIS AQ 4 µm column (150 x 4.6 mm, Grace Materials Technologies, Cranforth, UK) eluting with ammonium formate (10 mM, pH 2.7) and acetonitrile (v/v; 17:83 to 25:75 down to 17:83 over 6.0 mins) at a flow rate of 1.5 ml/min, passing through the UV-photo-diode array detector (UV-PDA; DAD 3000) set at 275 nm.

Peak identity was confirmed by measuring the retention time, spiking the sample with commercially available standards and determination of absorbance spectra using the photo diode array detector (PDA). Chromatographic data was generated by Chromeleon (Dionex software) and typical chromatograms generated for the commercially available standards in blood adsorbed onto tartaric acid treated filter papers. Trimethoprim elutes at 4.11 min and sulphamethoxazole at 6.06 min.

2.4. Materials for molecular Biology Techniques

2.4.1. DNA extraction

- Nuclease –free molecular grade water (Sigma, Aldrich, UK)
- Phosphate Buffered Saline (1% PBS tablet) each tablet dissolved in 200mls distilled water

- 0.5% saponin (Sigma, Aldrich, UK 0.5g of saponin dissolved in 100mls water)
- 6% Chelex-100 (50% Glycine, N-(carboxymethyl), reaction products with chloromethylated divinylbenzene-styrene polymer+35-50% of water) (Sigma, Aldrich, UK). 6g chelex dissolved in 100ml water)

2.4.2. PCR

- Nuclease-free molecular grade water (Sigma, Aldrich, UK)
- 10x NH4 Buffer (Bioline, UK): 160mM $NH_4)_2SO_4$, 670mM Tris-HCl (pH8.8 at 25°C) and stabilizers
- 500mM KCl Buffer (Bioline, UK): 50 mM KCl, 100 mM Tris-Cl (pH 8.8 at 25°C), 15 mM MgCl2 and stabilisers.
- 50mM MgCl2 (Bioline, UK): 160mM $NH_4)_2SO_4$, 670mM Tris-HCl (pH8.8 at 25°C), 15 mM MgCl2 and stabilizers
- 10µM of each primer (Eurofin, MWG Operon Germany): 10µl of stock primer (100pM) +90 µl water
- 5 µM of each probe (Eurofin, MWG Operon Germany): 5 µl stock probe (100pM) +95 µl water
- 10µM of each dNTP (Bioline, UK): 10µl of stock primer (100pM) +90 µl water
- 1 Unit Biotaq DNA polymerase (Bioline, UK)
- 10µM of each probe (Eurofin, MWG Operon Germany): 10 µl stock probe (100pM) +90 µl water

2.4.3. Detection of amplified DNA

- 1.2%, 1.5% and 2% agarose gel (Sigma, Aldrich, UK): agarous in 100ml 5xTBE
- 5xTBE (Tris-Borate–EDTA Sigma,Aldrich, UK): Tris (hydroxymethyl)naminomethane + Boric acid

2.4. 4. Purification and sequencing of amplified DNA products

- 10x FastAP Buffer (Fermentas, USA): 100mM Tris-HCl (pH 8.0 at 37°C), 50mM $MgCl_2$, 1 M KCl, 0.2% Triton X-100 and 1mg/ml BSA
- FastAP (Fermentas, USA): Thermosensitive alkaline phosphatase, supplied in 20mM Hepes-NaOH (ph 7.4), 1mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1% Triton X-100 and 50% (v/v) glycerol

- Exo-1 (Fermentas, USA): Exonuclease-1 supplied in 20mM Tris-HCl (pH 7.5), 0.1Mm EDTA, 1Mm DTT and 50% (v/v) glycerol
- BigDye Terminator v3.1 (Applied Biosystems, UK)
- 5x BigDye Sequencing Buffer (Applied Biosystems, UK)
- 3M NaOAc (Sodium acetate Sigma Aldrich)
- Absolute Ethanol (Analytical grade, Fisher Chemicals, UK)
- 70% Ethanol (70ml absolute Ethanol Analytical grade, Fisher Chemicals, UK + 30ml water)
- Hi-Di formamide (Applied Biosystems, UK)
- 1.5ml sterilised microcentrifuge tubes.

2.4. 5. Reference samples used

Plasmodium falciparum samples used as positive controls were obtained from the Malaria reference Lab (MRL).

- 3D7,
- 7G8
- International standard (IS)
- Nuclease –free molecular grade water was used as negative control

2.5. Molecular analysis using polymerase chain reaction (PCR)

2.5.1. DNA extraction using Chelex method

DNA was extracted from filter papers (Glass fibre Printed filtermat filter size 102x258mm. WALLAC Oy. Turku Finland) containing patients' blood using the Chelex extraction method. Approximately 7mm disc of blood spots were cut from the filter papers using a Harris Uni-Core hole punch into 96-deep well plates. Aseptic technique was observed by flaming in between punching of each sample to avoid contamination. 1ml of 0.5% Saponin freshly prepared by dissolving Saponin in 1x Phosphate Buffer Saline (PBS) was used to soak the filter paper spots in

the deep well plates, covered with aluminium seal and incubated overnight at 37 ^{o}C to release haemoglobin into the PBS while parasite DNA remained on the filter paper. Plate was centrifuged at 4000 rpm for 2minutes. Saponin and debris were removed and 1ml of freshly prepared 1x PBS was added to each well. This was centrifuged again and then washed with 1xPBS until the filter paper was cleared of haem. 150µl of 6% Chelex 100 was added to each well and heat sealed with a foil then incubated in water bath for 30 minutes. Plate was centrifuged at 4000rpm for 2minutes to spin down the Chelex and the remaining filter paper. Approximately 120µl of DNA supernatant was removed into sterile pre-labelled plates and stored in-20^{o}C freezer.

2.5.2. DNA extraction using QIAamp DNA Mini kit)

In the bid to extract more and cleaner DNA, the QIAamp mini kit was used to extract DNA from some filter paper samples. Using a single-hole paper punch, 6mm circle was punched out from a dried blood spot into a 1.5ml microcentrifuge tube containing 180 µl of Buffer ATL and incubated at 85 ^{o}C for 10mins. This was centrifuged briefly to remove drops from inside the lid. 20 µl of proteinase kinase K stock solution, vortexed and incubated at 56 ^{o}C for 1 hour and centrifuged again to remove drops from inside the lid. 200 µl of buffer AL was added to the sample, vortexed to mix thoroughly and incubated at 70 ^{o}C for 10 minutes, then centrifuged. 200 µl of absolute ethanol was added to the sample, mixed thoroughly by vortexing and centrifuged to remove drops from inside the lid. The mixture was carefully applied to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and centrifuged at 600x g (8000rpm) for 1 minute. Mini spin column was placed in a clean 2ml collection tube while the tube containing the filtrate was discarded. QIAamp Mini spin column was carefully opened and 500 µl Buffer AW1 added without wetting the rim. Cap was closed and centrifuged at 600 x g (8000rpm) for 1 minute. The QIAamp Mini spin column was placed in a clean 2ml collection tube and the collection tube containing the filtrate discarded. QIAamp Mini spin column was carefully opened and 500 µl Buffer AW2 added without wetting the rim. Cap was closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes. Column was placed in a new 2ml collection tube discarding the old collection tube containing the filtrate. This was centrifuged at full speed for 1 minute. QIAamp Mini spin column was placed in a clean 1.5ml Buffer AE or distilled water and incubated at room temperature for 1 minute,

then centrifuged at 6000 x g (8000rpm) for 1 minute. The eluate containing the extracted DNA was stored in -20^{o}C freezer.

2.5.3. Designing of primers

New Primers were designed for some samples where amplification was not possible with the regular primers using PCR Primer Design tool, (Eurofin, MWG Operon Germany). Primers flanking codon 1246 of the *pfmdr* gene and codons 431 and 540 of the *pfdhps* gene were designed. Gradient PCR was performed using standard *P. falciparum* controls 3D7 in order to ascertain the best conditions for the PCR. Primers were run at different annealing temperatures and PCR conditions. The primer that gave the best and clearer band on the UV agarose gel was chosen with the conditions used and was then adapted as the choice primer for the PCR. Primer sequences are listed in Table 2.1 below.

2.5.4. Amplification of DNA products using primers and Nested PCR

PCR amplifications were carried out to investigate the prevalence of parasites carrying molecular markers of resistance and/or tolerance before and following AL treatment in both the HIV-positive and the HIV-negative cohort. The various genes were analysed in the samples using nested PCR since nested PCR is much more sensitive for confirmation of *Plasmodium falciparum* parasitaemia infection (Snounou et al., 1993b). Different primers were employed for these reactions for both the nested, heminested and the unnested PCR reactions and purchased from Eurofins MWG, Operon Germany.

2.5.5. PCR protocol for small subunit ribosomal RNA (SSU rRNA) gene amplification

Amplification of the SSU rRNA gene of the *Plasmodium falciparum* was carried out on extracted DNA to assess the positivity of *P. falciparum* (Snounou et al., 1993a). Briefly described, 0.5 µl each of primers rplu5new and rplu6 (Eurofins, MWG Operon, Germany), consisting of primer mix that could be used to detect any of the 5 Plasmodium species were added into a 20 µl reaction mixture consisting of Nuclease-free water, 10.5 µl, 2.0µl 10x NH4 Reaction Buffer (Bioline, UK), 0.8 µl 50 mM $MgCl_2$, 0.5 µl 10mM dNTPs (Bioline, UK), 0.20µl BioTaq DNA Polymerase (Bioline, UK). 5 µl of DNA was added to each reaction mixture. PCR reactions was carried out under conditions and primers listed in Table 2.1 below. The amplified product was

nested using primers rfal1 and rfal2 in order to identify the *Plasmodium falciparum* species. The nested reaction consists of 14.5µl of water, 2.0µl 10x NH4 Reaction Buffer (Bioline, UK), 0.8 µl 50 mM $MgCl_2$, 0.5 µl 10mM dNTPs (Bioline, UK), , 0.5 µl each of primers rplu5new and rplu6 (Eurofins, MWG Operon, Germany), 0.20µl BioTaq DNA Polymerase (Bioline, UK) and 1 µl of nest 1 amplified product. PCR conditions and primer sequences used are listed in Table 2.1

2.5.6. PCR protocol for *pfmdr1* gene at codons 86, 184, 1034, 1042 and 1246.

Using nested PCR and employing outward and inward primers, DNA samples extracted on days 0, 3 and 28 samples that were positive by microscopy were screened for polymorphisms at codons 86, 184, 1034 and 1042 of the *pfmdr1* gene. The analysis were carried out on both pre and post treatment samples. Each unnested reaction mixture consisted of Nuclease-free water, 14.8 µl, 2.5µl 10x NH4 Reaction Buffer (Bioline, UK), 1.0 µl 50 mM $MgCl_2$, 0.5 µl 10mM dNTPs (Bioline, UK), 0.5 µl 10µM Forward Primer (Eurofins, MWG Operon, Germany), 0.5 µl 10µM Reverse primer (Eurofins, MWG Operon, Germany), 0.20µl BioTaq DNA Polymerase (Bioline, UK). 5 µl of DNA was added to each reaction mixture. However in the nested reaction, 18.8 µl of water was used and 1 µl of nest 1 amplified product. PCR conditions and primer sequences are listed below.

The outward primers FN1 and REVC1 and nested primers MDR2/1 and NEWREV1 (Humphreys et al., 2007). were used to screen for the polymorphisms at codons 86 and 184 while outer primers MDRF2F1 and MDRF2R1 and nested primers MDRF2F2 and MDRF2R2 (Humphreys et al, 2007) were employed for the screening of mutations for the region flanking 1034 and 1042.

Polymorphisms at codons 1246 were screened using the designed fragment 4 primers (F4N1(F), F4N1(R), F4N2(F) F4N2(R) as described above in addition to the primers MDRF4N1 and MDRF4R1 and MDRF4N2 (Dlamini et al., 2010). This was necessary because amplification was difficult in some samples due to very low parasitaemia since the patients were asymptomatic. Primers F4N1 (F) and F4N2(R) were used for the primary reaction while F4N2 (F) and F4N2(R) were employed for the nested reaction.

The primary reaction mixture consisted of 13.8 µl of nuclease-free water, 2.5µl 10x NH4 Reaction Buffer (Bioline, UK), 1.0 µl 50 mM $MgCl_2$, 0.5 µl 10mM dNTPs (Bioline, UK), 1.0 µl 10µM Forward Primer (Eurofins, MWG Operon, Germany), 1.0 µl 10µM Reverse primer

(Eurofins, MWG Operon, Germany), 0.20µl BioTaq DNA Polymerase (Bioline, UK) and 5 µl of DNA was added to each reaction mixture. 5 µl of DNA was added to each reaction mixture. However in the nested reaction, 17.8 µl of water was used instead and 1 µl of nest 1 amplified product. PCR conditions and primer sequences are listed below.

2.5.7. PCR protocol for *pfdhps* gene

Due to the low parasitaemia among our study participants and poor quality of DNA, various protocols were used in order to get a good yield of high quality DNA. DNA extracted from day0 samples were screened using different protocols and primers for the *dhps* gene for mutations flanking codons 431, 436, 437, 540, 581 and 613.

2.5.7.1. Protocol 1

A 25 µl reaction mixture containing, 14.8µl Nuclease-free water, 2.5µl 10x NH4 Reaction Buffer (Bioline, UK), 1.0 µl 50 mM $MgCl_2$, 0.5 µl 10mM dNTPs (Bioline, UK), 0.5 µl 10µM Forward Primer DHPSN1, 0.5 µl 10µM Reverse primer DHPSN2 (Pearce et al, 2003) 0.20µl BioTaq DNA Polymerase (Bioline, UK), and 5 µl of DNA was prepared for the primary PCR. The nested PCR mixture consisted of 18.8 µl Nuclease-free water and 1 µl of the nest 1 product with the forward primer DHPSR2 and the reverse primer DHPSR/ (Pearce et al, 2003).

2.5.7.2. Protocol 2

Primary PCR mixture consisted of 2.5 µl of DNA instead of the regular 5 µl, 15.3 µl nuclease-free water, 2.5 µl 10X KCL, 2.5 µl 2mM dNTPs 1.0 µl 10µM Forward Primer DHPSN1, 1.0 µl 10µM Reverse primer DHPSN2 (Pearce et al., 2003), 0.20µl BioTaq DNA Polymerase 5U/ µl (Bioline, UK). The nest 2 PCR reaction mixture consists of 16.8 µl water and 1.0 µl nest 1 product, 1.0 µl 10µM each of primers DHPSR2 and DHPS R/ in addition to the other materials as in nest1. Primer sequences and cycling conditions are listed below.

2.5.7.3. Protocol 3

The *dhps* gene was split into two fragments for the samples with very low DNA. Primers were designed to capture the polymorphisms at codons 431 and 540 respectively. Primary PCR mixture for the amplification for the region flanking codon 431 consisted of 14.8 µl Nuclease-

free water, 2.5µl 10x NH4 Reaction Buffer (Bioline, UK), 1.0 µl 50 mM $MgCl_2$, 0.5 µl 10mM dNTPs (Bioline, UK), 0.5 µl 10µM Forward Primer DHPSV1F, 0.5 µl 10µM Reverse primer DHPSV1R, 0.20µl BioTaq DNA Polymerase (Bioline, UK). 5 µl of DNA was added to each reaction mixture. However the second reaction was heminested since we used the same reverse primer DHPSV1R while the forward primer was DHPSV2F and consists of 18.8 µl of water and 1 µl of nest 1 amplified product for the 431 protocol. The 540 protocol reaction mixture for the primary reaction consists of 13.8µl Nuclease-free water, 2.5µl 10x NH4 Reaction Buffer (Bioline, UK), 1.0 µl 50 mM $MgCl_2$, 0.5 µl 10mM dNTPs (Bioline, UK), 1.0 µl 10µM Forward Primer DHPS540F1, 1.0 µl 10µM Reverse primer DHPS540R1, 0.20µl BioTaq DNA Polymerase (Bioline, UK) and 5 µl of DNA. The secondary reaction was also heminested consisting of forward primer DHPS540F, DHPS540R2 and 1.0 µl nest 1 product in addition to the other components. The primer sequences and cycling conditions are listed below Table 2.2.

2.5.8. Protocol for the *pfdhfr* gene

Primers DHFRM1 and DHFRM7 were employed for the detection of polymorphisms on the *dhfr* gene for the primary reaction, while DHFRM3 and DHFRM9 (Pearce et al., 2003) were used for the nested PCR. 25 µl of reaction mixture was prepared for both reactions but primary mixture contained 14.8 µl nuclease free water and 5 µl DNA, while the nested contained 17.8 µl and 1 µl DNA from nest 1 amplicon. Additionally, both mixtures consisted of 2.5µl 10x NH4 Reaction Buffer (Bioline, UK), 1.0 µl 50 mM $MgCl_2$, 0.5 µl 10mM dNTPs (Bioline, UK), 0.5 µl 10µM Forward Primer, 0.5 µl 10µM Reverse primer, 0.20µl BioTaq DNA Polymerase (Bioline, UK). Cycling conditions and primer sequences are listed in Table 2.2.

2.5.9. Genotypying of *pfcrt*

Due to the low parasitaemia of the samples, the DNA samples were first nested before using a multiplex double-labelled probe real time amplification method to analyse the samples. Employing forward primer CRTP1 and reverse primer CRTP2, *pfcrt* gene was amplified at 5' untranslated region (UTR) up to 88 to detect the wild type haplotype CVMNK and the other haplotypes CVIET and SVMNT commonly found at codons 72-76 on the *pfcrt* gene. A 25 µl reaction mixture consisting of 12.8 µl Nuclease-free water, 2.5µl 10x NH4 Reaction Buffer (Bioline, UK), 1.0 µl 50 mM $MgCl_2$, 2.5 µl 10mM dNTPs (Bioline, UK), 0.5 µl 10µM Forward Primer 0.5 µl 10µM Reverse primer, 0.20µl BioTaq DNA Polymerase (Bioline, UK), and 5 µl of

DNA was prepared for the primary PCR. Protocol and primer sequences are listed below in Table 2.2.

2.5.9.1. Real time PCR (qPCR) for *pfcrt* gene

Polymorphisms at codons 72, 74, 75 and 76 of the *pfcrt* genes were assessed using real time PCR protocol, primers and probes as described by (Gadalla et al., 2010). Briefly, a 25 µl master mix of 0.5µl each of probes FAM, JOE and ROX corresponding to haplotypes CVMNK, CVIET and SVMNT respectively, 0.375 µl primers CRTD1 and CRTD2, 15.55 µl nuclease-free water, 2.5µl 10x NH4 Reaction Buffer (Bioline, UK), 2.75 µl 50 mM $MgCl_2$, 0.5 µl 10mM dNTPs (Bioline, UK), 0.20µl BioTaq DNA Polymerase (Bioline, UK), and 1 µl of the nest 1 product was prepared for the qPCR. Three DNA positive samples 3D7, 7G8 and DD2 were used to provide the corresponding sequence desired for control. Nuclease-free water was used as negative control. 0.1ml strips of 4 tubes (Rotorgene) were used for the reaction. Tubes were inserted on a metal rack of 72 wells and placed on ice to prevent the fluorescence of the probes. Into each 0.1ml tube (Rotorgene) were added 24 µl of reaction mixture and subsequently 1 µl of nest 1 product. Each sample was duplicated to ensure accurate result. Reaction was carried out on the Rotorgene RG 3000 (Corbett Research, Australia). The cycle threshold (C_T) was set above the negative control and in the middle of the exponential phase of the positive controls. Then the C_T was determined for each sample accordingly (Gadalla et al, 2010). The probes and conditions used for the reaction are listed in Table 2.4.

2.6. Gel Electrophoresis of DNA amplification products

The amplified products were separated and visualized under gel electrophoresis using ethidium bromide. Different percentage agarose (Sigma, Aldrich, UK) gels depending on the size of the product being expected were prepared in Tris-Borate (TBE) and EDTA solution. This was stained with ethidium bromide (Sigma, Aldrich, UK) in order to identify the amplified bands of the DNA and poured into a gel tank. 5µl of each sample of DNA was mixed with 5µl of 5X loading buffer and loaded into the wells and ran along with hyperladder IV (Bioline, UK) to estimate the band size. After migration, the DNA was visualised by placing the gel under ultraviolet (UV) illuminator and the images were captured and recorded.

2.7. Purification of PCR products

Using EXOFAP (Fermentas, UK) protocol, products from the nested PCR were cleaned to remove the impurities and products like excess primers, dNTPs that could interfere with the sequence product. A 5µl mixture containing FasTAP buffer, FasTAP and Exo-1 nuclease prepared in proportions as specified in the protocol, was added to 5µl of nest 2 product in a PCR plate, vortexed briefly and reaction was carried out in the thermocycler at 37°C for I hour and 72°C for 15minutes.

2.8. Sequencing of PCR products

Amplified PCR products were sequenced using the BIG DYE sequencer protocol ABI PRISM Applied Biosystems as described below. Briefly, cleaned PCR products were directly sequenced in a bar-coded plate with 5x BigDye sequencing buffer and Big Dye^{TM} Terminator v3.1 in a mixture that includes double distilled water, either forward or backward primers with the BigDye and 5x BigDye sequencing buffer, (two reactions were prepared for each sample). The samples were run in a thermocycler machine using the BigDye protocol under the following conditions: 26 cycles 95°C for 0.30min, ramp 1 °C per second to 50.0°C, 55°C for 0.15min, ramp1 °C per second to 60.0°C, 60 °C for 4.0 min and , ramp1 °C per second to 96.0°C.

Following the BigDye reaction, a post-sequencing clean-up was carried out by preparing a mixture of double distilled water (24.5µl), absolute ethanol (62.5µl) and 3M sodium acetate (3.0µl). 90µl of this mixture was added to each 10µl reaction mixture in the 96-well plate, vortexed briefly and left in the fridge for 20 minutes to allow the extension products to precipitate. This was centrifuged at 4000rpm for 30 minutes at 4°C. The plate was inverted onto a blue paper roll after removing seal and then drained quickly in the centrifuge at 50rpm for 1minute. 150 µl of ice cold 70% ethanol was added and plate was covered with PCR seal and inverted five times then centrifuged again at 3000rpm for 10minutes at 4°C. Further draining of plate was done again inverted on a blue paper roll and centrifuged at 50rpm for 1 minute. Plate was removed and allowed to dry on the table. 10 µl of HiDi formamide (Applied Biosystems, UK) was added to each well. Plate was sealed and stored at -20°C and submitted for sequencing with ABI PRISM 3730 Automated Sequencer. Sequences were examined using CHROMAS software version 1.6.1 (Technelysium, Australia). Both forward and reverse sequences were

viewed and aligned using ClustalW software and the corresponding 3D7 gene from PlasmoDB.org as a standard reference clone to detect any mutation in the gene.

2.9. Parasite clearance time (PCT)

Using real time quantitative method, parasite clearance time was measured for some samples. Briefly described, a 20 µl master mix consisting of 0.2µM each of the probes (MWG-Biotech), 0.3 µM forward and reverse primers (Beshir et al., 2010b), 5.5mM MgCl2 buffer (Bioline), 0.3 µM dNTPs (Bioline), 10x NH4 buffer (Bioline), 1 unit of Biotaq DNA polymerase (Bioline), nuclease-free water (Sigma-Aldrich) and 5 µl DNA was added into tubes inserted on a metal rack of 72 wells and placed on ice to prevent the fluorescence of the probes. Parasite negative blood and nuclease-free water were used as negative control while the positive control was INT parasite DNA. The reactions were run in triplicates on a Rotorgene RG 3000 (Corbett, Australia) under the following conditions 95°C for 6minutes, 40 cycles of 95°C for 15secs and 60°C for 1 min. The probes are listed in Table 2.4.

2.10 Statistical Analysis

All data collected were entered into spread sheets and analysed in STATA version 11 (Stata Corp., Madison WI). Continuous variables that were normally distributed were analysed using student t-test while those that were not normally distributed and continuous data were analysed using Wilcoxon's rank sum test. Also categorical comparisons in 2-by-2 format were performed using the χ^2 distribution and Fischer's exact test where any value was less than 5. Mutations on the different genes were analysed to evaluate the associations on the alleles and the haplotypes using 95% CI around the odd ratios (OR). P-values below 0.05 were considered significant.

Table 2.3 PCR primer sequences and reaction conditions for the nested amplification of *pfdhps, pfdhfr, pfcrt* and *pfmdr1* genes.

Gene	Primers	Sequence	PCR product size	PCR conditions
pfcrt 72-76	Forward CRTP1	5'-CCGTTAATAATAAATACACGCAG--3'	546	94°C for 3mins / (94°C for 30s, 56°C for 30s, 62°C for 60s) x35cycles/ 62°C/5mins
	Reverse CRTP2	5'- CGGATGTTACAAAACTATAGTTACC-3'		
pfcrt 72-76	Forward CRTD1	5'-AGGTTCTTGTCTTGGTAAATTTGC-3'	164	95°C for 6min / (95°C for 15s – 55°C for 60s) x40cycles
	Reverse CRTD2	5'-CAAAACTATAGTTACCAATTTTG-3'		
pfmdr1 fragment1 86 and 184	Outer forward FN1/1	5'-ACA AAA AGA GTA CCG CTG AAT-3'	578	94°C for 3min / [94°C for 30s – 55°C for 30s – 65°C for 1 min] x30cycles / 65°C for 5min/15°C for 5min
	Outer reverse REV/C1	5'-AAA CGC AAG TAA TAC ATA AAG TC-3'		
Nested Frag 1	MDR2/1	5'-ACA AAA AGA GTA CCG CTG AAT-3'	534	94°C for 3min / [94°C for 30s – 55°C for 30s – 65°C for 1 min] x30cycles / 65°C for 5min/15°C for 5min
	NEWREV1	5'-AAA CGC AAG TAA TAC ATA AAG TC-3'		

Gene	Primers	Sequence	PCR product size	PCR conditions
primary Frag 3 1034, 1042	MDRF3N1	5'-GCA TTT TAT AAT ATG CAT ACT G-3'		94°C for 3min/[94°C for 30s-56°C for 60s-65°C for 50s]x30cycles/65°C for 5min/15°C for 5min
	MDRF3R1	5'-GGA TTT CAT AAA GTC ATC AAC-3'		
Nested Frag 3	MDRF3N2	5'-GGT TTA GAA GAT TAT TTC TGT A-3'		Same as primary
	MDRF3R1	5'-GCA TTT TAT AAT ATG CAT ACT G-3'		
Primary Frag 4 1246	MDRF4N1	5'-CAA ACC AAT CTG GAT CTG CAG-3'		94°C for 3min/[94°C for 30s-53°C for 60s-65°C for 40s]x30cycles/65°C for 5min/15°C for 5min
	MDRF4R1	5'-CAA TGT TGC ATC TTC TCT TCC-3'		
Nested Frag 4 1246	MDRF4N2	5'-GAT CTG CAG AAG ATT ATA CTG-3'	194	94°C for 3min/[94°C for 30s-55°C for 60s-65°C for 40s]x30cycles/65°C for 5min/15°C for 5min
	MDRF4R1	5'-CAA TGT TGC ATC TTC TCT TCC-3'		
Primary Frag 4 1246	F4N1(F)	5'- CAG CAA TCG TTG GAG AAA CAG	397	94°C for 3min/[94°C for 30s-55°C for 60s-65°C for 40s]x30cycles/65°C for 5min/15°C for 5min
	F4N1(R)	5' GCA GCA AAC TTA CTA ACA CG		
Nested Frag 4 1246	F4N2(F)	5' CAAACCAATCTGGATCTGCAGAAG		Same as primary
	F4N2(R)	5'-CAA TGT TGC ATC TTC TCT TCC-3'		

Gene	Primers	Sequence	PCR product size	PCR conditions
pfdhps 431,436,437, 540, 613	DHPSN1	5'- GATTCTTTTTCAGATGGAGG-3'	770	93°C for 5min/[93°C for 20s – 55°C for 30s–68°C for 75s] x40cycles / 65°C for 5min
	DHPSN2	5'- TTCCTCATGTAATTCATCTGA-3'		
	DHPS-R1	5'-AACCTAAACGTGCTGTTCAA-3'	711	93°C for 5min/[93°C for 30s – 56°C for 30s–68°C for 75s] x 30cycles / 72°C for 5min
Nested	DHPS-R/	5'-AATTGTGTGATTTGTCCACAA-3'		
431, 436, 437	DHPSV1F	5'-GCTTAAATGATATGATACCCG-3'	369	95^0C for 3min/[95°C for 30s-50°C for 30s-65^0C for 60s]x 39 cycles/65°C for 1min
	DHPSV1R	5'- CAGGTACTACTAAATCTCTTT-3'	135	
	DHPSV2F	5'-AACCTAAACGTGCTGTTCAA-3'		Same as primary
540, 580, 613	DHPS 540F1	5'- GAG GAA ATC CAC ATA CAA TG-3'	474	95^0C for 3min/[95°C for 30s-48°C for 45s-72^0C for 60s]x 39 cycles/72°C for 1min
	DHPS 540R1	5'- CGT CAT GAA CTC TTA TTA GA-3'		
	DHPS540R 2	5'-TCC TCA TGT AAT TCA TCT G-3'	372	95^0C for 3min/[95°C for 30s-48°C for 45s-72^0C for 60s]x 29 cycles/72°C for 1min
	DHPS 540F1	5'- GAG GAA ATC CAC ATA CAA TG-3'		
pfdhfr 50,51,59,108, 164	DHFR_M1	5'-TTTATGATGGAACAAGTCTGC-3'	650	93°C for 5min / (94°C for 30s – 54°C for 60s – 65°C for 60s] x41cycles/ 65°C for 5min/15°C for 5min
	DHFR_M7	5'-CTAGTATATACATCGCTAACA-3'		
	DHFR_M9	5'-CTGGAAAAAATACATCACATTCATATG-3'	594	95^0C for 5min/[93°C for 30s-56°C for 30s-68^0C for 75s]x 30 cycles/75°C for 5min

Gene	Primers	Sequence	PCR product size	PCR conditions
	DHFR_M3	5'- TGATGGAACAAGTCTGCGACGTT-3'		
Snounou	rPLU5new	5'- CYTGTTGTTGCCTTAAACTTC-3'		95^0C for 5min/[58^oC for 2min-72^oC for 2min-94^0C for 1min]x25 cycles/58^oC for 2min-72^oC for5min
	rPLU6	5'- TTAAAATTGTTGCAGTTAAAACG-3'		
	rFAL1	5'-TTAAACTGGTTTGGGAAAACCAAATATATT-3'	1200	95^0C for 5min/[58^oC for 1min-72^oC for 1min-94^0C for 1min]x30 cycles/58^oC for 1min-72^oC for5min
	rFAL2	5'- ACACAATAGACTCAATCATGACTACCCGTC-3'	205	

Table 2.4 Probes used to amplify *pfcrt* haplotypes *CVMNK, CVIET* and *SVMNT*

Primer/Probe	**5' Fluorophore**	**Sequence**	**3' Quencher**
crtCVMNK	FAM	TGT GTA ATG AAT AAA ATT TTT GCT AA	BHQ2
crtCVIET	JOE	TGT GTA ATT GAA ACA ATT TTT GCT AA	BHQ2
crtSVMNT	ROX	AGT GTA ATG AAT ACA ATT TTT GCT AA	BHQ2

Table 2.5 Primers and probes of gene fragments of HummTuBB used for the PCT

Primer/Probe	Sequence	Strand	Length	Primer Tm	Modification
HumTUUB_F1	TACATACCTTGAGGCGAGCA	forward	20	59.44	
HumTUUB _R1	GATCTGGTTGCCACATTGAC	reverse	20	58.94	
HumTUUB _P1	TGGATGTGCACGATTTCCCTCA	reverse	22	68.04	5'Fam - 3'Tamra
HumTUUB _F2	AAGGAGGTCGATGAGCAGAT	forward	20	58.85	
HumTUUB _R2	GCTGTCTTGACATTGTTGGG	reverse	20	59.14	
HumTUUB _P2	TTAACGTGCAGAACAAGAACAGCAGCT	forward	27	68.08	5'Joe - 3'Tamra
HumTUUB _F3	CACTTTCAAGGGAGGTGTCA	forward	20	58.69	
HumTUUB _R3	CAGATGGGACAGATCCTCCAG	reverse	20	59.05	
HumTUUB _P3	TCCATTTCAATCTCCCTCCAAGCTCT	forward	26	68.14	5'Rox - 3'Tamra

Chapter Three

Treatment of asymptomatic malaria-infection inHIV-positive and HIV-negative adults in Port Harcourt, Nigeria

3.1 Rationale

Artemether-lumefantrine (AL), the currently recommended therapy for malaria treatment, was introduced in Nigeria in 2005 as the first line regimen for uncomplicated malaria. Its use in Nigeria goes beyond prescription by physicians since the drug is easily obtained without prescription over the counter from pharmacies and medicine stores. Various studies (Falade et al., 2005, Falade et al., 2008, Happi et al., 2009) on the use of artemether-lumefantrine (AL) in the treatment of uncomplicated malaria in Nigeria have been reported all of which have established an association of either treatment failure or slow clearance of parasitaemia. As at date and time of our study, none of these studies involved the inclusion of HIV-subjects who are known to harbor more resistant parasites due to the increased drug pressure (Whitworth et al., 2000, French et al., 2001).

The overlap of malaria and HIV in sub-Saharan Africa is a major public health challenge and management of the co-infection may be compounded by potential drug-drug interactions that could result in the co-administration of antimalarials and antiretrovirals. Kredo et al, (2011) established an elevated lumefantrine level in HIV-infected adult volunteers receiving nevirapine who were not infected with malaria parasite but were treated with AL indicating an interaction between nevirapine (NVP) and AL. This report though very interesting has not been confirmed in malaria infected patients. There is paucity of data on AL malaria treatment of HIV-positive patients and this is particularly of importance in Nigeria especially in Rivers state, Niger Delta where there is a high prevalence (6.0%) of HIV seropositivity compared to the national prevalence of 4.1%. The study was designed to address this paucity and establish a current status of markers to resistance of AL among this vulnerable group of people in this very important region of the country.

Asymptomatic-malaria infection is very common in adults in endemic regions due to acquired immunity as a result of continued exposure to malaria infection. These asymptomatic *P. falciparum* carriers do not seek medical treatment and as such act as fundamental reservoirs of parasites thereby contributing to the persistent transmission of malaria (Zoghi et al., 2012). They remain infective longer than the treated symptomatic patients and the infections are 4-5

times more prevalent (Alves et al., 2005). Asymptomatic-malaria parasitaemia carriage is prevalent in sub-Saharan Africa (Bottius et al., 1996, Smith et al., 1994) and persons with long

term carriage are found in the population even during periods of low level transmission (Roper et al., 1996, Zwetyenga et al., 1999).

Diagnosis of asymptomatic-malaria is not standardised therefore the most widely used method is the identification of parasite in peripheral thick blood smears and the absence of clinical symptoms (Leoratti et al., 2008).

In clinical practice especially in sub-Saharan Africa, testing, treating and tracking of malaria is limited to symptomatic cases which are the clinical disease states hence majority of the antimalarial drugs used are targeted at the erythrocytic stage of the parasite. However some of these drugs have gametocytocidal properties. Therefore adequate action against the persistence of malaria in endemic regions should include the administration of gametocytocidal drugs as well as the incorporation of the LLINs, IRS and ITNs (Laishram et al., 2012). There is need to identify, treat and control asymptomatic-malaria which may trigger clinical disease and reduce immunity of the HIV patients thereby making them more susceptible to infections.

It has been suggested that community screening and treatment of asymptomatic-malaria with AL may reduce malaria transmission significantly (Kern et al., 2011) and treatment of the infective parasite reservoir of asymptomatic individuals may be an important intervention strategy (Laishram et al., 2012) and could also be a means of reducing malaria-associated morbidity (O'Meara et al., 2006). Though the immediate benefit of treating asymptomatic-malaria parasite carriers with AL is reduction of transmission, an additional benefit is that there may be fewer cases of clinical malaria in children below five years of age (Ogutu et al., 2010).

3.2 Asymptomatic-malaria infection in Nigeria

There are few studies on asymptomatic-malaria infection in Nigeria, and many of the studies are mostly in children and pregnant women. An earlier study in south-Western Nigeria reports, 26.5% prevalence (Salako et al, 1990), while a 22.5% prevalence was reported among school children in Kaduna, Northern Nigeria (Orogade et al, 2002) closely related to the 27.5% among school children in Port Harcourt (Jeremiah et al, 2007). However a study (Eke et al, 2006) reported a prevalence of 33.1% among adults in Aba, a town about 69 kilometres north of Port Harcourt. A more recent study (Otajevwo, 2013) among University students in mid-Western

Nigeria reports a very high prevalence of 76.8% and it is closely related to the 83.3% result obtained also among undergraduates in a Lagos university (Okwa and Ibidapo, 2010), and 80.4% in Abia state (Kalu et al, 2012). These later reports are quite alarming however the diagnosis was based on microscopy, which could have resulted in many false positives. This underlines the importance and relevance of the study of asymptomatic-malaria and its contribution to the spread of malaria in Nigeria, thereby necessitating the need for the treatment of asymptomatic-malaria infection.

The control of malaria in any endemic area would entail not only scaling up interventions in the areas of vector control but there is also need to prevent selective transmission of parasite resistant strains through the administration of drugs that can minimise gametocyte emergence (Sutherland et al., 2005). AL is a drug with known gametocytocidal properties and it is the first-line drug for the treatment of uncomplicated malaria in Nigeria hence the choice of its use in the study. This is an unmatched case-control study comparing HIV-positive adults attending ARV treatment clinic to HIV-negative healthy volunteers drawn from the hospital and University community comprising both staff and students but not hospital patients who were confirmed negative by HIV rapid serological test. Volunteers from both groups who were positive for *P. falciparum* by microscopy were treated with AL. The purpose of the study was to evaluate the antimalarial effect of AL treatment on asymptomatic *P. falciparum* infection in both groups of people in addition to the assessment of the molecular markers associated with AL. In this chapter, the parasitological study groups are described and compared and parasitological outcomes on day 3 and day 28 are presented. The results of the study will provide preliminary data that could form a basis for the assessment of drug resistance and treatment failure and could be contributory to the malarial control measures in Port Harcourt Nigeria.

3.3 Results

Patients who visited both hospitals came from all over the Niger Delta region since both are the two largest hospitals in the state. Finger prick blood samples were used for RDT and also to prepare thick film on Giemsa stained glass slide. Parasite count was made by counting the number of asexual forms of the parasites relative to number of WBCs assuming total WBCs of 8000/µl based on parasites counted over 100 high power visual fields. The results of the RDT

showed only 10% (46/459) positive. Using microscopy as the standard procedure for diagnosis, a total of 459 participants were screened for the study. Out of this number, 314 HIV-negative people were screened and 242 of these met the inclusion criteria and were found positive for *P.falciparum* and therefore recruited into the study.

In the HIV-positive arm of the study, 145 people with previously confirmed HIV infection attending either the UPTH or the BMH HIV adult clinics were screened for malaria by microscopy and 128 of these were found positive for *P. falciparum* and were recruited having met the inclusion criteria. The total number of people enrolled in the study from both hospitals based on positive microscopy was 370/459 (81%). A total of 459 people were screened on both arms. 238 people completed the study up to the 28 day follow-up as shown in the flow chart (Fig. 3.1). The baseline characteristics of study population are summarised in Table 3.1.

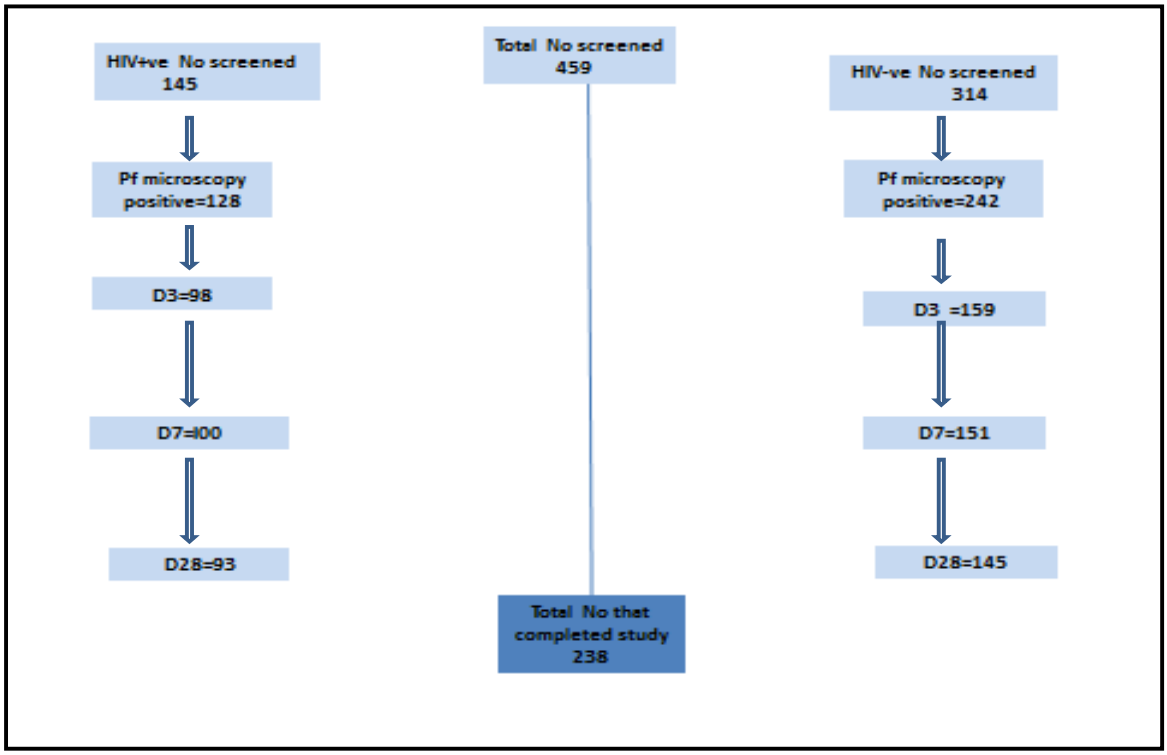


Figure 3.1 Flow chart of the study

Table 3.1 Baseline characteristics of study population

	HIV-positive	HIV-negative	Total
No. screened	145	314	459
MP positive	128	242	370
Mean PCV (%)	34.44 (16-50, SD:6.13)	37.09 (23-50, SD:5.32)	36 (16-50, SD:5.8)
Mean WBC ($x 10^9$/l)	4.74 (1.4-16.8, SD:1.9)	4.62 (1.3-10.9, SD:1.29	4.66(1.3-16.8, SD: 1.54)
Mean age (yrs)	37 (22-66) n=119	37 (17-65) n=225	35 (n=344)
Mean CD4 d0 (Cells/mm^3)	413 (32-1593, SD:253) N=96		
Mean Wt (Kg)	66.3 (40.92) SD:1.3, n=77	67.5 (25-100) SD:12.8 n=146	67 (25-100, SD:12.2) n=224
Mean parasite density (parasites/µl)	2530 (SD: 640.7, n=128)	1229 (SD: 180.1, n=242).	

3.3.1 Demographic and questionnaire data

Our cohorts of patients were from the two largest hospitals in Rivers State. Family and social history of the participants were obtained from the self-administered questionnaires given. The

results of the various pieces of information obtained were analysed using Excel and STATA and are presented below with the use of histograms.

3.3.1.1. HIV and Sex distribution

Analysis of the questionnaire data, our observation is that a higher proportion of our study subjects were female from the demography of the study as shown in Fig. 3.2 below.

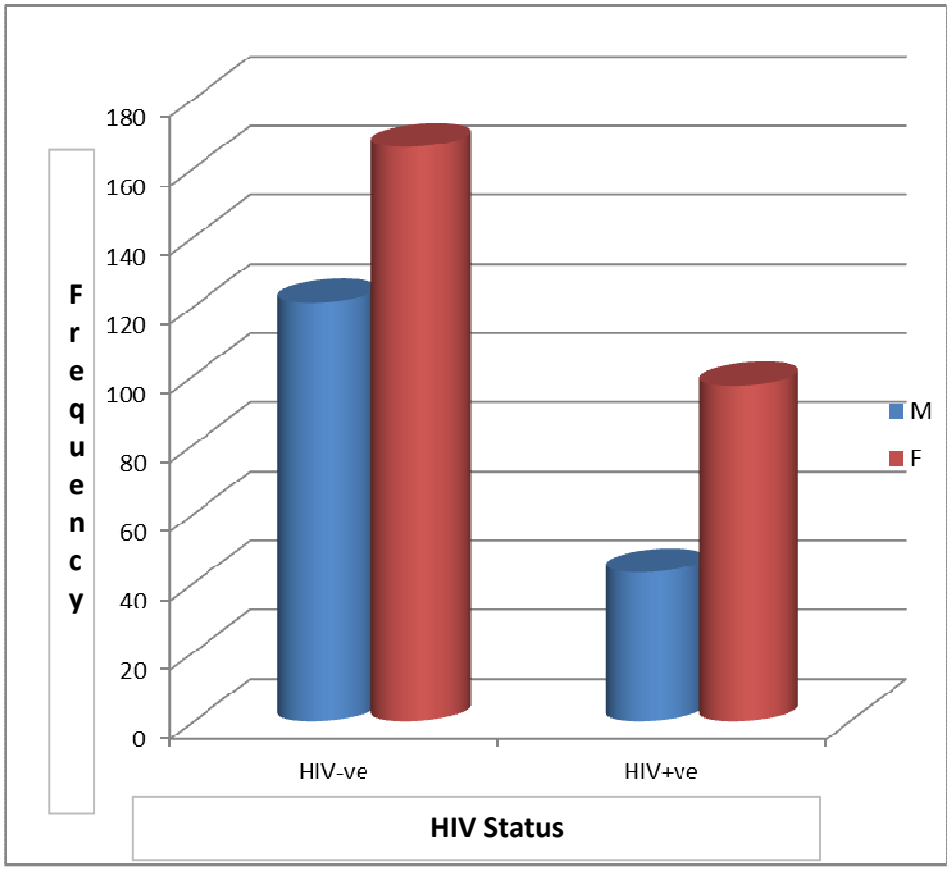


Figure 3.2 HIV status and Sex distribution

3.3.1.2. HIV and age distribution

The information obtained from the questionnaire data showed that the age range for the entire participants (n=343) was 16-67 with the mean age of 35. In the HIV group, the mean age was 37 in the range of 22-67. The highest prevalence of HIV was found among the age group 30-39, the lowest was among participants >60. There was no record of HIV positivity in the subjects below 20.

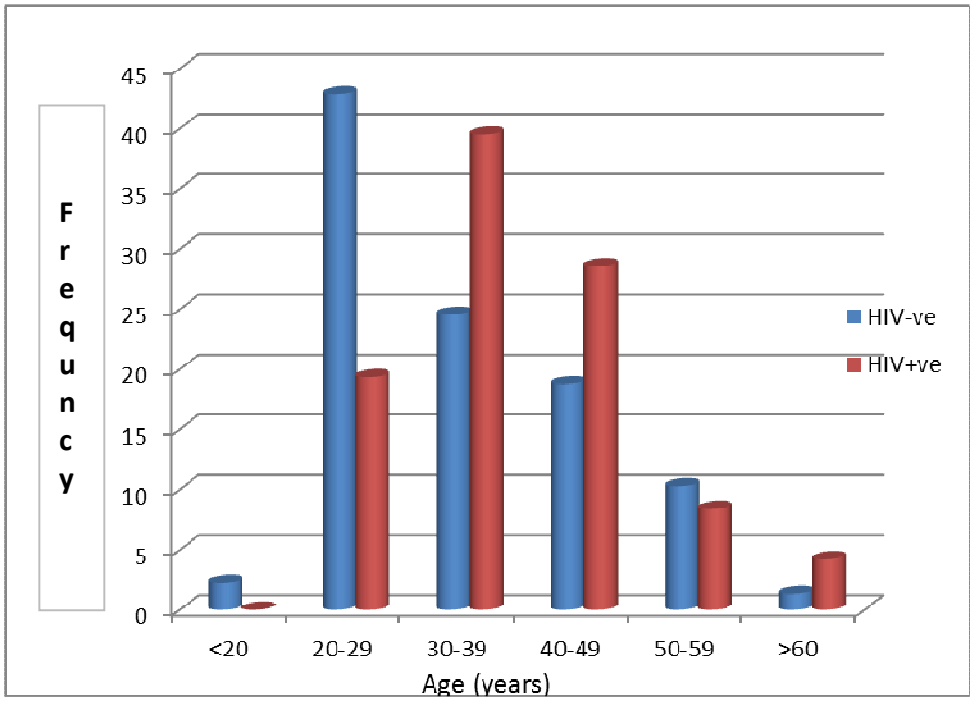


Figure 3.3 HIV status and age distribution

3.3.1.3. HIV and Education level

Analysis of our questionnaire data shows that the level of education in the two groups is different. There were more people with tertiary education in the HIV-negative group than the HIV-positive group probably because most of our HIV-negative recruits were from the University community of students and staff. Even though the HIV-positive clinic from where the HIV-people were recruited serves every class of persons, the results of the analysis of the HIV-positive group, using the chi square test, show that the level of education has a strong relationship to HIV status. ***(P=0.000)***. The highest percentage in the education level amongst the participants was found in those with secondary education (35.38 %).

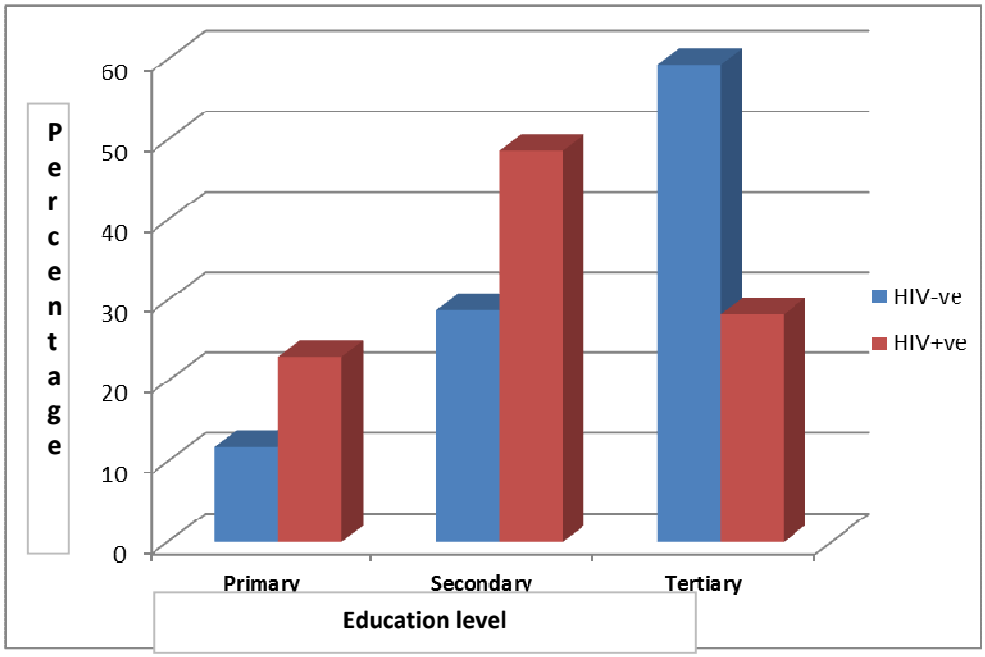


Figure 3.4 Level of education attained in the two groups

3.3.1.4. HIV and Occupation

Our study participants were recruited from various works of life including civil servants, traders, business men and women, teachers, oil workers and students. Considering the fact that the study venue was a University/ teaching hospital environment, many of our participants were students and these were mostly among the HIV-negative group. The survey of the occupation of our HIV participants revealed that the highest prevalence of HIV was found in traders (25.44%) followed by business men/women (21.05%), least in housewives (1.75%).

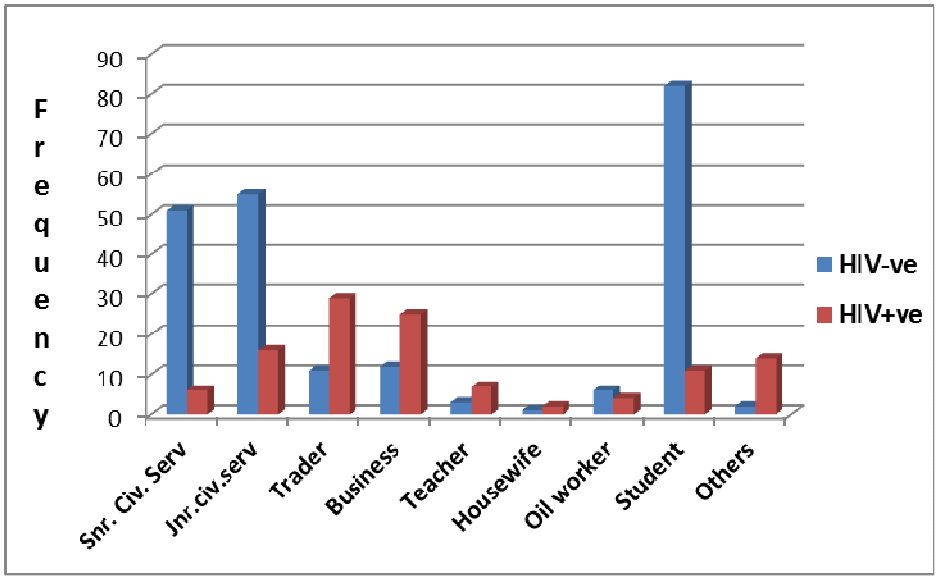


Figure 3.5 Distribution of Occupation among the study participants in the two groups.

3.3.1.5. HIV and Marital status

Our study participants were single or married; amongst who were those who were either divorced or widowed people. Results analysis showed that HIV positivity was more prevalent among married people.

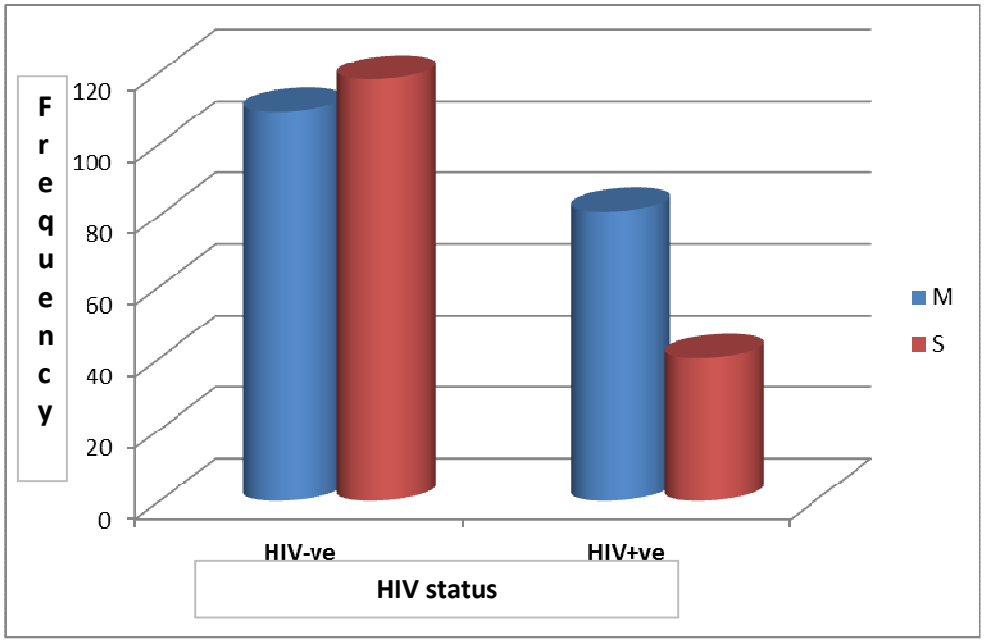


Figure 3.6 Relationship of HIV and marital status

3.3.1.6. Duration of HIV disease and duration of taking the ARV

Out of 103 people that responded to the question, 77.7% of the HIV subjects had been positive for greater than one year. Observation also showed that 56.7% of the HIV participants had been on ARVs for greater than 2 years duration.

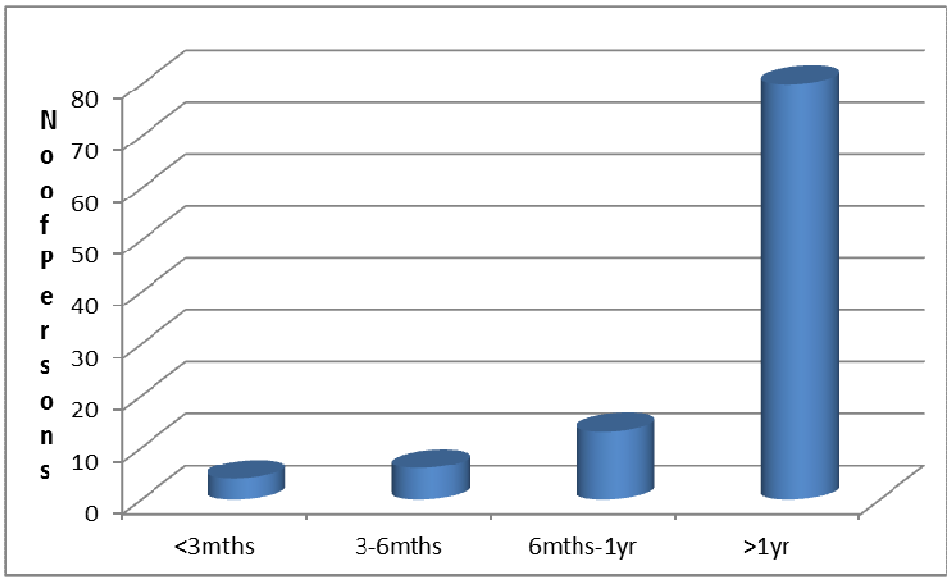


Figure 3.7 Number of HIV persons and their duration of the illness *N=103

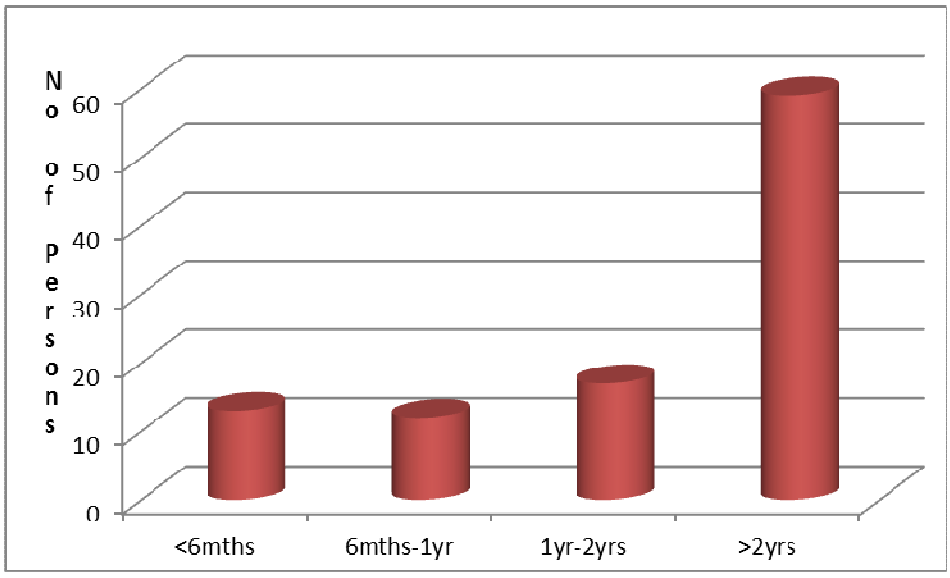


Figure 3.8 Number of HIV persons and their duration of taking ARV drugs *N=104

3.3.1.7. Insecticide treated net (ITN) and TS use

Result shows that ITN use is not common among our study participants, being that only 18.5% (58/313) out of the total number that responded to the question use ITN regularly. A higher percentage was found in the HIV group where it was almost double (14.1% vs 27.1%). Our observation on the basis of questionnaire responses is that only 27.2% of the HIV-positive people use TS regularly.

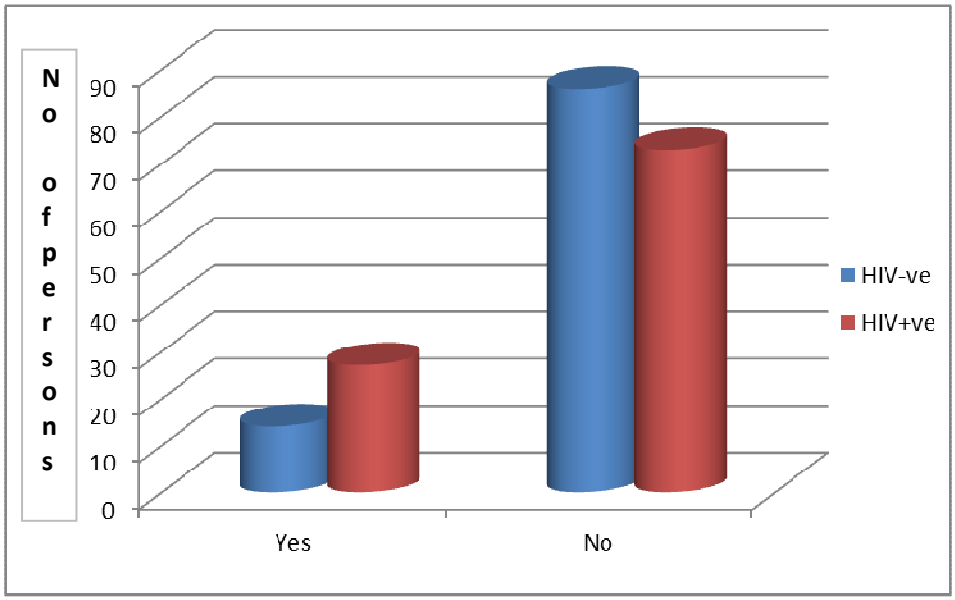


Figure 3.9 Use of Insecticide treated nets among study participants *N=313

3.3.1.8. Use of antimalarial drugs among study subjects

Self-medication is very common in Nigeria and this is seen from the table below. Out of 459 people screened for the study, 133 had taken antimalarial drug, while 44 had not taken. 282 did not indicate therefore there is no data on their previous usage of antimalarial drug. The percentages of people that had taken antimalarial drug within the last three months prior to the study were similar in both the HIV-positive and the HIV-negative control groups. Out of the 108 HIV-negative group that responded, 76.7% (83/108) had taken antimalarial drug while in the HIV-positive group, the percentage that had taken antimalarial was 72.5% (50/59). The results also indicates that 64% (85/133) of the persons that had taken antimalarial drug were *P.*

falciparum negative on day 0 and the remaining 36% (48/133) were positive despite the prior intake of antimalarial drug. Observation from the table below shows that the most commonly used medication was SP followed by AS and AL.

Table 3.2 Antimalarial drugs taken by subjects within the previous three months of the study

Antimalarial Drugs taken in the last 3 months	HIV-positive	HIV-negative	Total
None	19	25	44
AL	8	20	28
AQ	3	0	3
AQ+AL	1	0	1
AQ+AS	1	2	3
AQ+AS+AL	0	2	1
AS	10	19	29
AS+AL	1	0	1
CQ	2	1	3
CQ+SP+AL	1	0	1
PD	2	0	2
TS	2	0	2
SP	13	28	41
SP+AL	2	5	7
SP+AS	3	7	10
SP+AS+AL	1	0	1
Total	69	108	177

3.4 CD4 cell count Results

CD4 cell count results were obtained from 93 HIV-positive participants with a mean average 413 (SD 257; 11-1593). Paired peripheral blood CD4+ T-cell counts at both day 0 and day 28 were available for the 56 of these volunteers. Over 28 days following, we observed a mean increase of 107.3 cells per µl (95% CI 53.8 – 160.8; ***P = 0.0002***) in 68% (38/56) of the people. This was observed in patients PCR-positive for *P. falciparum* (N = 14) and those PCR negative (N=24). However, in 32% (18/56) of these volunteers, there was a reduction in the CD4 cell count results.

Table 3.3 CD4 range and PCR positivity for *P. falciparum*

CD4 cell count Range	Frequency	Number *P. falciparum* PCR positive
<200	16	6
200-349	21	8
350-500	32	15
>500	24	14
Total	93	43

The overall mean Cd4 cell count obtained among our subjects was 413cells/µl. The women had a higher mean of 432 (32-1593, sd 283) compared to 369 (82-623, sd 163) for the men.

3.5. WBC/PCV Results

The WBC for the HIV-positive group was found to be 4.74 x 10^9/1 (range 1.4-16.8) SD 1.90 consistent with the result 4.51± 1.82 x 10^9/1 (range 0.9-8.2 x 10^9/1) previously obtained by Erhabor et al, (2004) among HIV patients in Port Harcourt. The study reports mean haemoglobin (Hb) of 10.25 ±1.97g/dl (range 6.31-14.2 g/dl) for HIV-positive patients and this approximates to about 30.75 PCV but there was no report of Hb values in the HIV-negative people. In the present study, PCV was measured to establish the Hb levels. Since malaria is a major cause of anaemia in endemic areas (Lee et al., 2008) the measure of Hb is very important because it provides information on the clinical state or severity of the infection.

Table 3.4 Mean PCV results for both groups

Mean PCV	D0	D28
HIV-negative	36.8 (25-50, sd=4.5): n=134	39 (30-47, sd=7.0): n=5
HIV-positive	34.8 (16-50, sd=5.7): n=107	33 (28-38, sd=7.1): n=2

3.6. PCR diagnosis result

Using the PCR as a more reliable test for parasite presence, we observed that a high proportion of enrolees (61.1%) were actually aparasitaemic. Using Fischer's exact test, there was evidence to show that HIV-positive people were more likely to be parasitaemic at day 0 (P = 0.006). This reflects the higher parasite densities and the likelihood of parasites being correctly identified by the screening microscopists. Follow up results up to days 3 and 28 days after treatment revealed that the HIV-positive group were also more parasitaemic. This was more evident on day 28 (**OR=2.6; P=0.007**).

We analysed the first 180 samples using small subunit ribosomal RNA (SSU rRNA) gene amplification nested PCR protocol (Snounou et al., 1993a) to assess the sensitivity compared to *pfmdr1.* Our results (Table 3.6) show that nested PCR amplifying fragment 1 *pfmdr1* showed more positive results than the 18S ribosomal subunit and it is well validated in our laboratory for sequencing, so we chose to use it to identify positives. The higher *Pfmdr1* positivity was not due to contamination since all the negative controls in the PCR were negative. Comparing this with the *pfdhps* gene, we found that 89.5% of *pfmdr1* negatives were negative as well by *pfdhps*, while 62.5% of *pfmdr1* positives were also positive by *pfdhps* (Table 3.7). RDT results were very poor and unreliable, only 8% (12/150) of the first 150 samples were positive using the First Response kit.

Table 3.5 PCR Results for days 0, 3 and 28

	Microscopy	PCR +ve (D0)	PCR +ve (D3)	PCR +ve (D28)
HIV+ve	**128**	**62/128 (48.4%)**	**16/62 (25.8%)**	**23/62 (37.0%)**
HIV-ve	**242**	**82/242 (33.9%)**	**8/82 (9.8%)**	**14/82 (18.3%)**
Total	**370**	**144/370 (38.9%)**	**24**	**37**

Table 3.6 Small subunit ribosomal RNA (SSU rRNA) gene amplification of DNA from pre-treatment (day 0) samples

Method Used		SSU rRNA assay		
		Positive (n)	Negative (n)	Total
Pfmdr1 assay	Positive (n)	15	35	50
	Negative (n)	4	126	130
	Total (N)	19	161	180

Table 3.7 Comparison of *pfdhps* and *pfmdr1* assay sensitivity

	***Pfdhps* assay**	
mdr1	**Positive (%)**	**Negative (%)**
Negative	10.5	89.5
Positive	62.5	37.5

3.6.1. Parasite carriage and HIV status duration

Analysis of the pre and post-treatment PCR results using *mdr1* 86 results, shows that HIV-positive subjects who had indicated having HIV sickness duration for greater than one year were found to be more parasitaemic on both day 0 and day 28 as seen in Table 3.8.

Table 3.8 Duration of HIV status and parasitemia carriage.

Sickness Duration	Mdr 86 (d0)	Mdr 86 (d28)
1. less 3 months	2	0
2. 3mths -6mths	2	0
3. 6mths-1yr	1	1
4. greater than 1yr	28	6
Total	33	7

This observation was also made among study subjects who had been on ARV drugs for greater than 2 years as shown in Table 3.9.

Table 3.9 Duration of ARV and parasitaemia carriage

ARV Duration	Mdr 86 (d0)	Mdr 86 (d28)
1. less than 6 months	3	0
2. 6mths -1yr	2	2
3. 1yr-2yrs	7	0
4. greater than 2yrs	21	5
Total	33	7

Analysis of the various genes amplification of the samples revealed that the prior use of antimalarial drugs by some of the study participants affected the presentation of the haplotypes obtained as shown in Table 3.10

Table 3.10 Antimalarial drugs taken by participants with the presenting haplotypes from *mdr1, crt, dhps* and *dhfr* genes

Sample ID	HIV status	Antimalarial	d0_haplotype	d3_haplotype	d28_haplotype
4	1	AS	NYD+CVIET+IRNI		
7	1	SP	VAG+NYD+IRNI		
10	1	PD	VAG+NFD		
12	1	SP+AS	VAG+NYD+YYD+IRNI	NY	
13	1	SP	VAG+NYD+NFD+CVIET+IRNI		
14	1	AL	NYD+CVIET+IRNI		
22	1	AL	NYD		
23	1	AL	NYD		
24	1	AS	VAG+NYD+IRNI		
26	1	CQ+SP+AL	ISG+IRNI		
27	1	AS+AL	ISG+NYD+CVIET		

Sample ID	HIV status	Antimalarial	d0_haplotype	d3_haplotype	d28_haplotype
30	1	SP	IAG+NYD+IRNI		
34	1	SP+AL+AS	IAG+VAG+NYD+NFD+IRNI		
36	1	SP	IAG+NYD+IRNI	NFD	
37	1	TS	CVIET+IRNI		
38	1	SP	CVMNK		
42	1	AS	ISG+CVIET		
43	1	SP		NYD	NYD
45	1	CQ	IRNI		
49	1	CQ			NYD
50	0	AL	ISG+IRNI		
54	1	AL	ISG+VSG		
66	0	SP+AS	VAG+YFD+IRNI		
76	0	AS	IAG+NYY=IRNI		
77	0	AL	VAG+NFD+CVIET+IRNI		
84	0	AL	NYD		
86	0	AL	NYD		
97	0	SP+AL+TS	ISG		
99	1	AS	ISG+NY+NFD		
108	0	AL	CVIET		
127	1	SP	ISG+NYD		NYD
137	0	AS	ISG		
147	1	SP+AL+TS	NFD	NYD	
171	0	AQ	NYD+IRNI		
207	0	SP+AL	NY+CVIET		
210	1	AQ+AS	NFD+CVIET		
253	1	SP	ISG+CVIET		

Sample ID	HIV status	Antimalarial	d0_haplotype	d3_haplotype	d28_haplotype
326	0	SP	NYD+IAG+CVIET+IRNI		
372	0	SP	CVMNK		
374	0	AS	CVMNK		
383	0	SP+AS	CVMNK		
388	0	SP	CVMNK		
413	0	SP	CVMNK		
420	0	SP	CVMNK		

3.7. Discussion

In this study, results before and 28 days after treatment revealed that the HIV-positive group were more parasitaemic as shown in Table 3.5. This corroborates with studies that HIV-positive people harbour more parasitaemia (Withworth et al, 2000; Shah et al, 2006). The 48.4% parasitaemia observed in the HIV-positive group on day 0 was very close to the 46.7% reported by Ukibe et al, (2010) but was however much higher than that observed (2.11%) by (Akinbo and Omoregie, 2012) in a study on HIV-positive asymptomatic malaria adults in Benin City, Nigeria and equally higher than the 24% prevalence reported by (Iroezindu et al., 2012) among HIV adults living in Jos, North central Nigeria. Parasitaemia prevalence among the HIV-negative group in the Jos study was 9% at variance with the 33.9% obtained in our result. Our result was not a population-based study and was limited by small sample size. Also we do not know whether the Jos result was based on PCR. The slightly lower percentage of parasitemia obtained in the Jos study could be due to the fact that this was just detection by microscopy knowing that PCR sensitivity is about 100 times higher than microscopy (Snounou et al, 1993a, Snounou et al, 1993b). Additionally the increased rainfall and the mangrove vegetation of the Niger Delta (our study area) compared to the Guinea savannah in the Jos area could also be confounding factors for the higher prevalence obtained in the study.

Our HIV participants were predominantly females consistent with demographics of HIV/AIDS with many published reports (Anafi et al, 2008; (Ekouevi et al., 2013, Collins et al., 2009, Hwang

et al., 2012, Abdool Karim et al., 1992). They were comprised of 69.3% females and 30.7% males. This agrees with the report (NARHS, 2007) of higher prevalence among women due to higher vulnerability and infections in all age groups. One of the key drivers in HIV distribution is the entrenched danger of inequalities and inequities. This is often seen in the areas of economic dependency for women, because in most societies men have greater control and access to productive resources (FRN, 2012). As a result of this, women do not have right to determine sex choice or right over her own body. In sub-Saharan Africa, 61% of the people living with HIV/AIDS are women (WHO, 2009b). This is visibly seen as the feminization of AIDS where for every HIV-positive young man, there are three HIV-positive young women. Reports have shown that a young woman in Africa is up to eight times more likely to acquire HIV than a young man (UNAIDS, 2010). In Nigeria the prevalence rate in females is 4.0% compared to 3.2% in men (FRN GARPR, 2012). The other aspect of this imbalance is seen in the area of violence against women and girls which include forced sex, rape, forced marriages, physical assault and humiliation or intimidation (ICA, 2006).

Demographic data obtained from the present study, revealed highest prevalence of HIV in the age range of 30-34 years corresponding to the UNGASS, 2010 report that HIV/AIDS was highest in the age range 30-34 years in the urban region. This was also established in the HIV/AIDS prevalence disaggregated age and sex report with the highest prevalence in the age group 30-39 in both males and females (NARHS, 2007), in consonance with the results obtained.

On the basis of questionnaire responses, our observation is that education has an influence on the serostatus of our HIV-positive subjects. The influence of education as seen from the result is an important factor that should be addressed with regards to the spread of HIV**.** The result shows that a greater percentage (54%) of our HIV study participants were those with secondary education. National results report that highest prevalence of HIV in women is found among those with primary and secondary education (FMOH, 2010). This class of people are mainly junior workers and they mostly commute between the villages and the city since they may not be able to maintain their families in the city. This invariably exposes them to higher sexual risk behaviour. The females in this group are not able to determine sex choice because of economic dependence on the men and societal pressure. Also they are more likely to be un-informed of the health facilities available to them.

Migration in addition to some other factors has been reported as one of the key driving factors of HIV (Abdool Karim et al, 1992; UNDP, 2010). Traders and business people by the nature of their occupation are migrants so they are more exposed to HIV. The report (Husken and Heck, 2012) of higher vulnerability of female fish traders in Zambia to HIV, due to the fact that they regularly travel to remote fishing camps to purchase fish, highlights this issue. In Malawi, AIDS is believed to have spread along major trading and transporting centres (Guebbels and Bowie, 2006). Another group of people who are migratory are students. The slightly high prevalence among the students is indicative of this lifestyle. HIV/AIDS is a disease that is driven by poverty and this explains why the unemployment is a major contributing factor to increased HIV prevalence. The data shows that the junior civil servants and the unemployed group in this study also have relatively high prevalence and this is consistent with the 3.1% prevalence of HIV infection among unemployed individuals previously reported in Port Harcourt, Nigeria (Ejele et al, 2005d).

Significant continued unfaithfulness by both partners in the marriage relationship is a major factor that exposes both partners to increased risk of HIV, hence the higher prevalence seen among married people. This is inter-related to the economic dependence of women on their husbands for sustenance thereby not being able to determine sex choice even where unfaithfulness is very evident. The cultural aspect of polygamy in the society places additional pressure on the women resulting to 'inbreeding' within the family and spread of HIV in the society. The relatively high number of widowed people in the group is a further indication of the consequences of this lifestyle in the society.

The result of the study revealed that a greater majority of our HIV subjects (77.7%) have been established as being HIV positive for longer than one year and thereby many of them have been on long-term ARV therapy, the longest duration of the persons on ARV being greater than two years (56.7%). Analysis of results shows that participants who have been established to be HIV-positive for greater than one year and who also had been on ARV for a longer duration were more parasitaemic before and after treatment as seen in Table 3.7. The most probable explanation of this result may be that the longer duration of HIV status has possibly reduced the immunity of the participants such that they are more likely to harbour parasites. Despite the longer duration of taking ARVs, these patients still harboured more parasites even after treatment with AL.

The indiscriminate use of antimalarial drugs ranging from artemisinin monotherapy to the use of ACTs and other combination drugs from the study is a major issue in Nigeria. This calls for more awareness on the dangers of self-medication with the implicating consequences of increased drug resistance. The result shows that this indiscriminate use of antimalarial drug affected the percentage of parasitaemia/ the low parasitaemia recorded in many of the samples, hence it is not very possible to properly establish the correct percentage of *Plasmodium falciparum* parasitaemia in the study area. Of more serious concern is the increased possibility of antimalarial drug resistance as a result of increased drug pressure. This was evidenced by the various haplotypes presented from the four different gene sequences analysed (Table 3.10).

The mean CD4 cell count obtained among our subjects was 413cells/µl consistent with earlier result 415cells/µl reported by Fehintola et al, (2012). However it was at variance from an earlier value of 314 cells/µl obtained in HIV adults in Port Harcourt (Erhabor et al, 2005). The study did not classify the gender of the subjects so we are not able to make adequate comparison, however the report stated that healthy women had higher values than the men. In our results, the mean CD4 count for women was 432 (sd 283), and 369 (sd 163) for the men. This corroborates many reports that have demonstrated that women have higher CD4 values than men (Bussmann et al., 2004, Lugada et al., 2004, Maini et al., 1996, Mair et al., 2008). The observation of higher prevalence of parasitaemia among patients with CD4 cell count 350-500 is at variance with other studies that have indicated an inverse relationship between CD4 cell count and parasitaemia level. We do not have any explanation to this however we are aware that many factors influence CD4 count cells and some of the factors include time of sample collection and sex. To the best of our abilities and within the circumstances of our study, we took the samples at the times of presentation of the participants but some of the samples were not analysed at the right time due to the peculiar challenges we encountered in the course of the study. This may have contributed to the disparity in the results.

The increased CD4 cell count results 28days after treatment with AL suggests that there was an improvement in the immune status of the patients after treatment with AL since an improved CD4 result is a good measure of immune status of patients (Kublin et al., 2005, Patnaik et al., 2005, Mermin et al., 2006, Mair et al., 2008). Use of CD4 cell count level as a measure for monitoring of ART has been proved to be a good measure in resource limit areas where viral

load measure is not economical due to limited financial empowerment (Jourdain et al., 2013). The improved CD4 cell count after AL administration goes to underline the need for the treatment of asymptomatic-malaria infection thereby reducing the potential to progress to clinical stage of malaria.

Observation of the PCV levels which were measured to establish the Hb levels, shows that HIV-negative control group had higher mean PCV level than their HIV-positive counterparts. This agrees with the report of an earlier study of haematological parameters in Port Harcourt, Nigeria (Erhabor., et al., 2004)

Despite the higher use of ITN, the HIV-positive subjects were still more parasitaemic than their negative counterpart before and after treatment in contrast to studies that have established that use of ITN reduced malaria infection in people living with HIV/AIDS (PLWHA) in Nigeria (Olowookere, et al, 2013). Our observation on the basis of questionnaire responses is that only 27.2% of the HIV-positive people use TS regularly.

3.8 Findings and recommendations

Treatment of asymptomatic *P. falciparum* malaria is of primary importance as stated earlier for the management and control of malaria in endemic areas especially in HIV- positive patients. In sub-Saharan Africa, testing, treating and tracking of malaria is limited to symptomatic cases. Studies on AL, the first-line treatment in Nigeria have neither included asymptomatic-*P. falciparum* carriers nor HIV-positive patients. This study was able to show the presence of asymptomatic- malaria parasitaemia carriers among the study participants in both HIV-positive and HIV-negative people in this area of the country where there has been little or no published data. As at the time and date of our study, treatment of asymptomatic-*P. falciparum* malaria infection especially among HIV-positive subjects have not been reported in Nigeria. The results of the study contribute to the limited data on the following: AL use, the malaria/HIV interaction in addition to the drug–drug interactions between ARVs and antimalarial drugs in Port Harcourt, Niger Delta region of Nigeria and will serve as a template for larger studies.

The use of PCR which is a very powerful tool for detecting asymptomatic-malaria infection is not feasible in the field hence we depended absolutely on microscopy for diagnosis. This resulted in very poor recruitment because of the several many false positives we recorded.

Parasitological data obtained from microscopy has revealed a very great need for well-established study involving the training of personnel, better diagnostic measures and enabling environment to obtain data that will show the true picture of malaria prevalence in the environment and could help influence policy towards achieving the goal of the elimination of malaria.

The result of the study was able to show that there is significant presence of asymptomatic-malaria infection in both HIV-positive and HIV-negative people in the study area and highlights the very important need for the study of malaria epidemiology and molecular markers of resistance in the Niger Delta area. Due to its peculiar terrain and vegetation being mostly surrounded by creeks and rivers there is a great need to invest in studies in this area especially with the high number of migrant workers in this region due to the high level of oil and gas resources in the area.

There is need to identify, treat and control asymptomatic-malaria which triggers the clinical disease and reduces the immunity of the HIV patients thereby making them more susceptible to infections resulting in increased morbidity and mortality in HIV patients and consequently increased presence of resistant strain of parasites in the environment.

Key drivers like education, poverty, marital status, age that play relevant roles in HIV prevalence have been highlighted from this study. There is need for more aggressive awareness campaigns against the socio-cultural and socio-economic factors that promote HIV spread in our environment.

The indiscriminate use and misuse of antimalarial drugs as seen from the study is a major area of concern with regards to the role it plays in the spread of antimalarial drug resistance. Enforcement of laws that will discourage these attitudes by stakeholders and governing bodies will help to prolong the life of the ACTs so that it does not end up like chloroquine and the older antimalarial drugs.

Even though chemotherapy is one of the major control measures for the treatment of malaria, other control measures such as the use of ITNs, IRS, SMC, larvicides and use of window netting should be promoted and encouraged in the environment so as to reduce the burden of malaria in the country. The study showed that only 18.5% of the study participants use ITN, a very poor

number for an endemic region. The poor use of TS chemoprophylaxis among HIV subjects in the study area is a very important issue that needs to be addressed in order to improve mortality and reduce co-morbidity of malaria in HIV-positive subjects. National guidelines recommend the use of TS for prophylaxis against opportunistic infections both in adults and children.

3.9 Limitations to our study

Being the first time the study was carried out in our environment, we were faced with numerous challenges some of which we were not able to surmount. There were several factors that imposed significant limitations on this study and therefore led to some flaws in our results as shown below.

A major weakness of our study was the poor quality of enrolment microscopic diagnosis, such that the majority of participants had infact failed a major inclusion criterion. Although microscopy for years has been the gold standard for diagnosis of malaria, but in many developing countries, it is not very reliable because the microscopists are not sufficiently trained and the reagents may be of poor quality. Although we employed senior microscopists who were attached to the hospital, they failed to make appropriate diagnosis as we had many false positives. This proved to be a major flaw in our study, and had two main impacts; first the study was greatly underpowered 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 malarial 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 (Bousema et al., 2006, Sutherland et al., 2005). Nevertheless, a recently described persistence of asexual parasites in asymptomatic Ghanaian school children treated with ACT, suggests that sub-clinical parasitaemia may be more difficult to clear than previously thought (Dinko et al, 2013).

Constraint of funds resulted in our not being able to train microscopists specifically for the research and could not employ enough microscopists as well as phlebotomists therefore we depended on the hospital staff.

During the period of sample collection, there were several adverse conditions such as kidnapping of doctors which resulted to many industrial actions by the hospital staff thereby leading to the disruption of the study severally especially in the area of sample analysis and the necessary follow-up for some patients. A major aspect of this was in the analysis of CD4 cell count levels where the accuracy of the result is influenced by the time of collection and storage of sample as established by the significant decrease in CD4 count from baseline by as much as 10% at day 4 in a Ugandan study (Kabengera et al., 1994). Due to the industrial actions, many of our samples were not analysed on the days of collection but were stored and analysed later. This may have resulted in reduced levels as was observed in many of the day 28 CD4 results being lower than the day 0 results. On some of these days the hospital was closed down such that we could not collect samples on those days and consequently we have a lot of incomplete data.

Another major factor that impeded on sample collection, storage and analysis was that of frequent power outages in the hospital. Here, the frequent power outages affected the storage of some samples, filter papers and the RDT kits further contributing to the poor yield of results obtained.

The terrain of the city of Port Harcourt especially in and around the Choba area where the Teaching Hospital is located is rather bumpy, leading to frequent traffic congestion on the roads. This made it very difficult for some of our participants to present themselves on follow-up days and equally difficult for us to visit their homes to collect the samples ourselves.

We experienced frequent break down of the machines and equipment that were deployed for sample analysis due to the fact that we did not have access to especially dedicated ones for research purposes. Since we had to use the same ones that were used for routine hospital work, some of the machines were overworked leading to frequent break downs. This ultimately resulted in disruption of adequate sample collection and analysis.

Part of our limitations was the nature of filter paper used. Our observation was that in the course of extraction of DNA using Chelex method, some of the filter papers were degraded and therefore we could not adequately extract the DNA. There was a possibility of retained chelex in the supernatant of the DNA used for the PCR and this would affect the quality of DNA extracted.

Notwithstanding the limitations, the work has provided a template for larger studies of AL use and the malaria/HIV interactions in the environment particularly in the creek regions of the area where we did not include in our studies. Moreover, even though the study is not a population survey, information obtained contributes to the demographic data available in the assessment of HIV/AIDS in the region.

Chapter Four

Pharmacokinetics of lumefantrine in HIV-positive and HIV-negative adults based on day 7 blood concentrations

Part of the work described in this chapter has been published in the Antimicrob. Agents Chemother. September 2013 57:9 4146-4150

4.1 Rationale

Malaria /HIV interaction has been ignored as a major public health issue and particularly so in areas of endemic malaria where there is also geographic overlap with HIV such as are obtained in sub-Saharan Africa. Of particular importance is the potential drug-drug interactions involved in the management of the co-infections as a result of co-administration of the drugs for the treatment of both infections which have not been adequately investigated. The mortality and morbidity of the two diseases is already high, and if the interaction of the treatment undermines the efficacy of the antimalarial drug, it will increase the outcome risks and may require the need to modify or could result in the elevation of the concentration of the antimalarial leading to a possible increased toxicity. On the other hand, the elevation may be an advantage in enhancing the antimalarial effect especially with antiretrovirals that possess antimalarial activity (Parikh et al., 2005, Nsanzabana and Rosenthal, 2011, Soyinka et al., 2010).

Increased use of the highly active antiretroviral therapy (HAART) means a higher potential of drug-drug interactions and yet not much is known about these interactions because there have been very few publications on this very important subject. Classes of antiretroviral drugs include the neucloside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, integrase inhibitors and chemokine receptor anatagonists. However the first-line drugs currently in use in Nigeria involve the use of 2 neucloside reverse transcriptase inhibitors (NRTIs) and 1 non-nucleoside reverse transcriptase inhibitors (NNRTI), the most common being a fixed dose combination of lamivudine, zidovudine and nevirapine. In situations of adverse drug reaction to nevirapine the patient is given efavirenz which is another NNRTI.

There are reports of drug-drug interactions between ARVs and the antimalarial quinine in Nigeria. Nevirapine was reported to cause a reduced exposure of quinine (Soyinka et al., 2009, Uriel and Lewthwaite, 2011) while the administration with ritonavir resulted in its four-fold increase (Soyinka et al, 2010). Quinine is used in Nigeria as a second line drug for severe malaria. Fehintola et al, (2012) reported an increase in artesunate exposure in asymptomatic-malaria HIV-infected Nigerian adults on nevirapine who were given artesunate-amodiaquine.

The choice of antimalarial drug for the treatment of HIV-positive patients therefore is of utmost importance considering the dangers of co-morbidity but sufficient pharmacokinetic and parasitological evidence to make this choice is currently lacking. Our study is the first involving parasitological implication.

Table 4.1 Antimalarial and antiretroviral interactions available in literature

Study	Drugs used	Subjects	Outcome
Khaliq et al, 2001	Mefloquine + ritonavir	Healthy volunteers	Decreased exposure of ritonavir
German et al, 2007	Artesunate-amodiaquine + efavirenz	Healthy volunteers	Increased exposure of amoadiaquine and decreased exposure to DEAQ
German et al, 2009.	Artemether-lumefantrine + lopinavir-ritonavir	HIV-un-infected healthy volunteers	2-3 fold increase in lumefantrine exposure
Soyinka et al, 2009	Quinine + nevirapine	Healthy volunteers	Significant decrease in quinine concentration
Soyinka et al, 2010	Quinine +ritonavir	Healthy volunteers	4-fold increase in quinine concentration
Soyinka & Onyeji, 2010	Proguanil + efavirenz	Healthy volunteers	Increased exposure of proguanil
Kredo et al, 2011	Artemether-lumefantrine + nevirapine	HIV-infected patients without malaria on nevirapine therapy	Increased day 7 lumefantrine concentrations and decreased artemether and DHA exposure
Fehintola et al, 2012	Artesuante-amodiaquine + nevirapine	HIV-positive adults without malaria	Increased exposure to DHA and artesunate
Huang et al, 2012	Artemether-lumefantrine + efavirenz	Healthy volunteers	Reduced exposure of DHA and day 7 lumefantrine level
Byakika-Kibwika et al, 2012a	Aretmether-lumefantrine+ efavirenz or nevirapine	HIV-infected adults	Reduced exposure to DHA, artemether, lumefantrine and nevirapine
Byakika-Kibwika et al, 2012b	Aretmether-lumefantrine+ lopinavir-ritonavir	HIV-infected adults	Increased lumefantrine exposure, but decreased artemether exposure
Kakuda et al, 2013	Artemether-lumefantrine + etravirine or darunavir/ritonavir	Healthy volunteers	Reduced plasma concentration of artemether , lumefantrine and DHA
Chijioke-Nwauche et al, 2013	Artemether-lumefantrine + nevirapine	HIV-positive asymptomatic - malaria positive and negative adults	Elevated day 7 lumefantrine concentration
Tommasi et al, 2011	Atovaquone/proguanil + etravirine/saquinavir	HIV-positive adult in malaria prophylaxis	Marked increase in etravirine and saquinavir concentrations
Scarsi et al, 2014	Artesunate + amodiaquine	HIV-positive adults without malaria	Reduced exposure to amodiaquine and desethyl amodiaquine (DEAQ)

4.2 Pharmacology of lumefantrine and nevirapine

Lumefantrine is a racemic mixture of a synthetic flourene derivative of the aryl amino-alcohol family. Lumefantrine has a similar mechanism of action to halofantrine probably due to similarities in structure and pharmacokinetic properties, but unlike halofantrine, lumefantrine is very safe as it does not prolong the electrocardiographic QT interval (van Vugt et al., 1999). It was first synthesized and registered in China and is now commercially available only in co-formulated product with artemether as coartemether (Annerberg et al., 2005). Peak plasma concentrations are observed 6-8 hours after oral administrations. Lumefantrine is metabolized mainly by CYP P450, 3A4 isoform to its active metabolite desbutyl-lumefantrine (Ezzet et al, 1998) and it is eliminated very slowly with a terminal half-life of 4-6 days in *P. falciparum* malaria and about 2-3 days in healthy individuals (WHO, 2008). Concentration of lumefantrine persists in plasma for >20days (Ezzet et al, 1998).

Nevirapine in a non-nucleoside reverse transcriptase inhibitor and acts by blocking the polymerisation of viral RNA to DNA through its binding to the enzyme reverse transcriptase. It is well absorbed after oral administration. With a biovailabilty >90%, its maximum concentration is reached 4 hours after the oral dose. It is widely distributed in the body, highly lipophyllic and about 60% bound to plasma proteins. Metabolism of Nevirapine is primarily through the induction of CYP 450 enzyme mainly 3A4 isoform but also 2B6 to its major metabolites 2-hydroxynevirapine and 3-hydroxynevirapine and excretion is primarily through the kidney. It induces the CYP3A4 and 2B6 by approximately 20-25% which leads to auto-induction. The auto-induction leads to approximately 1.5 to 2 fold increase in the clearance of NVP which continues in longer duration of treatment. In addition to this, there is a decrease in the terminal half-life from 45 hours after a single dose to about 25-30 hours following multiple dosing and during steady state dosing (Cooper and van Heesjvik, 2009). Even though NVP is primarily an inducer of CYP 450, it may also inhibit the system.

A previous study (German et al., 2009) on drug-drug interactions in ARVs assayed the levels of lumefantrine, artemether, dihydroartemisin (DHA) and the protease inhibitors lopinavir/ritonavir in the co-administration of the drugs in healthy volunteers. They reported a non-significant trend towards the decrease of artemether when co-administered with lopinavir/ritonavir and a decrease (P=0.02) in DHA. However there was 2-3 fold increase in the

lumefantrine concentration but the concentrations of LPV and RTV were not significantly altered. None of the participants were parasitaemia-positive.

After our study design protocol was approved and recruitment commenced, published reports of similar studies became available. The study by, Kredo et al, (2011) examined the impact of nevirapine on artemether-lumefantrine pharmacodynamics in 18 HIV-infected adult volunteers receiving nevirapine and 18 naive controls who were not infected with malaria parasite. They reported evidence of enhanced lumefantrine levels. This interesting result is yet to be confirmed in a large study with malaria-infected patients. The result is at variance with the more recent study also among HIV-infected adults in Uganda who did not harbour malaria parasites (Byakika-Kibwika et al., 2012) which reported a reduced exposure to artemether, DHA, lumefantrine and NVP, however there was no difference in day 7 lumefantrine concentrations with NVP. A more recent study among healthy volunteers involving AL co-administration with a single dose of etravirine a similar drug to NVP established a reduction in artemether and DHA after 8hours and 13% of lumefantrine after 264 hours (Kakuda et al, 2013).

4.3 Results

167 study participants (68 HIV-positive and 99 HIV-negative) who were confirmed *P. falciparum* positive had their day 7 samples collected during follow-up. Nested PCR performed to confirm microscopy revealed that 78.1% were actually aparasitaemic (Table 4.1). Analysis of the day 7 lumefantrine concentrations in the study, showed elevated levels of lumefantrine in the HIV-positive subjects as reported in the paper (Chijioke-Nwauche et al., 2013). Initial expectation was that there would be lower lumefantrine levels in HIV-positive participants since nevirapine is an enzyme inducer. However, contrary to the expectation, our results revealed higher levels in agreement with Kredo et al, (2011). Despite the higher lumefantrine concentration in the HIV-positive participants, there was no advantage in the parasite clearance over the HIV-negative control group. HIV status was found to have a significant effect on the lumefantrine concentration 7 days after treatment (Wilcoxon rank sum test z=2.830, *P*=0.0046).

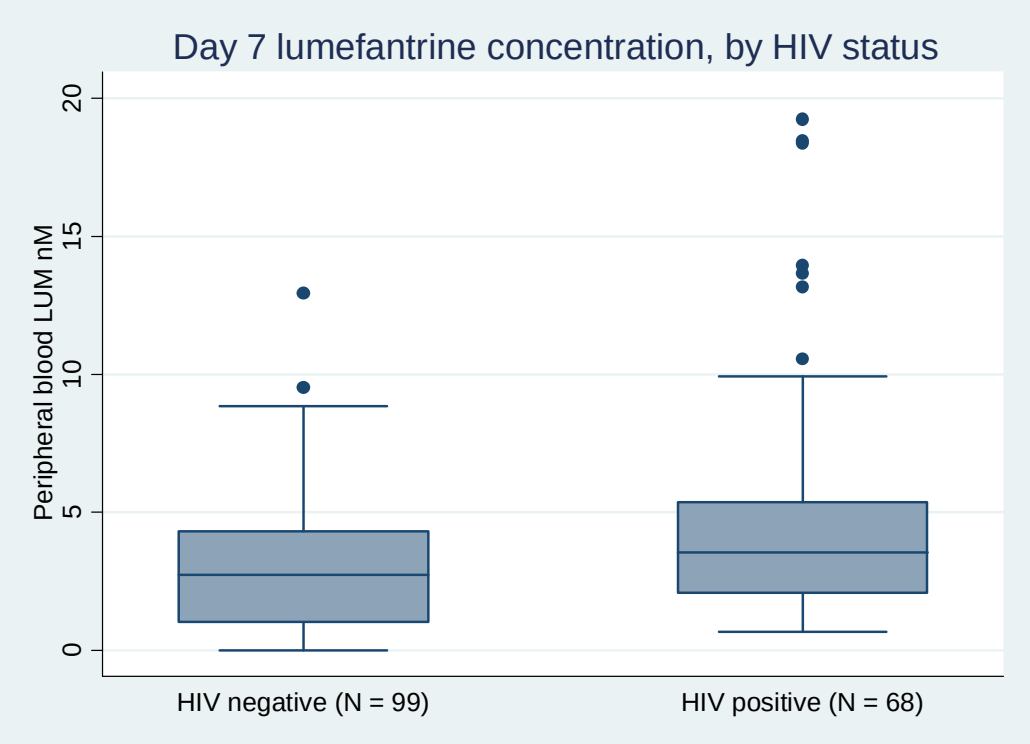


Figure 4.1 Day 7 lumefantrine concentration in AL-treated participants. Mid-line of each box-plot is the median, with the edges of the box representing the inter-quartile interval. Whiskers delineate the 5th and 95th percentile. Lumefantrine was below the normal limits of detection in five individuals, all in the HIV-negative group.

*mean day 7 concentration for HIV-negative arm=2.75µM (equivalent to 1454.59ng/mL); HIV-positive arm=3.55 µM (equivalent to 1877.74ng/mL)

Table 4.2 Parasite carriage by microscopy and PCR in 68 HIV-positive and 99 HIV-negative individuals

HIV status	No of individuals PCR positive for *P.falciparum* on day		
	0	3	28
Positive *(n=68)*	20	12	12
Negative (n=99)	17	8	12

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

Assessment of day 0 parasitaemia using qPCR for some samples (both HIV-positive and HIV-negative) did not show any evidence that higher starting parasitaemia increased the likelihood of an individual remaining PCR-positive for *P. falciparum* on day 3.

Table 4.3 Summary of initial parasitaemia of both HIV-positive and HIV-negative participants.

Patients	Parasitemia on D0			
	Min	**Max**	**Mean**	**Median**
d3 -ve (n=10)	0.98	33.22	5.80323	5.50
d3 +ve (n=7)	1.48	94.61	8.95335	5.29

4.4 Discussion

The paucity of data in respect of drug-drug interactions between ARVs and antimalarial drugs makes it a little difficult to make conclusions however certain patterns are expected *a priori.* The result from the study (German et al., 2009) of 2-3 fold increase in lumefantrine level in co-administration of AL with lopinavir/ritonavir is expected *a priori* since ritonavir is a potent CYP3A inhibitor.

The recent study (Huang et al., 2012) indicated a significant reduction in day 7 lumefantrine concentration in co-admnistration with efavirenz in malaria un-infected participants similar to the findings of Byakika-Kibwika et al, (2012) in Uganda. The difference in result obtained could be as a result of the co-infection with malaria in some of our participants in addition to the concurrent use of nevirapine by our participants for duration greater than 6 months. Efavirenz and nevirapine are known inducers of cytochrome P450 3A4 and both act through the same route, however nevirapine additionally has an ability to auto-induce itself. The auto-induction leads to approximately 1.5 to 2-fold increase in the rate of clearance of NVP which is maintained in longer duration of treatment. In addition to this, there is a decrease in the

terminal half-life from 45 hours after a single dose to about 25-30 hours following multiple dosing (Cooper and van Heeswijk, 2007). In the HIV patients, this decrease in terminal half-life and the 1.5 to 2 fold increase in the clearance of nevirapine could result in a reduced concentration of the drug present in the system, thereby making less of the drug available to induce the enzyme so that there will be higher concentration of lumefantrine not metabolised but rather remain present in the blood.

A small number of *P. falciparum*-infected HIV patients failed to clear parasitaemia in our study despite the apparent elevated lumefantrine concentration in peripheral blood. This could be due to the same issue of the host immune status (WHO, 2006a). Byakika-Kibwika et al, (2012) infers that the difference between the lumefantrine in their study and that of Kredo et al (2011) could be due to inter-individual variability as a result of differing genetics thereby underlining the importance of the assessment and influence of HIV status on pharmacokinetics of drugs. Additionally they opined that this was in contrast to the Kredo study where the subjects had CD4 cell counts >200 cells/mm^3 and were on stable ART making the subjects somewhat similar to ours. Our study participants were on stable ART for durations longer than 6 months and had a mean CD4 cell count of 411 cells/mm^3 (sd 257) with a very wide range between 11-1593.

Another possible reason for the failure to clear the parasites despite the apparent increased lumefantrine concentration could be due to a possible reduced exposure of artemether and DHA as established in the Ugandan study (Byakika-Kibwika et al., 2012). Artemether and DHA exert their pharmacological properties in the first 48hours of treatment to clear the parasite biomass leaving behind the residual parasites to the longer acting partner drug lumefantrine (Ezzet et al., 1998, Djimdé and Lefèvre, 2009). Our observation was that by day 3 and 28, the HIV-positive people had more parasites that were not cleared than the HIV-negative group (day3: OR: 3.2, P=0.011; day 28: OR: 2.86, 0.007). This may suggest that these parasites were not cleared by artemether or DHA and were therefore left to be cleared by the partner drug lumefantrine, so that even with a higher lumefantrine level the parasites that escaped the artemether were still not cleared probably suggesting that the inability to do so was a result of slow clearance of artemisinin (Dondorp et al., 2009).

Report (Farnert et al., 2012) of AL failure in a returning Japanese worker from Tanzania despite adequate lumefantrine level is another case which cannot easily be explained. The authors however suggest that the failure could be due to reduced sensitivity to lumefantrine even though the suboptimal concentration of desbutyl-lumefantrine (the metabolite of lumefantrine) and a missed dose could not be ruled out. Even though, this result was not obtained in HIV-positive patients, it brings to light the possibility of the influence of this metabolite in parasite clearance.

The scope of our study neither includes the assessment of parasite clearance time nor the measurement of the concentrations of artemether or DHA. Moreover since the subjects in our study were asymptomatic-malaria adults with very low parasitaemia, measurement of parasite clearance is very difficult. This is easier in symptomatic clinically ill patients with high parasitaemia concentration. Also we did not measure the level of desbutyl-lumefantrine which has been shown to exhibit more potent in vitro antimalarial activity than lumefantrine and could possibly be a better marker for assessment of treatment failure of AL (Wong et al., 2011). As a result of this incomplete information therefore we cannot conclusively state any definite reason for the inability to clear parasite despite the higher lumefantrine concentration. A larger and more detailed pharmacokinetic study will throw more light on these unanswered questions.

With regards to the influence of food intake on the measured lumefantrine concentrations, even though the administration of AL was unsupervised, I do not think this has any impact on the lumefantrine concentration of the HIV-positive people since the situation was similar on both arms of the study. Moreover the mean lumefantrine levels in both arms exceeded the 270ng/ml used as a threshold concentration to predict treatment failure (Ezzet et al, 1998). It is also known that AL has high cure rate irrespective of being given under supervision with food or not (Piola et al., 2005, Dunyo et al., 2011). Moreover, according to Djimde and Lefevre, (2009) standard African diets or even breast milk are sufficient to meet the need of very small dietary fat necessary to ensure adequate lumefantrine absorption.

One of the areas of concern about the parasite clearance could be the parasitaemia level at the onset of the study before treatment, whether the initial parasitaemia could be a confounding factor. In order to evaluate the baseline parasitaemia of study participants, assessment of day 0

parasitaemia using qPCR was performed for some samples both of HIV-positive and HIV-negative (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+). Established primers (Beshir et al., 2010b) were used to amplify both the parasite gene and the human gene. The baseline parasitaemia on both the samples that cleared parasites and those that did not on day 3 (Table 4.2) were very low and there was no significant difference in their levels so I propose that initial parasitaemia is not a cofounding factor.

In conclusion, among the few studies on drug-drug interactions between ARVs and antimalarials, the question about treatment failure in HIV-infected patients on ARVs treated with artemisinin has not been addressed and therefore none has been able to establish the impact of these interactions on the parasite clearance. Many issues raised from the present study still remain unanswered so there is need to design further studies to provide answers to questions about what happens to the nevirapine, the possible influence it has on parasitological outcome and why the lumefantrine levels are increased. Our study was an attempt to answer some of the questions; unfortunately our sample size does not have the power to address these issues conclusively.

Even though, we were able to show that there was an elevated day 7 lumefantrine concentrations in HIV-positive NVP-treated participants compared to their HIV-negative counterpart who did not receive NVP, we found no evidence of parasitological benefit of the higher plasma levels of lumefantrine. This is a preliminary study and the results are consistent with the reports of Kredo et al, (2011). This is the first study of NVP-AL interactions in both malaria and HIV co-infected patients. Therefore we advocate the need for further studies on the ARV and antimalarial interaction in malaria/HIV co-infected patients detailing full pharmacokinetic study in relation to parasite clearance.

4.5 Limitations to the study

The major limitation was the inability to calculate parasite clearance time (PCT) and this is closely related to the low parasitaemia of the study participants since they were asymptomatic-malaria adult people. As result of this we could not calculate parasite reduction ratio so as to associate the levels of lumefantrine with parasite clearance.

Chapter Five

Polymorphisms in the *pfmdr1* and *pfcrt* gene before and after treatment with artemether-lumefantrine

5.1 Introduction

The emergence of artemisinin resistance has become a major threat to the treatment of malaria. As at date, the artemisinins stand as the mainstay of treatment of uncomplicated malaria since the failure of chloroquine, partly because of the rapid beneficial reduction of parasite biomass in the first 48 hours of malaria infection. Studies (Noedl et al., 2008, Dondorp et al., 2009, O'Brien et al., 2011, Malmberg et al., 2013a), have demonstrated reduced susceptibility of *Plasmodium falciparum* to artemisinin derivatives leading to delayed clearance. The implication of this is that the partner drugs such as lumefantrine, amodiaquine, piperaquine, mefloquine also are more likely to develop resistance because of the higher parasite biomass exposed to these drugs.

Single nucleotide polymorphisms of the *pfmdr1* gene particularly the 86N allele have been associated with *in vivo* resistance to lumefantrine in several studies (Sisowath et al, 2005; Humphreys et al, 2007; Lekana-Douki et al, 2011; Gadalla et al, 2011; Malmberg et al, 2013) and less sensitivity to artemisinin *in vitro* (Duraisingh et al, 2000a; 2000b). Studies on artemether/lumefantrine (AL) have established an association of the NFD (86, 184, 1246) haplotype (Humphreys et al., 2007, Happi et al., 2009) with decreased *in vitro* lumefantrine sensitivity and also *in vivo* post treatment of Ugandan children (Dokomajilar et al., 2006). The haplotype is associated with treatment failure. A more recent study by (Gadalla et al., 2011) reported the selection of the haplotype in samples collected from Sudan 14 days after treatment with AL.

Plasmodium falciparum strains sensitive to chloroquine show the ancestral haplotype CVMNK at amino acids 72-76 on the *pfcrt* gene. The most common variant of this gene, has three substitutions and encodes the haplotype CVIET, but *in vitro* experiments suggest that crucial change at the K76T codon is the most important. (Fidock et al., 2000, Djimde et al., 2001a, Warhurst, 2003). Increased prevalence of 76K in countries where chloroquine has been withdrawn for a long time indicates a restoration of chloroquine sensitivity in these areas (Wang et al, 2005; Laufer et al, 2010). *Pfcrt* modulates parasite susceptibility to a variety of drugs such as quinine, mefloquine and artemisinins (Sidhu et al., 2002) whose sensitivity are equally affected by mutations or change in copy number in the *mdr*1 gene (Cooper et al., 2002, Cooper et al., 2005). Studies (Basco and Le Bras, 1993, Duraisingh et al., 2000a) reported a

cross-resistance and inter–relationship with an inverse sensitivity to CQ among these drugs thereby emphasizing a relationship between *pfcrt* and *pfmdr1* genes. This inverse relationship is also seen between CQ and lumefantrine, where the decrease in lumefantrine activity is associated with the selecton of wild types *pfcrt*-76K and *pfmdr1*-86N, suggesting that the use of AL is likely to lead to the selection of more CQ-susceptible parasites (Mwai et al., 2009, Sisowath et al., 2009, Eyase et al., 2013).

The involvement of modified *pfcrt* gene 72-76 in clinical AQ failure (Beshir et al., 2010a, Sa and Twu, 2010) and the role of *pfcrt* modifications in conferring hypersensitivity to MFQ (a partner drug in ACT) and halofantrine (also structurally related to lumefantrine) (Johnson et al., 2004) calls for a close monitoring of this marker in areas where AL is in use especially with the reports of replacement in *pfmdr1* of the mutant allele 86Y (Tyrosine) by the wild type 86N (Asparagine) or the reversal to 86F (Phenylalanine) in some regions of Africa, (Kublin et al., 2003, Dlamini et al., 2010, Gadalla et al., 2011). This association is believed to be as a result of removal of CQ pressure.

Sutherland et al, (2002) in a study of Gambian children also reported that gametocytes emerging after treatment with CQ carry a mixture of the mutant strain of the *pfmdr1-86* and that of the *pfcrt-76* gene confirming the linkage disequilibrum shown earlier in Nigeria (Adagu and Warhurst, 2001). The study further emphasises the importance of determining the alleles of the genes associated with resistance in the analysis of CQR at population level and shows that other genotypic changes such as *pfmdr1* mutations can be advantageous to the resistant parasites or may be essential in some parasite populations (Warhurst, 2001b). Further relationship is in the fact that wild type *pfcrt* 76K and *pfmdr*1 86N alleles have been associated with a five-fold increase in lumefantrine inhibitory concentration (IC50) *in vitro* and also found to be selected for after treatment with AL *in vivo* in Kenya and Sudan (Mwai et al., 2009, Gadalla et al., 2011).

CQR was first reported in South-Western Nigeria in 1977 (Olatunde, 1977) and it increased by 2005 to 96% in the six geo-political zones of the country, hence the ban on CQ and subsequent adoption of ACTs as first-line treatment. However the use of Chloroquine (CQ) for self-medication against malaria is still very much in practice in Nigeria despite this ban. In spite of the National policy on ACT, a report indicates that 70% of children were treated with

chloroquine or SP (NPC & NMCP, 2012). This is because CQ is cheap and is easily obtained from pharmacies and medicine stores without prescription. A more recent study (Efunshile et. al, 2011) of the resistance pattern of CQ in Nigerian children reported 96.9% of the *pfcrt* 76T allele thereby indicating that CQ resistance is still an important issue in the country.

In the light of the reports (Kublin et al., 2003, Laufer et al., 2006) of the return of chloroquine-senstive strains of *P. falciparum* parasites twelve years after withdrawal of CQ, there is need to monitor molecular marker status for this drug which has been very effective in the past so as to ascertain its present resistant status in Nigeria. The increasing use of Amodiaquine in Nigeria for monotherapy (field observation) as well as it being a partner drug of artesunate-amodiaquine, the second line ACT in Nigeria calls for a close monitoring of the SVMNT haplotype which is generally implicated in amodiaquine resistance (Warhurst, 2003, Sa et al., 2009, Beshir et al., 2010a).

In our pilot study with DNA samples from imported cases of Nigerian origin, obtained from the Malaria reference laboratory (MRL), we detected the NFD haplotype in 20% of cases. No study to date has focused on the prevalence of this haplotype in Nigerian HIV-positive patients treated with AL. Our study evaluated this haplotype in this vulnerable group which are of great importance in the spread of antimalarial drug resistance because of their tendency to harbor higher parasitaemia (ter Kuile et al., 2004). This chapter provides some data and information on the prevalence baseline marker in this area on which future regular surveillance may be based.

The present study analysed the pre-and post-treatment samples collected for the presence of *pfmdr1* mutations to assess any allele, mutation or haplotype of interest with regards to the response to treatment with AL to identify any genotype selected after treatment, particularly the wild type allele 86N of the *pfmdr1* gene which has been associated with decreased sensitivity to lumefantrine *in vitro* (Duraisingh et al., 2000a) and a strong indicator of AL treatment failure or slow clearance (Sisowath et al., 2005, Humphreys et al., 2007).

This chapter therefore deals with the investigation of the polymorphisms of *Pfcrt* genes among HIV-positive and HIV-negative adults and information of the possible associations between point mutations on the *pfmdr1* gene. The implications of these findings in the management of CQR and amodiaquine resistance in the environment are discussed.

5.2 Rationale

The ACTs (AL and AS-AQ) have both been established to have high cure rates for the treatment of uncomplicated malaria in Nigeria (Meremikwu et al., 2006, Sowunmi et al., 2007). Therefore monitoring of the resistance markers related to the drugs is of vital importance in order to sustain their use in Nigeria. Since the adoption of AL as the first-line treatment for uncomplicated malaria, various studies (Falade et al., 2005, Falade et al., 2008, Happi et al., 2009) on its use in Nigeria have been reported all of which have established an association of either treatment failure or slow clearance with polymorphisms in the *pfmdr1* gene. As at date and time of our study, none of these studies involved the inclusion of HIV-infected subjects. There are no published studies on AL malaria treatment of HIV-positive patients in Nigeria. This situation is highlighted in Rivers state, Niger Delta region of Nigeria, where there is a high prevalence (6.0%) of HIV infection compared to the national prevalence of 4.1% (FRN, 2012). The study was designed to address this paucity of data and establish the current status of markers to resistance of AL in this very important region of the country.

5.3 Results

A total of 459 participants who visited both hospitals were interviewed. Participants came from different parts of the Niger Delta region. Out of this number, the HIV-negative people screened were 314 and 242 of these were found positive for *Plasmodium falciparum* by microscopy, while 145 HIV-positive people were screened and 128 who were positive by microscopy were recruited. The total number of filter papers collected on day 0 was 391. 21 persons out of the 391 people who gave their samples decided to opt out of the study because they were not willing to be finger pricked any more therefore the number of people enrolled in the study was reduced to 370.

5.3.1 *Pfmdr1* polymorphisms

DNA was extracted from the 391 filter papers collected and screened by PCR for the mutations on the *pfmdr1* gene. A total of 68, 66 and 79 out of these samples were successfully amplified by PCR and mutant sequences obtained at codons 86, 184 and 1246 on the *pfmdr1* gene respectively. All the samples showed only wild type on codons 1034 and 1042. Molecular analysis of the sequences showed a predominance of the wild type asparagine (86N) allele with

a 75% (51/68) prevalence compared to 13.2% (9/68) of tyrosine (86Y) in a pure form and 12% (8/68) of mixture or other alleles on day 0 (Table 5.1). Post-treatment samples collected during follow-up on days 3 and 28 were analysed for mutations on the same loci, and the sequences show similar pattern with 96.9% (31/32) 86N and a reduction of the 86Y to 3.1% (1/32) on day 3.

Baseline distribution of polymorphisms at locus 184 showed a high prevalence of 184Y (53%, 35/66) in its pure form as against 36.4% (24/66) of 184F. This pattern was maintained at day 3 after treatment (Table 5.2) while at codon 1246 there was a higher prevalence of 1246D (90.1% ,80/88) and 5.7%, 5/88 of 1246Y in pure form on day 0. None of the post-treatment samples harboured any mixed allele on locus 184 or 1246 (Tables 5.2 & 5. 3).

Table 5.1 Distribution of 86 polymorphism on days 0, 3 and 28 among HIV-positive and HIV-negative groups.

		N	Y	NY	F	FY	Total
D0	**HIV+ve**	**31**	**5**	**1**	**0**	**0**	**37**
	HIV-ve	**20**	**4**	**1**	**1**	**5**	**31**
D3	**HIV+ve**	**22**	**1**	**0**	**0**	**0**	**23**
	HIV-ve	**9**	**0**	**0**	**0**	**0**	**9**
D28	**HIV+ve**	**7**	**0**	**0**	**0**	**0**	**7**
	HIV-ve	**1**	**0**	**0**	**0**	**0**	**1**

Table 5.2 Distribution of 184 polymorphism on days 0, 3 and 28 among HIV-positive and HIV-negative groups.

		F	Y	FY	Total
D0	HIV+ve	10	23	3	36
	HIV-ve	14	12	4	30
D3	HIV+ve	4	19	0	23
	HIV-ve	5	4	0	9
D28	HIV+ve	4	3	0	7
	HIV-ve	0	0	0	0

Table 5.3 Distribution of 1246 polymorphism on days 0, 3 and 28 among HIV-positive and HIV-negative groups.

		D	Y	DY	Total	Total
D0	HIV+ve	38	1	1	40	
	HIV-ve	42	4	2	48	88
D3	HIV+ve	19	1	0	20	
	HIV-ve	11	3	0	14	34
D28	HIV+ve	16	3	0	19	
	HIV-ve	9	0	0	9	28

Analysis of the sequence for the selection of *pfmdr1* on different loci, revealed a very strong selection for the *mdr*86N wild type conferring resistance against AL with an **OR** of 8.77 (CI=1.2-380; **P**=0.016,) at day 3 after treatment. Further analysis also showed such selection in the HIV-positive group but this was not significant with an **OR** of 3.05 (**CI=0.8-12.85 P=0.063**).

However with the allele *mdr*86F, analysis showed strong evidence that HIV-negative group have a higher probability of harbouring this polymorphism. (**P=0.0051**).

Assessment of the day 0 pre-treatment samples, results showed 12 different haplotypes either in single or mixed forms. The most prevalent haplotype of the pre-treatment sample was the NYD (40%) in the pure form but also in combination with other haplotypes followed by (13%) of NFD (Table 3). This pattern was similar in both the HIV-positive and the control group. Post-treatment samples on days 3 and 28 revealed only NYD and NFD haplotypes with NFD being slightly higher than the NYD on day 28. A rare haplotype 'FFD' was detected in the pre-treatment samples day 0, this was as result of the polymorphism 86F which has recently been reported in Swaziland and Afghanistan (Dlamini et al, 2010; Beshir et al, 2010).

Table 5.4 *mdr*1 Haplotypes on days 0, 3 and 28

		NFD	NYD	NFD/NYD	Others	Total
D0	**HIV+ve**	**2**	**14**	**3**	**2**	**21**
	HIV-ve	**2**	**4**	**1**	**5**	**12**
D3	**HIV+ve**	**2**	**12**	**0**	**2**	**16**
	HIV-ve	**4**	**4**	**0**	**1**	**9**
	HIV+ve	**3**	**3**	**0**	**0**	**6**
D28	**HIV-ve**	**1**	**0**	**0**	**0**	**1**

Using the STATA case control study analysis for the results, the following were observed

- A very strong selection for *mdr*86N wild type after treatment at day3.

  OR ratio of 8.77; 95% CI: 1.2-380; **P**=**0.016**

- Weak evidence to show this selection in HIV-positive group

  OR: 3.05; 95% CI: 0 .8-12.85; **P=0.063**

- A strong evidence that HIV-positive patients had a higher probability of carrying the NYD haplotype **OR**: 4.8; 95% CI: 1.37-17-3; **P=0.005**

- A weak evidence to show that there is a selection of NFD at D28

  OR: 4.2; 95% CI: 0.549-33.470; **P=0.085**

- There was no evidence of selection of NFD at D3

- No evidence for selection for 86N at day 28 from the analysis

5.3.2 *Pfcrt* polymorphisms

Results of our study show that in the day 0 pre-treatment samples, *pfcrt*76T is still more prevalent (72.6%) than the 76K in the study area and the HIV-positive harbour more of this mutant group than the HIV-negative hence the wild type *Crt*76K was found to be less common among the HIV group (Table 5.5) however this difference was not significant (OR: 2.5; 95**% CI:** 0.824-7.95; **P= 0.0722**). A larger study may probably give a more significant result.

Only two *pfcrt 72-76* haplotypes: the wild type CVMNK and the mutant type CVIET in their single and mixed forms were observed in both the pre-treatment samples but both presented as single forms in the post-treatment samples. There was no presence of the minor haplotype CVMNT (Vieira et al, 2004) which has been previously reported in isolates from South-Western Nigeria (Gbotosho et al., 2012), Papua New Guinea (Mehlotra et al., 2001) Brazil and Peruvian

Amazon (Huaman et al., 2004, Fidock et al., 2000). Also we did not detect any SVMNT the haplotype commonly associated with AQ resistance (Beshir et al., 2010a, Sa and Twu, 2010, Fidock et al., 2000) which later has also been reported in Angola (Gama et al., 2010) and in Ghana (Mehlotra et al., 2008).

Table 5.5 Distribution of *crt* polymorphisms from day 0 samples

	***crt*76T**	***crt*76K**	**Total**
HIV-ve	**30**	**14**	**44**
HIV+ve	**22**	**6**	**28**

In analysing the associations between the *pfmdr*186 and *pfcrt*76 alleles, we found no evidence of an association between *mdr*86N and *crt*76K, neither was there any between *mdr*86Y and *crt*76T.

Table 5.6 Haplotypes of *pfcrt* gene

		CVMNK	**CVIET**	**CMNK/CVIET**	**Total**
D0	**HIV+ve**	**6**	**20**	**2**	**28**
	HIV-ve	**22**	**15**	**8**	**45**
D28	**HIV+ve**	**1**	**4**	**0**	**5**
	HIV-ve	**2**	**3**	**0**	**5**

5.3.3 New mutations

Sequence analysis of the gene samples to detect mutations flanking codons 86, 184, 1034, 1042 1nd 1246 using Chromas software, revealed three novel non-synonymus mutations in three pre-treated samples on the *pfmdr*1 gene at amino acid positions 73, 97 and 164 (Fig. 5. 4). The control sample 3D7 is depicted by the consensus chromatogram while the samples with the mutations are identified by their numbers. These chromatograms were confirmed on the Geneious software.

Sample 059: Serine to Leucine **(S97L)**

Change in nucleotide from Cytosine to Thymine at position 290 resulted in amino acid change from serine to leucine **(S97L)**

Sample 207A: Phenylalanine to Serine **(F73S)**

Nucleotide change from Thymine to Cytosine at position 218 resulted in amino acid from phenyl alanine to serine **(F73S)**

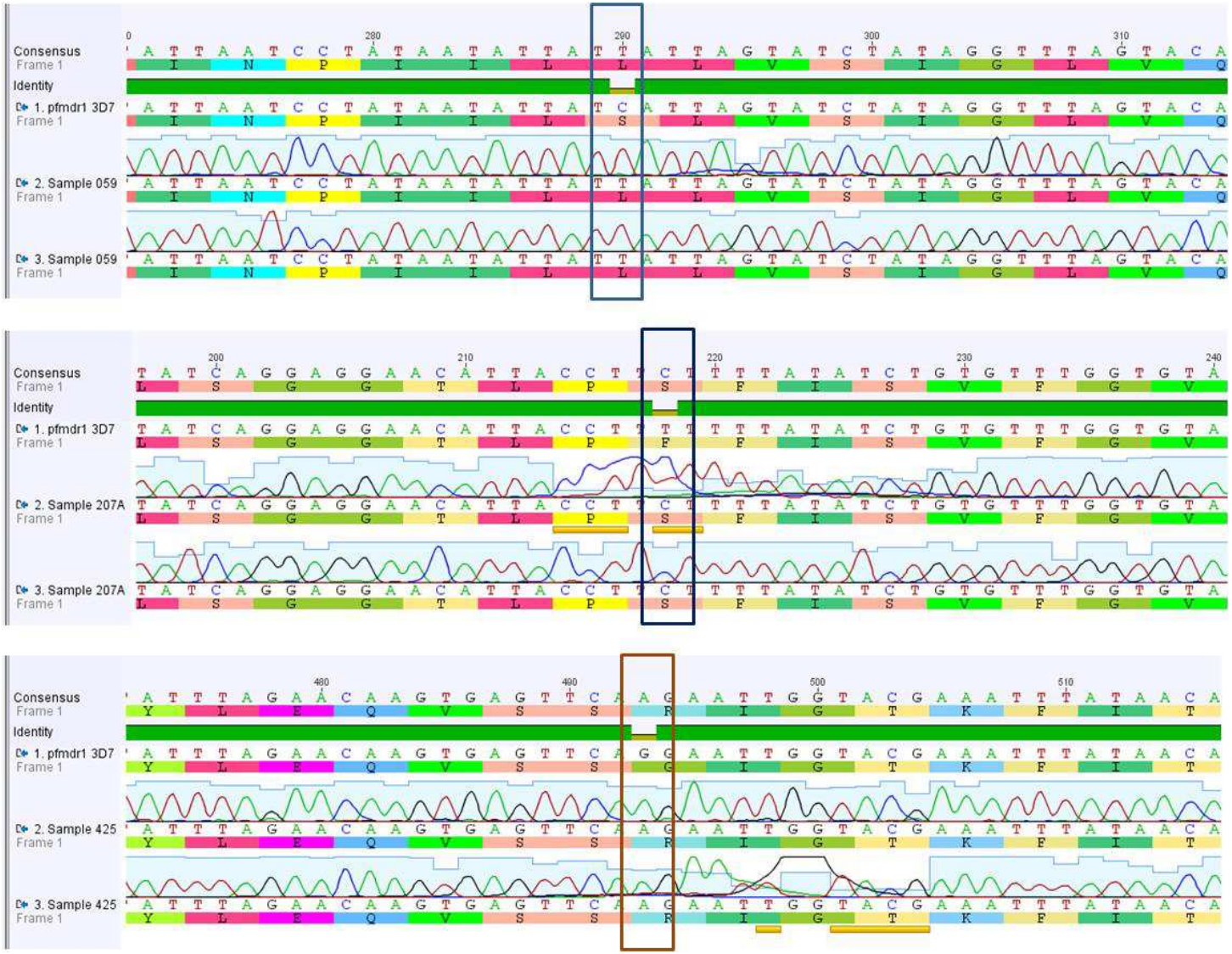
Sample 425: Glycine to Arginine **(G165R)**

Nucleotide change from Guanine to Adenine at position 493 resulted in amino acid change from glycine to arginine **(G165R)**

The three new mutations on the *pfmdr1* gene at positions F73S, S97L and F165R are all apparently associated with the 86Y and the 184Y.

Figure 5.1 New mutations in samples 059, 207A and 425

Chromatograms are from Geneious ver. 5.5.5



5.3.4 Parasite Clearance.

Using the PCR as a more reliable tool, we assessed the parasite clearance after treatment with AL based on positivity on any gene, we observed that 74.3% (107/144) of the samples that were PCR-positive on day 0 had cleared their parasites by day 3 while 25.7% (37/144) of the participants still harboured parasites that were detected by day 28 and 62.2% (23/37) of these were from the HIV-positive patients (Table 5.7).

The HIV-positive group had a lower parasite clearance by days 3 and 28. By day 3, HIV-positive group still harboured 25.8% (16/62) of the initial parasites and by day 28, 37.1% (23/62) of the parasites were yet to be cleared. On the whole, HIV-positive participants had 62.9% (39/62) clearance while the HIV-negative control group had a clearance of 82.9% (68/82).

There is evidence from day 3 positivity to show that HIV-positive patients are slower to clear sub-patent parasites after AL treatment (**CI=1.177-9.340; OR=3.22; P=0.011**). This was more evident on day 28 with a higher P value (**CI=1.239-6.727; OR=2.86; P=0.007**). The total parasite clearance was 74% (107/144).

Table 5.7 Parasite clearance at days 3 and 28 in the different groups

	Day 0	Day 3	Day 28
HIV+ve	**62**	**16**	**23**
HIV-ve	**82**	**8**	**14**
Total	**144**	**24**	**37**

5.4 Discussions

The results of the study reveal a high prevalence of 86N in pre-treatment samples and its very strong selection after treatment at day 3 with an OR ratio of 8.77 (P=0.016, CI=1.2-380). There is also weak evidence to show this in the HIV subjects after stratification (Table 5.1, P=0.063). A larger sample size with a higher power may provide a clarification.

The prevalence of 86N has been suggested as a first step to lumefantrine tolerance and its selection as a strong indicator of AL treatment failure or slow clearance (Sisowath et al., 2005, Humphreys et al., 2007, Mwai et al., 2009). There was no evidence of this selection at day 28.

Mutant allele 86Y has been associated with enhanced sensitivity to AL and resistance to CQ and AQ in West African countries like Nigeria, Guinea Bissau and The Gambia, (Adagu et al, 1995a, 1997; Adagu et al., 1996, Duraisingh et al, 1997); and also in Kenya and Thailand (Spalding et al., 2010, Lopes et al., 2002). In the result of this study, there was a 23.5% (16/68) prevalence of 86Y at day 0 both in the pure and mixed form but only one allele of 86Y remained by day 3 out of the 16 originally present at day 0 thereby showing that the fitness of the parasite carrying 86Y mutation was greatly reduced after treatment with AL. It has been proposed that the 86Y exerts its effect in the presence of *crt*76T thereby increasing the fitness of parasite resistance (Djimde et al., 2001a, Wellems and Plowe, 2001, Tinto et al., 2003). On the other hand, there is increased sensitivity of Plasmodium parasites to arylaminoalcohols with the presence of the mutant 86Y and its presence is favourable for chloroquine resistance (Duraisingh et al., 2000a).

The absence of mutant in positions 1034 and 1042 is in agreement with the study of (Folarin et al., 2008) in south-Western Nigeria. These mutations are well known in South America where it has been shown with mutant *pfcrt* to confer high grade resistance to chloroquine (Zalis et al., 1998, Huaman et al., 2004). Earlier studies in Nigerian isolates (Adagu et al, 1995a, (Adagu et al., 1995, Happi et al., 2003), did not detect the 1246Y mutation which has been associated with CQR and AQR in South American and East African isolates (Foote et al., 1990, Zalis et al., 1998, Holmgren et al., 2007, Dokomajilar et al., 2006, Humphreys et al., 2007).

Our results showed that the NFD haplotype which has been associated with AL slow clearance, increased tolerance and treatment failure (Dokomajilar et al., 2006, Sisowath et al., 2007, Humphreys et al., 2007) was found to be more prevalent in HIV-positive patients however the haplotype NYD had a higher prevalence in the overall population. The slight selection of NFD haplotype by day 28 is indication that AL tolerance or failure is not very evident in this part of Nigeria even among the HIV-positive people. This could be probably because AL has not been in long-term use in Nigeria, having been introduced in 2005. However there is need for close monitoring of AL use especially following the report of the NFD haplotype in South-Western Nigeria among children (Happi et al, 2009).

The high prevalence of 76T in pre-treatment samples is consistent with high rates usually obtained in chloroquine-resistant high endemic areas (Djimde et al., 2001a, Duah et al., 2007, Mlambo et al., 2007). In contrast to an earlier study (Folarin et al, 2008) of children treated with CQ, there were 30% (3/10) of crt76K in the post-treatment (day 28) sample which interestingly had been found at day 0 to contain the mutant 76T, 2 being isolates from HIV-negative participants while the third was from a HIV-positive patient. The presence of the wild type 76K in the post-treatment samples possibly indicates the gradual restoration of chloroquine-senstive strains with the longer use of AL in Nigeria in contrast to Folarin's study in 2008 which was carried out not many years after the introduction of ACTs (AL or ASAQ) as first-line treatment.

Our results show an unexpected presence of six cases of the N86F in the pre-treatment samples and these are all from the HIV-negative people. This polymorphism was reported in clinical isolates from Afghanistan (Beshir et al., 2010a) and in samples from Swaziland (Dlamini et al., 2010). These mutations mostly presented as a mixed infection with 86Y. The significance of this is not clear but suggests the need for investigations of new mutations for close monitoring of antimalarial drug resistance. The possible explanation of this is that the withdrawal of CQ may result in the change from tyrosine (Y) which is associated with CQR to phenylalanine (F) because they are more structurally related and are both aromatic in nature. Selection of 86N is more prevalent where AL is in use. It is noteworthy that the HIV-positive group had a higher prevalence of this allele while the HIV-negative group had a higher prevalence of the 86Y in both pure and mixed form.

From this study, there is evidence to show that among adult patients with asymptomatic-malaria infection in Port Harcourt Nigeria, the HIV-negative control group had a higher parasite clearance than their HIV-positive counterparts (Table 5.7; CI: 1.239-6.727; OR: 2.86; P=0.007). Parasite clearance is dependent on factors such as host factors, the parasite as well as initial parasitaemia and drug factors (White, 1997, Anderson et al., 2010). A major host factor that plays a role is host immunity (Djimde et al., 2003) hence adults in endemic regions who have acquired immunity by virtue of several exposures (Whitworth et al., 2000) are able to clear their parasites even without treatment. The immunosuppression that is prevalent in HIV-positive persons may lower the immunity acquired as adults thereby reducing their ability to clear parasite. This is probably why the HIV-positive patients had higher parasitaemia at the end of treatment. It has been hypothesised that chronic asymptomatic-malaria infections are non-immunogenic (Dinko et al., 2013) as

against symptomatic infections (Djimde et al, 2003; Diallo et al, 2007). This could possibly explain why there was a high percentage of sub-patent parasites that remained uncleared in our study participants by day 28 as against the high parasite clearance (93.9%) previously reported in AL-treated symptomatic malaria infants in Ibadan Nigeria (Falade et al., 2005). Our results show that even in semi-immune persons, there is inability to completely clear sub-patent parasites after treatment with AL as also observed in the Ghanaian children treated with dihydroartemisinin and piperaquine in the Dinko et al (2013) study. These results were not coordinated with microscopy readings since our initial diagnosis based on microscopy was flawed by very many false positives. Follow-up studies with microscopy parasite clearance and PCR is urgently needed in addition to *in vitro* drug resistance studies to demonstrate these associations.

The CQR mutant *crt*76T was found to be more prevalent than *crt*76K in our study area despite the official change to ACT in 2005 and this was higher in the HIV-positive group. The high prevalence of this allele corroborates the recent study in Lagos, South-west Nigeria (Efunshile et al., 2011) indicating a 96% prevalence of *crt*76T. High prevalence of *crt*76T is not surprising since this is still obtainable in most parts of Africa where CQ is still in use. The higher prevalence of the *crt*76T (though not significant) in the HIV-positive group before and after treatment is suggestive of the fact that the HIV-infected people have more selection of the mutant allele CVIET after treatment with AL possibly due to increased drug pressure. A larger study may indicate a more significant result.

The continued use of a drug in a locality confers a selective advantage to any parasites carrying resistant genes for that drug and leads to higher transmission rates of the drug resistant genes (Handunnetti et al., 1996, Sutherland et al., 2002, Hallett et al., 2006). This explains the high prevalence of *crt*76T, despite the official change from chloroquine use to ACTs because chloroquine use is still very common in Nigeria. One can infer that the presence of molecular markers of chloroquine resistance keeps AL efficacy up (Sutherland et al., 2011, Raman et al., 2011). For efficient control of antimalarial drug resistance, there is need to reduce the drug pressure through improving the ways the drugs are used especially in the face of self-medication and misuse of drugs.

The result of our field work study shows that AL was the third most commonly used antimalarial by our study participants, albeit without prescription. Adding up this finding from the field observation and selection of *mdr*86N, there is therefore need for close monitoring of AL use in order to assess the level of resistance being developed in Nigeria.

Further studies are needed to confirm these findings especially since there is no published report of AL efficacy in the Niger Delta region. This is particularly important with regards to the HIV-infected persons who exhibited lower parasite clearance. Our summation is that further studies be carried out with a larger sample size among HIV-infected malaria patients so as establish the impact of the *mdr*86N and *crt*76T selection as well as the parasite clearance following treatment with AL in order to curtail the threat to AL efficacy in the area.

The findings of this study therefore serve as additional information to the scarce data available on the use of AL in Nigerian adults especially in the Niger Delta where there is no published data, and should help to form a data basis of calculating sample size for a larger study.

5.5 Limitations to the study

The study aimed to investigate the molecular markers related to AL treatment with particular reference to polymorphisms on the *pfmdr1* gene. In order to ascertain whether the polymorphisms that remain after 28 days treatment are recrudescent parasites or new infections, there is need to genotype for polymorphic regions of surface antigens *msp1* and *msp2* in the pre and post treatment isolates. Therefore one major limitation to this study was the inability to do this PCR correction. This was due to poor yield of DNA because of very low parasitaemia. As a result of this we could not differentiate between new infections and recrudescence. Additionally the scope of our study did not include the investigating of copy numbers another factor associated with AL treatment failure.

Chapter Six

Antifolates and their molecular markers in asymptomatic-malaria Nigerian adults. Drug assays for trimethoprim; any relationship with dhps_431V?

6.1. Rationale

Antifolate drugs are very important in malaria control because of their use in the prophylaxis of malaria in people at higher risks of malaria infection such as pregnant women, children, infants and HIV-infected persons. The concept of preventive treatment of malaria intermittently (IPT) as recommended by WHO (2004; 2006; 2009) for the treatment and prevention of malaria infection or clinical episodes in vulnerable persons has been proved to be very efficacious in several studies in infants (IPTi) (Schellenberg et al., 2005, Gosling et al., 2009, Aponte et al., 2009). IPT in pregnancy (IPTp) helps prevent maternal anaemia and reduces neonatal mortality (Shulman et al., 1999, Menendez et al., 2010). It was also found to improve cognitive ability of semi-immune Kenyan school children and reduced annual incidence rate of clinical malaria in children in Mali (Clarke et al., 2008, Dicko et al., 2008). The drug of choice for intermittent treatment is Sulfadoxine/pyrimethamine (SP), however later studies have included a partner other drug in addition to SP. This was evidenced by the recent study in Senegal with the use of AQ+SP which reported lower incidence of parasiatemia in areas where it was used for seasonal malaria chemoprevention (SMC), formerly IPTc (Lo et al., 2013). IPT was adopted in Nigeria in 2005 as part of the national guidelines for the prevention of malaria in pregnant women (IPTp) in line with the WHO recommendation that pregnant women living in high transmission areas should receive at least two doses of SP for IPTp after quickening, the first noted movement of the foetus, (FMOH, 2005). However studies have shown that IPTp use among pregnant women in Nigeria is still very low (Akinleye et al., 2009, Efunshile et al., 2011, Ugwu et al., 2013).

Malaria in pregnancy and the impact of placental *Plasmodium falciparum* infection with consequent low birth weight (LBW), maternal anaemia, intrauterine growth restriction and preterm delivery (Steketee et al., 2001) is a major concern in sub-Saharan Africa. The non-compliant use of SP-IPT therefore will invariably increase the burden of these risks contributing substantially to infant deaths globally in the malaria-endemic regions.

In addition to its use in intermittent preventive treatment, SP is also given in combination with AQ (AQSP) and this combination has been found to be more efficacious than artemether-lumefantrine (AL) regimen in preventing recurrent P*. falciparum* malaria in Burkina Faso (Zongo et al, 2007).

6.2. Resistance to antifolates

Considering the importance of antifolates in IPT, there is a great need of the understanding and monitoring of resistance in the areas where they are in use. Since the introduction of SP in the early 1980s as a result of chloroquine failure (Roper et al., 2003), there have been increased reports of resistance to this drug especially in East and southern Africa. Pyrimethamine resistance was earlier recorded and this was subsequently followed by sulfadoxine resistance in the early 90s (Omar et al., 2001, Kublin et al., 2002, Naidoo and Roper, 2010). Substitutions on the target enzymes *dhfr* and *dhp*s have long been established as source of resistance in these drugs (Cowman et al., 1988, Kublin et al., 2002, Bwijo et al., 2003, Karema et al., 2010). In contrast to the principle of accumulation or efflux of drugs by transporters as obtained in the quinolones, resistance to the antifolates is due to altered affinity at the drug target as a result of single or multiple mutations on the genes (Wang et al., 1997).

Triple mutations in the *dhfr* gene at codons N51I, C59R and S108N have been associated with reduced efficacy of pyrimethamine and the additional mutation on the 164 codon from Isoleucine to Leucine (I164L) results in the highest level of resistance to pyrimethamine (Ahmed et al., 2006). However the *dhfr* I164L is not very common in Africa but has only been reported in some parts of East Africa (Nzila et al., 2005, Juliano et al., 2008).

Similarly, mutations at various points on codons 436, 437, 540, 581 and 613 on the *dhps* gene in various combinations especially result in decreased sulfadoxine efficacy. Acquisition of the quintiple mutants (*dhfr* N51I/C59R/S108N/*dhps* A437G/K540E) is associated with increased risk of SP resistance and treatment failure (Kublin et al., 2002) as found in some parts of East Africa. The occurrence of *dhps* A581G in addition to A437G or A437G+ K540E confers, higher resistance to sulfadoxine (Naidoo and Roper, 2010).

A non-synonymous mutation at position 431 of the *dhps* gene involving a change from isoleucine to valine (I431V) was observed in three isolates from imported cases of *P. falciparum* of Nigerian origin in UK (Sutherland et al., 2009). Preliminary data from the study shows that in several cases the mutation I431V was found with three other mutations S437G, A581G and A613S presenting as VSGKGS and VAGKGS; and rarely occurred with A581 presenting the haplotype VAGKAA. We hypothesize that the use of trimethoprim/sulfamethoxazole (TS) prophylaxis in HIV-positive patients may be placing

additional sulfonamide selective pressure for new alleles at the *pfdhps* locus that are not directly related to antimalarial use (Sutherland et al, 2009). The study was designed based on the hypothesis that HIV-positive patients are infected with parasites that are selected by the drugs they take, including ARV and TS, therefore their *pfdhps* genotypes will differ from that of the HIV-negative patients. We therefore decided to measure and compare the prevalence of different *dhps* haplotypes including I431V in the two different groups and look for any evidence of association with recent use of TS.

The misuse of antibiotics and antimalarial drugs is very common in Nigeria. Despite the ban of SP and the subsequent adoption of ACTs as first-line treatment for malaria, its use is still very common in Nigeria being one of the cheapest antimalarials available. Furthermore, TS a structurally related drug to SP which is usually given to HIV-positive people for prophylaxis against opportunistic infections, shows cross resistance with pyrimethamine and trimethoprim in *in vitro P. falciparum* culture (Iyer et al., 2001, Khalil et al., 2003). It is the cheapest antibiotic in use in Nigeria and can easily be obtained over the counter without prescription. Its misuse in the country could result in increased incidence of resistance to SP and therefore endanger the prophylactic use of SP for IPTp, IPTi and IPTc programmes.

The SNP at codon 540 on the *dhps* gene is responsible for increased resistance to SP. A cut-off of 50% prevalence for *dhp*s K540E mutation has been recommended by the WHO above which SP-IPTi should not be implemented in any country (Naidoo and Roper, 2010). In East Africa, the use of SP has been greatly affected due to the high prevalence of this mutation in many countries such as Uganda (Staedke et al., 2004), Kenya (Zhong et al., 2008, Bonizzoni et al., 2009), Ethiopia (Alifrangis et al., 2009), and Rwanda (Karema et al., 2010) contrary to the situation in West Africa where there is low resistance to SP as result of low incidence of the K540E (Naidoo and Roper, 2010).

There is paucity of data on the prevalence of *dhps* K540E and *dhfr* I164L in Nigeria, however a 23.8% prevalence of *dhps* K540E was reported in western Nigeria among children less than 5 years (Happi et al., 2005). Based on this we decided to assess the prevalence of K540E and the presence if any of *dhfr* 164L and *dhps* 581G in Port Harcourt, in the Niger Delta region. IPTp was adopted in Nigeria in 2005 as part of the national guidelines. Since then, there has been little or no information about its use or the monitoring of the K540E with regards to its effect on IPTp programme in Nigeria. Moreover WHO (2009) advocates the monitoring of the prevalence of mutations associated with SP resistance and the

determination of the predictive value of mutation at *dhps* 540 or the quintuple mutation haplotype in different settings and populations.

Studies (Shah et al, 2006, Whitworth et al, 2000, French et al, 2001) have shown that HIV subjects have increased incidence of clinical malaria and antimalarial treatment failure. Therefore they are more likely to carry more resistant parasites because of increased drug pressure. Although the presence of these markers may not affect AL efficacy, it may however affect the transmissibility of the resistant strains of the parasite thereby reducing the efficacy of SP prophylaxis in areas where it is used in combination with artesunate. Furthermore, if the newly emerging mutations in Africa *dhfr*164L and *dhps* 581G are found in conjunction with the *dhfr* triple and *dhps* double mutants there will be reduced sensitivity to SP thereby endangering the use of SP for both IPTi and IPTp (Naidoo and Roper, 2011).

This chapter deals with the investigations of the presence of *dhps* I431V in the HIV-positive group as well as establish any relationship between its prevalence and TS use in addition to their HIV status. Additionally, the prevalence of *dhps* K540E and *dhfr* I164L and any other mutations of interest in both genes were investigated in the two groups.

6.3 Results

Out of 391 filter papers obtained from the participants on day 0, amplification of the *dhps* gene using PCR yielded 67 positive results; however, some of the sequence was not complete for all the codons of interest. Molecular analysis of the sequences showed a predominance of the mutant A437G (94%). The mutation on codon 431 Isoleucine change to Valine (I431V) previously detected only in Nigerian isolates (Sutherland et al., 2009) was found in 19 samples (28.79%) and 2 of these were in mixed form with the wild type Isoleucine (I+V). The prevalences of all the alleles are shown in Table 6.1.

Table 6.1 Distribution of *dhps* polymorphisms

Allele	Prevalence
I431V	19/66 (28.8%)
S436A/F	26/66 (39.4%)
A437G	62/66 (94%)
K540E	0/67 (0%)
A581G	14/65 (21.5%)
A613S	15/65 (23.1%)

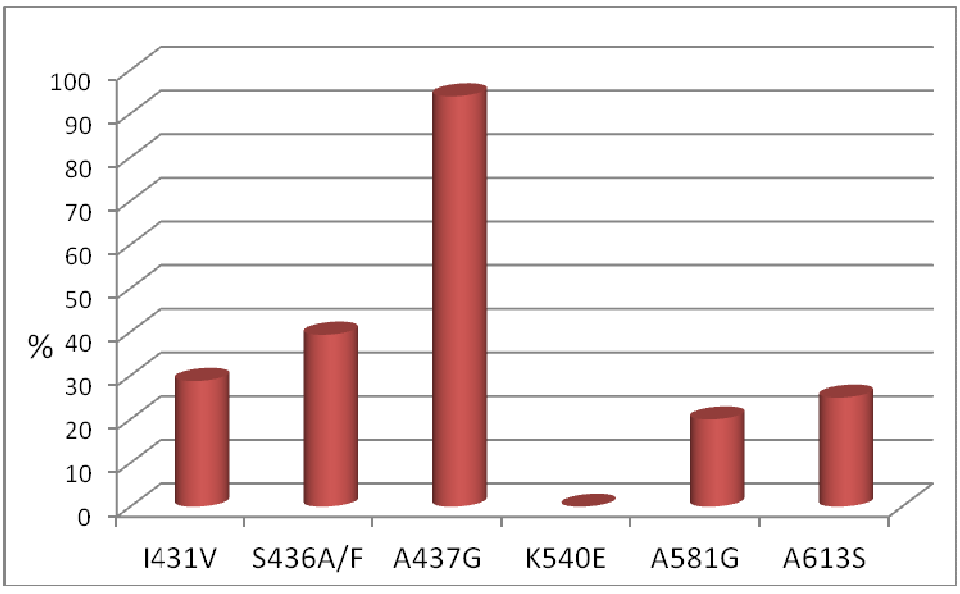


Figure 6.1 Polymorphisms on the *dhps* gene

Analysis of the sequences revealed the absence of K540E. All the samples retained the K540 wild type.

6.3.1 Association of the alleles on the *dhps* gene

The newly described mutation I431V was observed in 19 samples (28.8%) and 2 of these were mixed with Isoleucine (I+V). The overall prevalence of I431V was 28.8% (n=19/66) while that of A437G was 94%. The 431V was found to have a strong association with the 613S. Analysis of the different alleles of the *dhps* gene showed pair-wise associations between the alleles as shown below:

431V and 613S OR: 7.6; 95% CI: 1.716- 35.303; **P=0.0012 (N=61)**

581G and 613S 95% CI: 15.669-8352; **P=0.0000 (N=64)**

436A and 581G OR: 23.57; 95% CI: 2.732-1052.709; **P=0.0002 (N=58)**

436A and 613S OR: 37.92; 95% CI: 4.501-1655.282 **P=0.000 (N=61)**

There was no special association of the 437G mutations with HIV status. Our earlier hypothesis is that HIV-positive patients are infected with parasites that are selected by the drug they take and therefore their response to the drug will differ from that of the HIV-negative patients. We expected a difference in the outcome of the mutations. Our results show that there was no association between the HIV status and the 431V mutation. Using chi square test, we compared the frequency of mutations at position 431 on both arms and we found no difference (**P=0.876**). Out of all the polymorphisms in the 613, there was no 581G that did not present in combination with 613S. We could not calculate the **OR** however the P value =0.000.

6.3.2 Haplotypes of the *dhps* gene

Our results show a variety of different antifolate resistance-associated haplotypes on the *dhps* genes. The prevalences of the various haplotypes are presented in the Table 6.2 below. The most common *pfdhps* haplotype is the ISGKAA with 50.8% followed by the VAGKGS.

Table 6.2 *Dhps* haplotypes

Haplotype	Frequency	Percentage
VAGKGS	8	12.3
ISGKAA	33	50.8
VAGKAA	7	10.8
VAGKAS	1	1.5
IAGKAA	4	6.2
VSGKAA	1	1.5
ISAKAA	4	6.2
IAGKAS	2	3.1
VSGKAA	1	1.5
ISAKGA	1	1.5
IFAKAS	1	1.5
ISGKGS	1	1.5
IAGKGS	1	1.5
Total	65	100

***mixed genotypes were included in both categories; therefore total frequency is greater than the total number (59).**

Nucleotide sequences of the *dhps* gene showed mixed infections in some samples and these were more prevalent in the HIV-positive samples. Fig 6.2 and 6.3 show two HIV-positive samples with mixed infections (at positions 436 and 437). We did not observe any difference in the prevalence of mutant alleles of the *dhps* gene between the HIV-positive individuals and the HIV-negative control group.

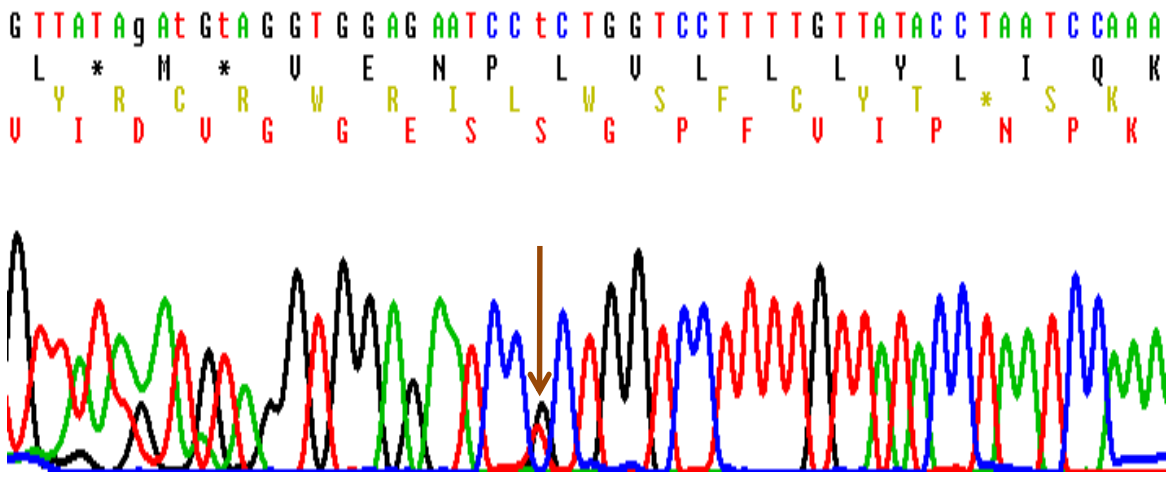


Figure 6.2 Diagram showing the chromatogram of mixed infection at position 436 in a HIV-positive patient

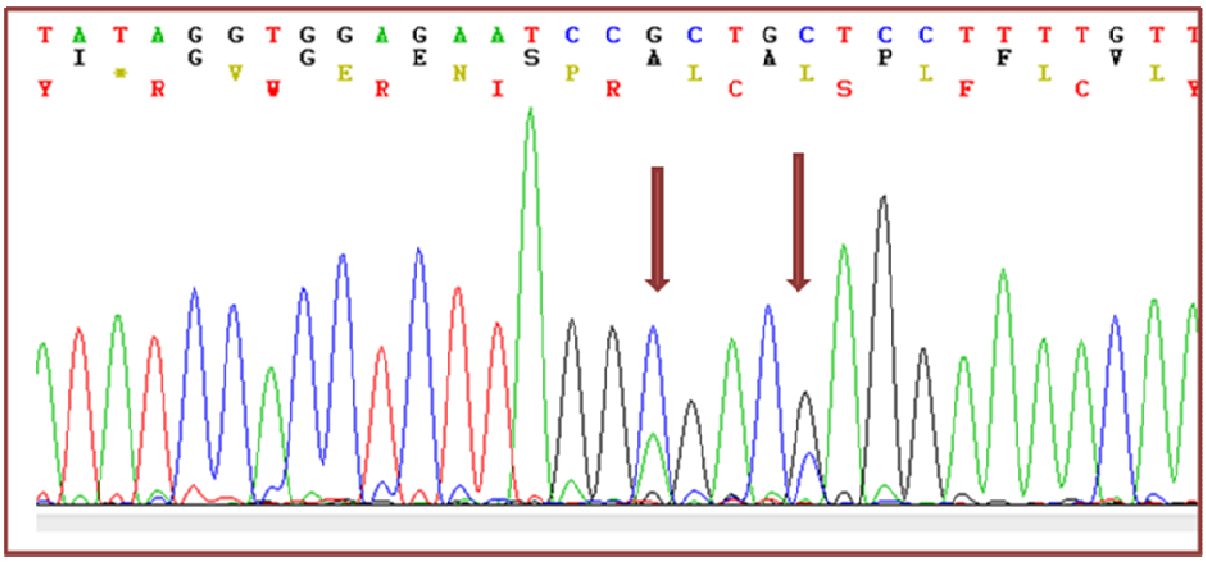


Figure 6.3 Diagram showing the chromatogram of mixed infection at positions 436 and 437 in a HIV-positive patient

6.3.3 New mutations

In addition to the known polymorphisms on the *dhps* gene, we observed two novel synonymus polymorphisms. A change at nucleotide 1732 from thymine to cytosine (T1732C) did not result to any change in the amino acid which remained leucine (L), and at position 1878 from adenine to guanine (A1878G) while retaining the same amino acid Glutamine (G). These polymorphisms occurred as mixed infection in a single HIV-negative patient.

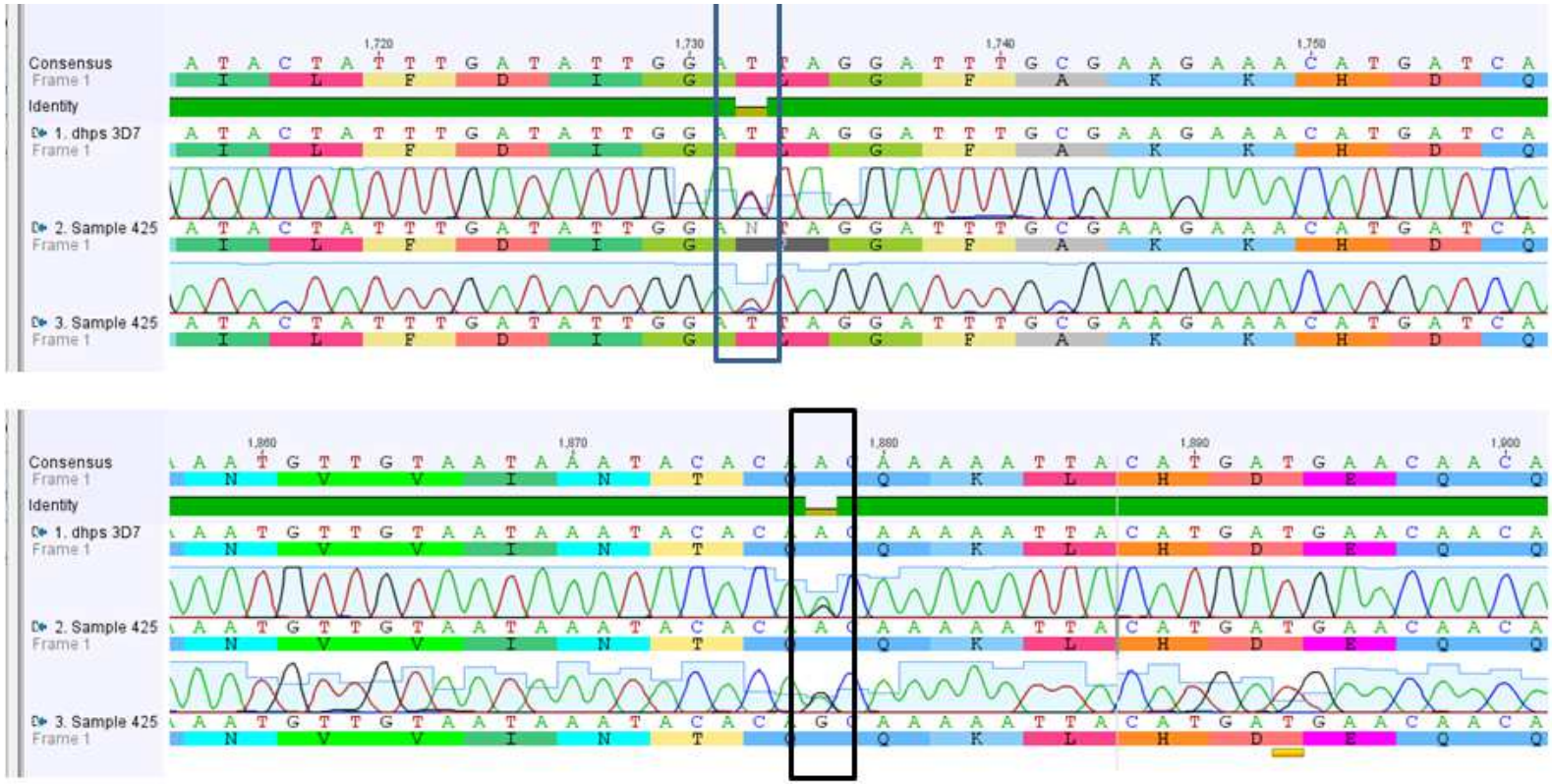


Figure 6.4 Chromatogram from Geneious of sample 425 showing two synonymus mutations with mixed infection at two different positions in the same sample

6.3.4 *Dhfr* polymorphisms

On the *dhfr* gene we were able to amplify only 48 full gene fragments encompassing the loci of interest. Table 6.3 shows the various haplotypes obtained on the different genes. Molecular analysis of the sequences showed a predominance of the triple mutant (N51I, C59R, S108N) with a 95.83% prevalence. No single isolate carried the pure wild type haplotype NCSI instead there was a haplotype consisting of a mixture of wild type and mutant alleles (NCNI) and a single case of both the mutant haplotype and haplotype mixture of wild type and mutant allele.

The predominant haplotype was IRNI (codons 51, 59, 108 and 164). All the samples showed only wild type on codon 164, so there was no quadruple mutant. There were a total of three different combinations of the *dhfr* mutations in all the sequenced samples. The S108N allele had the highest prevalence (97.9%).

Table 6.3 D*hfr* haplotype

Type	Frequency	Percentage
ICNI/IRNI	1	2.08
IRNI	46	95.83
NCNI	1	2.08
Total	48	100

*N=48

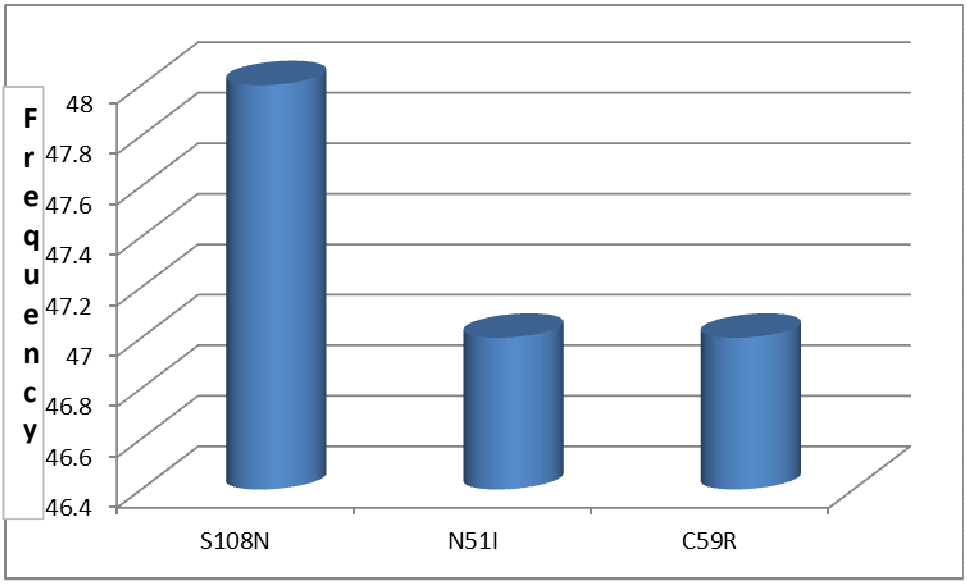


Figure 6.5 Distribution of the polymorphisms on the *dhfr* gene showing the allele S108N with the highest prevalence.

6.4 Trimethoprim/Sulphamethoxazole (TS) assay

Towards the completion of our studies, Dr. Harparkash Kaur who did the lumefantrine assay developed a protocol for detecting trimethoprim and sulphalmethoxazole (TS) in the laboratory. Day 7 samples collected for estimation of lumefantrine on filter papers pre-treated with 0.75M tartaric acid were used. Participants identified as positive for either component of TS are presented in Table 6.4 (N = 18). The remaining 158 participants tested were negative for both drugs.

Table 6.4 TS assay result

Sample ID	Trimethoprim conc	SMX conc	HIV status	Reported TS use
86	3.19	59.28	0	ND
104	0.00	24.30	0	ND
46	0.00	20.96	1	N
150	0.00	19.06	1	ND
51	3.08	12.81	1	N
48	0.00	10.45	1	N
41	0.91	8.00	1	N
49	0.00	7.13	1	N
164	0.00	6.35	1	N
103	0.00	2.63	0	ND
101	0.00	2.29	0	ND
121	0.00	2.09	0	ND
153	0.00	2.01	1	N
152	0.00	1.77	0	ND
192	0.00	1.06	0	ND
165	0.00	0.81	1	N
167	0.00	0.53	1	N
169	0.00	0.03	1	Y

*Legend: HIV status: 0=Negative; 1=Positive; Reported TS use: N=no; Y=yes; ND=no data

Observation of our results shows that detection of the TS did not match with the questionnaire answers obtained from participants. Out of the 176 samples analysed, only 18 samples were identified to have presence of either trimethoprim or sulphamethoxazole and only 3 of these 18 had the presence of trimethoprim detected. Analysis of the different alleles of the *dhps* gene based on our initial hypothesis that the TS being taken by HIV-positive people may be placing sulphonamide selective pressure for alleles at the *dhps* loci showed pair-wise associations between the *dhps* 437 and TS presence.

Table 6.5 Pair-wise association between TS positivity and *dhps* 437

TSpos	**437A**	**437G**	**Total**
0	3	30	33
1	1	1	2
Total	4	31	35

*N=35

Pair-wise relationship between TS and *dhps*437A shows that participants with detectable TS on day 7 suggesting recent use were more likely to carry the allele form of Alanine (dhps 437A) rather than the glycine 437G. (**P=0.077, OR=10.0 and CI= 0.096-809**).

Using same analysis for the 431 allele, we observed that there was no relationship established as shown in Table 6.6

Table 6.6 Pair-wise relationship between TS and *dhps*431

TSpos	**431I**	**431 I+V**	**431V**	**Total**
0	26	2	5	33
1	2	0	0	2
Total	28	2	5	35

6.5 Discussions

Contrary to our hypothesis that there will probably be an increased number of the 431V mutations among HIV-positive people because of the use of TS for prophylaxis, the result of the study showed no difference in the prevalence of this mutation. This could be attributed to the fact that very few of the HIV-positive people were actually taking TS regularly. Our observation from the field work in this study is that self-reported TS use among these subjects in the Niger Delta region, Nigeria is very low. Out of the 128 microscopy positive people recruited, only 27.7% (28/101) of the 128 reported regular use of TS. Analysis of the TS assay revealed no relationship between the *dhps*431 and TS use thereby disproving our hypothesis of any relationship of this allele to TS use.

Some of the HIV subjects were placed on TS at the initiation of ARV therapy but discontinued the use thereafter. Analysis of the results from the assay for detection of TS, showed that that detection of the TS did not match with the questionnaire answers as mentioned earlier. Therefore TS was not routinely used by our HIV participants. This was similar to the observation made in the study (Walker et al., 2010) among HIV-infected adults in Uganda and Zimbabwe studies where TS use was not strictly adhered to in some centres.

The insignificant relationship between TS and *dhps*437A (**P=0.077**) from our study is potentially of interest with a need for larger studies with development of better protocol for measuring TS. This is our first attempt to measure this drug so there is need for optimising the method for collection of TS. It may be more appropriate to collect the samples on the pre-treatment day so as to avoid any possible interaction with any drug during treatment.

The high prevalence of the single mutant A437G observed in the study did not confer higher degree of resistance since it only occurred in the absence of K540E required for high level resistance to SP (Brooks et al., 1994). Despite the high prevalence of HIV in the region and the indiscriminate use of SP as observed from the questionnaire data, there was no evidence of K540E observed in the samples even in the HIV cohort; all the samples retained the 540K wild type. However, we cannot rule out the possibility of its presence in a larger sample size.

The absence of K540E is in contrast to the 23% reported in children in Ibadan Western Nigeria by Happi et al, (2005). This implies that the use of SP for prophylaxis is not under

threat in Nigeria since the cut-off threshold for the use of SP is 50% prevalence of K540E. Therefore this authenticates/supports the continued use of SP for prophylaxis in IPTp, IPTi, IPTc. However, there is need for close monitoring of this polymorphism since its presence has been reported by Sutherland et al., (2009) in Ghana a neighbouring country to Nigeria. The absence of 540E shows that the *dhfr* has been more selected than *dhps* because of the long period of use of pyrimethamine as monotherapy for prophylaxis in Nigeria and subsequent loss of its efficacy (Nahlen et al., 1989) before it was used in combination with sulfadoxine.

To the best of our records and search, a single presence of 436F found among the HIV cohort is the first of this reported case in Nigeria since this has not been previously described. This may indicate an early sign of new mutations in the *dhps* gene being introduced into Nigeria as result of co-infection in the HIV patient. It is noteworthy that this same patient harboured a mixed infection in the 86 and 184 codons of the *mdr1* gene.

Out of the 48 *dhfr* sequences of our samples, none had the quintuple mutant (*dhfr* N51I/C59R/S108N and *dhps* A437G/K540E) which is considered a molecular marker for SP resistance and treatment failure (Bwijo et al, 2003; Kublin et al, 2002). However 20.8% (10/48) had the *dhfr* mutants N51I/C59R/S108N and *dhps* A437G with the absence of K540E. The low prevalence of these mutants in our samples could possibly be the reason why SP resistance is not very high in our study site, hence its continued effective use for IPTp in Nigeria.

The absence of *dhfr*I164L is in agreement with the report that *dhfr*I164L is very rare in sub-Saharan Africa, as all the report are from East Africa and Madagascar (Alker et al., 2005, Lynch et al., 2008, Hamel et al., 2008, Andriantsoanirina et al., 2011). This mutation found mostly in South East Asia and South America has been associated with proguanil as well as in conjunction with pyrimethamine (Ochong et al., 2008). The two drugs are no longer in use in Nigeria even though pyrimethamine monotherapy had long been in use for prophylaxis but has lost its prophylactic effect against maternal malaria (Nahlen et al., 1989, Falade et al., 2007). The abundance of the triple mutant is an indication of pyrimethamine resistance (Ahmed et al., 2006).

In our study only one of the *dhps* haplotypes (ISGKGS) had a combination of S436A, A581G and A613S in association with A437G. These four mutations are associated with increased

levels of *in vitro* resistance to SP (Triglia et al., 1997, Wang et al., 1997). With these four being very few in our samples, it shows that SP resistance is still not very high.

There was a very high prevalence of 437G (94%, 61/66) in the entire samples amplified as usually obtained in areas of high use of sulphonamides.

The presence of 581G allele in our study is probably an indication of sustained presence in Nigeria since it was first reported among isolates of Nigerian origin imported to the UK (Sutherland et al., 2009). This observation calls for a closer monitoring of the use of SP in Nigeria so as to ensure the continued use of SP for prophylaxis in Nigeria.

The triple mutant (N51I/C59R/S108N) of the *dhfr* gene was the most prevalent with a percentage of 95.83% frequency as shown in table 6.3 above. The absence of the quadruple mutation as a result of no presence of I164L, probably explains why the antifolates are still useful in Nigeria because the quadruple mutations show the highest level of antifolate resistance (Peterson et al., 1988, Wang et al., 1997).

In conclusion, the results from our small sample sized study showed no difference in the prevalence of 431V mutant between the HIV-positive and HIV-negative control group. However, this does not rule out the possibility of any difference in a larger sample size. Our result is similar to the report in study among HIV-infected and HIV-uninfected women on SP (Newman et al., 2009) in Uganda. There is need for larger studies involving the assessment of *pfdhps* and *pfdhfr* mutations in both symptomatic and asymptomatic-malaria HIV-positive individuals, pregnant women and children leaving in the study area particularly the surveillance of *dhps* K540E in order to ensure the continued use of SP for IPT in the environment especially with the report of its presence in the nearby country Ghana. Although the significance of the 431V mutation with regards to antifolate resistance has not been established, there is need for population studies of this mutation so as to ascertain its prevalence and possibly any relationship or association with the use of antifolate drugs in the area.

6.6 Limitations to the study

The inability to measure the levels of TS in the samples is a major limitation because we are not able to assess if there was any interaction with AL. Being the first attempt to measure the level, there is need for optimising the method of collecting samples with TS as well as assaying or detecting the presence of trimethoprim and sulphamethoxazole.

Chapter Seven

General discussions and conclusions

7.1 General discussions

Malaria still remains major public health challenge in endemic regions especially in sub-Saharan Africa where it has a geographical overlap with HIV. Recent efforts to control malaria have contributed to reducing the prevalence of disease through various control measures. WHO (2011) reports a 17% decline in number of malaria cases and a 26% reduction in malaria-specific child mortality between 2000 and 2011. According to Dr. Margaret Chan, Director–General of World Health Organisation, an estimated 1.1 million malaria deaths were averted as a result of a scale-up of malaria interventions (WHO, 2012b). Despite the progress made, the war against malaria is yet to be won. Malaria still takes the life of an African child every minute (WHO, 2012b). The situation is made worse in HIV-infected persons because of immunosuppression. HIV affects the prognosis of malaria and vice versa (Whitworth et al., 2000., French et al., 2001, Kublin et al, 2005, Mermin et al, 2006).

The impact of malaria on public health and the overwhelming financial burden especially in sub-Saharan Africa cannot be overemphasized. The effort to reach targets for malaria elimination and eradication should be intensified in endemic regions. To achieve this, high burden countries with large populations like Nigeria and Democratic Republic of Congo should be encouraged or empowered to deal with the issues and challenges limiting the achievement of this goal. With a population of 162 million and being the eighth most populous country in the world and the highest in Africa, the problem of malaria in Nigeria is a global problem especially with the high level of migration by Nigerians to the western world.

Resistance to antimalarial drugs by *Plasmodium* parasites has become a major health problem since the first resistance to chloroquine was recorded in Thailand and Cambodia in the late 1950s (WHO, 2006). The current antimalarial policy of artemisinin combination therapy for the treatment of malaria, as advocated by the WHO, has contributed to reductions in morbidity and mortality caused by malaria. However various studies (Noedl et, al 2008, Dondorp et al, 2009) indicate that resistance to artemisinin or its derivative artemether (Jambou et al, 2005, Dokomajilar et al, 2006) has posed a great threat to the continued advancement of malaria control. This calls for active monitoring of the efficacy of the ACTs in order to prolong their life and sustain their use. Monitoring of drug efficacy is multifaceted and involves both *in vivo* and *in vitro* methods. *In vivo* methods have been accepted as gold standard but this is time consuming and very expensive. The employment

of molecular markers have proved very effective as tools of surveillance (Djimde et al, 2001a) in measuring parasite drug sensitivity as well as drug susceptibility (Laufer et al, 2006) and in informing policy (Mugittu et al., 2004). The report of the reemergence of chloroquine-senstive parasites in areas where it had been withdrawn (Kublin et al., 2003) is another proof of the importance of monitoring of molecular markers of resistance. The information obtained from such surveillance will be beneficial to informing policy when and where there may be need for a change in treatment policy.

7.2 Malaria/HIV co-infection in Nigeria.

Malaria is holoendemic in Nigeria and has a geographical overlap with HIV. There is paucity of malaria prevalence data in Rivers State. However a cross sectional study among pregnant women attending antenatal clinics reports a 26% prevalence of *P. falciparum* in Rivers State. (Wogu et al, 2013). The results of our study show a positive predictive value of the microscopy results with 80% of parasitaemia. This was found to be much higher than the PCR result. There is urgent need to undertake a cross-sectional study of asymptomatic-malaria and HIV-co-infected adults in order to measure the prevalence of malaria in these subjects considering the fact that prevalence of HIV in the area (6.0%) is higher than the national prevalence of 4.6% (NACA, 2011).

Management of co-infection of malaria and HIV is of great challenge to public health especially with regards to drug-drug interactions. The first line treatment of HIV-positive patients in Nigeria is a combination of 1 non-nucleoside reverse transcriptase (nevirapine) and 2 nucleoside reverse transcriptase inhibitors (lamivudine and zidovudine). Artemether-lumefantrine is the first line treatment for uncomplicated malaria. There are no published data on the possible drug-drug interactions of these commonly used antiretroviral drugs and the antimalarial regimens in use in our study area.

The study was designed to investigate any associations at enrolment between drug resistant *P. falciparum* and the use of medication for either HIV treatment or prevention of opportunistic infections with particular reference to the use of TS prophylaxis. Additionally, the study was to evaluate the use of AL in the environment of Port Harcourt, Niger Delta among HIV co-infected individuals, assess the possible drug-drug interactions between the ARVs and antimalarials as well as survey the possible differences in the molecular markers of resistance between the two groups.

7.3 Summary of results

7.3.1 Demographic and field results

Data collected from the questionnaires described socio-economic and socio-cultural factors such as age, sex, occupation, marital status and education that affect the spread and prevalence of HIV. In consonance with previous studies, the present study observed the relationship between sex, marital status, age and education and HIV status. Analysis of the questionnaire data revealed that our HIV-subjects consisted mainly of females, married people, traders, those with secondary education and people in the age range of 30-39.

The study is the first from our study area to assess CD4 cell count among HIV-positive participants treated for malaria. We were able to demonstrate that treatment of asymptomatic-*P. falciparum* malaria infection improved the CD4 cell count in 38/56 HIV-positive participants who had paired peripheral CD4 cell counts for days 0 and 28. There is need for further detailed study of the impact of treatment of confirmed asymptomatic-*P. falciparum* malaria infection on CD4, since our study over-diagnosed malaria infection by a significant amount.

Three different diagnostic methods for identifying parasitaemia positive samples were employed. Results show that microscopy and RDT methods were very poor for correct diagnosis in our study site, and the most reliable was post hoc PCR. Hence our statistical analysis was based on PCR results, but was hampered by inappropriate recruitment of volunteers lacking detectable *P. falciparum* infection, leading to loss of statistical power in the analysis. Correct malaria diagnosis is critical for proper recruitment and enrolment of study participants, and future studies need to overcome this obstacle.

7.3.2 Pharmacokinetic studies

Results of our pharmacokinetic study show elevated plasma levels of day7 lumefantrine concentration in nevirapine–treated HIV-positive patients and corroborates the finding of Kredo et al, (2011) in a study among HIV-positive volunteers in South Africa who were administered AL. This suggests a possible drug-drug interaction between lumefantrine and nevirapine. Although our HIV-positive patients had higher lumefantrine levels, we found no evidence of a parasitological benefit of higher plasma levels of lumefantrine. In fact, we provided weak evidence that HIV-positive patients are slower to clear sub-patent infections

after AL treatment. This indicates a possible perturbation of the immune system of the patients.

Another factor that has been reported to influence variability in individual drug response and drug disposition is genetic polymorphism of the host (Owen, 2006; Owen et al, 2006). Other studies (Stohr et al, 2008; Mahungu et al, 2009b) report that plasma concentrations of nevirapine were influenced by ethnicity in addition to weight, gender and underlying hepatic diseases. These factors are closely related to the immune system of the host. Our study participants were all of African ethnicity, but specific tribal groupings and individual weights were not taken into consideration in the analysis. It is possible that these factors may have affected the disposition and response of nevirapine such that an unexpected elevation of lumefantrine occurred. In a study of drug-drug interactions in selected ARV combinations on the pharmacokinetics of maraviroc, Pozniak et al, (2008) reported a 1.5 fold increase of maraviroc in the nevirapine containing regimen. This report is contrary to the expected inducing action of nevirapine.

Another possible reason for the failure to clear the parasites despite the apparent increased lumefantrine concentration could be due to a possible reduced exposure of artemether and DHA as established in a recent Ugandan study (Byakika-Kibwika et al., 2012). Artemether and DHA exert their pharmacological properties in the first 48hours of treatment to clear the parasite biomass leaving behind the residual parasites to the longer acting partner drug lumefantrine (Ezzet et al., 1998, Djimdé and Lefèvre, 2009). The study did not include the measurement of artemether and dihydroartemisinin levels. The knowledge of these values may provide more information on parasite clearance. We therefore advocate further studies that will include more detailed pharmacokinetic study that will incorporate the measurements of artemether and DHA.

The inability of the elevated lumefantrine concentration to provide additional parasitological benefit could also mean that a possible plateau of the lumefantrine concentration has been reached beyond which there would be no increase in effecting parasite clearance. However, the study also revealed persistence of sub-patent parasites even among treated volunteers in the HIV-negative control group. This is similar to the report by Dinko et al, (2013) on Ghanaian children who retained sub-patent parasites after treatment of asymptomatic-malaria infection with the ACT dihydroartemisinin - piperaquine implying that in chronic low grade malaria infection the immune system may be compromised.

The proportion of volunteers displaying complete PCR-detectable parasite clearance at day 28 based on PCR positives at day 0 was 74% (104/144). This is in contrast to previous reports of 93.9% successful clearance by microscopy (Falade et al., 2005). However it must be noted that Falade's study was carried out among children with symptomatic *P. falciparum* malaria infection and at the early period of adoption of AL as first-line treatment in Nigeria, at which time parasite susceptibility will have been very high. The current thesis describes a preliminary study involving AL use and parasite clearance in HIV-positive persons and there were many confounding factors to the result especially with regards to poor microscopy, and missing data on follow-up days.

Asymptomatic malaria parasitaemia carriage is prevalent in sub-Saharan Africa (Bottius et al., 1996, Smith et al., 1994) and carriers remain infective longer than the treated symptomatic patients, and thus serve as good reservoirs for further transmission of malaria parasites (Alves et al., 2005, Zoghi et al., 2012). There is an urgent need for studies involving asymptomatic-*P. falciparum* infection carriers, who may represent an obstacle to proper malaria control. We also propose that future studies should extend follow-up to 42 days, since studies have reported recrudescence of recurrent parasitaemia after 42days (Yeka et al., 2008).

We therefore advocate a detailed kinetic study with a larger sample size involving both symptomatic and asymptomatic malaria infected persons in addition to measuring parasite clearance time so that adequate comparisons can be made. There is also need to measure the level of the metabolite desbutyl-lumefantrine which has been shown to possess a higher antimalarial activity than the parent compound *in vitro* (Wong et al, 2011). This is the first such study of ARV antimalarial interactions in malaria–infected individuals.

Although we were not able to ascertain by our PCR method whether the remaining parasites not cleared by AL were asexual stage parasites or gametocytes, the presence of either is important in malaria control since asexual parasites are responsible for the clinical symptoms while the gametocytes are involved in transmission. Also we were not able to distinguish between new infections and recrudescence. Further expanded studies in the area will involve this aspect of research.

7.3.3 Molecular markers of genes associated with Artemether-lumefantrine

An important observation from the result of our study is the strong selection of the mdr86N allele among our study samples which was also evident in the HIV-positive arm alone. This allele has been suggested as a first step to lumefantrine tolerance and its selection as a strong indicator of AL treatment failure or slow clearance (Sisowath et al., 2005, Humphreys et al., 2007, Mwai et al., 2009). This therefore calls for a close monitoring of this allele in order to monitor the efficacy of AL in the area. The accompanying haplotype NFD equally associated with slow clearance to AL was found to have a slight selection at day 28 and was more prevalent in HIV-positive patients however the haplotype NYD had a higher prevalence in the overall population. There is need for close monitoring of AL use especially following the report of the NFD haplotype in South-Western Nigeria among children (Happi et al, 2009).

The *crt*76T mutant allele implicated in chloroquine resistance was found to be in very high prevalence in our study area despite the official withdrawal of chloroquine indicating that its use may still be common in the environment. This was also seen from the field observations in the self-reported use of antimalarial drug among participants. This allele seems to exhibit an inverse relationship with AL efficacy because it is usually found in areas of AL efficacy (Sutherland et al., 2011, Raman et al., 2011) while the wild type allele *crt*76K has been implicated in recurrent infections following AL treatment (Sisowath et al., 2009). Future studies of ARV-antimalarial interactions in this area should also measure the prevalence of detectable chloroquine in peripheral blood samples taken at enrolment.

The recent study in Tanzania (Malmberg et al., 2013b) established an inverse relationship between day 7 lumefantrine concentration and the NFD haplotype, stating that samples with the NFD haplotype had lower levels of lumefantrine concentrations and were able to withstand estimated lumefantrine concentration 15-fold higher than those with YYY haplotype. We were not able to associate the 86N polymorphism nor the NFD haplotype with lumefantrine concentration because parasite genotyping was not carried out at the time of drug measurement (day 7), however we advocate that further studies should incorporate the evaluation of this relationship. Since *pfmdr1* and *pfcrt* genes are both involved in modulation of resistance in *P. falciparum* malaria, routine and close monitoring of these molecular markers is critical in order to preserve and sustain the efficacy of ACTs.

Three new polymorphisms which have not been previously reported were detected in three of our study samples in addition to the rare *mdr*86F reported in Swaziland (Dlamini et al., 2010). It is of interest that these new mutations were all found among HIV-negative participants. The implications of these mutations are not clear but may indicate polymorphic drug pressure on this gene by the various drugs being taken in the environment. Data from our questionnaire show that AL is the third most commonly used drug by the participants, albeit without prescription. This calls for urgent actions to be taken with regards to enforcing the policies of antimalarial drug use in the country, including appropriate diagnosis.

7.3.4 Relationship of polymorphisms with antifolate markers.

The results of our study showed no difference in the prevalence of molecular markers related to antifolate drugs between the HIV-positive and HIV-negative control group. This does not support our former hypothesis that HIV-positive people may harbour parasites different from their HIV-negative counterparts because of the drugs they take, in particular TS. This observation is similar to the results observed from the study among HIV-infected and HIV-uninfected women taking SP for IPTp in Uganda and Zimbabwe and HIV-infected women taking TS for prophylaxis (Newman et al., 2009).

Analysis of our trimethoprim-sulphamethoxazole (TS) showed weak evidence of a relationship between *dhps*437A and TS use. This result is of interest and requires further studies employing larger sample size, in addition to optimisation of the sampling protocol and method for the recovery of trimethoprim and sulphamethoxazole from field samples. This study is a first attempt of this assay, which was not available at the time we designed the study protocol, and the data are thus preliminary. The presence of a single case of the rare *dhps*436F from a HIV-positive participant is of interest since to our knowledge there has been no previous report of this mutation in Nigeria.

A result of importance from our results is the absence of *dhps* K540E in the small sample sized-population studied. The K540E is very relevant in the decision of continuous use of SP for intermittent treatment of malaria. The study data thus suggest that SP use is not under threat yet in the study area, however there should be a close monitoring of the use since there has been a previous report of this mutation among children in western Nigeria (Happi et al, 2005) and in nearby Ghana (Sutherland et al, 2009). Another important discovery from our study is the absence of *dhfr* I164L mutation in our samples. This

mutation has been associated with resistance to proguanil and is also implicated in SP resistance because of the pyrimethamine content in SP (Ochong et al., 2008, Andriantsoanirina et al., 2011). Its absence in conjunction with the absence of *dhps* K540E gives credibility to the continued use of SP for IPT in our environment, with the caveat that larger surveys are needed to confirm the absence of these two alleles.

The findings of this study therefore will serve as additional information to the limited data available on the use of AL in Nigerian adults and provides a template for sample size calculation for future larger studies. More studies involving children and pregnant women in multiple sites are needed in order to confirm the findings from the study especially since there is no published report of AL use, molecular markers associated with it and drug-drug interactions between antimalarials and ARVs in the Niger Delta region. There is also need for *in vitro* sensitivity assays of antimalarial drugs in use for prophylaxis and treatment especially the ACTs.

7.4 Summary of findings

This is a preliminary study and first of its kind to investigate drug-drug interactions between ARVs and the antimalarial drug AL in HIV-positive patients co-infected with *P. falciparum.* We were able to also provide a limited analysis in relation to parasite clearance. We present the first attempt to assay the presence of trimethoprim and sulphamethoxazole from blood spots on filter papers collected from individuals treated for malaria. The results of the study could be summarised as follows:

- Day 7 peripheral blood levels of lumefantine were significantly higher in nevirapine-treated people.
- There was no evidence of a parasitological benefit of the higher blood levels of lumefantrine
- Overall parasite clearance on day 28 after administration of AL was 74.3% (107/144). HIV-positive persons had a lower parasite clearance (OR:2.86: P=0.007)
- Strong selection of *mdr*86N at day3 after treatment with AL
- Weak evidence of selection of NFD haplotype at day 28
- Absence of mutation at codons 1034 and 1042 on the *mdr* gene
- High prevalence of *crt*76T in the study area, especially in the HIV-group

- Absence of *dhps* 540E and *dhfr* 164L
- A single isolate with the *dhps* 436F allele.
- No difference in the prevalence of antifolate markers between the HIV-positive and HIV-negative group.
- Presence of new mutations on the *mdr1* gene and the *dhps* gene.
- Artemether-lumefantrine treatment improved CD4+ counts in HIV-positive people with and without PCR-positive parasitaemia.

7.5 Limitations to the study

As previously mentioned in chapter 3, the major weakness of our study was the poor quality of enrolment microscopic diagnosis, such that the study was under-powered because of a very small sample size. Prior to the study, there was unavailability of data to be used as a framework for sample size calculation. Other factors include many industrial strikes by hospital staff during sample collection, frequent power outages leading to poor storage and even loss of some collected samples, frequent breakdown of machines and equipment used for sample analysis and transportation difficulties for both study participants and our team of workers thereby impinging on our follow-up studies.

Notwithstanding the limitations, the work has provided a template for future larger studies of AL use and the malaria/HIV interactions in the environment.

7.6 Conclusions and Recommendations

According to the summary proceedings from the 3rd Annual malaria control programme review of Ethiopia and Nigeria (2012), the major challenges to malaria control in Nigeria include insufficient baseline on malaria prevalence, weak national surveillance system, limited availability of ACTs and RDTs, microscopy for malaria diagnosis and high rates of treatment seeking in the private sector (pharmacies, drug vendors, shops). In order to win the war of malaria control in Nigeria, these key factors must be addressed. The limitations to our study with regards to poor microscopy, and thus incompleteness of data, have emphasised this need. The strong selection of the *mdr*86N on day 3 after treatment with AL and the slight selection of NFD haplotype both of which have been implicated in AL treatment failure and slow clearance have shown that there is obvious threat on the efficacy of AL in the study area. There is therefore urgent need for active surveillance

studies to be carried out especially for molecular markers of ACTs and other drugs of relevance in the control of malaria in Nigeria, particularly in the Niger Delta where there is paucity of published data on malaria studies.

Observation from field work has revealed that malaria treatment in Nigeria is mostly done without confirmatory laboratory diagnosis and there are also high rates of treatment seeking in the private sector as mentioned previously. This contributes to the misuse and poor administration of antimalarial drug thereby increasing the possibility of increased drug resistance and drug-drug interactions. There was no co-ordinated effort between the health workers with regards to research into antimalarial drug resistance and surveillance of molecular markers related to the drugs. The present study can provide a good platform for the initiating of a properly organised research team with particular reference to active monitoring and surveillance of molecular markers especially to the ACTs and SP so as to sustain or prolong their use in the area and advise policy where need may arise for change in policy. The platform should also incorporate the malaria/HIV interaction studies with special emphasis on the potential drug-drug interactions considering the very high prevalence of HIV in the environment.

There is need to invest in machineries that will encourage proper diagnosis such as the Loop-mediated Amplification kit (LAMP) which have been proven to show greater accuracy over microscopy and requires minimal training in addition to requiring less time compared to PCR and is equally cheaper (Polley et al., 2013) or where not applicable proper training and external quality assurance for microscopy and use of RDT before treatment. There is also need for community health education for improved and efficient health services.

Review of the literature has revealed that many studies on antimalarial drug resistance in Nigeria were carried out in the western part of the country, close to the major urban centres. This may not represent a true picture of the situation in the entire country. The high level of migration in the Niger Delta as a result of the large deposit of the natural resource of oil and gas in the area and the strategic position of Nigeria in the global map of both HIV and malaria therefore calls for investment on increased and expanded study in multiple sites in order to achieve the millennial development goal of malaria elimination. Every effort to improve malaria control in this area will be beneficial globally.

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Annexe1. LSHTM ethics approval

LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

ETHICS COMMITTEE

APPROVAL FORM
Application number: **5817**

Name of Principal Investigator	**Ifeyinwa Chijioke-Nwauche**
Faculty	**Infectious and Tropical Diseases**
Head of Faculty	**Professor Simon Croft**

Title: **Efficacy of artemether-lumaefantrine (AL) in the clearance of malaria infections in HIV-positive and HIV-negative Nigerian adults.**

This application is approved by the Committee.

Chair of the Ethics Committee .....

Date .....2 December 2010.....

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form.

Annexe 2. Ethics approval from BMH for the field work study

RIVERS STATE HOSPITALS MANAGEMENT BOARD

BRAITHWAITE MEMORIAL SPECIALIST HOSPITAL

P.M.B. 5084, PORT HARCOURT, RIVERS STATE

Your Ref: _____

Our Ref: BMSH/ADM/44/VOL.III/ 69

25th March, 2011.

Mrs. Ifeyinwa Ch'jioke – Nwauche
Immunology Unit
Department of Infections & Tropical Diseases
London School of Hygiene & Tropical Medicine
London.

Ethics Approval
Title of Study: "The Efficacy of artemether/ lumefantrine (coartem) in the clearance of malaria infections in HIV-positive and HIV-negative Nigerian adults".

In respect of the above study, the following documents were received:

- Application letter
- Study protocol
- Information sheet and consent form for patients

These were reviewed by the Ethics Committee at its meeting held on the 10th of December, 2010 and approved for the study to take place at the Braithwaite Memorial Specialist Hospital.

HOSPITAL MANAGEMENT BOARD

ABO, O. A (MS)
For: Chairman
Ethics & Research Committee

Annexe 3. Ethics approval from UPTH for the field work study

UNIVERSITY OF PORT HARCOURT TEACHING HOSPITAL

P.M.B. 6173, PORT HARCOURT - website: www.upthnigeria.org

CHAIRMAN
PROF. RICHARD P. KING
B.Sc, M.Sc, Ph.D, MNES, MEcSN

DIRECTOR OF ADMINISTRATION
B. AMAOMU-JUMBO (MRS.)
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CHIEF MEDICAL DIRECTOR
PROF. AARON C. OJULE
MBBS, M.sc, FMCPath. MNIM

CHAIRMAN, MEDICAL ADVISORY COMMITTEE
DR. CHRISTIE N. MATO
MB.BS, PgDA, FMCA, FWACS, FICS

14TH JULY, 2010

UPTH/CS&T/118/VOL XII/151

Mrs. Ifeyinwa Chijioke Nwauche
Immunology Unit
Department of Infectious and Tropical Diseases
London School of Hygiene and Tropical Medicine
London.

THE EFFICACY ARTEMETHER-LUMEFANTRINE IN THE CLEARANCE OF MALARIA INFECTIONS IN HIV-POSITIVE AND HIV-NEGATIVE NIGERIAN ADULTS

I write with pleasure to inform you that the Ethics Committee considered your proposal titled above and gave approval for you to proceed with the research work.

You are however expected to submit three (3) hard bound copies to the undersigned on completion.

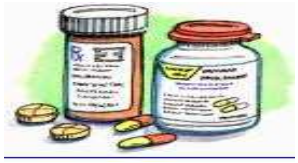
Barr. (Mrs.) D. Abbey
For: Chairman Ethics Committee

Annexe 4. Information sheet

Pharm(Mrs). Ify Chijioke-Nwauche
0802 323 5061
Dr. C. A . Nwauche
0802 314 8155

This study is in collaboration with The London School of Hygiene and Tropical Medicine, London.

A study of the efficacy of Coartem in the clearance of malaria infections in HIV-positive and HIV-negative Nigerian adults



Introduction:

Malaria is an important illness caused by Plasmodium transmitted through the bite of female anopheles mosquito. Patients with malaria in our country are treated first with the antimalarial drug "Coartem".

However if the malaria parasite becomes resistant to the drug, there is a chance the treatment may not work well.

Therefore it is important to know when this may start happening so we can give you a different treatment.

The possibility of resistance may be investigated using certain techniques and requires small amount of blood.

What will be done during the study?

If you agree to take part in the study, you will be asked to give a small blood sample from your arm today, 3rd day and 4 weeks after today to monitor your progress. We will also take few drops of blood from your finger today, tomorrow and 2 days after today to ensure that the drug is clearing the parasite. The diagnosis will be made by a quick dipstick method and confirmed by microscopical examination. Some of the blood will be put on a special type of paper to find out if the parasites show signs of resistance. We will tell you the result of the test. If tested positive, you will be treated with Coartem.

Do I have to take part?

No. You do not have to take part and you can withdraw anytime if you do not want to take the drug.

Will the study bring me any discomfort?

You will have some discomfort for a short time when blood will be collected but it will not last long. We will also test for HIV using a standard HIV kit.

How will I benefit from the study?

You will get free treatment of the malaria now or any other episode within the study period of 2-3 months. Your participation will also help us to find out if parasites in Port Harcourt are resistant in future to Coartem.

Annexe 5. Consent form

MALARIA CHEMOTHERAPY STUDY **CONSENT FORM**

Efficacy of Artemether-Lumefantrine (AL) In the Clearance of Malaria infections in HIV-positive and HIV-negative Nigerian adults.

Name: ----------Study ID: ----------
Phone Num: ----------

House Address: ----------

"I have understood the verbal explanation concerning this study and I understand what will be required of me and what will happen to me if I take part in it"

"My questions concerning the study have been answered.

"I understand that at any time I may withdraw from this study anytime I want without affecting my normal care"

"I agree to take part in this study"

Name/signature of Patient *Date*

Witness's signature *Date*

Annexe 6. Questionnaire for HIV-positive participants

Malaria Chemotherapy: HIV-positive group questionnaire

The Efficacy of Artemether-Lumefantrine (AL) In the Clearance of Malaria infections in HIV-positive and HIV-negative Nigerian adults.

Study ID: _____

Wt: _____

Ht: _____

Date of Birth: _____

Please enter the appropriate number in the boxes

Gender: Male (1); Female (2) ☐

Religion: Christianity (1); Muslim (2); Traditional religion (3); others (4) ☐

Marital status: married/single/ divorced /widowed ☐

Level of education: Primary (1); secondary (2); tertiary (3) ☐

Occupation: Snr. Civil servant (1); Jnr civil servant (2); Trader (3); Business man/woman (4); Teacher (5); Housewife (6); Oil worker (7); Student (8); Others (9). ☐

Spouse' occupation: Snr. Civil servant (1); Jnr civil servant (2); Trader (3); Business man/woman (4); Teacher (5) Housewife (6); Oil worker (7); Student (8); Others (9). ☐

When did you fall ill?

< 3 months (1); 3-6 months (2); 6 months-1 yr (3); > 1 year (4) ☐

When did you seek medical help?

< 3 months (1); 3-6 months (2); 6 months-1 yr (3); > 1 year (4) ☐

When did you register in the HIV clinic?

< 3 months (1); 3-6 months (2); 6 months-1 yr (3); > 1 year (4) ☐

How many family members are aware of your condition?

All (1); father (2); mother (3); spouse (4) children (5); siblings (6); None (7) ☐

How long have you taken the ARV drugs?

> 6mths (1); 6mths-1yr (2); 1yr-2yrs (3); > 2yrs (4) ☐

What medicines have you taken in the last 3 months?

Medicine Name	Start Date	Still taking
CQ		
Camoquine		
Fansidar		
Metakelfin		
Maloxine		
Artesunate		
Coartem		
Lonart		
Paracetamol		
Septrin		
Zidovudine		
Lamivudine		
Stavudine		
Nevirapine		
Efavirenz		

Please write any other medication and the reason for taking it

_____

Please enter the appropriate number in the boxes: 1=yes; 2=no

Have you been taking your Septrin regularly? ☐

How often do you have malaria?

Monthly (1); once in 3 months (2); once in 6 months (3); once a year (4); rarely (5) ☐

Do you have any symptoms now? ☐

Do you use

Insecticide ☐

Insecticide treated net ☐

Mosquito repellent ☐

Mosquito coil ☐

Window netting in your house ☐

Is there any child in the house that has malaria now? ☐

Thank you for answering the questions

Annexe 7. Questionnaire for HIV-Negative participants (Control group)

Malaria Chemotherapy: HIV-negative group questionnaire

The Efficacy of Artemether-Lumefantrine (AL) In the Clearance of Malaria infections in HIV-positive and HIV-negative Nigerian adults.

Study ID: _____

Wt: _____

Ht: _____

Date of Birth: _____

Please enter the appropriate number in the boxes

Gender: Male (1); Female (2) ☐

Religion: Christianity (1); Muslim (2); Traditional religion (3); others (4) ☐

Marital status: married/single/ divorced /widowed ☐

Level of education: Primary (1); secondary (2); tertiary (3) ☐

Occupation: Snr. Civil servant (1); Jnr civil servant (2); Trader (3); Business man/woman (4); Teacher (5); Housewife (6); Oil worker (7); Student (8); Others (9). ☐

Spouse' occupation: Snr. Civil servant (1); Jnr civil servant (2); Trader (3); Business man/woman (4); Teacher (5) Housewife (6); Oil worker (7); Student (8); Others (9). ☐

How often do you have malaria?

Monthly (1); once in 3 months (2); once in 6 months (3); once a year (4) ☐

Please enter the appropriate number in the boxes: 1=yes; 2=no

Do you have symptoms now? ☐

Do you useInsecticide ☐

Insecticide treated net ☐

Mosquito repellent ☐

Mosquito coil ☐

Window netting in your house ☐

Is there any child in the house that has malaria now?

What medicines have you taken in the last 3 months?

Medicine Name	**Start Date**	**Still taking**
CQ		
Camoquine		
Fansidar		
Metakelfin		
Maloxine		
Artesunate		
Coartem		
Lonart		
Paracetamol		
Septrin		

Please write any other medication and the reason for taking it

_____

Thank you for answering the questions

Annexe 8. Published paper

Ifeyinwa Chijioke-Nwauche, Albert van Wyk, Chijioke Nwauche, Khalid B. Beshir, Harparkash Kaur, Colin J. Sutherland
HIV-Positive Nigerian Adults Harbor Significantly Higher Serum Lumefantrine Levels than HIV-Negative Individuals Seven Days after Treatment for Plasmodium falciparum Infection
Antimicrobial Agents and Chemotherapy p. 4146–4150

AAC Journals.ASM.org

HIV-Positive Nigerian Adults Harbor Significantly Higher Serum Lumefantrine Levels than HIV-Negative Individuals Seven Days after Treatment for *Plasmodium falciparum* Infection

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Management of coinfection 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 metabolized 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 parasitemia 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.

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 coinfection is thought to contribute to 3 million additional malaria cases, higher malaria parasite densities in immunosuppressed 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 nonartemisinin partner drug. The most widely used such combination is artemether plus lumefantrine (coartemether; 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 to 3 h after oral administration (4); elimination half-life is estimated at approximately 1 h. There is thus only limited opportunity for DHA to participate in drug-drug interactions. Lumefantrine is partially metabolized to desbutyl-lumefantrine, predominantly through CYP3A4, reaching peak plasma levels approximately 10 h after oral administration, and is then cleared slowly, showing a terminal half-life of 4 to 6 days in *P. falciparum* malaria cases (5–9). Oral bioavailability of lumefantrine is variable and highly dependent on administration with fatty foods (5, 9, 10).

The antiretroviral drug nevirapine (NVP) is a nonnucleoside reverse transcriptase inhibitor that is well absorbed after oral administration with >90% bioavailability, generally achieved about 4 h after oral dosing, and has a long half-life (11). NVP is extensively metabolized 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 metabolized through this route. This raises the possibility of significant drug-drug interactions of NVP with lumefantrine and other antimalarials (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 naive 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, 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 prev-

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alence of HIV infection (7.4% of the population), and malaria transmission is hyperendemic. 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 hypothesized 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 socioeconomic conditions contribute to a high estimated population prevalence of HIV infection of 7.4% (15). Malaria is holoendemic in Nigeria, with transmission all year round, but malaria cases are most common during the rainy season, from April to September, with peaks of rain and intense transmission between May and July. Annual rainfall averages more than 3,550 mm 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, which is 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 posttreatment. 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: 16 to 65 years of age, 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, United Kingdom.

Enrolled patients were treated with AL (Coartem; Novartis Pharma, Nigeria) according to the manufacturer's dosing regimen: 4 tablets twice daily for 3 days for persons with a weight of >35 kg. Patients were advised to eat before taking the tablets. Most of the patients took their first dose at the site, having been preinformed 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, 100 μl of blood was measured using a pipette and dropped on a filter paper (glass microfiber paper; Fisherbrand FB59431) pretreated with 0.75 M tartaric acid (Fisher Scientific). The papers were allowed to air dry and were 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 analyzed for lumefantrine using liquid chromatography mass spectrometry (LCMS; Thermo Finnigan LCQ instrument) by 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 3-μm C_{18} column (4.6 by 150 mm, with 120-Å pore size, fitted with a guard column) and eluted with ammonium formate (20 mM, pH 2.7) and methanol (vol/vol; 85:15) isocratically at a flow rate of 500 μl/min. The column temperature was maintained at 35°C. The electrospray ionization (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 to 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 transition of *m/z* 530 to 512. The lower limit of detection was determined to be 0.1 μg/ml, the lower limit of quantification was 1.0 μg/ml, and the upper limit of quantification was 20.0 μg/ml.

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

Data were entered into spreadsheets and analyzed in STATA 11 (Stata Corp., Madison WI). Continuous data were compared between groups using Wilcoxon's rank sum test, while categorical comparisons in 2-by-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. A total of 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 days 3, 7, and 28 for repeat blood sampling.

To confirm microscopic diagnosis of *P. falciparum* parasitemia at enrollment, 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 enrollees (78.1%) were found to be aparasitemic 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 (odds ratio [OR], 5.56; 95% confidence interval [CI], 1.76 to 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 parasitemic at day 0 (OR, 2.05; 95% CI, 0.917 to 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. falcip-*

TABLE 1 Parasite carriage by microscopy and PCR in 68 HIV-positive and 99 HIV-negative individuals[a]

HIV status	No. of individuals (%) PCR positive for *P. falciparum* on day:		
	0	3	28
Positive ($n = 68$[b])	17 (29.9)	8	12
Negative ($n = 99$)	20 (17.2)	12	12

[a] All 167 individuals were reported as positive for *P. falciparum* parasites on microscopic examination of thick blood films.
[b] Sixty-seven of these individuals were receiving daily nevirapine antiretroviral therapy, and one received efavirenz. All HIV-positive patients also received the nucleoside reverse transcriptase inhibitors lamivudine and zidovudine.

arum at day 3 and/or day 28 after AL treatment than were HIV-negative individuals (OR, 1.75; 95% CI, 0.776 to 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 score = 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, 0.08 μM, all in the HIV-negative group) and log transformed as described above. Departure from the normal distribution was then marginally nonsignificant ($z = 1.594$, $P = 0.054$). After consideration of these findings, we decided to take the conservative approach of using only nonparametric methods 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 rank sum test $z = -3.270$, $P = 0.0011$), with a median concentration in the HIV-negative group of 2.75 μM (interquartile range [IQR], 1.03 to 4.31) and in the HIV-positive group of 3.55 μM (IQR, 2.07 to 5.37) (Fig. 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 after AL treatment ($z = -2.830$, $P = 0.0046$).

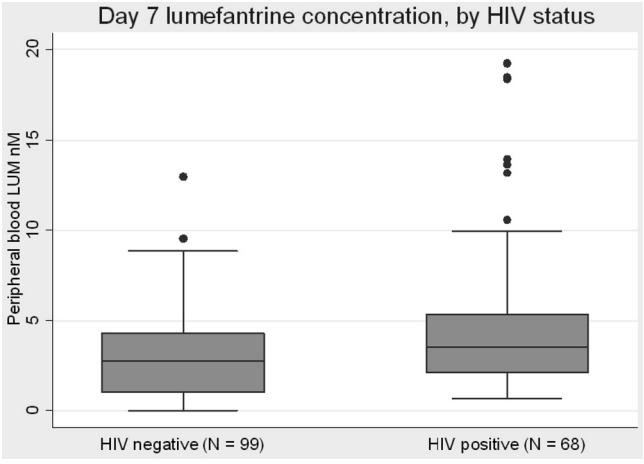

FIG 1 Day 7 lumefantrine concentration in AL-treated participants. Midline of each box plot is the median, with the edges of the box representing the interquartile interval. Whiskers delineate the 5th and 95th percentiles. Lumefantrine concentration was below the normal limits of detection in five individuals, all in the HIV-negative group (see the text).

As many of our subjects were shown to be parasite negative by PCR, we tested for any effect of parasitemia on lumefantrine concentrations at day 7. Overall, in all 166 evaluable individuals, PCR-positive parasitemia at day 0 was not associated with any difference in day 7 lumefantrine concentration in our sample set (37 positive versus 129 negative individuals). There was a weak association between day 3 PCR-detected parasitemia and higher lumefantrine concentrations ($z = -2.305$, $P = 0.021$), 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 parasitemia on either or both day 3 and day 28, compared to 22.7% of HIV-negative individuals (OR, 1.75; 95% CI, 0.776 to 3.95; $P = 0.141$). Assessment of day 0 parasitemia using qPCR was performed for 8 individuals (including 5 who were HIV positive) who were subsequently PCR positive on day 3, and 15 who had cleared parasites by day 3 (including 9 who were HIV positive). This exploratory analysis did not provide any evidence that higher starting parasitemia increased the likelihood of an individual remaining PCR positive for *P. falciparum* on day 3 (Wilcoxon rank sum test $z = -0.904$, $P = 0.37$).

DISCUSSION

The coformulated combination of artemether, a sesquiterpene lactone derived from the natural compound artemisinin, with the aryl amino-alcohol lumefantrine, as a systemic racemic fluorene mixture, has become the most widely distributed and available antiretroviral chemotherapy throughout Africa. As antiretroviral 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 than treated adults who were HIV test negative and not receiving NVP. However, we found no evidence that submicroscopic 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 parasitemia 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 metabolized 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* parasitemia at day 3 and/or day 28, compared to less than a quarter of the control group. This difference, which suggests that perturbation of the immune system in HIV infection may have some impact on antimalarial effectiveness 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 posttreatment parasitemia versus age, weight, gender, and educational attainment found no evidence of confounding by any of these parameters (data not shown). The recent observation that coadministration 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 enrollment microscopic diagnosis, such that the majority of participants had in fact failed a major inclusion criterion. This had two main impacts. First, the study was greatly underpowered to evaluate any parasitological outcomes, as so many participants were actually uninfected (with *P. falciparum*). Second, we were not able to analyze 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 PCRs on posttreatment 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 artemisinin combination therapy, suggesting that subclinical parasitemia 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 harbor 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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