DOLUTEGRAVIR-ASSOCIATED RESISTANCE GENE MUTATIONS AMONG PEOPLE LIVING WITH HUMAN IMMUNODEFICIENCY VIRUS IN MALAWI

By

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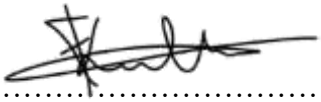
A dissertation Submitted to the University of Zambia in Partial Fulfillment of the Requirements of the Award for the Degree of Master in One Health Laboratory Diagnostic.

The University of Zambia

Lusaka

DECLARATION

I, Felistus Zumazuma Kanjira Hussein, do hereby declare that this dissertation entitled; "Dolutegravir-Associated Resistance Gene Mutations among People Living with Human Immunodeficiency Virus Patients In Malawi" is my original work and has not been submitted to any institution before. All sources have been thoroughly acknowledged.

Signature  ……. Date 17 March 2025

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CERTIFICATE OF APPROVAL

The University of Zambia has approved this dissertation by Felistus Kanjira Hussein as partial fulfillment of the requirements for the award of the degree of Masters in One Health Laboratory Diagnostic.

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ABSTRACT

The emergence of drug resistance among Human Immunodeficiency Virus (HIV) patients poses a significant challenge to the success of antiretroviral therapy (ART) programs globally. In Malawi, where HIV prevalence remains a major public health concern, the incorporation of Dolutegravir (DTG) into first-line ART regimens has demonstrated significant promise in improving treatment outcomes. However, the development of resistance to DTG threatens these advancements, necessitating continuous monitoring and adjustments in treatment strategies. This retrospective study identified HIV drug resistance mutations associated with DTG use among individuals living with HIV in Malawi. Data from HIV-positive individuals aged 15 to 60 years, undergoing routine ART monitoring, were evaluated. Patients with high viral loads (≥ 1000 copies/ml) were included, and HIV-1 pol gene sequences were examined to identify mutations linked to DTG resistance. Phylogenetic analysis was performed to assess the relatedness of samples with resistance mutations. A total of 91 sequences from various districts in Malawi were reviewed, comprising 60.4% females and 39.6% males. DTG resistance mutations were found 15.4% of samples, with females accounting for 57.1% and males for 42.9% of those affected. G118R/R, G140A, Q148H/R, and R263K mutations associated DTG resistance, to varying levels, were identified. Among the 14 cases, 50% exhibited high-level resistance, 28% intermediate resistance, and 14.3% low resistance potential. The findings indicate a widespread distribution of DTG resistance mutations across Malawi, with evidence of both isolated and clustered patterns of resistance mutation from different districts emergence suggesting transmission of resistance within the communities. These DTG resistance mutations impact treatment outcomes by reducing the drug's binding efficiency to the integrase enzyme, which is essential for HIV replication inhibition. Mutations such as G118R and Q148H/R significantly impair DTG’s potency, potentially leading to treatment failure, virological rebound, and an increased risk of onward transmission of drug-resistant HIV strains. Additionally, these mutations can limit future ART options by conferring cross-resistance to other Integrase Strand Transfer Inhibitors (InSTIs), complicating treatment regimens and increasing healthcare costs.

DEDICATION

Thanking the Lord almighty for He is good and His faithfulness endures to all generations. I dedicate this dissertation to my family, whose unwavering support and encouragement have been my anchor throughout this challenging journey, my husband Patrick Hussein, my daughter Abrianna, my sister Nellie Kanjira for her support in taking care of Abrianna, my mother Helen Zumazuma and my siblings for their support during the period of writing this dissertation.

This work is a reflection of the collective efforts and support of all those who have played a role in my academic journey. Thank you for being the pillars of my success.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ART	Anti-Retroviral Therapy
ARV	Anti-Retroviral
BIC	Bictegravir
CAB	Cabotegravir
DNA	Deoxyribonucleic Acid
DRM	Drug Resistance Mutation
DTG	Dolutegravir
EVG	Elvitegravir
HIV	Human Immunodeficiency Virus
HIVDR	Human immunodeficiency virus drug resistance
InSTI	Integrase Strand Transfer Inhibitor
LMIC	Low and Middle-Income Countries
NHSRC	National Health Science Research Committee
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
PI	Protease Inhibitors
PHIM	Public Health Institute of Malawi
Pol	Polymerase
RAV	Raltegravir
RNA	Ribonucleic Acid
RT	Reverse Transcriptase

SSA	Sub-Saharan Africa
VF	Virological Failure
VL	Viral Load
VS	Viral Suppression
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Over the past 20 years, antiretroviral therapy (ART) has played a pivotal role in the global response to the HIV/AIDS epidemic, preventing an estimated 16 million deaths related to HIV/AIDS (Forsythe et al., 2019). ART works by suppressing the replication of the HIV virus, thus improving the quality of life and life expectancy of individuals living with HIV. However, despite the progress made in ART provision, the emergence of drug resistance to ART has become a growing concern. Drug-resistant HIV strains are increasingly being reported, which complicates treatment regimens and could potentially undermine the gains made in managing the HIV epidemic (Zaki et al., 2020). Resistance to commonly used ART medications such as non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) has been observed, especially in regions with high ART coverage (Wang, De Clercq and Li, 2019).

Dolutegravir (DTG) , an antiretroviral that is a second-generation integrase strand transfer inhibitor (InSTI), is recommended in first and second-line antiretroviral therapy by the World Health Organization (WHO) (Ngoufack Jagni Semengue *et al.*, 2022). Although DTG is highly effective in managing HIV, cases of resistance have been documented. Resistance typically arises from mutations in the integrase enzyme, which diminish the drug's capacity to inhibit HIV replication. These mutations may develop due to suboptimal adherence to treatment or through the natural selection of resistant viral strains (Oliveira et al., 2015).

The mutations R263K**,** G118R**,** and H51Y are associated with resistance to dolutegravir (DTG), an integrase strand transfer inhibitor used in HIV-1 treatment. The R263K mutation confers low to moderate DTG resistance but significantly impairs viral replication capacity, making it less likely for the virus to thrive (Oliveira et al., 2015). Similarly, G118R causes a substantial reduction in viral fitness and integration efficiency while conferring high-level resistance to DTG and other integrase inhibitors (Brenner *et al.*, 2016). In contrast, H51Y contributes minimally to resistance on its own but can

enhance resistance and partially compensate for fitness defects when paired with other mutations such as R263K (Oliveira et al., 2015).

According to a study by Kouamou (2019), adolescents and young adults provide considerable problems for treatment programs around the world, especially in low- and middle-income countries (LMICs) where virological failure is a result of weak health systems, decreased adherence, and restricted access to viral load monitoring. The 2016 WHO definition of virological failure refers to viral loads greater than 1000 copies/mL following two consecutive viral load measurements in three months, with adherence to treatment regimen after the first viral load test. These high failure rates result in drug resistance, rising morbidity and mortality, and the possibility of transmitting resistant viruses to sexual partners and newborns. Data on DTG HIV drug resistance (HIVDR) in Africa is still limited (van Oosterhout *et al.*, 2022).

Malawi remains significantly impacted by HIV, with approximately 991,600 individuals living with the virus as of 2023 (CDC, 2023). The adult HIV prevalence rate among those aged 15 to 49 has decreased to 7.1% in 2022 (CDC, 2023). Antiretroviral therapy has been crucial in reducing HIV-related morbidity, mortality, and new infections. However, the emergence of DTG resistance threatens the efficacy of ART programs. A study conducted between November 2020 and September 2021 identified DTG resistance in 8 out of 27 individuals (30%) experiencing virological failure (Malawi Ministry of Health, 2022). Another study reported 24 cases of DTG resistance among 89 individuals with confirmed VF, highlighting the prevalence of resistance within Malawi's HIV program (Mbunkah et al., 2023). Studies have concluded that DTG resistance is present in Malawi and highlights the importance of continued monitoring for DTG resistance in Malawi and other settings where the drug is being used (Schramm *et al.*, 2022; van Oosterhout *et al.*, 2022). These findings emphasize the need for improved monitoring and strategies to mitigate drug resistance to sustain ART effectiveness. Monitoring the effectiveness of antiretroviral therapy is crucial for ensuring viral load suppression in individuals. Sequencing plays a vital role in studying persistent viral measures in patients with low or suppressed viral loads, helping to identify resistance and predict future virological failure effectively (Taiwo *et al.*, 2011).

This retrospective study aimed to assess the occurrence of HIV drug-resistance mutations linked to DTG use in various districts of Malawi using secondary data.

1.2 Problem Statement

This retrospective study addresses the limited data on HIV drug resistance to DTG in Malawi, a cornerstone of the country's ART regimen due to its high barrier to resistance, potency, and tolerability. Despite its advantages, emerging studies have reported cases of DTG resistance. Since Malawi's transition to DTG-based regimens in 2019, only two articles have explored DTG resistance (Schramm et al., 2022; Van Oosterhout et al., 2022), while earlier studies on HIV drug resistance primarily focused on transmitted resistance without detailed analysis of resistance mutations (Wadonda-Kabondo et al., 2012). There is a pressing need to evaluate and monitor DTG resistance mutations in individuals with increase in HIV viral load to guide appropriate treatment adjustments. This study emphasizes identifying of DTG resistance mutations within the country and includes a phylogenetic analysis to explore the evolutionary relationships and transmission dynamics of resistant HIV strains. Such insights are critical for safeguarding the effectiveness of DTG-based regimens, ensuring optimal patient outcomes, and informing future treatment strategies

1.3. Study Justification

Dolutegravir, a WHO-recommended first-line antiretroviral drug, has been widely used since 2019. While it is highly effective, the emergence of resistance to DTG presents a significant challenge to HIV treatment efforts. Drug resistance can result in treatment failure, fewer therapeutic options, and disease progression with increased susceptibility to opportunistic infections, transmission of resistant HIV strains, higher healthcare costs, and difficulties in achieving viral suppression. Limited studies within the country have identified cases of DTG resistance (Schramm et al., 2022). These findings highlight the critical need for continuous monitoring of drug resistance in HIV treatment programs and the development of targeted strategies to address emerging resistance patterns (van Oosterhout et al., 2022).

1.4 General Objectives

1.4.1 Main Objective

The main objective of the study was to analyze and identify the presence of HIV drug-resistance mutations associated with the use of DTG in districts of Malawi.

1.4.2 Specific Objectives

1. Analyze for DTG resistance mutations in virus samples from people living with HIV.
2. Establish a phylogenetic relationship of determined HIV DTG-resistant strains.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

The Human Immunodeficiency Virus is a retrovirus that attacks the immune system, specifically targeting CD4+ T cells, which play a crucial role in immune defense. If left untreated, HIV progressively weakens the immune system, leading to Acquired Immunodeficiency Syndrome (AIDS), a condition characterized by severe immune suppression and increased susceptibility to opportunistic infections (UNAIDS, 2023). HIV is primarily transmitted through unprotected sexual contact, blood transfusions, shared needles, and from mother to child during childbirth or breastfeeding (WHO, 2023). Since its discovery in the early 1980s, HIV has become a global pandemic, with approximately 39 million people living with the virus worldwide as of 2022 (UNAIDS, 2023). Advances in ART have significantly improved the prognosis for individuals living with HIV, transforming it from a fatal disease to a manageable chronic condition (Deeks et al., 2013).

2.2 HIV Evolution and Genetic Diversity

HIV-1 evolves rapidly due to the high mutation rate of its reverse transcriptase enzyme, which lacks proofreading capabilities, leading to extensive genetic diversity (Liu et al., 2020). This genetic variability allows the virus to adapt to host immune responses and antiretroviral therapies. Since its emergence in human populations, HIV-1 has accumulated substantial genetic diversity, with the common ancestor of HIV-1 group M strains dating back to the 1920s (Korber et al., 2000). The zoonotic origin of HIV is well-established, with HIV-1 closely related to Simian Immunodeficiency Viruses (SIVs) found in wild chimpanzees and gorillas in west-central Africa (Sharp & Hahn, 2010). Phylogenetic analyses indicate that HIV-1 originated from chimpanzees, with at least four independent cross-species transmission events to humans, some potentially involving gorillas (Sharp & Hahn, 2010).

The cross-species transmission of HIV-1 likely occurred through exposure to infected primate blood, particularly during hunting and butchering practices. Once in humans, the virus adapted and spread, with evidence suggesting that early urbanization in Kinshasa and Brazzaville, coupled with colonial-era medical practices such as unsterile injections, facilitated its expansion (Faria et al., 2014). HIV-1 first emerged in the Democratic Republic of the Congo and southern Cameroon in the early 1920s, but AIDS was formally recognized in the United States in 1981. HIV-1 group M is responsible for the majority of global infections, while other groups, including N, O, and P, remain geographically restricted (Robertson et al., 2000). In contrast, HIV-2, which was identified in 1986 and is more closely related to SIVs from sooty mangabeys, has a lower transmission rate and is primarily localized to West Africa, where it generally causes a less aggressive form of the disease (De Silva et al., 2008).

Despite its extensive genetic diversity, HIV-1 remains largely susceptible to ART, which effectively suppresses viral replication and slows disease progression. However, drug resistance mutations can emerge under suboptimal treatment conditions, necessitating continuous monitoring and regimen adjustments (Pennings, 2013). The ongoing evolution of HIV-1 highlights the importance of molecular epidemiology in tracking viral transmission networks and optimizing therapeutic strategies

2.3 Global Impact of HIV

Human Immunodeficiency Virus has had a significant global impact since its identification in the 1980s (Turner and Summers, 1999). According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), as of 2020, approximately 38 million people have been infected with HIV, and nearly 32 million people have died from AIDS-related illnesses since the epidemic began. HIV/AIDS has strained healthcare systems globally, particularly in resource-limited settings.

The disease requires long-term medical management, including ART, which has improved life expectancy and quality of life for people living with HIV. However, access to treatment remains a challenge in many regions, leading to increased morbidity and mortality. Social and economic consequences of HIV/AIDS affect young adults in the

most productive years, leading to a loss of workforce and productivity. The impact of HIV/AIDS varies across regions and countries. Sub-Saharan Africa remains the most affected region, accounting for the newest HIV infections and AIDS-related deaths. However, other regions, such as Eastern Europe, Central Asia, and certain parts of Asia and the Americas, also face significant challenges in addressing the HIV/AIDS epidemic (UNAIDS, 2022).

Resistance to HIV antiretroviral drugs reduces the effectiveness of these medications in controlling the virus. As a result, viral loads can increase, leading to faster disease progression and complicating infection management. The emergence of drug resistance in HIV/AIDS has profound implications for both individuals and public health. It requires the use of more costly second and third-line antiretroviral medications, which are often less accessible and more expensive. This places significant strain on healthcare systems and elevates the financial burden on individuals, communities, and governments (WHO, 2021)

According to the WHO, by the end of 2023, approximately 39.9 million people globally were living with HIV, with 1.3 million new infections reported that year (UNAIDS, 2023). In Malawi, as of March 2023, a total of 949,158 people had been diagnosed with HIV (AIDS Malawi, 2023). The Southern region of Malawi has the highest HIV prevalence rate at 10.1%, followed by the Central region at 7.4%, and the Northern region with the lowest at 4.1% (UNAIDS, 2023). Despite significant progress in ART access, HIV remains a leading cause of hospital admissions in sub-Saharan Africa, with early mortality rates of about 31% (Gupta-Wright et al., 2020). Globally, the number of new HIV infections has decreased by 60% since the peak in 1995, but the challenge of reducing new infections continues, as 1.3 million new infections and 630,000 AIDS-related deaths were reported in 2023 (UNAIDS, 2023)..

2.4 HIV Antiretroviral Therapy

As of 2019, approximately 18 million people living with HIV (PLWH) in sub-Saharan Africa (SSA) were receiving ART (McCluskey *et al.*, 2021). With increasing access to ART, drug resistance to ART is also rising. There have been a growing number of anti-retroviral drugs developed that target HIV. ART usually consists of a combination of three

or more antiretroviral drugs from different drug classes, such as non-nucleoside reverse transcriptase inhibitors (NNRTIs) with drugs such as Efavirenzd/tenofovir/emtricitabine/plus abacavirc/lamivudine under it, nucleoside reverse transcriptase inhibitors (NRTIs) which comprises of Atazanavir plus tenofovir/emtricitabine/Darunavir/abacavir. Under Protease Inhibitors (PIs) we have drugs such as Efavirenzd/tenofovir/emtricitabine, and InSTIs are DTG, tenofovir/emtricitabine/ Elvitegravir/ abacavirc/lamivudine and entry inhibitors. The combination of drugs used in ART is tailored to each individual's unique medical history, viral load, and other factors. (Günthard *et al.*, 2014).

The most recent generation of ART, HIV-1 InSTIs, has altered the HIV-1 treatment landscape globally. An article by Zhao in 2022 stated that InSTIs are the latest approved drug class to treat HIV infection. Specifically, InSTIs stop antiretroviral activity by blocking the integration of HIV proviral DNA into the genetic material of host cells. There are currently four approved drugs belonging to this therapeutic class: raltegravir, elvitegravir, DTG, and bictegravir.

DTG, a second-generation InSTI with a stronger genetic barrier to resistance, is a successful therapy for both treatment-naïve patients and those who have viruses resistant to raltegravir or elvitegravir (Castagna *et al.*, 2014). Because of the mutations that build up in the viral genome and permit viral replication to go on in the presence of antiretroviral (ARVs), virological failure can occur in HIV-1-infected people.

2.5 Dolutegravir Resistance and Associated Mutations

The HIV-1 genetic variability arises from many viral variants infecting the same cell through recombination, the accumulation of proviral variations throughout infection, and the high rate of HIV-1 reverse transcriptase (RT) processing mistakes (Clutter *et al.*, 2016a). The development of drug resistance, which emerges in a substantial proportion of treated individuals, threatens to compromise treatment and eradication strategies. Blassel *et al* 2021, state that “Drug Resistance_Mutations (DRMs) usually arise in HIV1 due to antiretroviral treatment pressure, leading to viral rebound and treatment failure. Furthermore, according to Blassel *et al* 2021, drug-resistant HIV variants can be transmitted to treatment-naive individuals and further spread throughout the population

over time, HIV can develop resistance to almost all drugs based on mutations that are usually located within the coding regions of the enzymes that serve as drug targets”

Resistance to DTG can occur when mutations in the HIV-1 virus develop that allow it to evade the drugs mechanism of action. The most common mutation associated with dolutegravir resistance is called the R263K mutation, which occurs in the integrase gene of HIV-1. This mutation reduces the potency of DTG by about 3- to 4- fold and can emerge rapidly if treatment with the drug is not successful. Other mutations associated with DTG resistance include H51Y, E138K, G14OS/A/C, Y143R/C/H, Q148H/R/KNI55H and, G118R (Wensing *et al.*, 2022). Figure 2.3 displays major and minor mutations associated with InSTIs classified by the International Antiviral Society–USA (IAS–USA) drug resistance mutations.

H51Y is a rare nonpolymorphic accessory mutation for the other mutations. It is caused by Raltegravir (RAL), DTG, Elvitegravir (EVG), and Cabotegravir (CAB) in vivo and by EVG, DTG, and EVG in vitro. G118R is a nonpolymorphic mutation that has been found in a considerable number of patients experiencing emergent HIVDR and virological failure while on a DTG-containing regimen; it has hardly ever been seen in patients receiving other InSTIs. It is linked to a 2-3 fold reduction in susceptibility to BIC and a 5–10 fold reduction in susceptibility to RAL, EVG, DTG, and CAB. Patients undergoing RAL, EVG, CAB, and DTG are frequently selected for nonpolymorphic accessory resistance mutations, including E138K/A/T.

Common nonpolymorphic accessory mutations, G140S/A/C, typically coexist with Q14 8H/R/K.When taken alone, they are linked to a roughly fivefold decrease in EVG suscep tibility, but their phenotypic influence on the other InSTIs is minimal.

They confer >100-fold lower susceptibility to RAL and EVG, roughly 10fold reduced susceptibility to CAB, and typically 2 to 5-fold reduced sensitivity to DTG and BIC when combined with Q148 mutations (https://hivdb.stanford.edu/dr-summary/resistance-notes/INSTI).

G140R, is a very uncommon mutation with a decreased potential for replication that has only been documented in one CAB recipient and not in any of the more than 2500 In

STI recipieorphic accessory mutations, that usually occur with Q148H/R/K. Alone, they are associated with about 5-fold reduced EVG susceptibility, but have little phenotypic impact on the remaining InSTIs. In combination with Q148 mutations they confer >100-fold reduced susceptibility to RAL and EVG, about 10-fold reduced susceptibility to CAB, and usually 2- to 5-fold reduced susceptibility to DTG and BIC. G140R is an extremely rare mutation with reduced replication capacity that has been reported in one person receiving CAB and not in any other of the more than 2500 persons receiving an InSTI. Nonpolymorphic mutations Q148H/K/R have been identified in humans receiving RAL, EVG, CAB, and DTG. Although Q148R may occur alone, Q148H/K always occur in combination with accessory mutations (http://hivdb.stanford.edu).

These mutations can occur either alone or in combination and result in potency reduction (Rhee *et al.*, 2019). In a 2019 observational study that was conducted in Chiradzulu (Malawi), 98% of the more than one thousand three hundred Malawians who switched to a DTG-based regimen and were evaluated six months later had suppressed VL. DTG resistance due to Arg263Lys or Gly118Arg mutation was found in two participants with proven virological failure (Schramm *et al.*, 2022). Another study' that was done in Malawi resulted in 8 incidences of DTG resistance mutations among twenty-seven people living with HIV and had virological failure, (30%) with R263K, E138K, H51Y, E157Q, S147G and Q95K mutations (van Oosterhout *et al.*, 2022). These results may indicate that DTG resistance is widespread in Malawi.

DTG resistance mutations in HIV-1 have significant biological and clinical implications, including reduced drug efficacy, potential treatment failure, and the risk of transmitting resistant strains. These mutations can diminish the virus's susceptibility to DTG, compromise viral fitness, and create cross-resistance to other integrase inhibitors, thereby limiting treatment options. Consequently, regular resistance monitoring and the development of new antiretroviral drugs are essential to manage and mitigate these challenges effectively (Hunt, 2019; Castagna *et al.*, 2019; Tzou *et al.*, 2021).

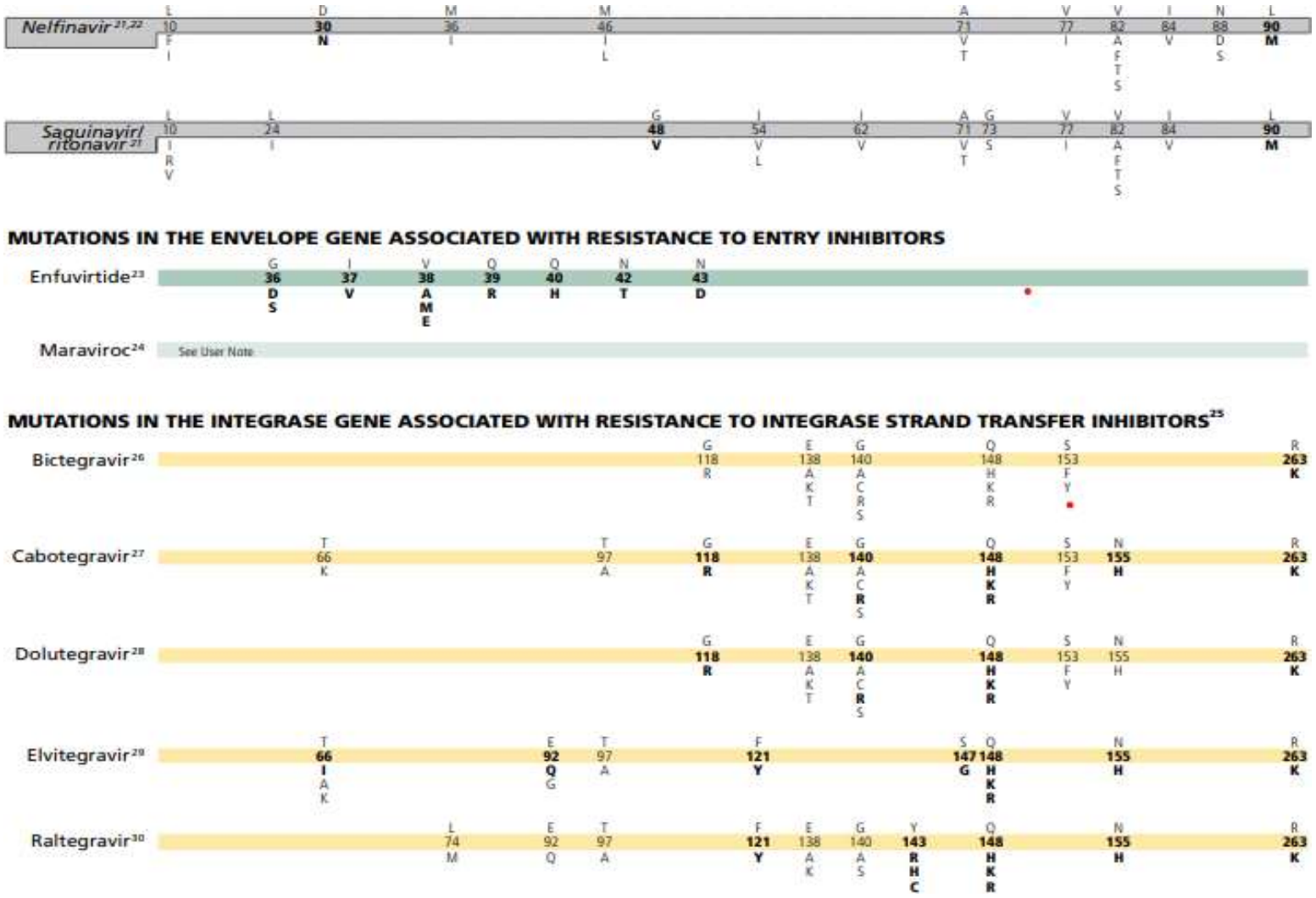


Figure 2.1: Mutations in Dolutegravir and other integrase inhibitors gene (Wensing et al., 2022)

2.6 Drug Resistance in Relation to ART Resistance and Factors that lead to the Development of ART Resistance.

Drug resistance mutations are specific mutations that occur in the HIV genome that are associated with a reduced in vitro and in vivo activity of ARV drugs. Drug resistance mutations are specific and generally emerge in the gene (Günthard *et al*., 2019). Many factors influence the development of resistance, these elements include the low genetic barrier that certain ARVs have to the development of resistance, drug-drug interactions, improper prescribing procedures, disruptions in the drug supply, low patient retention, and the absence of regular viral load monitoring (Bertagnolio *et al*., 2012). Expanding access to antiretroviral medication has been shown to be a successful strategy in lowering mortality and enhancing quality of life for individuals living with HIV (PLHIV) however Sub-Saharan Africa has the highest rate of new HIV infections, despite widespread access to ART (Zazzi, Hu and Prosperi, 2018).

Potency, tolerability, ease of adherence, and, in particular, a medium-to-high genetic barrier to resistance are all combined in the majority of currently advised treatment regimens. As a result, in high-income nations where the most cutting-edge treatment regimens have been utilized more frequently, the prevalence and incidence of emergent drug resistance have decreased recently (Frentz *et al*., 2014; Rhee et al., 2015; Schultze *et al*., 2015). Conversely, the introduction of ART in low- and middle-income nations has gradually increased the number of patients receiving treatment; however, less tolerant, and lower genetic barrier regimens have been employed extensively, which has created the conditions for the selection of drug-resistant variants (Bertagnolio *et al.*, 2012). It has been reported that several low- and middle-income nations, the prevalence of drug resistance has increased dramatically in recent years.

2.7 HIV 1 Drug Resistance Testing and Interpretation

HIV-1 drug resistance testing can be performed phenotypically or genotypically. Standard genotypic resistance testing (SGRT) involves the use of dideoxy-terminator Sanger sequencing of Protease (PI), Reverse Transcriptase (RT), and integrase to identify established clinically significant DRMs. SGRT is performed on Polymerase Chain Reaction (PCR) products directly amplified from cDNA that have been reverse-

transcribed from plasma RNA. Because the virus population in an individual is typically heterogeneous, it is common for more than one nucleotide (i.e. mixture) to be present in a given position on Sanger sequence electropherograms. Standardization between laboratories for studies of drug resistance has been facilitated through the use of software tools such as the Stanford database, International AIDS Society and Los Alamos sequence database that apply a consistent approach to identifying mixed bases (Clutter *et al.*, 2016b).

The Stanford HIV Drug Resistance Database (HIVDB) provides an online genotypic resistance interpretation program to help clinicians and laboratories interpret HIV-1 genotypic resistance tests (http://hivdb.stanford.edu). The program accepts RT, protease, and/or integrase sequences submitted by the user and returns a list of penalty scores for each DRM and an estimate of reduced susceptibility for each ARV(Clutter *et al*., 2016b). To determine the possibility of a technical artifact that could confuse the interpretation of a sequence, either regional or overall, sequences go through a quality control study. The quality control analysis identifies four types of positions: (1) those with frame shifts or stop codons; (2) highly ambiguous nucleotides; (3) evidence of 3F-mediated G-to-A, hyper mutation and/or APOBEC3G; and (4) positions with mutations found in the Stanford HIV Drug Resistance Database at remarkably low prevalence.

In an article by Tang *et al*., 2012, the estimation of ARV susceptibility is derived by adding the mutation penalty scores for each of the mutations present within a submitted sequence. Susceptibility estimations can be categorized into one of five groups or viewed as a continuous variable equal to the total of the mutation penalty scores. Susceptible: A total score of 0–9 indicates no evidence of decreased ARV susceptibility when compared to a wild-type virus. Potential low-level resistance: the sequence that was supplied certainly encodes a virus that is entirely susceptible, but it also has mutations that could suggest prior exposure to an ARV (total score of 10–14). Low-level resistance: Patients with viruses containing the submitted mutations may have a less than-ideal virological response to antiretroviral therapy, or the virus encoded by the submitted sequence may have decreased in vitro ARV susceptibility to receiving ARV medication (total score of 15–30). Intermediate resistance (total score of 30-59) is an ARV resistance level that is higher

than low-level resistance but lower than high-level resistance. An ARV with intermediate resistance to the virus should only be taken in conjunction with other active medications that are scarce or if the ARV has a strong genetic barrier to resistance (such as some ritonavir-boosted inhibitors). High-level resistance: the changes found in the virus's submitted sequence are comparable to those seen in viruses with the highest concentrations of in vitro drug resistance (total score >60) (Tang, Liu and Shafer, 2012). High-level resistance to DTG is conferred by several mutations in HIV integrase. Q148H/R and G140S in combination with mutations L74I/M, E92Q, T97A, E138A/K, G140A, or N155H are linked to 5-to 20-fold reduced DTG susceptibility102 and reduced virological suppression in patients, according to 100,101 cross-resistance studies with raltegravir- and elvitegravir-resistant virus (Wensing *et al.*, 2022).

2.8 Interpretation of Dolutegravir Resistance Mutations

Stanford HIV database, version 9.0 genotypic resistance system (https://hivdb.stanford.edu/hivdb/by-sequences/) was used to analyze sample Integrase sequences isolated from individuals with viral load > 1000 copies /mL for mutations related to DTG resistance. The Stanford HIV Drug Resistance Database (https://hivdb.stanford.edu/) is the largest public repository and most widely used online resource for HIV drug resistance. It currently comprises, ~450 000 sequences (reverse transcriptase, protease, or integrase) from ~200 000 patients with treatment status, from all around the world with ~60 000 results of drug susceptibility assays from HIV-1 virus isolates (Blassel *et al.*, 2021). Susceptibility (score below 10), prospective low (10–14), low (15–29), intermediate (30–59), or high (≥60) drug resistance levels were classified using the Stanford HIV Database version 9.·0 and the Stanford HIVdb algorithm(https://hivdb.stanford.edu/hivdb/by-sequences/). The HIVdb system developed by Stanford University in California operates on a set of rules. It includes scores and explanatory notes for each mutation related to drug resistance. The system calculates a total score for each drug by summing up the scores of all mutations known to confer resistance to that drug. Based on this score, the system categorizes the level of resistance into one of five levels: susceptible, potential low-level resistance, low-level resistance, intermediate resistance, and high-level resistance (Liu and Shafer, 2006).

2.9 Relationship between the Occurrences of Drug Resistance with Viral Load

The interplay between HIV drug resistance and viral load is a critical area of research with significant implications for clinical management. Viral load suppression, a key objective of antiretroviral therapy (ART), directly influences the development of drug resistance. Effective suppression minimizes viral replication, thereby reducing the likelihood of resistance mutations (World Health Organization, 2023).

A viral load threshold of 1000 copies/mL is particularly significant in the context of HIV treatment monitoring. According to the World Health Organization (WHO), a persistent viral load above 1000 copies/mL while on ART is a key indicator of virological failure and signals the potential emergence of drug-resistant strains (WHO, 2023). This threshold has been widely adopted as a standard for initiating confirmatory testing and resistance analysis to guide subsequent treatment decisions (Tang & Shafer, 2012).

Patients with resistance-associated mutations often exhibit higher viral loads compared to those without such mutations, further emphasizing the role of inadequate viral suppression in fostering drug resistance (Prasetyo et al., 2020). In particular, when viral load exceeds 1000 copies/mL, it creates an environment conducive to the selection of resistant strains, especially when adherence to treatment is inconsistent ((Bangsberg, Moss and Deeks, 2004)). Furthermore, the relationship between adherence, resistance, and viral load varies among different classes of antiretroviral drugs, reflecting diverse mechanisms of action and resistance pathways ((Paton *et al.*, 2006)).

Routine viral load monitoring, especially with the critical threshold of 1000 copies/mL, is paramount for early detection of treatment failure and resistance development. Timely intervention, such as switching to alternative regimens, is essential to maintain therapeutic efficacy and mitigate further resistance (WHO, 2023).

Dolutegravir, an InSTI, has shown great efficacy in controlling HIV replication when used alongside combination ART. The combination of Tenofovir, Lamivudine, and DTG (TLD) is recommended as the initial treatment for adults with HIV. This regimen is proven to effectively suppress viral load, is cost-efficient, well tolerated by patients, and has a

high resistance barrier. Nevertheless, there are challenges with DTG due to circulating HIV integrase genotypes linked to drug resistance, posing obstacles to its effectiveness (Bwire et al., 2023).

2.10 Phylogenetic Analysis

Phylogenetic analyses can reveal aspects of HIV evolution that other methods cannot, such as "where and when" and even "how" infections are spreading around the world (Castro-Nallar *et al.*, 2012). The short generation durations and high mutation rates of HIV viruses lead them to rapidly amass genetic diversity. These variants are used by phylogenetic inference methods to reconstruct phylogenies (phylogenetic trees) using recent sequencing data. The tips of the tree correspond to the viral sequences at the time of sampling, and the base of the tree represents the ancestral lineage. Advancing in time involves progressing from the beginning to the end. A branching node in the phylogeny is what is used to illustrate the speciation of a lineage. Such a split can be viewed as a virus spreading to a new person when sampling is dense, and the entire tree is a rough approximation of the transmission tree (Leitner et al., 1996).

Various research endeavors have employed phylogenetic alongside comprehensive clinical and epidemiological information to investigate the emergence of new infections. Fisher *et al*. revealed that as much as 30% of fresh infections originated from individuals in the highly contagious primary stage of the disease. Brenner *et al*. utilized phylogenetic clustering analysis on a population of HIV-infected individuals in Quebec to indicate that early-stage infections might contribute significantly to onward transmissions (Zhukova *et al*., 2017). The HIV-1 pol gene has enough variety, according to a 2004 study by Hue *et al.*, to allow for the phylogenetic reconstruction of transmissions, which is crucial in the age of antiretroviral medication selection pressure.

Phylogenetic analysis in the study can aid in enabling the surveillance and monitoring of DTG resistance on a broader scale. By analyzing HIV sequences from different geographical regions, the study can detect the emergence of resistance in different populations and track its spread over time. The opportunity exists in conjunction with contact tracing to identify circumstances where multiple HIV transmissions are occurring or individuals are unknowingly transmitting HIV to multiple recipients if reliable

phylogenetic linkages can be obtained using the pol sequences analyzed as part of routine drug resistance testing.

CHAPTER THREE

3.0 METHODS AND MATERIALS

3.1 Study Design

This retrospective study evaluated genotypic resistance profiles in individuals experiencing virological failure on DTG-based therapy regimens in Malawi. For this study virological failure was defined according to Malawi HIV guidelines as a second viral load (VL) result of >1000 copies/mL after three months of intensive adherence support. Samples eligible for HIV drug resistance (HIVDR) testing were identified from the Malawi Laboratory Information Management System (LIMS). Data was collected for the period from December 2022 to January 2024, during which 91 out of 140 samples from individuals on DTG-based regimens met the criteria for the study.

Genotypic resistance analysis targeted the integrase IN, PR, and RT regions of the pol gene. Resistance-associated mutations were classified using the 2019 IAS-USA updated drug resistance mutations list. Subtype assignments were confirmed through phylogenetic analysis. Data from the National Drug Resistance Database were utilized to perform sequence alignment and mutation identification.

The primary outcome was the presence of resistance mutations compromising DTG efficacy.

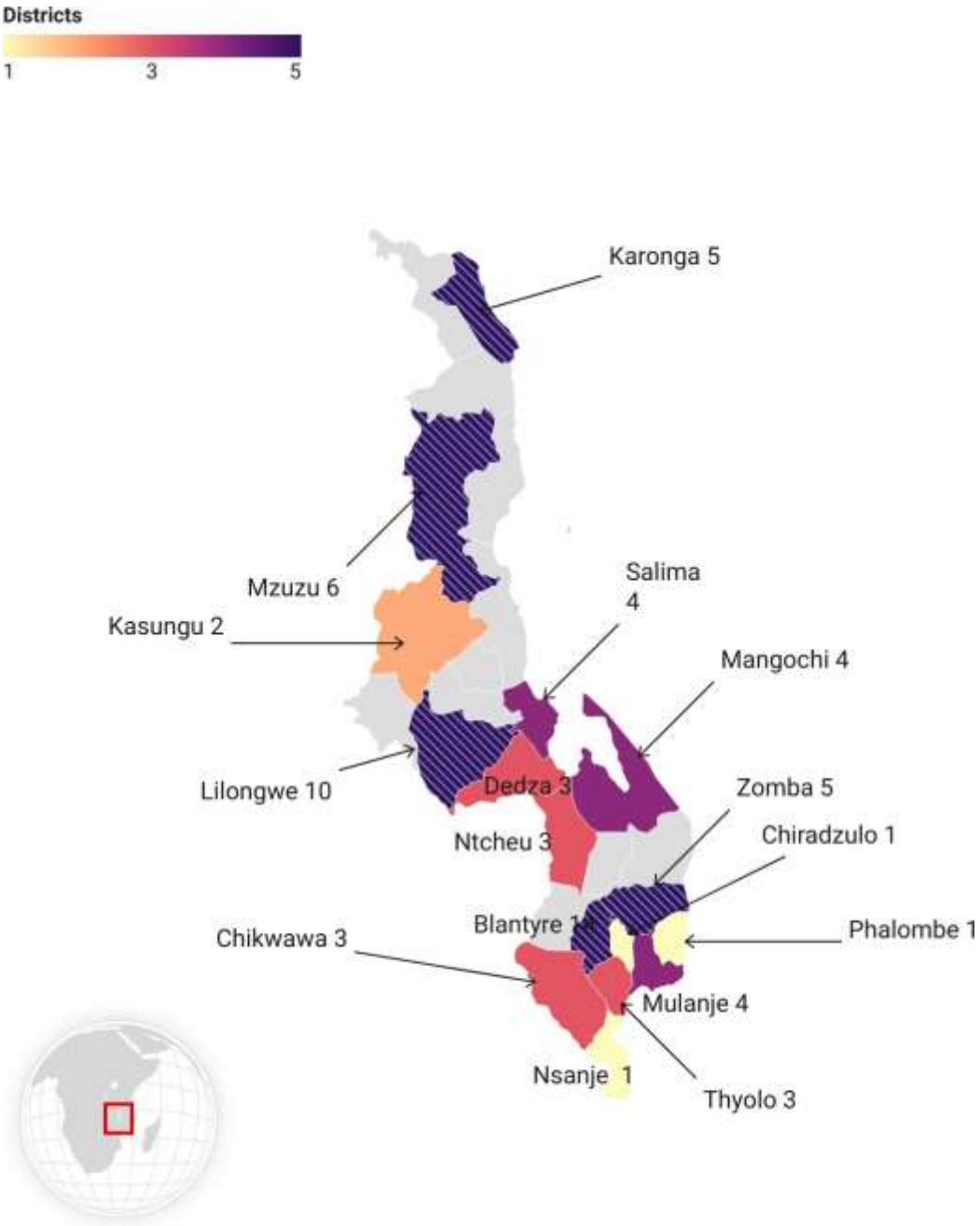


Figure 3.1: Map of Malawi, Highlighted are the districts of sample origin and number of samples obtained from the named districts. The number of samples from each districts are indicated by the color code above the map and numbers against each named district (Map drawn using Datawrapper*)*

3.2 Target Population

The study targeted samples with HIV viral load ≥1000 copies/mLs that were on DTG treatment.

3.3 Sampling Techniques

In this retrospective study with a small sample size, a purposive sampling technique was used to select male and female participants aged 15–60 years, a demographic at higher risk of HIV infection and varied ART response (Miti et al., 2020; UNAIDS, 2022). The criteria used in this study is as follows;

Inclusion criteria: Patients experiencing virological failure, defined as a repeated viral load exceeding 1000 copies/ml despite good adherence in the preceding three months (Heller et al., 2019). A targeted or repeated viral load exceeding 1000 copies/ml, coupled with good adherence in the preceding three months, indicates confirmed therapy failure (Heller et al., 2019). Only individuals with available sequence data were included to enable the detection of DTG resistance mutations, providing critical insights for clinical management and public health interventions (WHO, 2021).

Exclusion Criteria: The study excluded data from individuals who were below 15 years of age and above 60 years. Data with viral load less than 1000 copies/ml was excluded. Individuals with incomplete or missing data relevant to the study were excluded. The study excluded individuals who were not on DTG regimen, as the study focused on identifying resistance mutations among those who were on DTG regimen.

3.4 Data Analysis

3.4.1 Multiple Sequences Alignment and Phylogenetic Tree

The sequence data, formatted in Fasta, underwent further analysis to assess phylogenetic relatedness, focusing on HIV-1 pol genes targeting the reverse transcriptase, protease, and integrase domains (Kaye et al., 2008). One hundred and forty sequences were retrieved from the PHIM database for phylogenetic analysis, however, for the analysis, only a subset comprising ninety-one sample sequencing results were utilized. Forty-nine results were excluded, as they did not meet the study criteria. Three DTG-positive sample sequences

were of suboptimal quality and were therefore excluded from phylogenetic analysis. HIV-1 pol sequence sets were partitioned using Genetyx SEQ to exclude sequences falling outside the study criteria. The AliView application tools (available at www.github.com/AliView) were subsequently used to combine the sequences with reference sequences of the HIV pol gene from Zambia and Malawi. These reference sequences were from Gene bank database. Geneious was used to visualize and analyze the alignments (https://www.geneious.com). The sequences were aligned using Muscle alignment (http://www.drive5.com/muscle). Alignment inspection, gaps trimming and conversion of the alignment were done using Geneious Prime v2022.0.1

Maximum likelihood was used for tree construction. The tree was constructed using IQ-TREE, with the model test indicating the use of the GTR+F (Generalized Time Reversible model with empirical base frequencies) model based on the data. To evaluate the reliability of the branching order, 1000 bootstrap replicates were performed. Branches representing partitions replicated in less than 50% of the bootstrap replicates were condensed. Since no outgroup sequence was available, midpoint rooting was applied to root the tree. Additionally, the tree was ladderized to enhance topology and visualization (http://www.iqtree.org/doc/Substitution-Models.The resulting ML tree file was modified using Interactive Tree of Life (iTOL) v5, an online tool for visualizing and annotating phylogenetic trees (Letunic & Bork, 2021**)** (https://itol.embl.de).

3.4.2 Statistical Analysis

The study utilized Stata version 18 and Microsoft Excel for statistical analysis. Descriptive statistics, including frequencies and percentages, were used to summarize demographic characteristics and drug resistance mutations (DRMs). The incidence of HIV drug resistance (HIVDR) was assessed by calculating the proportion of patient samples with DTG-resistant mutations among successfully genotyped sequences. A chi-square test was performed to evaluate the association between DRMs, viral load (VL), and participant characteristics, with statistical significance set at $P < 0.05$.

3.5 Ethical Considerations

Institutional approval was granted by the University of Zambia (Appendix B). Furthermore the study received ethical approval from the National Health Science Research Committee of Malawi under protocol #23/12/2377 (Appendix D).

CHAPTER FOUR

4.0 RESULTS

4.1 Sample Data Distribution per District.

Retrieved sample data came from 16 out of 28 districts, representing 57.1% of the total districts in Malawi. Out of 91 sample datasets retrieved, the highest number was from Blantyre with 14 samples while Phalombe, Chiladzuro and Nsanje had the lowest count with just one sample each. There were 21 samples (23%) with no traceable codes to indicate their district source or of origin. The data included nine districts from the southern region, representing a high number.

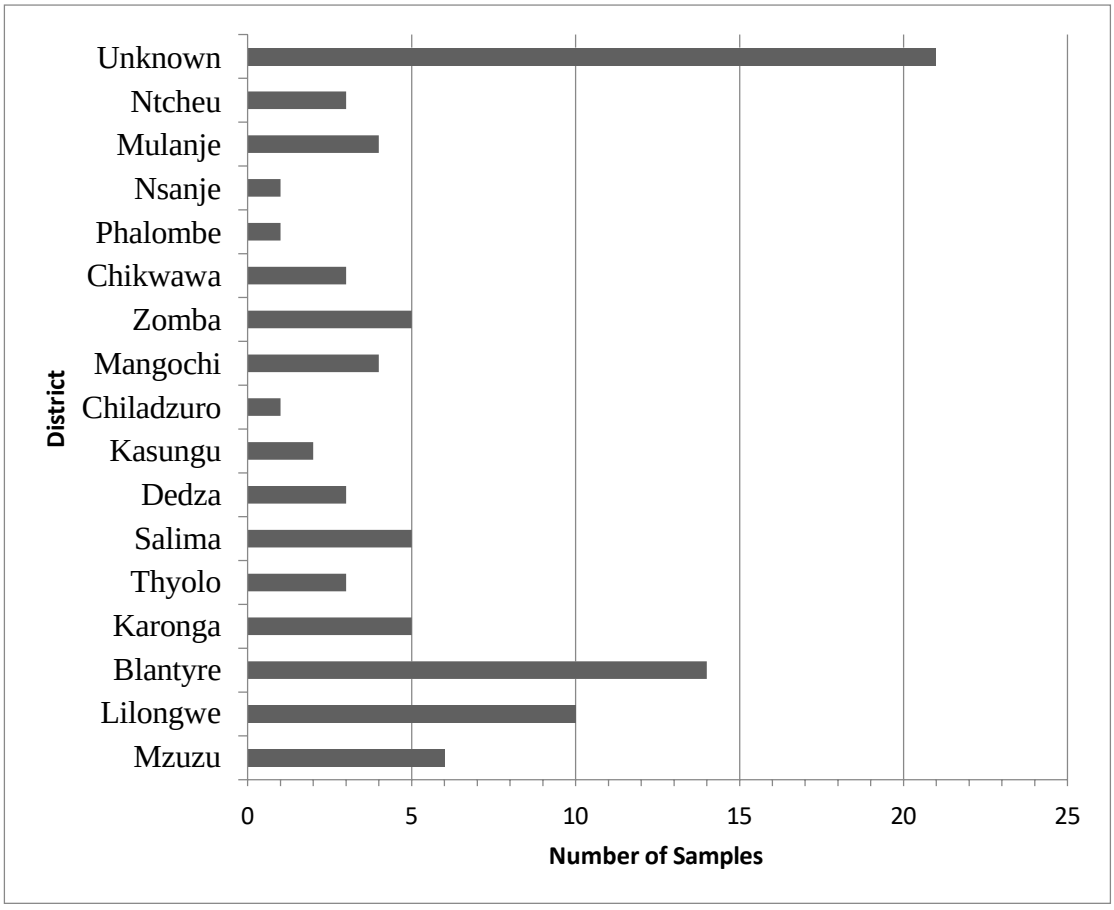


Figure 4.1: Number of samples per district whose data were retrieved from the LIMS (PHIM - LIMS).

4.2 Demographic Characteristics

The data constituted 60.4 % (n=55) of female patients, while males accounted for 39.6% (n=36). In terms of age distribution, 51.6% of the participants were aged between 15 and 29 years, with females making up 48.9% and males accounting for 51.1%. Of the 48.4% of the samples comprising individuals aged 30 to 60 years, 29.5% were males and 70.5% were females (see Figure 4.2)

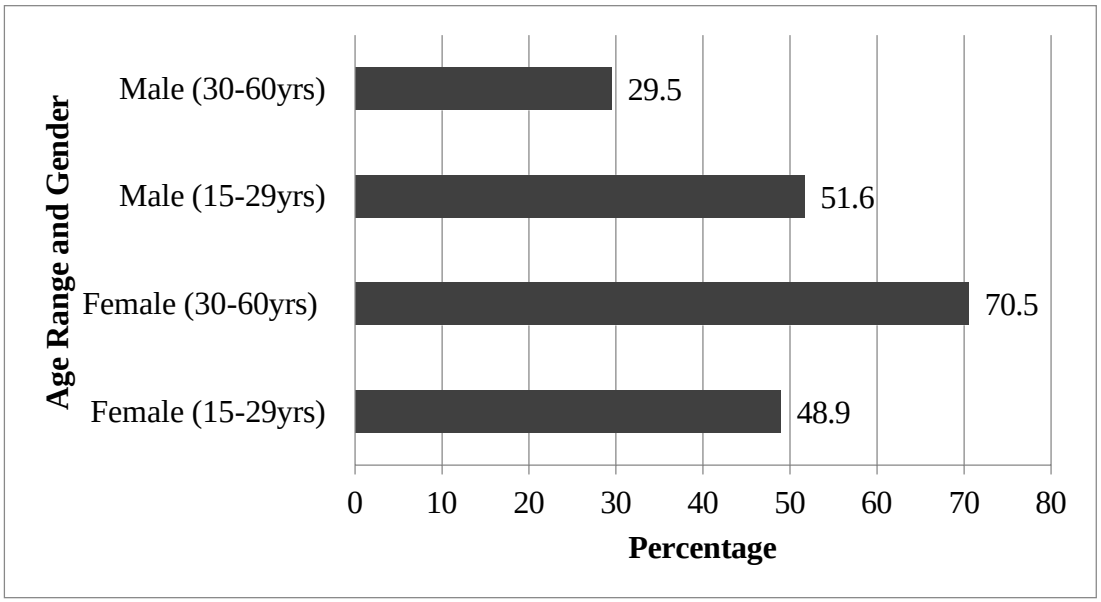


Figure 4.2: Gender and age range distribution of patients. A represents the gender and age group segregation while B represents gender distribution within age groups.

4.3 Geographical Distribution of DTG Resistance Mutations

DTG resistance mutations were in 15.4% (n = 91) of the samples reviewed. These samples were from Phalombe, Lilongwe Karonga, Zomba, Blantyre, Nsanje, and Mzuzu districts. Three samples had no known district source with Lilongwe having highest number of positive samples.

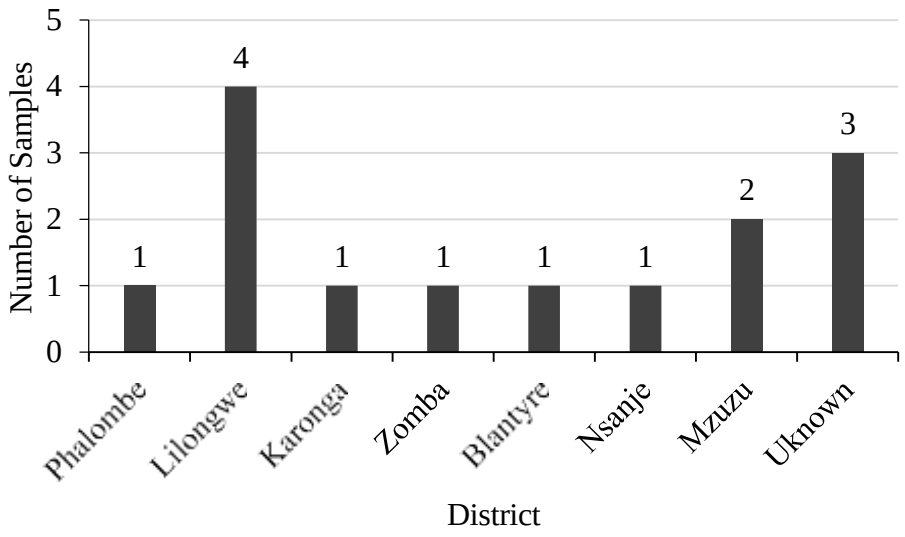


Figure 4.3: Geographical distribution of samples with DTG resistance mutations

4.4 Resistance Level of Categorization

Out of 91 samples, 14 were found to have resistance mutations related to DTG and were categorized based on the drug resistance levels as defined by the Stanford database. Seven out of 14 samples with DTG resistances were classified as high resistance. (Table 4.1) of DTG resistance levels in sample results.

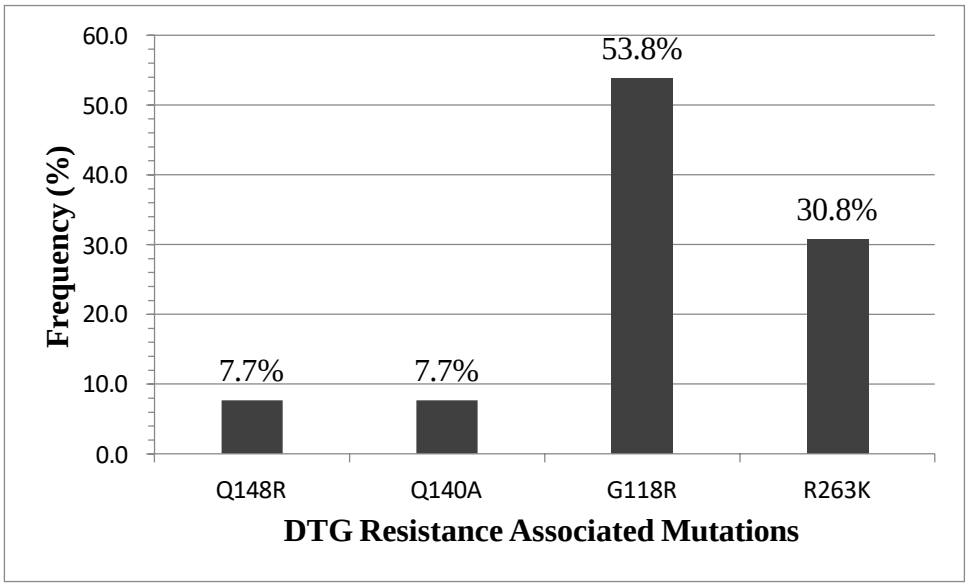
Table 4.1: Resistance Level of Categorization

Level of Resistance	Number of Samples	Percentage
High level resistance	7	7.7
Intermediate resistance	5	5.5
Potential low level	2	2.2
Susceptible	77	84.6
Total	91	100.0

4.5 DTG Mutations within the Country

Several mutations have been associated with resistance to DTG (Figure 4.5A). The frequency of specific genetic mutations associated with DTG resistance within the dataset was therefore ascertained The major mutation of G118R had the highest frequency of 53.8% (n=7) followed by R263K with a frequency of 30.8% (n=4). DTG resistance mutations, G140A and Q148R, had the lowest frequencies (Figure 4.5A and Appendix B). The four major mutations associated with DTG resistance (G118R, R263K, G140A and Q148R) were found in Lilongwe district but the other districts exhibited less of these mutations with different frequencies. However, despite the presence of these main DTG resistance determinant mutations, there were accessory mutations (H51Y, K103N, M184V), which could potentially contribute to resistance levels, in Lilongwe as was the case for other districts under study (Figure 4.5B).

A



B

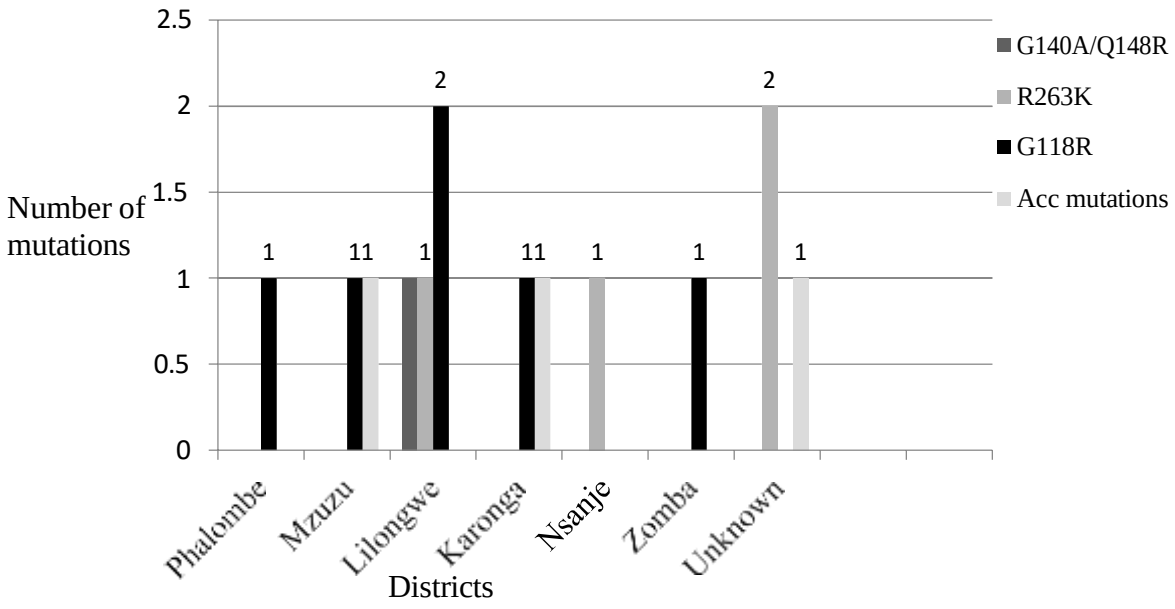


Figure 4.4: The frequency of (A) major InSTI mutations found in the study associated with DTG Resistance and (B) Distribution of mutations per district. Acc mutations- accessory mutation (H51Y, K103N and M184V).

4.6 Distribution of DTG Resistance Mutation Frequencies According to Gender and Age

Samples from female patients exhibited a relatively higher number of DTG resistance mutations compared to those from male patients. Distribution was n of DTG resistance mutations in females, 8.8% (n = 8) and 6.6% (n=6) of the samples from males had the DTG resistance mutation (Figure 4.6A). There was no association (P = 0.8) between gender and the presence of the DTG resistance mutation. Although more samples 8.8% (n=8) with DTG resistance mutations were found in the 30 to 60 age range compared to the 15 to 29 age range, which had 6.6% (n=6) (Figure 4.6B) of the samples, there was no association (p = 0.5) between age range and the presence of DTG resistance mutations. It is intriguing to observe that the proportions of DTG resistance mutations were identical when considering gender and age ranges (Appendix B)

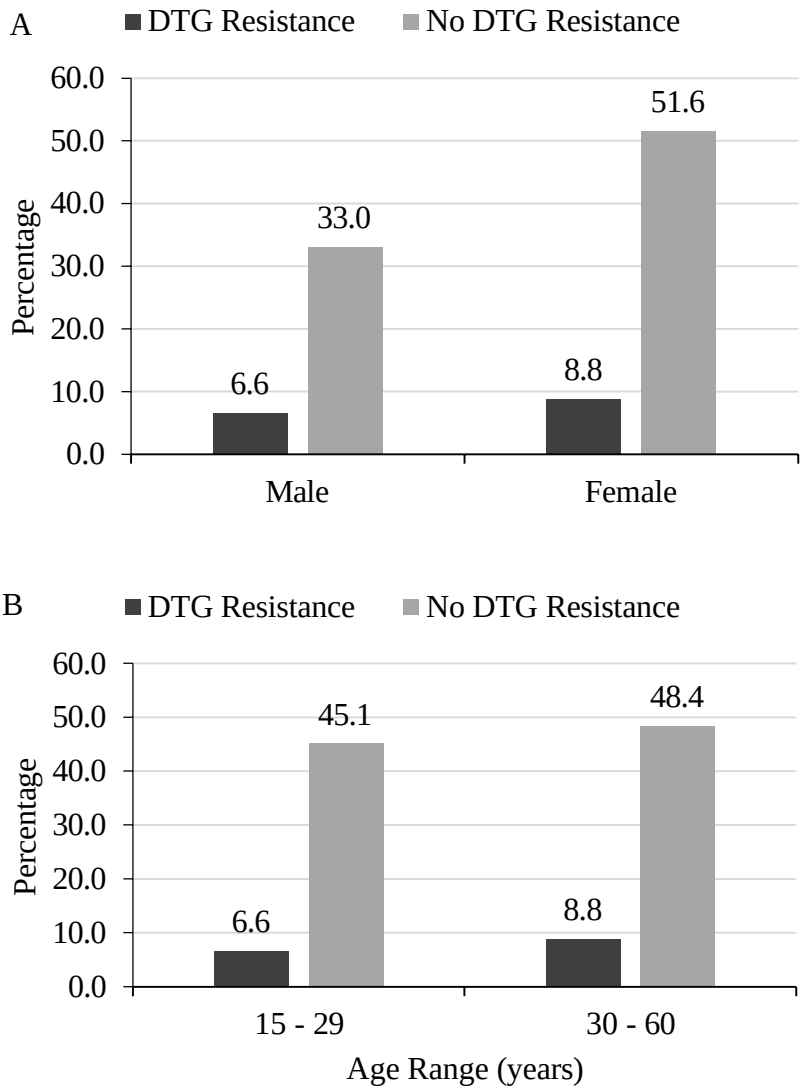


Figure 4.5: Comparison of the presence of DTG resistance mutation based on (A) gender and (B) two age ranges

4.7 DTG Mutations and Viral Load Results

A viral load of 1000 copies/ml or more was the inclusion criterion for sample data analyzed for DTG resistance mutations. All 91 samples had a VL greater than 1000 copies/ml. More samples were retrieved from other categories than the 1000 to 3499 copy number category, with the highest number of samples in the 5000 copies/ml or more categories. None of the samples with a viral load between 1000 and 3499 copies/ml

showed DTG resistance mutations from this studies results (Figure 4.7). The other three higher viral load categories had a comparable number of samples with DTG resistance mutations. However, there was no association (P = 0.576) between viral load and the occurrence of DTG resistance mutations.

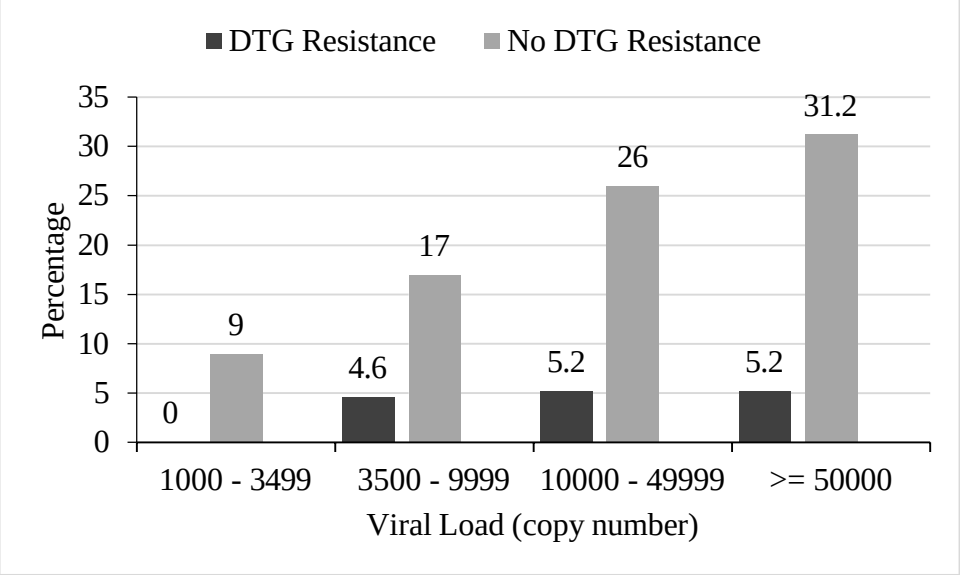


Figure 4.6: Occurrence of DTG resistance mutations according to viral load

4.8 Non DTG Resistance Mutations

While the study aimed at analyzing samples for DTG resistance mutations, other ARV drug resistance mutations were identified (Table 4.2). This indicated that the samples analyzed could be exhibiting multidrug resistance. Nineteen sample results were found to have more than one ARV drug resistance associated mutations.

Table 4.2: Resistance mutations to other classes of antiretroviral (ARV) drugs, including Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), Nucleoside Reverse Transcriptase Inhibitors (NRTIs), and Protease Inhibitors (PIs.)

CLASS OF ARV DRUGS	MUTATIONS
NNRTI	V108I, Y181C, H221Y, K70KE, G190A, V106M, G190A, F227L, K103N, E138EG, P22H, A98G and K101E.
NRTI	M41L, D67T, T69G, L74I, T215F K219E, D67N, T215I, K219Q, D67DN, Y69DE K70R, M184V, T215V and K219Q
PI	M46MI, I54V, L90M and 184V

4.9 Phylogenetic Relationship of Sample Sequences Analyzed for Drug Resistance.

The mid-point rooted maximum likelihood phylogenetic tree (Figure 4.8) illustrates the relationships among the DTG positive sequences. This phylogenetic analysis is based on the pol gene and visualized using itol phylogeny, highlighting the genetic diversity and relatedness of the DTG positive sequences, which are labeled dtg at the end of the code. Additionally, two reference strains from Zambia (KM04991) and Malawi (AY958320.1), while controls used in the analysis are depicted with code NHR309. The analysis revealed two major clusters originating from the root. Notably, two DTG positive samples from different districts clustered together in one group, while the remaining DTG positive sequences formed a separate cluster, which further subdivided into additional clusters. (Figure 4.8)

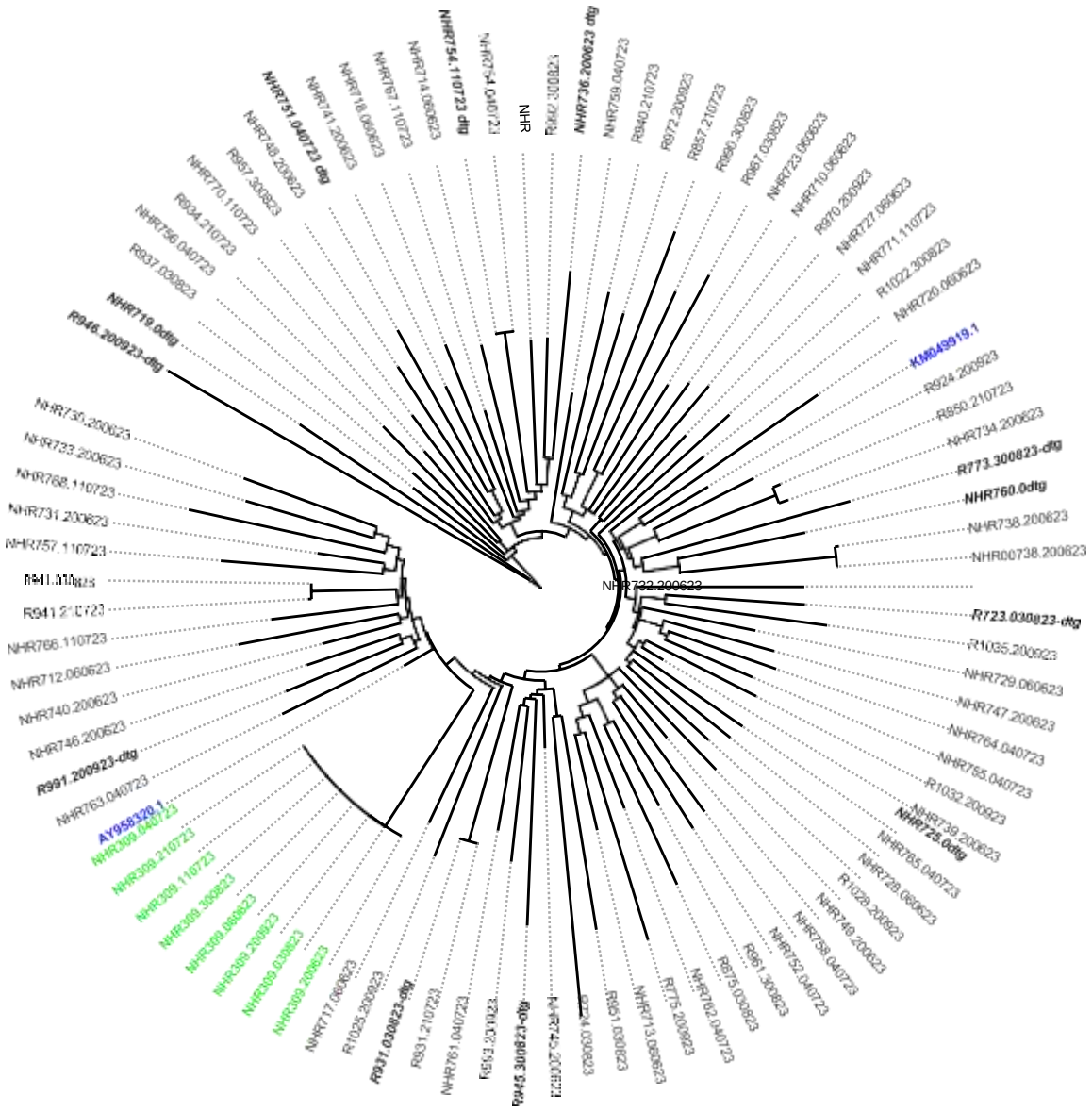


Figure 4.7: Phylogenetic relationship of HIV isolates from participants on DTG therapy. The sequences targeted regions of the HIV-1 pol gene specifically reverse transcriptase, integrase, and protease. Control sequences are denoted with code NHR309, and highlighted in green within the tree. Additionally, reference strains of HIV-1 subtypes from Zambia and Malawi are incorporated into the analysis. Samples that tested positive for DTG resistance are highlighted in red and labeled dtg.

CHAPTER FIVE

5.0 DISCUSSION

The study data retrieved originated from 16 out of 28 districts in Malawi, accounting for 57.1% of the total districts. A total of 91 sample datasets were analyzed at the Lilongwe Reference Laboratory on DTG. However, there was considerable variation in the number of samples per district. Blantyre had the highest sample count, contributing 14 samples (15.4% of the total), while Phalombe, Chiladzulu, and Nsanje had the lowest, with only one sample each. Notably, 21 samples (23%) lacked traceable codes to identify their district of origin, which presents a gap in data completeness and could affect regional analysis. The distribution across Malawi's regions was uneven, with nine out of the 16 represented districts coming from the southern region, indicating a higher sample contribution from this area. This aligns with national HIV prevalence trends, as studies have shown that the southern region has the highest HIV prevalence in Malawi at 13.2%, compared to the national average of 9% (Nutor *et al.*, 2020). The central and northern regions had relatively fewer districts contributing data. Further analysis is needed to determine whether the observed distribution is due to population density, healthcare facility accessibility, or other logistical factors influencing sample collection and submission to the reference laboratory.

The emergence of resistance to DTG, a key component of HIV treatment, poses a significant challenge to its long-term effectiveness. In this study, DTG resistance mutations were identified in 15.4% (14 out of 91) of the analyzed samples, with Lilongwe exhibiting the highest incidence. However, these mutations were distributed across multiple districts, underscoring the widespread nature of resistance. This finding aligns with previous studies, such as Rhee et al. (2020), which reported an increasing prevalence of InSTI resistance mutations in low- and middle-income countries. Similarly, studies from Uganda and South Africa have highlighted the emergence of DTG resistance, particularly among individuals with prior ART exposure or treatment failure (Wang et al., 2021; Moyo et al., 2022).

The frequency of DTG resistance observed in this study (15.4%) is lower than that reported by van Oosterhout et al. (2022), where 24.2% (8 out of 33) of samples exhibited resistance. The predominant major mutations associated with DTG resistance in patients on a DTG-containing regimen were G118R/R (50%), G140A (7.1%), Q148H/R (7.1%), and R263K (28.6%), as illustrated in Figure 4.5. These findings are consistent with prior research, which has identified R263K and G118R as the most common InSTI resistance mutations among individuals experiencing virological failure on a DTG-containing regimen (Rhee et al., 2019; Tao et al., 2023).

The captured data offers a detailed portrayal of sample distribution across multiple districts in Malawi, illuminating the breadth and depth of the research endeavor. Notably, the dataset encompasses a spectrum of regions, from populous urban centers like Lilongwe and Blantyre to locales such as Karonga and Thyolo. Interestingly, while some districts, like Salima and Mulanje, contributed relatively moderate sample sizes, others like Chikwawa and Phalombe exhibited relatively lower sampling rates. Moreover, the presence of a substantial number of samples categorized as "unknown" underscores potential challenges in sample identification or documentation and localized application of the results (Figure 4.1).

The data from the study had females comprising the highest proportion of the population at 60.4%, while males accounted for 39.6%. The age range of 15 to 29 years constituted 51.6% of the population, with those aged 30–60 years making up 48.4%. High sampling of female data is consistent with findings from other studies (Miti et al., 2020). This trend may be attributed to factors such as higher healthcare-seeking behavior among women, routine antenatal HIV testing, and engagement in prevention of mother-to-child transmission (PMTCT) programs, which increase their likelihood of being diagnosed and enrolled in treatment and monitoring programs (UNAIDS, 2021).

DTG resistance mutations were identified in 15.4% of the individuals analyzed, as depicted in Figure 4.3. These mutations were observed across various districts, with Lilongwe showing the highest incidence of these mutations. The study underscores that DTG resistance mutations are not localized to a single area but are spread across all regions. This widespread distribution of resistance mutations highlights the need for

enhanced surveillance and tailored treatment strategies to effectively manage and prevent the spread of resistant HIV strains.

Individuals with DTG resistance mutations were categorized based on resistance levels using the Stanford HIV Drug Resistance Database reporting criteria (Stanford University, n.d.) (Figure 4.4). Among those, exhibiting high-level resistance associated with major mutations, G118R was identified in 50% of cases, with a resistance score ranging from 80% to 85%. G118R is a non-polymorphic mutation commonly observed in individuals experiencing virological failure and emerging HIVDR while on DTG-containing regimens (Rhee et al., 2019). Notably, this mutation is rarely reported in individuals receiving other InSTIs (Wainberg & Han, 2019). Studies have shown that G118R is linked to a 5- to 10-fold reduction in susceptibility to RAV, EVG, DTG, and CAB, as well as a 2- to 3-fold reduction in susceptibility to BIC ((Tsiang *et al.*, 2016)).

In the present study, only 7.1% (n = 7) of participants exhibited high-level resistance, with a resistance score of 100%, attributed to the presence of G140A/Q148R mutations. The G140S/A/C mutations are non-polymorphic accessory mutations that typically co-occur with Q148H/R/K (Malet et al., 2022). The Q148R/G140A combination has been associated with over a 100-fold reduction in susceptibility to RAV and EVG and typically results in a 2- to 5-fold reduction in susceptibility to DTG and BIC (Hachiya et al., 2017)

This study found that 28.6% (n = 4) of cases with intermediate resistance carried the R263K mutation among the 14 DTG-resistant cases. This non-polymorphic mutation is selected in vitro by EVG, DTG), BIC, and CAB and is frequently w in individuals experiencing VF and emerging HIVDR while on DTG-containing regimens (Rhee et al., 2019). These findings are consistent with previous studies indicating that R263K is a key mutation conferring reduced susceptibility to DTG and other InSTIs (Quashie *et al.*, 2012). Additionally, 14.3% (n = 2) of cases were classified under low potential resistance mutations. While these cases did not exhibit major DTG resistance mutations, they harbored accessory InSTI mutations such as H51Y, a rare non-polymorphic mutation. Prior research has shown that H51Y is selected in vitro by EVG and DTG, though its impact on clinical resistance remains uncertain (Stanford University, n.d.). The

identification of such mutations aligns with findings from other studies monitoring VF in patients on DTG-containing regimens (Rhee et al., 2019).

Understanding the prevalence and implications of DTG-associated mutations is crucial for optimizing treatment strategies. The ability of DTG to maintain efficacy in individuals with prior ART exposure, as well as in monotherapy and dual therapy regimens, is an important factor in determining the risk of VF and the emergence of DTG resistance (Paredes et al., 2019).

Among individuals with DTG resistance mutations, 57.1% were female, while 42.9% were male. DTG resistance mutations were observed across both genders and all age groups; however, females and individuals aged 30 to 60 years had higher proportions of these mutations. These findings align with previous studies indicating that demographic factors such as sex and age may influence HIV drug resistance patterns, although the extent of this influence remains debated (Gupta et al., 2019; WHO, 2021).

In the analyzed dataset, 6.6% (n = 6) of males exhibited DTG resistance mutations, compared to 8.8% (n = 8) of females. Although there appeared to be a higher representation of females and individuals aged 30 to 60 years among those with resistance mutations, statistical analysis showed no significant association between DTG resistance and gender or age groups (χ^2, P = 0.449). These results are consistent with prior research, which has similarly found no strong correlation between demographic factors and InSTI resistance (Rhee et al., 2020). Consequently, this suggests that other biological, clinical, or treatment-related factors may play a more substantial role in determining the presence of DTG resistance mutations in this population. Further investigation is warranted to explore potential contributors such as treatment adherence, viral load dynamics, and prior antiretroviral exposure (Paredes et al., 2019).

Most sample results in this study had viral loads greater than 10,000 copies (Fig 4.7). DTG resistance mutations were found in samples with viral loads above 3500. However, there was no statistically significant association between DTG resistance and viral load ((χ^2, P > 0.05) a notable observation pertained to numerous samples exhibiting a viral load surpassing 32,000 (48.4%), yet devoid of any detectable DRM which is similar to results

obtained in a study that was done in Tanzania (Kamori *et al.*, 2023). Consequently, it makes sense to suggest that inadequate adherence may be the cause of elevated viraemia in certain patients without any discernible DRMs. The phenomena of reversion of HIV to wild type in the absence of enough medication pressure may make interpreting HIVDR results more difficult. Reversion to HIV wild type has been reported in a number of contexts and is recognized as a huddle interpreting HIVDR data (Bwire *et al.*, 2023).

Apart from the DTG resistance mutations, several other HIV drug resistances associated mutations, were in 50.4% of the sample sequences analyzed. The most prevalent type mutations found were NNRTI mutations 42.9% ,this is similar to most of the studies that have been conducted where by NNRTI mutations are higher (Rugemalila *et al.*, 2023). Thus policy recommendations to adopt InSTIs-based regimens were prompted by the rising occurrence of NNRTI resistance mutations in both ART-naive people (Kouamou, Inzaule and Manasa, 2021). PI resistance mutations were notably infrequent and this is consistent with other studies that have been conducted (Kamori *et a*l., 2023), but in contrast to a study that was conducted in Malawi where no PI mutations were present (van Oosterhout *et al.*, 2022). In this study the number of sample data with multiple mutations associated with resistance was nineteen representing 41.3%. of the population.

The phylogenetic analysis identified two major clusters or clades. One of these clusters further sub branched into additional clusters, indicating significant sequence diversity among the samples. The use of control samples in phylogenetic analysis is crucial for validating experimental procedures, providing a baseline for comparison, identifying contaminants, and increasing statistical confidence. In this study, all eight control samples (highlighted in green in Figure 4.8) formed a single cluster, demonstrating complete sequence concordance. This reinforces the reliability and accuracy of the phylogenetic analysis, ensuring that the observed genetic relationships among the DTG positive sequences are accurate and free from methodological errors (Hillis and Dixon, 1991).

The phylogenetic analysis showed that some of the study's sequences from Malawi clustered with the Zambian reference sequence (KM049919.1 in blue). This clustering may indicate potential genetic relatedness and cross-border transmission of HIV strains between Malawi and Zambia. Research has demonstrated that such cross-border genetic

similarities can arise due to human mobility and regional migration patterns. For instance, Faria *et al*. (2014) illustrated how international travel and migration play significant roles in the geographic and genetic dispersal of HIV, emphasizing the importance of coordinated regional public health strategies to manage and mitigate the spread of the virus.

The observation that two DTG-positive samples, R946 and N719 (Figure 4.8), cluster closely together despite originating from different districts highlights the potential for phylogenetic analysis to reveal unexpected genetic relationships. This finding aligns with previous research, which demonstrates that geographic separation does not always correlate with genetic divergence due to factors such as migration and human movement patterns. For instance, a study by Grenfell *et al.* (2004) on the spread of infectious diseases using phylogenetic methods underscores that genetic clustering can occur over wide geographic areas, reflecting complex patterns of transmission rather than simple geographic proximity.

Sporadic high resistance level DTG-positive samples, such as sequence P967, were noted to have mutations that did not share common ancestry with other high resistance level sequences in the tree (Figure 4.8). This finding is similar to observations from a cross-sectional study conducted in Tanzania among adolescents (Rugemalila *et al.*, 2023). The observed mutations could signify individual adaptations to treatment among drug-experienced patients, highlighting the complex dynamics of resistance development in different contexts (Rugemalila *et al.,* 2023.

The observation that DTG-resistant sequences are widely dispersed, with occasional clusters such as sequences R773 and R760, reflects a complex landscape of genetic resistance. Studies have shown that the distribution of resistance mutations can be highly variable, with both isolated and clustered mutations indicating diverse evolutionary pressures and transmission dynamics. For example, research by Rhee *et al.* (2004) demonstrated that HIV drug resistance mutations could occur in both isolated and clustered forms, influenced by factors like treatment history and viral evolution. Similarly, a study by Zur Wiesch *et al*. (2011) highlighted that the spatial and temporal dispersion of resistance mutations can provide insights into the patterns of drug resistance

development and transmission within populations. This scattered pattern of resistance in DTG-positive sequences emphasizes the need for comprehensive surveillance to understand the mechanisms driving resistance and to inform effective treatment strategies.

These scenarios underscore the importance of considering treatment histories and transmission dynamics in understanding the emergence and spread of drug resistance mutations. In the analysis, the identification of phylogenetic clustering involving three or more sequences from patients who share at least one resistance-associated polymorphism was deemed as evidence supporting the presence of a drug-resistant viral lineage. This approach helps discern the potential development and transmission of resistance within the studied population.

5.1 Study Limitations

It is worth mentioning that the current investigation encountered certain limitations. The limited dataset significantly affects the research by reducing the statistical power and generalizability of the findings. With fewer samples, it becomes challenging to accurately, identify patterns or trends in mutations, increasing the risk of bias and limiting the ability to draw robust conclusions. The study-utilized results from samples that were analyzed through Sanger sequencing. Conventional population-based Sanger sequencing effectively identifies the genotypes of dominant variants within a patient's viral population; however, its sensitivity is limited, making it incapable of detecting resistant minority variants that constitute less than approximately 20% of the total viral population. In contrast, next-generation sequencing (NGS)-based methodologies not only reduce sequencing costs but also facilitate accurate and specific identification of resistant variants that constitute approximately 2% of the viral population (Blassel *et al.*, 2021).

Furthermore, being a retrospective study, it relied on pre-existing data, which had limited control over the consistency of sample collection methods and data accuracy and the study only provides a snapshot of DTG resistance but does not track changes over time in individuals, which could reveal the progression of resistance or mutation patterns.

Data on adherence, treatment duration, and CD4 cell count were not collected due to their unavailability across the entire dataset. Thus, the study cannot attribute these factors as

contributors to emergence of DTG resistance. Nevertheless, within the country's HIV programming, there is a priority placed on enhanced adherence counseling when treatment failure is suspected (van Oosterhout *et al.*, 2022).

Owing to the absence of data regarding the district of origin for certain sequences, the comprehensive population of resistance categorized by district could not be presented.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

The identification of DTG resistance mutations in 15.4% of the study population, including major mutations such as G118R, R263K, G140A, and Q148H/R, highlights a critical challenge for Malawi's ART program. These mutations reduce the efficacy of DTG-based regimens, potentially leading to treatment failure, increased viral load, and higher transmission rates. The presence of accessory mutations like E138K and T66A further raises concerns about cross-resistance to other integrase inhibitors, limiting future treatment options. The study underscores the urgent need to strengthen HIVDR surveillance in Malawi to safeguard the effectiveness of DTG-based regimens. Without proactive monitoring and policy adjustments, the emergence of resistance could compromise treatment success and HIV epidemic control efforts. Implementing robust resistance testing methodologies and integrating them into routine clinical care will be essential in sustaining ART efficacy, optimizing patient management, and shaping future policy decisions for long-term program sustainability.

6.2 Recommendations

Expanding DTG resistance testing nationwide, especially in remote regions, is vital to ensure all HIV patients can receive timely testing and treatment adjustments. Improved access allows healthcare providers to better tailor regimens, enhancing patient outcomes and curbing drug-resistant HIV. Supporting research on DTG resistance in Malawi informs treatment strategies and ensures evidence-based decision-making. Scaling up testing after piloting, with phylogenetic analysis, helps identify resistance mutations, track the spread of resistant strains, and optimize treatments. This comprehensive approach aids in controlling transmission and supports global surveillance efforts, guiding public health responses and antiretroviral therapy development. Correct entry or completion of information by health care centers in the different districts should be included as the revelation of many samples not being allocated to any district is concerning.

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APPENDICES

Appendix A: Data Collection Tool Template

ID	CD4	DTG Results	Total score	Major DTG mutation	Minor DTG mutation	Sample type	Viral load results (if known)	VL in log

Appendix B: DTG Mutations Rate and Demographic Data DTG Positive Samples of Samples within the Study

ID	SEX	AGE	DTG MAJOR MUTATIONS	RESISTANCE LEVEL	OTHER MUTATIONS	VIRAL LOAD	DISTRICT
1	F	15	**G118R,**	High resistance level (80%)	T66A, E138K	6265	Phalombe
2	F	26	**G140A,Q148R**	High resistance level (100%)	S147G, E138K, H221Y,V106M,G190A,F227L, L90M, M41L,E44D,D67NV75M,F77L,M184V,L210W ,T215Y	494,000	Lilongwe
3	M	23	**G118R**	Intermediate resistance level (50%)		6736	Lilongwe
4	M	18	**G118R**	High resistance level (85%)	T66TA.E138K	276,648	Karonga
5	M	53	**G118R**	High resistance level (80%	T66A, E157Q, E138K, K103N,P225H,K238T, D67N,K70R,M184V,K219Q	22300	Lilongwe
6	F	15	**R263K**	Intermediate resistance level (30%)	G48GA, E44ED,M184V	225000	

ID	SEX	AGE	DTG MAJOR MUTATIONS	RESISTANCE LEVEL	OTHER MUTATIONS	VIRAL LOAD	DISTRICT
7	F	34	**R263K**	Intermediate resistance level (40%)	Q95K,E157Q, V108I,Y181C,G190A, M41L,D67T,T69G,L74I,M184V,T215F,K219E	861379	Lilongwe
8	F	16	**R263K**	Intermediate resistance level (30%)	K103S,G190A,P225H,K238T, D67N,K70R,M184V,T215I,K219Q	7910	
9	F	36	**G118R**	High resistance level (85%)	T66I, L74M,E138K, A98G,K101E,V108I,Y181C,G190A,H221Y, M41L,E44D,D67G,S68G,K70KNT,M184V,T215F,K219Q	24996	Zomba
10	M	43	**G118R**	High resistance level (80%)	L74M,E157Q, T66A,E138K, V179D,Y188L, M41L,M184V	7454	Blantyre
11	M	44		Potential low-level resistance (10%)	H51Y, K103N, M184V	23900	
12	F	40	**R263K**	Intermediate resistance level (30%)	K101E,E138Q,V179D,G190A,P225H, D67N,T1215TAIV,K219E	62311	Nsanje

ID	SEX	AGE	DTG MAJOR MUTATIONS	RESISTANCE LEVEL	OTHER MUTATIONS	VIRAL LOAD	DISTRICT
13	M	53		Potential low level resistance (10%)	H51Y,Q95K, K101H,E138Q,Y181C,G190A, M46I, A62V,S68G,K70S,V75I,F77L,Y115F,F116Y,Q151M,M184V	19268	Mzuzu
14	F	50	**G118R**	High resistance level (85%)	T66I, EA38A,	22858	Mzuzu

Appendix C: Institutional Approval

THE UNIVERSITY OF ZAMBIA
SCHOOL OF VETERINARY MEDICINE
OFFICE OF THE ASSISTANT DEAN (POSTGRADUATE)

Telephone: 293727
Telegrams: UNZA LUSAKA
Zambia
Telex: UNZALU ZA 44370
Fax: 293727/253952
School Fax: 293727
Vet. Clinic Telephone: 291515

P.O. Box 32379
Lusaka,

Your Ref:

Our Ref:

22nd August 2023

Felistus Kanjira Zumazuma Hussein
Biomedical Studies Department
School of Veterinary Medicine
University of Zambia
P.O. Box 32379
LUSAKA

Dear Felistus Kanjira Zumazuma Hussein,

RE: APPROVAL OF RESEARCH PROPOSAL

At the meeting of the School Board of Graduate Studies held on 10th August 2023, your research proposal entitled ***'Detection of Human Immunodeficiency Dolutegravir Resistance Gene Mutations Among Adults In Selected Districts Of Malawi -A Retrospective Study'*** was tabled and discussed. I am therefore pleased to inform you that the research proposal was subsequently approved by the Board.

On behalf of the Board, I wish you success as you apply for ethical approval and carry on with you research activities.

Yours sincerely

Dr Chisoni Mumba

Appendix D: Reference Laboratory Approval Letter

Tel No.: +265 757 851
Fax No.: +265 757 853
www.malawipublichealth.org
/publichealthinstituteofmalawi

PHIM
PUBLIC HEALTH INSTITUTE OF MALAWI
Community Health Sciences Unit
P/Bag 65
Lilongwe
Malawi

REF/PHIM/NPHL/20/2023 **20th September, 2023**

TO WHOM IT MAY CONCERN

Dear Sir/Madam

SUPPORT LETTER FOR FELISTUS KANJIRA

This letter confirms that Felistus Kanjira whose research is entitled **DETECTION OF HUMAN IMMUNODEFICIENCY DOLUTEGRAVIR RESISTANCE GENE MUTATIONS AMONG ADULTS IN SELECTED DISTRICTS OF MALAWI (A RETROSPECTIVE STUDY** will collect her data here at Public Health Institute of Malawi. The Institution has granted her the permission to enable her to collect the required information relevant to her study.

Joseph Bitilinyu- Bangoh

DEPUTY DIRECTOR - PUBLICHEALTH INSTITUTE OF MALAWI (NPHL)

For **SECRETARY FOR HEALTH**

Appendix E Malawi Ethical Approval Letter

Telephone: + 265 789 400
Facsimile: + 265 789 431

All Communications should be addressed to:

The Secretary for Health

In reply please quote No. MED 4/36c
MINISTRY OF HEALTH
P.O. BOX 30377
LILONGWE 3
MALAWI

6th December, 2023

Felistus Zamazema Kanjira Hussein
Thyolo District Hospital

Dear Sir/Madam

RE: Protocol #23/12/4277: Ref: Detection of Human Immunodeficiency Dolutegravir Resistance Gene Mutations Among Adults in Selected Districts of Malawi (A Retrospective Study)

Thank you for the above titled proposal that you submitted to the National Health Sciences Research Committee (NHSRC) for review. Please be advised that the NHSRC has **reviewed** and **approved** your application to conduct the above titled study.

- **APPROVAL NUMBER : 4277**
- The above details should be used on all correspondences, consent forms and documents as appropriate.
- **APPROVAL DATE : 06/12/2023**
- **EXPIRATION DATE : 05/12/2024**
  This approval expires on **05/12/2024**. After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the NHSRC Secretariat should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING:** All serious problems having to do with subject safety must be reported to the NHSRC within 10 working days using standard forms obtainable from the NHSRC Secretariat.
- **MODIFICATIONS:** Prior NHSRC approval using forms obtainable from the NHSRC Secretariat is required before implementing any changes in the protocol (including changes in the consent documents). You may not use any other consent documents besides those approved by the NHSRC.
- **TERMINATION OF STUDY:** On termination of a study, a report has to be submitted to the NHSRC using standard forms obtainable from the NHSRC Secretariat.
- **QUESTIONS:** Please contact the NHSRC on phone number *+265 999397913* or by email on mohdocentre@gmail.com.
- **OTHER:** Please be reminded to send in copies of your final research results for our records (Health Research Database).

Kind regards from the NHSRC Secretariat.

National Health Sciences Research Committee
2023 -12- 06
APPROVED

.....

For: CHAIRPERSON, NATIONAL HEALTH SCIENCES RESEARCH COMMITTEE

Promoting Ethical Conduct of Research

Appendix F: Malawi Ethical Approval Certificate

CERTIFICATE OF ETHICS APPROVAL

This is to certify that the National Health Science Research Committee has reviewed and approved the study titled;

Study Title: Protocol #23/12/4277: Ref: Detection of Human Immunodeficiency Dolutegravir Resistance Gene Mutations Among Adults in Selected Districts of Malawi (A Retrospective Study)

Investigator: Felistus Kanjira Hussein

StartDate: 08/12/2023

End Date: 05/12/2024

Date of issue: ---06/12/2023.....

Dr. Martias Joshua
Chairperson-NHSRC

National Health Sciences Research Committee
2023 -12- 06
APPROVED

Mr. Billy Nyambalo
NHSRC -Administrator