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UNITÉ DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE

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PREDICTIVE EFFICACY OF THE NEW NUCLEOSIDE REVERSE TRANSCRIPTASE TRANSLOCATION INHIBITOR (ISLATRAVIR), AND POTENTIAL REVERSE TRANSCRIPTASE ANALOGS FOR COMBINATION, AMONG HIV TREATMENT-EXPERIENCED PATIENTS IN CAMEROON

THESIS

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DEDICATION

To my Parents,

Mr. TSAPI PIERRE
&
Mrs. TCHOUANWA ROSALIE

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LIST OF ABBREVIATIONS AND ACRONYMS

3TC	2',3'-didéoxy-3'-thiacytidine (Lamivudine)
ABC	Abacavir
AIDS	Acquired Immuno-Deficiency Syndrome
ART	Antiretroviral Therapy
ATV/r	Atazanavir boosted ritonavir
ARV	Antiretroviral
AZT	Zidovudine
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CDC	Center for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic acid
CIRCB	*Centre International de Référence "Chantal BIYA" pour la recherche sur la prévention et la prise en charge du VIH/SIDA*
CNLS	*Comité National de Lutte contre le SIDA*
CRF	Circulating Recombinant Forms
CXCR4	C-X-C chemokine receptor type 4
D4T	2'-3'-didéhydro-2'-3'-didéoxythymidine (Stavudine)
DdI	Didanosine
DdNTP	Dideoxynucleotide
DNA	Deoxyribonucleic Acid
DNTP	Deoxynucleotide Triphosphate
DRMs	Drug Resistance Mutations
DRV/r	Darunavir boosted with ritonavir
DTG	Dolutegravir
EDTA	Ethylene Diamine Tetra-acetic Acid
EFV	Efavirenz
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
FTC	2',3'-dideoxy-5-fluoro-3'-thiacytidine (Emtricitabine)
GRT	Genotypic Resistance Testing

HIV	Human Immunodeficiency Virus
HIVdb	HIV database
IAS	International AIDS Society
IDV	Indinavir
INSTI	Integrase Strand Transfer Inhibitors
IQR	Interquartile range
LPV/r	Protease Inhibitor Lopinavir boosted
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NVP	Nevirapine
PCR	Polymerase Chain Reaction
PLHIV	People Leaving with HIV
PI	Protease Inhibitor
PI/r	Protease Inhibitor Ritonavir boosted
Pol	Polyprotein
PR	Protease
RLS(C)	Resource Limited Settings (Countries)
LMICs	Low and Middle Income Countries
RAL	Raltegravir
RNA	Ribonucleic Acid
RPV	Rilpivirine
RT	Reverse Transcriptase
SIDA	Syndrome d'Immuno-Déficience Aquis
TAM	Thymidine Analog Mutations
TDF	Tenofovir Disoproxil Fumarate
UNAIDS	The Joint United Nations Program on HIV/AIDS
VIH	Virus de l'Immunodéficience Humain
VL	Viral Load
WHO	World Health Organization

ABSTRACT

Islatravir (ISL) is a novel antiretroviral that inhibits HIV-1 reverse transcriptase through multiple mechanisms. The M184V mutation, known to reduce ISL's in vitro activity, could arise from prolonged exposure to nucleoside (NRTI) and non-nucleoside (NNRTI) reverse transcriptase inhibitors. Islatravir (ISL) is a new antiretroviral that inhibits HIV-1 reverse transcriptase through multiple mechanisms.

Using the CIRCB Antiviral Resistance Evaluation (CIRCB-CARE) database in Cameroon, 1170 sequences were analyzed from patients failing first-line ART (2NNRTI+NRTI, n=671), second-line (2NNRTI+PI/r, n=470) and third-line ART (2NNRTI+INI=29). HIV-1 sequences were analyzed using Seqscape.v.2.6 and drug-resistance mutations (DRMs) were interpreted using Stanford HIVdb.v9. Patterns of pairwise interactions were analyzed between M184V and other NRTI and NNRTI DRMs. Fisher's exact test was performed to assess difference in the co-occurrence of the mutated residues.

Median (IQR) age of the study-population was 40[28-47] years; 61.0% female; with a broad diversity of HIV-1 non-B: CRF02_AG (60.6%), A (10.51%), G (5.81%), and 29 other viral clades (24.0%). The study population showed a high prevalence of resistance to NRTIs (77.4%) and NNRTIs (49.1%). The most frequent NRTIs-DRMs were M184V/I (83.3%), M41L (25.0%), and T215FY (36.8%), while the most present NNRTIs-DRMs included K103NS (53.3%), Y181CIV (27.7%), and G190ASE (22.2%). Covariation patterns between M184V and other DRMs were assessed, and after adjusting ($P< 0.001$) in first-line ART failure, M184V significantly co-varied with K70R, L74I, and M41L for NRTIs, and K103N and G190A for NNRTIs. In second-line failure, the covariation with M184V extended to T215Y, M41L, and D67N for NRTIs, and G190A, K103N, and K103S for NNRTIs. No significant covariation with M184V was observed in third-line treatment failures. Following these covariations, potentially active antiretrovirals to combine with islatravir were TDF (partial efficacy) and Doravirine (fully active).-

Overall, high rate of resistance to nucleos(t)ide and non-nucleos(t)ide reverse-transcriptase inhibitors has been observed in people failing first-, second-, and third-line ART in Cameroon. Thus, Islatravir would serve as an additional therapeutic weapon, if combined preferentially with TDF and Doravirine after first- or second-line ART failure in LMICs sharing similar programmatic challenges like Cameroon.

Keywords: HIV**,** ART, Islatravir, Long acting, Drug resistance.

RESUME

Islatravir (ISL) est un nouvel antirétroviral qui inhibe la transcriptase inverse du VIH-1 par plusieurs mécanismes. La mutation M184V, connue pour réduire l'activité in vitro de l'ISL, pourrait survenir suite à une exposition prolongée aux inhibiteurs de la transcriptase inverse nucléosidiques (NRTI) et non nucléosidiques (NNRTI).

À partir de la base de données CIRCB-CARE au Cameroun, 1170 séquences ont été analysées chez des patients échouant à un traitement antirétroviral de première (n=671), deuxième (n=470) et troisième lignes (n=29). Les séquences VIH-1 ont été analysées avec Seqscape.v.2.6 et les mutations de résistance (DRMs) interprétées à l'aide de Stanford HIVdb.v9. Des analyses de covariation par paires ont été réalisées entre M184V et d'autres nutations de resistance aux INTI et INNTI, avec un test exact de Fisher pour évaluer la co-occurrence des résidus mutés.

L'âge médian (IQR) de la population étudiée était de 40 [28-47] ans ; 61,0 % étaient des femmes ; avec une grande diversité de VIH-1 non-B : CRF02_AG (60,6 %), A (10,51 %), G (5,81 %) et 29 autres clades viraux (24,0 %). La population étudiée a montré une forte prévalence de résistance aux NRTIs (77,4 %) et aux NNRTIs (49,2 %). Les DRMs NRTIs les plus fréquentes étaient M184V/I (83,3 %), M41L (25,0 %) et T215FY (36,8 %), tandis que les mutations de résistance aux NNRTIs les plus présentes comprenaient K103NS (53,3 %), Y181CIV (27,7 %) et G190ASE (22,2 %). Les modèles de covariation entre M184V et d'autres mutations ont été évalués, et après ajustement ($P < 0,001$) dans l'échec du traitement de première ligne, M184V a significativement covarié avec K70R, L74I et M41L pour les NRTIs, et K103N et G190A pour les NNRTIs. Dans l'échec de la deuxième ligne, la covariation avec M184V s'est étendue à T215Y, M41L et D67N pour les NRTIs, et G190A, K103N et K103S pour les NNRTIs. Aucune covariation significative avec M184V n'a été observée dans les échecs de traitement de troisième ligne. Suite à ces covariations, les antirétroviraux potentiellement actifs à combiner avec islatravir étaient TDF (efficacité partielle) et Doravirine (entièrement actif).

Dans l'ensemble, un taux élevé de résistance aux inhibiteurs de la transcriptase inverse nucléosidiques et non nucléosidiques a été observé chez les personnes échouant aux traitements de première, deuxième et troisième lignes au Cameroun. Ainsi, l'Islatravir pourrait servir d'arme thérapeutique supplémentaire, s'il est combiné préférentiellement avec TDF et Doravirine après un échec du traitement ART de première ou deuxième ligne dans les pays à revenu faible ou intermédiaire partageant des défis programmatiques similaires à ceux du Cameroun.

Mots-clés : VIH, TARV, Islatravir, Action prolongée, Résistance antirétrovirale.

Introduction

INTRODUCTION

Improvements in antiretroviral therapy (ART) have played a crucial role in decreasing morbidity, mortality, and the transmission of HIV-1. However, HIV continues to pose a significant threat, particularly in Western and Central Africa, where an estimated 130,000 [100,000-170,000] out of the 630,000 [500,000-820,000] AIDS-related deaths worldwide occurred in 2023 despite increased access to antiretroviral therapy(ART)**(Global HIV & AIDS Statistics—Fact Sheet (2024))**. This region remains disproportionately affected by the pandemic. Current efforts to achieve viral suppression, a crucial component of the "Test and Treat" strategy in resource-limited settings, face challenges **(Fokam *et al.,* 2019)**. While ART has expanded, viral suppression rates remain below the desired 95% target, currently at 89.2% for adults **(Fokam *et al.,* 2019)**.

Factors contributing to this suboptimal rate include treatment non-adherence **(Røge *et al.,* 2004)**, interruptions in therapy **(Meresse *et al.,* 2014)**, and high initial viral load **(Sigaloff *et al.,* 2011)**. These factors increase the risk of developing drug-resistant viral strains, further complicating treatment efforts **(Cortez & Maldarelli, 2011)**. To achieve the global goal of HIV elimination by 2030, a continued focus on improving viral suppression rates is essential, and therefore requires a comprehensive approach to address the underlying causes of poor therapeutic outcomes, such as enhancing adherence to treatment, mitigating treatment interruptions, and managing high baseline viral load **(Conway, 2007)**. This highlights the urging necessity to reinforce treatment adherence and strengthen the genetic barrier of newly conceived ARV drugs as the recommended HIV-1 treatment and prevention strategies rely on good adherence to daily administration; thus, agents with more favorable and extended dosing schedules would be of benefit **(Conway, 2007)**. Novel agents with improved safety, tolerability profiles and a high barrier to the selection of resistance are needed, as existing agents can be associated with long-term toxicities, as well as a potential for selection of drug-resistant HIV-1 variant. Long-acting antiretroviral drugs (LAAR) are capable of being administered on a monthly or less frequent basis, giving them the potential to improve adherence to therapy and extend opportunities for therapeutic or prophylactic intervention to underserved patient populations **(Conway, 2007; Spreen *et al.,* 2013)**.

Islatravir (ISL) is the first nucleoside reverse transcriptase translocation inhibitor (NRTTI), with structural and mechanistic features that distinguish it from currently marketed antiretroviral drugs. Its action is rooted on three chemical structured components (3′-OH group, 4′-ethynyl group, and a 2-fluoro group) that contribute to its unique profile **(Michailidis *et al.,* 2009, 2014; Schürmann *et al.,* 2020)**. The fluoro-group inhibits islatravir metabolism and contributes to its long half-life, giving a 56 mg injection a half-life of 198h and ISL-eluting implant projected to release adequate ISL for more than 52 weeks **(Randolph *et al.,* 2021)**. Islatravir, by these capacities, is a promising approach especially for resource-limited settings (RLS). However, despite the strong genetic barrier of ISL compared to other ARV drugs, some viral resistance selection studies **(Cilento *et al.,* 2021; Diamond *et al.,* 2022)** showed that Islatravir is not immune to resistance. These studies identified some mutations and combinations of mutations within RT that were shown to alter susceptibility to ISL in vitro. M184V and M184I conferred the largest fold reductions in potency to ISL (fold-change (FC) of 6.2 and 6.8, respectively) compared to wild type, and only combinations of variants containing M184I or M184V conferred reduced potencies greater than 6.8-fold **(Diamond *et al.,* 2022).** The effect of M184V/I highlights the importance of the cross-resistance within the class of NRTIs and Islatravir (NNRTI) which increases the risk of resistance to ISL among ISL-naive patients with previous exposure to NRTI. HIV drug resistance (HIVDR) is prevalent in Cameroon, with 89.1% of patients failing first-line ART exhibit HIVDR, with 83.2% harboring the M184V mutation **(Takou *et al.,* 2019)**. Pre-treatment drug resistance (PDR) is also common, with a 15.0% rate, primarily driven by NNRTI PDR (12.4%) **(Fokam *et al.,* 2023)**.

Therefore, regarding the long exposure and the high-level resistance to NRTIs in Cameroon, a resource limited setting where clinical trials are not yet performed for this new drug, there is the need to evaluate and monitor the co-occurrence of M184V and other major reverse transcriptase mutations, providing preliminary data on the efficacy of ISL and best potential treatment combinations among HIV treated patients failing ART in Cameroon. Here, we report the results of a cross-sectional analytical study designed to assess the level of efficacy of ISL and the best potentially active RTIs for combination with ISL in Cameroon and similar resources limited settings.

Research Questions

- What would be the rate of efficacy of islatravir in Cameroon?
- What would be the potential RTIs for combination with ISL among PLHIV previously exposed to NRTI and NNRTI.

Research Hypothesis

In Cameroon, despite the high rate of exposure to reverse transcriptase inhibitors, Islatravir would keep a good rate of efficacy, especially in combination with NRTIs and NNRTIs among patients failing ART.

Objectives of the Proposed study

Main objective

Evaluate the predictive efficacy rate of Islatravir among HIV patients previously exposed to NRTIs and propose potential RTIs for combination.

Specific Objectives

i. Determine the prevalence of NRTI and NNRTI major drug resistance mutations among patients previously exposed to reverse transcriptase inhibitors;
ii. Analyze the covariation between M184V/I and other major NRTI and NNRTI drug resistance mutations;
iii. Determine the viral susceptibility rate to islatravir and major RTIs among patients with previous exposure to RTIs;
iv. Propose potential NRTIs and NNRTIs for combination with Islatravir.

Chapter One:
Literature Review

CHAPTER 1: LITERATURE REVIEW

I- GENERALITIES ON THE HUMAN IMMUNO-DEFICIENCY VIRUS

1. History:

In the 1920s, simian immunodeficiency virus (SIV) was identified in African non-human primates. The first documented case of an AIDS-like syndrome in a human occurred in Kinshasa, Democratic Republic of Congo, around 1959. and later spread along trade and travel routes, reaching Haiti, the Caribbean, and the United States in the 1960s and 1970s. International travel from the United States helped the virus spread globally **(Gao *et al.,* 1999; History, 2021)**

It was on June 5, 1981 that CDC first published a report describing the need for a drug, pentamidine, to treat five previously healthy young men who had sex with men (MSM), suffering from a deadly disease called *Pneumocystis carinii* pneumonia (PCP). Just after this report, health officials signaled a spike in cases of Kaposi's sarcoma, which was a rare deadly diseases associated to immune suppression **(Fauci, 2003; Volberding, 1986)**, in this same population type in New York**(CDC, 1981; Gottlieb *et al.,* 1981; Sencer, 2021).**

Initially referred to as "gay disease" for many years, the disease was later named AIDS (acquired immunodeficiency syndrome) in 1982 **(Fauci, 2003)**. One year after, in 1983, a research team led by Luc Montagnier highlighted in a published note, the relationship between the human T-lymphocytic virus I (HLTV-1) (the retrovirus) and AIDS, precising that role of this virus in the etiology of the disease was still to be figured out **(Barré-Sinoussi *et al.,* 1983)**. It's in 1984 that Robert C. Gallo and collaborators published papers with virological and epidemiological evidence that HTLV, today HIV, was the cause of AIDS **(Gallo, 2002; Montagnier, 2002)**. In 1986, A novel human retrovirus, HIV-2, with bio-morphological similarities with HIV-1, but different envelope glycoproteins, was isolated from two AIDS patients from West Africa by François Clavel and his team **(Clavel *et al.,* 1986)**. HIV-2 is predominant in West Africa, and in their publication, Marlink *et al.* showed that compared to HIV-1, HIV-2 showed lower: infectivity, transmission, viral burden, and the disease-free survival time was longer **(Marlink *et al.,* 1994).**

2. HIV infection

a. Epidemiology

The global epidemiology of AIDS has undergone significant changes over the past several decades, due to advances in prevention, treatment, and care. In 2022, 1.3 million people were newly

infected with HIV, compared to 3.2 million people in 1995.Women and girls accounted for 46% of all new infections in 2022. An estimated 39 million people worldwide were living with HIV, and 630,000 people died from AIDS-related illnesses **(UNAIDS, 2023)**. The median prevalence among the adult population (ages 15-49) was 0.7% in 2022 **(*UNAIDS Global AIDS Update 2022*, n.d.).** HIV-2 is less common than HIV-1, and it is mainly found in West Africa. HIV-1 is the most common type of HIV worldwide, accounting for approximately 95% of all HIV infections. It is found in all regions of the world, but it is most prevalent in sub-Saharan Africa **(Marlink *et al.,* 1994)**.

Africa has been disproportionately affected by the HIV/AIDS epidemic, the Sub-Saharan part remaining by far the most affected with more than 80% of Africa's living subjects **(*UNAIDS 2023 Global HIV & AIDS Statistics — Fact Sheet.Pdf*, n.d.)**. It is, however, important to highlight that the Middle-East and North-Africa region noted a drastic increase in the number of new HIV infections between 2015 and 2021, according to UNAIDS 2022 global AIDS update. In 2022, an estimated 25.6 million people in Africa were living with HIV, representing more than 70% of the global record, of whom 90% knew their status, and 760,000 people were newly infected **(WHO, 2023a)**.

According to UNAIDS, the HIV prevalence rate among the population aged 15-64 in Cameroon is 2.6 in 2022 **(UNAIDS, 2022)**. However, the latest information on HIV prevalence in Cameroon indicates that rate would be 2.1% as of 2023 **(MINSANTE, 2023)**, showing a drop of ~50% over 14 years as prevalence fell from 5.4% in 2004, to 4.3% in 2011, and 2.7% in 2018, passing through 3.4% in 2019 and 3.1% in 2020 **(GPC, 2024; Nwaha, 2022)**. The number of people, aged 15 and over, living with HIV (PLHIV) in Cameroon was estimated at 450,000 and the number of new HIV cases at 6,500 in 2022 **(WHO, 2023b)**.

b. Transmission modes

HIV is transmitted through contact with infected bodily fluids, such as blood, semen, vaginal or rectal fluids, and breast milk. Three modes of transmission of the virus are described:

- **Unprotected sex**

Unprotected sex is the most common way that HIV is transmitted especially if there are ulcers or breaks in the skin or mucous membrane. This includes vaginal, anal, and oral sex. Either partner can get HIV during sex, but the risk of HIV transmission is highest during anal sex, followed by

vaginal sex and then oral sex. During vaginal sex, HIV in vaginal fluid, semen or blood can enter a person's body through the tissue that lines the vagina or cervix, as well as through the opening at the tip of the penis(urethra), the foreskin, or cuts or sores. During anal sex, there is more risk in being the bottom than being the top. HIV can enter the body through the rectum, the opening at the tip of the penis(urethra), the foreskin, or cuts or sores on the penis **(CDC, 2024)**.

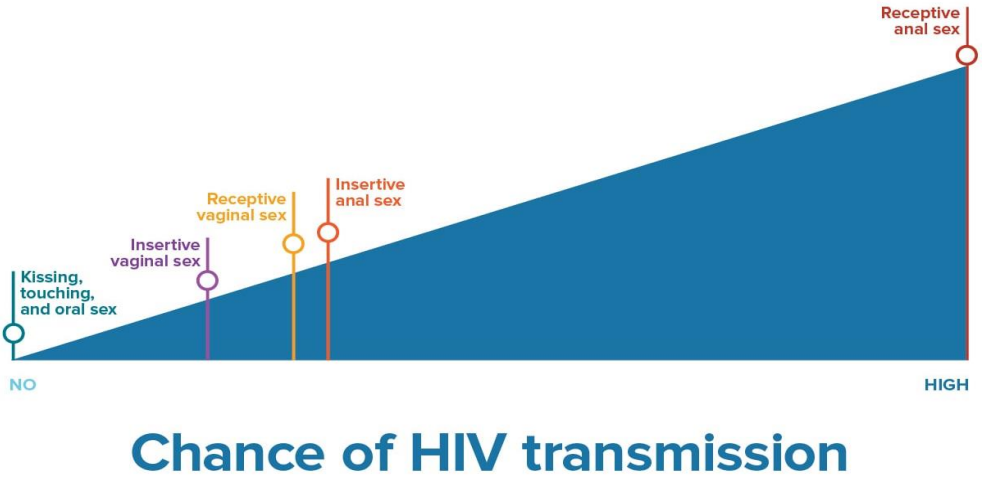


Figure 1: The chance of getting HIV per sex act if you have sex with someone who has HIV and is not on HIV treatment ***(*CDC, 2024*)***.

- **Blood-to-Blood contact:**

Blood-to-blood transmission of HIV occurs when infected blood comes into contact with the bloodstream of an uninfected person. This can happen in a number of ways, including: Sharing needles or other drug paraphernalia, getting a tattoo or piercing with contaminated equipment, or receiving a blood transfusion from an infected donor. The latter one is very rare since all donated blood is screened for HIV, but transmission is still possible depending on the diagnostic method used, since detectable antibodies may not be present in the donor's blood yet or equally inadequate blood transfusion policies, like national donor bank **(CDC, 2024; Ribeiro, 2011)**.

- **Mother-to-Child/ Perinatal transmission**

Mother-to-child transmission (MTCT) of HIV occurs when a pregnant woman living with HIV passes the virus to her child during pregnancy, labor, delivery, or breastfeeding. IT represents the major route through which pediatric populations acquire HIV-1 infection **(Friedland & Klein, 1987)**. A study, published in 2017, carried out in Bamenda, Cameroon showed that the prevalence of HIV among HIV-exposed infants was 7.1% **(Fondoh & Mom, 2017)**, which same as national

7.0% in 2023 obtained by Ka'e and collaborators **(Ka'e *et al.*, 2023)**. There are three main types of MTCT:

- **Antepartum transmission:** This occurs during pregnancy, when the virus crosses the placenta from the mother to the fetus.
- **Intrapartum transmission:** This occurs during labor and delivery, when the baby comes into contact with the mother's infected blood or vaginal fluids.
- **Postpartum transmission:** This occurs after birth, through breastfeeding. Avoidance of breast feeding prevents postpartum transmission of HIV, but this is balances. against the multiple benefits that breast feeding offers. In resource-poor countries, breast feeding is strongly associated with reduced infant morbidity and improved child survival(UNICEF, n.d.).

Without antiviral treatment, the risk of transmission of HIV from infected mothers to their children is approximately 15-30% during pregnancy and labor, with an additional 10-20% transmission risk attributed to prolonged breast feeding**(John & Kreiss, 1996)**.

c. Clinical evolution

In the absence of treatment, HIV progressively follows its course in the body and can be followed using two biological indicators that progress in opposite directions. HIV infection progresses through three distinct successive stages (Figure 2):

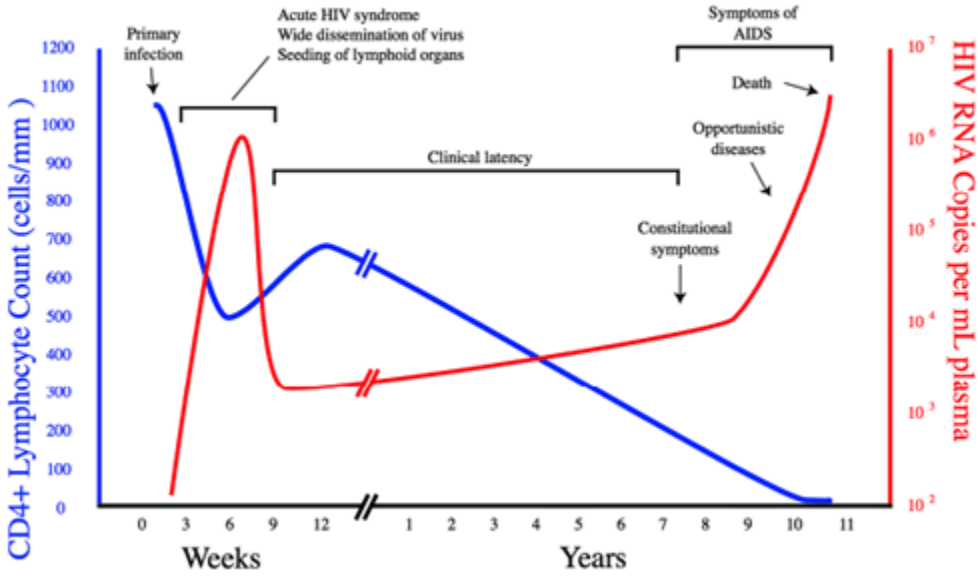


Figure 2: A generalized graph of the relationship between HIV copies (viral load) and CD4 counts over the average course of untreated HIV infection ***(*Nhlap, 2019*)***.

i. Primo-infection or Acute infection

Also called the acute phase, it is the initial period that follows the entry of HIV in the organism. This stage typically within 2-4 after exposure, during which the virus rapidly replicates and spreads throughout the body attacking and destroying infection-fighting CD4 cells (CD4 T lymphocyte) of the immune system, causing flu-like symptoms, such as: fever, large tender lymph nodes, throat inflammation, a rash, headache, tiredness, and/or sores of the mouth and genitals, in 40-90% of cases and may go unnoticed or be mistaken for another illness **(NIH, 2022)**.

ii. Clinical latency or Asymptomatic phase

After the primo-infection stage, the virus enters a period of latency, during which it remains dormant within the body's immune cells. Without treatment, this stage can last for several years or even decades, though it may advance faster in some people. During latency, the infected individual may not experience any symptoms or have only mild, non-specific symptoms. However, the virus continues to replicate at low levels, gradually weakening the immune system **(NIH, 2021)**.

iii. Symptomatic or AIDS

If left untreated, HIV infection eventually progresses to AIDS (acquired immunodeficiency syndrome), the most advanced and severe stage of the disease. AIDS is characterized by a severely weakened immune system with CD4 count of less than 200 cells/mm3, making the body vulnerable to opportunistic infections and certain types of cancer. Common AIDS-defining illnesses include *Pneumocystis carinii* pneumonia (PCP), Kaposi's sarcoma, and HIV-associated neurocognitive disorders (HAND)**(NIH, 2021)**. Without treatment, people with AIDS typically survive about 3 years.

3. HIV Characteristics

a. Taxonomy

HIV is a retrovirus (Baltimore class VI) that belongs to the family *Retroviridae*, subfamily *Orthoretrovirinae*, and genus Lentivirus. It is classified into two main types: HIV-1 which is most common and responsible the HIV pandemic, and HIV-2 which is less aggressive and mainly found in West Africa **(COL, 2024)**.

HIV is classified based on its genetic characteristics, particularly the sequence of its envelope glycoproteins (gp120 and gp41). HIV-1 is further classified into three groups (M, N, and

O) and several subtypes (A-D, F-H, J-K). Group M (for Main) is the most common and is responsible for the majority of HIV infections worldwide **(GACB, 2016)**.

b. HIV Structure

HIV is a complex retrovirus with a unique structure that enables it to infect and replicate within human immune cells. Its diameter lies between 80 and 120nm **(Fig. 3)**.

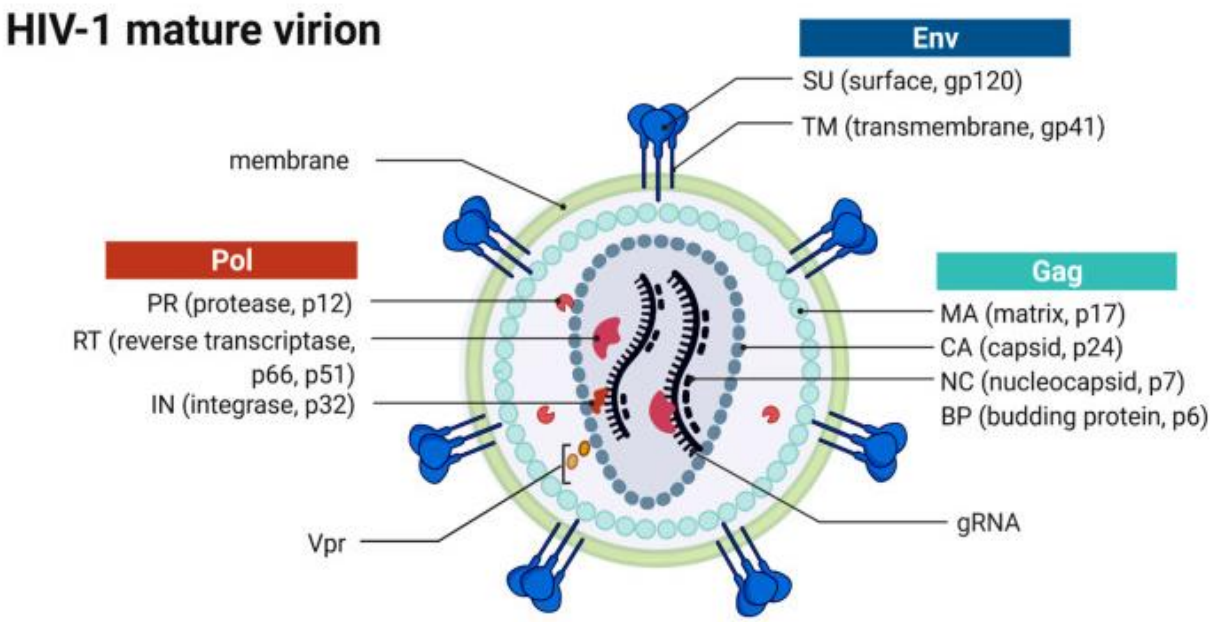


Figure 3: HIV-1 structure **(van Heuvel *et al.*, 2022).**

The virus particle consists of a cone-shaped core surrounded by a lipid envelope. The core contains the virus's genetic material, which consists of two identical copies of positive-sense single-stranded RNA. The RNA is packaged with several proteins, including the **nucleocapsid protein** (NC, p7), which protects the RNA genome, and the reverse transcriptase enzyme (p66/p51), which is essential for converting the RNA genome into double-stranded DNA. The capsid, in turn, is surrounded by the viral envelope that is composed of a lipid bilayer derived from the host cell membrane. It contains several viral proteins, including the **envelope glycoproteins** (gp120 for HIV-1 and gp110 for HIV-2), **transmembrane glycoproteins** (gp41 for HIV-1 and gp46 for HIV-2) which are responsible for binding to and entering host cells.

In addition to the core and envelope, HIV also contains several other structural components, like: the matrix protein (MA, p17 for HIV-1 and p16 for HIV-2) which plays a role in virus assembly and budding, the capsid protein (CA, p24 proteins for HIV-1 and p26 for HIV-2) which lines up

forming the cone-shaped core, the integrase enzyme(p32) which is responsible for integrating the viral DNA genome into the host cell's DNA **(GACB, 2016)**.

c. HIV-1 genome structure

The HIV-1 genome is a complex structure that comprises two single-stranded RNA molecules encapsulated within a protein capsid. These RNA molecules are approximately 9.75 kb in length and contain nine genes encoding 15 viral proteins. These genes are grouped as follows **(Fig. 4)**:

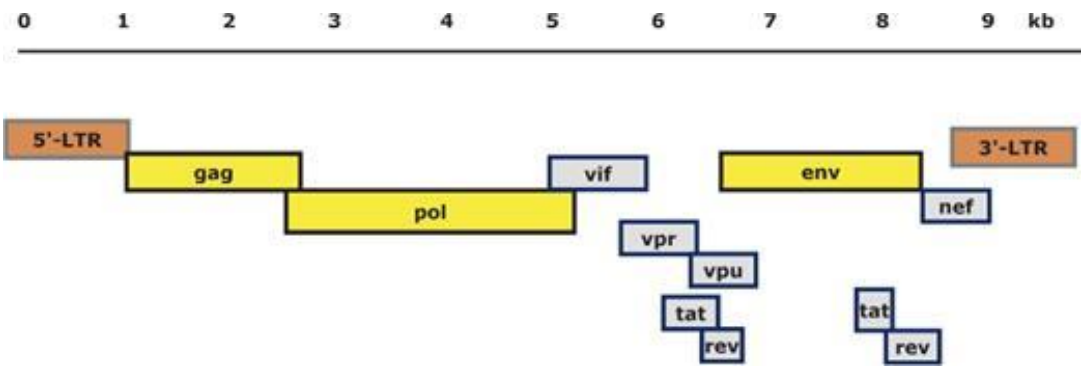


Figure 4: Structure and organization of the HIV-1 genome ***(*GACB, 2016).**

- **Structural Genes (gag, pol, env)**
  - **gag** (Group-specific antigen) gene encodes the precursor proteins that make up the viral capsid (p24), nucleocapsid (p7), and matrix (p17).
  - **pol** (Polymerase) gene encodes enzymes crucial for the viral life cycle, including: Reverse transcriptase (RT), Integrase (IN) and Protease (PR)
  - **env** (Envelope) gene encodes the envelope glycoproteins gp120 and gp41, which are embedded in the viral envelope. Gp120 mediates attachment to host cell receptors (CD4 and CCR5/CXCR4), while gp41 facilitates fusion with the host cell membrane.
- **Regulatory Genes (tat, rev, nef)**
  - **tat** (Trans-activator of transcription) gene encodes the Tat protein, which binds to the TAR element on viral RNA, significantly enhancing transcription of viral genes.
  - **rev** (Regulator of expression of virion proteins) gene encodes the Rev protein, which regulates the splicing and transport of viral RNA, enabling the expression of late viral proteins (structural proteins).

- **nef** (Negative regulatory factor) gene encodes the Nef protein, which downregulates CD4 and MHC class I expression on infected cells, helping the virus evade the immune system.

❖ **Accessory Genes (vif, vpr, vpu)**

- **vif** (Viral infectivity factor) gene encodes the Vif protein, which counteracts the antiviral activity of the host cell protein APOBEC3G, protecting the viral genome from degradation.
- **vpr** (Viral protein R) gene encodes the Vpr protein, which plays roles in nuclear import of the preintegration complex, cell cycle arrest, and regulation of viral gene expression.
- **vpu** (Viral protein U) gene encodes the Vpu protein, which enhances viral release from infected cells and downregulates CD4 expression.

The HIV-1 genome is flanked by two LTRs (Long Terminal Repeats), which are identical non-coding sequences of DNA. These regions contain promoters and enhancers that regulate viral gene expression.

d. Genetic variability

Two types of HIV are known, HIV-1 and HIV-2. HIV-1, exhibits high genetic variability due to its high mutation rate and ability to recombine, leading to its classification into various groups, subtypes, and recombinant forms. HIV-1 includes 4 groups, **M (main)**, which is by far the most common in the world and further splits into 9 clades called “subtypes” (A, B, C, D, F, G, H, J, K) based on genetic differences, **group O (outlier)** located in west-central Africa and most common in Cameroon **(Cahn *et al.,* 2019), N (New, non-M, non-O)**, and **P** which was discovered in a Cameroonian woman in 2009. The are also “unique recombinant forms”, URF, and “circulating recombinant forms, CRF, that are increasingly common. Recombinant forms of HIV-1 arise when different subtypes co-infect a single individual and mix their genetic material (genetic recombination), CRF02_AG is the most prevalent in Africa and in Cameroon **(Roquebert *et al.,* 2009)**. HIV-2 on the other comprises 8 subtypes, A to H **(Santiago *et al.,* 2005)**.

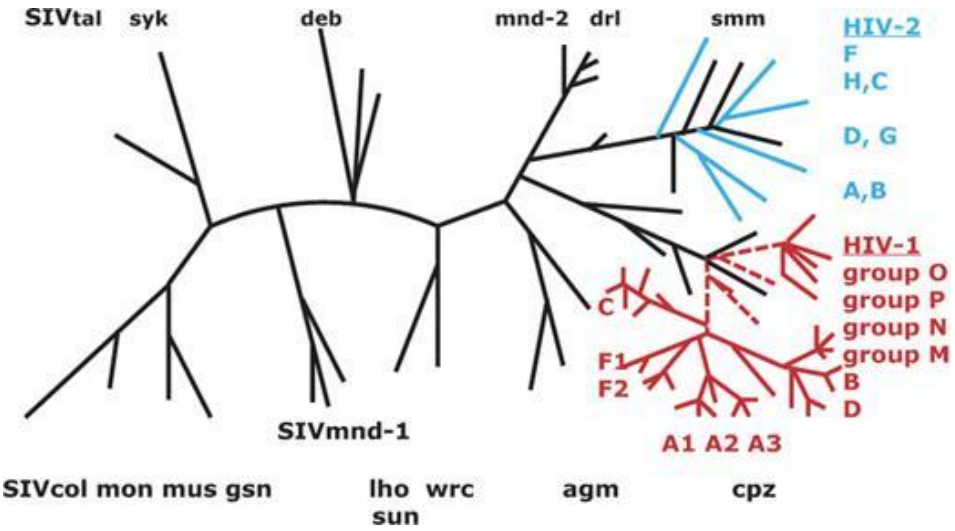


Figure 5: Schema of the phylogenetic tree showing the diversity of HIV **(GACB, 2016).**

4. Target cells and replication cycle of HIV

a. Target cells

HIV-1 primarily targets cells of the immune system, particularly CD4+ T cells, also known as helper T cells, which play a crucial role in the body's immune response to infection. When naïve T lymphocytes are infected, they proliferate, multiply and differentiate into CD8+ and CD4+ cells. The latter one has a predominant role in the immune system as it plays a pivotal role in the coordination and activation of other immune cells to fight off infections. Once these cells are activated, they grow as well, multiply and track the cause for their activation, while activating other cells, through direct contact or cytokines. However, as they carry their function, activated cells that carry HIV receptors at their surface get infected, and, in turn, each represent a kick-off point for the dissemination of the virus **(Chan & Kim, 1998)**.

The reason for this viral tropism lies in the virus's surface structure and interaction with specific host cell receptors. Specifically, gp120 binds to the CD4 receptor which is almost exclusively expressed on the surface of CD4+ T cells, T-helper cells, monocytes, macrophages, and dendritic cells. After gp120 binds, gp41 undergoes a conformational change, allowing it to fuse with the host cell membrane, facilitating the entry of the virus into the cell. HIV equally use chemokine receptors for their access into the cell. Though the use of co-receptors alone does not explain viral tropism, Macrophage(M)-trophic strains of HIV use the **β-chemokine** receptor,

CCR5, are called **R5 viruses** and can thus replicate in both macrophages and CD4+ T-cells. T-trophic strains that replicate in CD4+ T-cells as well as in macrophages and use **α-chemokine** receptor, **CXCR4,** called **X4.** Dual-trophic strains are able to use both CCR5 and CXCR4 as co-receptors **(Berger *et al.,* 1998)**.

b. Replication cycle of HIV

The principle of HIV replication solely relies on the invasion of the host immune cells and the diversion of their machinery in favor of the active production of viral copies. The HIV replication cycle is separated into 7 steps, from the attachment to the liberation of the new virion. These steps can, however, be grouped into **three** main phases **(early, intermediate and late)** as follows:

- **Attachment and entry**

The HIV enters macrophages and CD4+ T-cells by the adsorption of its surface glycoproteins (gp120, gp41) to the receptors (CD4, CCR5, CXCR4) of these ones on the surface of the target cells (Macrophages, CD4+ T-cell), enabling the fusion of the viral envelope with the target cell membrane and the release of the viral capsid into the cell. This is followed by the injection, into the cell's cytoplasm, of the viral RNA and various enzymes, including reverse transcriptase, integrase, ribonuclease, and protease **(Chan & Kim, 1998)**.

- **Transcription and replication**

Shortly after the viral capsid and its content are liberated in the cell, reverse transcriptase (RT), which is an RNA-dependent DNA polymerase, takes in charge the positive-sense single-stranded viral RNA genome and copies it into its complementary single-stranded DNA (**cDNA, also called "pro-virus"**). This step is highly mutagenic due to the error-prone nature of RT, and is responsible for the genetic variability of the future viral population. The single strand viral cDNA is then converted by cellular DNA polymerases into a double stranded cDNA, enabling its integration its integration into the host cell DNA by integrase **(IN).** Once in the cell's DNA, the provirus replicates alongside the cell's DNA replication and progressively diverting the cell's transcription machinery to focus on the active production of viral mRNA, which are then translated into viral proteins, the building blocks of new virions. Some of this RNA will become the genome of the new virions **(van Heuvel *et al.,* 2022)**.

➢ **Liberation and maturation**

In this last phase of HIV replication, the synthesized proteins as well as viral RNA move towards the surface of the cell and are assembled into immature virions (non-infectious). The Env polyprotein (gp160) undergoes maturation and is cleaved in the two HIV envelope glycoproteins, gp41 and gp120. These two move and anchor to the cell membrane while the Gag (p55) and Gag-Pol (p160) polyproteins associate with the inner surface of the cell membrane as the new virion begin to bud. After budding, the virion remains immature till the gag polyproteins get cleaved into the matrix, capsid and nucleocapsid proteins by the viral protease **(PR)** pack **(van Heuvel *et al.*, 2022).**

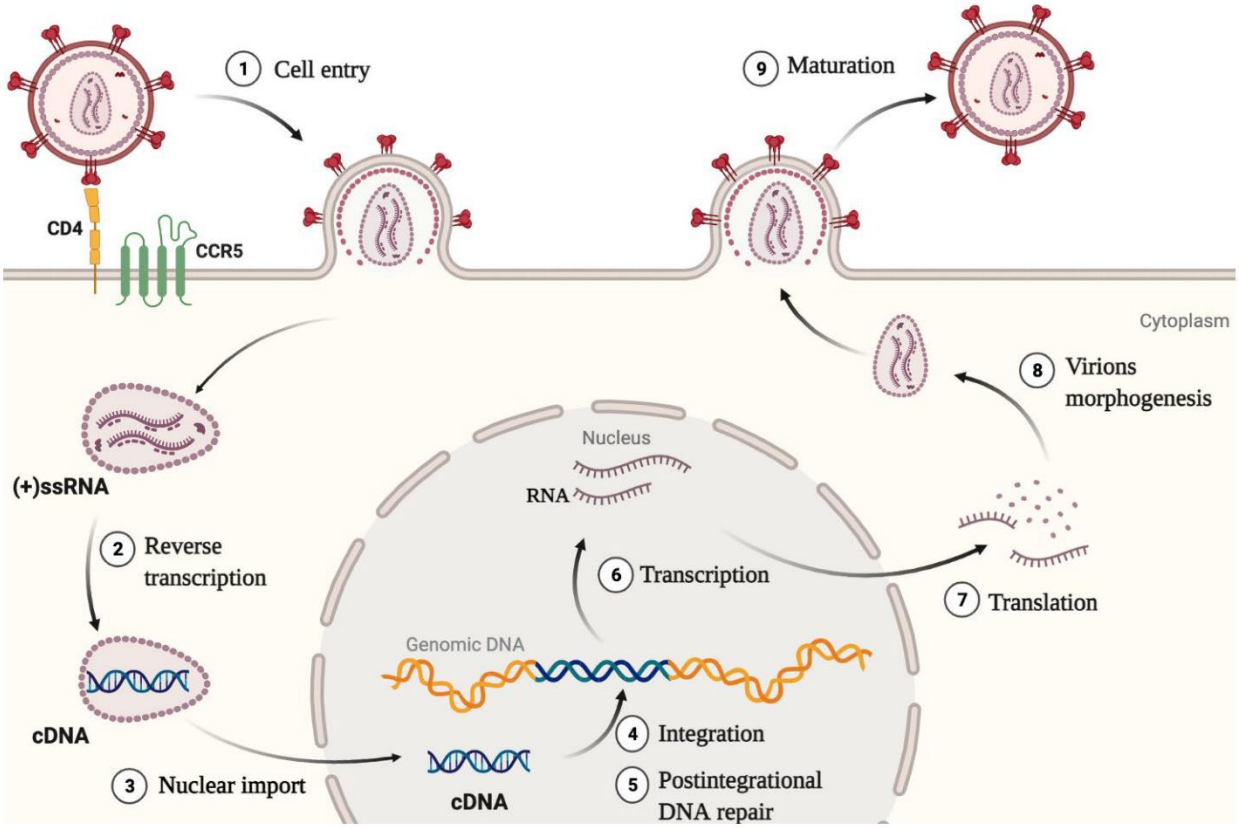


Figure 6: Schematic overview of the HIV-1 replication cycle **(van Heuvel *et al.*, 2022).**

5. Antiretroviral treatment

An antiretroviral (ARV) is a drug that is used to treat an infection that is caused by a retrovirus. ARV inhibit the multiplication of HIV, thereby reducing the viral burden, maintaining the immune

function and preventing opportunistic infections that often lead to death. This therefore reduces to zero the risk of transmission as long as the individual maintains and undetectable viral load **(Eisinger *et al.,* 2019; Moore & Chaisson, 1999).**

Since antiretroviral therapy (ART) is not a cure, it has to be taken for life while ensuring proper observance. ART is a combination of two to three ARV from at least two classes, which are grouped according to the stage of the viral cycle that they inhibit. HAART reduce the chances of viral evasion by creating multiple obstacles **(Smyth *et al.,* 2012).** ARVs are therefore grouped as follows:

5.1. Entry inhibitors:

HIV entry inhibitors are a class of antiviral drugs that prevent HIV from entering human cells. They are used to treat HIV infection and prevent transmission of the virus. Entry inhibitors work by binding to specific proteins on the surface of HIV, blocking the virus from attaching to and entering human cells. Some examples of entry inhibitors include:

5.1.1. Fusion inhibitors, FIs:

Enfuvirtide (Fuzeon®) is the first and only drug available, it was approved in 2003 **(Robertson, 2003)** to be administered as subcutaneous injections, twice daily, in patients with an advanced stage of disease progression and were resistant to other drug classes. It is a 36 amino acid synthetic peptide, derived from a conserved region of gp41, HR-2, that specifically inhibits the function of the gp41 transmembrane glycoprotein of HIV-1. HIV-1 binding begins with the interaction of the viral gp120 with the CD4 receptor of the cell, causing the unveiling of the transmembrane gp41. The gp41 can be sub-divided into N and C-terminal heptad repeats, HR-1 and HR-2 respectively, which normally connect, inducing conformational changes required for the fusion of the viral envelope and cell membrane. Enfuvirtide which is a biomimetic of HR-2, acts by binding to HR-1, inhibiting the connection between HR-1 and HR-2, thereby blocking the fusion of HIV with the host cell membrane **(Ashkenazi *et al.,* 2011)**. Today enfuvirtide (T-20) exist in optimized (second generation), T-1249, and more potent (third generation), T-2635, forms with increased stability and potency against HIV-1 wild strains and fusion inhibitor resistant strains **(Dwyer *et al.,* 2007)**.

5.1.2. Chemokine receptor 5 (CCR5) antagonist:

CCR5-antagonists were the first class of ARV that had as target the host cell and not the virus. **Maraviroc,** FDA approved in 2007 **(*Prezista* | *European Medicines Agency*, n.d.)**, is the only marketed drug in this class, taken as daily oral tablet. Maraviroc blocks the interaction between HIV and the CCR5 co-receptor on human cells by binding to the CCR5 co-receptor, inducing a conformational change that prevents its recognition by viral gp120. This mechanism limits its action to CCR5-trophic (R5) HIV infections. However, due to a possible shift in tropism which allows HIV to target an alternative co-receptor such as CXCR4 or individuals that present a mutation in the CCR5 delta gene.

5.2. Integrase strand transfer inhibitors, INSTIs or INIs:

Integrase is the latest enzyme to be targeted by ART, and Raltegravir was the first drug of this class that received FDA approval in 2007 **(Research, n.d.)**. HIV-1 IN is a 288 amino acid long and comprises three domains. An N-terminal zinc-binding motif, HHCC, the catalytic core domain (CCD), and a C-terminal domain. After HIV-1 DNA is reverse transcribed into double-stranded DNA, the viral enzyme integrase begins a multi-step integration process. First, integrase cleaves dinucleotides from each 3' end of the viral DNA, leaving overhangs. Still bound to the DNA, integrase then circularizes the viral genome and facilitates its entry into the host cell's nucleus. Finally, within the nucleus, integrase catalyzes the insertion of the viral DNA into the host chromosome, a step called the "strand-transfer reaction" **(Pommier *et al.,* 2005)**. Integrase inhibitors block this activity by binding to the active site of integrase, thereby preventing the viral DNA from being incorporated into the host cell DNA. All drugs in this class so far are integrase strand transfer inhibitors (INSTIs) due to their ability to block the strand transfer step of viral DNA integration. Raltegravir was the first drug in the INSTI class and was approved for twice-daily administration. Meanwhile, a once-daily formulation was approved in 2017. Elvitegravir followed in 2012, then dolutegravir in 2013 and today the most commonly used with bictegravir **(Pau & George, 2014; Scarsi *et al.,* 2020).** The newest INSTI, cabotegravir, represents an alternative to oral administration of life-long antiretroviral therapy with the availability of a long-acting injectable formulation **(Scarsi *et al.,* 2020)**.

5.3. Protease inhibitors, PI:

After the mRNA is translated into viral proteins, the third virally encoded enzyme, namely HIV protease, also known as aspartyl-protease, is required to cleave a viral polyprotein precursor into individual mature proteins that assemble to form the mature virion. Protease inhibitors are peptide-like chemicals that competitively inhibit the action of the viral aspartyl-protease by binding to its active site which contains the catalytic residues responsible for cleavage. They are designed to mimic the transition state of the protease's substrates, inhibiting its activity, and by binding to the active site, the inhibitor blocks the catalytic residues and prevents them from interacting with the substrate, thereby preventing the protease enzyme from cleaving the Gag and Gag-Pol polyproteins. This inhibition of cleavage prevents the maturation of HIV-1 virions, rendering them non-infectious and unable to replicate. There are actually 9 PIs available on the US market and Saquinavir was the first that was discovered. Following that discovery, other HIV protease inhibitors were designed using the same principle: **ritonavir, saquinavir, lopinavir, fosamprenavir, tipranavir, atazanavir, and darunavir**. **Indinavir** and **nelfinavir** has known diminished uses mainly due to large pill burden and intolerable side effects **(Guide to Protease Inhibitors for HIV, 2023)**.

5.4. Reverse transcriptase inhibitors, RTIs:

Since RT is responsible for converting the viral RNA genome into DNA, which can then be converted to a double-stranded DNA by the host cell DNA polymerase, this makes it a selective target for inhibition. RTIs work by binding to RT and interfering with its ability to synthesize DNA. RTIs work by binding to RT and interfering with its ability to synthesize DNA. Currently there are two classes of RTIs in clinical use, NRTIs and NNRTIs.

5.4.1. Nucleoside and Nucleotide reverse transcriptase inhibitors, NNRTIs:

NRTIs were the first class of ARV to be discovered, with zidovudine been the very first drug approved in 1987 **(Robertson, 2003)**. These drugs are analogs of natural nucleosides or nucleotides and lack the 3’-OH and/or contain a modified sugar moiety. An NRTI is converted to a dNTP (deoxynucleotide triphosphate) analog by a phosphorylation performed by the kinases of the host cell, and then RT catalytically incorporates the drug as an NRTI monophosphate at the 3’-end of the growing viral DNA strand. They compete with natural nucleotides for incorporation into

the growing DNA strand, but once incorporated, they cause chain termination because they lack the 3'-OH group that is necessary for further elongation of the DNA strand. These NRTIs are **zidovudine** (AZT), **emtricitabine** (FTC), **lamivudine** (3 TC), **zalcitabine** (ddC), **abacavir** (ABC), **didanosine** (ddI), **stavudine** (d4T), and two **tenofovir** prodrug derivatives, 5′-disoproxil fumarate (TDF) and Tenofovir alafenamide (TAF) **(Singh & Das, 2022).**

5.4.2. Non-nucleoside reverse transcriptase inhibitors, NNRTIs:

NNRTIs are non-competitive inhibitors, meaning that they do not compete with natural nucleotides for incorporation into the growing DNA strand. All NNRTIs bind to an allosteric site near the active site of RT, called the NNRTI binding pocket, causing a structural change in RT that disrupts the enzyme's active site and inhibits its ability to properly bind to the RNA template and polymerize the new DNA. NNRTIs are often classified into generations based on their potency, resistance profile, and side effects: first generation comprising efavirenz(EFV), nevirapine(NVP), and delavirdine(DLV), second generation with etravirine(ETR), and Rilpivirine(RPV), and third generation with Doravirine, FDA approved in August 2018 **(Das & Arnold, 2013; HIV/AIDS Drugs | NIH, n.d.)**.

5.4.3. Nucleoside reverse transcriptase translocation inhibitors, NRTTIs:

This is a new class of antivirals, Islatravir or ISL (or 4’-ethynyl-2-fluoro-2’-deoxyadenosine [EFdA]) being the first agent of this group. It is orally available, long-acting antiviral, being actually tested as ART against HIV-1 in phase 3. Current HIV treatments are single-tablet regimens that are taken orally once daily, but challenges remain in reaching UNAIDS goals, especially with respect to treatment adherence and viral suppression in some parts of the world (WHO, n.d.). HIV treatment regimens that minimize pill burden, reduce dosing frequency, and improve convenience might improve adherence and, consequently, long-term outcomes **(Clinicalinfo, 2023; Conway, 2007)**. Recent and potential advances in HIV therapy include simplification of oral once-daily regimens from three to two drugs, injectable formulations that facilitate infrequent dosing, and new classes of medication with differentiated mechanisms of action **(Cihlar & Fordyce, 2016)**. Two-drug regimens, like dolutegravir plus rilpivirine and dolutegravir plus lamivudine, have demonstrated non-inferiority to the standard three-drug regimens in specific populations **(Cahn *et al.,* 2019; Llibre *et al.,* 2018)**. Islatravir has characteristics that potentially allow it to manifest all of these potential advances.

- **Mechanism of action of ISL:**

ISL has structural and mechanistic features that distinguish it from currently marketed antiretroviral drugs. It has three chemical structure components that confers its unique profile: a **3′-OH group**, also found in naturally occurring nucleotides, which is associated with the very high binding affinity for the reverse transcriptase; a **4′-ethynyl group**, responsible for the ability of islatravir to block primer translocation and cause immediate chain termination; and a **2-fluoro group**, which inhibits islatravir metabolism and contributes to its long half-life. If translocation does occur, islatravir also acts as a delayed chain terminator. The active form of islatravir, islatravir-triphosphate, is efficiently incorporated onto the end of the viral DNA chain but leads to mismatched primers that are difficult to extend. The multiple mechanisms of action, coupled with the high binding affinity to reverse transcriptase, translate to robust antiviral efficacy and has a potency up to several orders of magnitude higher than that of NRTIs **(Michailidis *et al.,* 2009, 2014; Schürmann *et al.,* 2020).**

- **Long-acting properties and efficacy:**

In a study of phase 1b trial performed with 30 treatment-naïve patients, single oral doses of 0.5 to 30 mg of ISL reduced plasma HIV RNA levels by up to 1.7 $\log_{10}$ copies/mL at 10 days, suggesting that weekly dosing and manufacturing of long-acting formulation may be possible **(Schürmann *et al.,* 2020)**. When 56 mg of ISL is administered by subcutaneous injection, its $T_{1/2}$ is 198 h and ISL-eluting implant is projected to release adequate ISL-TP for more than 52 weeks **(Randolph *et al.,* 2021)**. The phase 2b dose-ranging trial of ISL combined with doravirine (DOR) was performed to evaluate its efficacy and safety for treatment-naïve patients.

5.5. Capsid inhibitor

Lenacapavir is the first drug of a class of drugs called capsid inhibitors to be FDA-approved in December 2022 for treating HIV/AIDS in people with limited treatment options **(Mushtaq & Kazi, 2023).** Lenacapavir disrupts two main stages of the viral cycle. After HIV binds to the cell, the fusion of the viral envelope and the cell membrane releases the viral capsid, there by unveiling lenacapavir targets. Lenacapavir binds to the capsid and blocks its disassembly, causing the viral genome to be trapped inside. Lenacapavir equally blocks the capsid's maturation process through its binding to the N-terminal domain of the capsid protein (CA), preventing viral assembly and

release. Lenacapavir is a “long-acting” drug with a very long half-life, taken by mouth or by subcutaneous injection. The most common side effects include reactions at the injection site and nausea **(*Gilead Sciences : SUNLENCA® (Lenacapavir)*, n.d.)**.

6. HIV-1 resistance testing

There are two ways to test for HIV drug resistance: phenotypic and genotypic. **Phenotypic testing** directly measures how effective a drug is against a patient's specific HIV strain grown in a lab. Susceptibility is usually reported as the ARV concentration that inhibits HIV-1 replication by 50% (IC50), and is reported as a "fold change"(ratio of IC50s), which compares While remaining the most accurate (gold standard) and comprehensive method, it is costly, time-consuming, and technically complex, making it impractical for routine clinical use (especially in low clinical setting (LCS)), limiting its use to research, drug development, and complex clinical scenarios **(Petropoulos *et al.*, 2000)**.

However, the development of **resistance genotyping** in the 1990s, driven by the need for more rapid and cost-effective methods, quickly surpassed phenotypic testing. Genotyping offers quicker turnaround times and greater precision in pinpointing specific mutations, making it the preferred approach. This shift has significantly improved treatment outcomes and allowed for more effective and “individualized” care for people living with HIV.

The test uses sequencing techniques to sequence and examine specific regions of the HIV genome like those that code for key enzymes like RT, PR and IN. These sequences are compared to a "wild-type" reference sequence, and any differences are identified and interpreted based on a vast database of known resistance mutations **(Woods *et al.*, 2012)**. Resistance genotyping, therefore, relies on previously characterized mutations and may miss novel resistance mechanisms. Additionally, unlike phenotypic studies, it does not provide information on the degree of resistance conferred by a particular mutation, but however revolutionized ART **(Clutter *et al.*, 2016)**. The principle, advantages and limits of each sequencing technique are developed below.

6.1. Sanger sequencing

Sanger sequencing, also known as chain termination sequencing, was developed by Frederick Sanger in the 1970s. It relies on the incorporation of modified nucleotides, called dideoxynucleotides (ddNTPs), into a growing DNA strand. Unlike regular nucleotides (dNTPs),

ddNTPs lack a 3'-hydroxyl group, which is essential for the formation of the phosphodiester bond that links nucleotides together. These ddNTPs therefore halt the elongation process, causing the generation of a mixture of DNA fragments of varying lengths **(Sanger *et al.*, 1977)**.

While early DNA sequencing relied on manual interpretation of fragment migration patterns on agarose gels, the advent of automated Sanger sequencing revolutionized the field. This automation, facilitated by fluorescently-labeled primers first, then, ddNTPs and capillary electrophoresis, enabled rapid and accurate sequence determination, making it a cornerstone of routine diagnostic procedures **(Smith *et al.*, 1989)**.

This technology, however, has its limitations, including insufficient sensitivity to detect a mutation or variant present at less than 20% of the viral population under study. Added to that, it is limited to relatively short reads (~300-850 bp), making it unsuitable for sequencing large fragments, justifying the use of many pair of primers to cover the enter fragment during a sequence cycle **(Ek *et al.*, 2017)**.

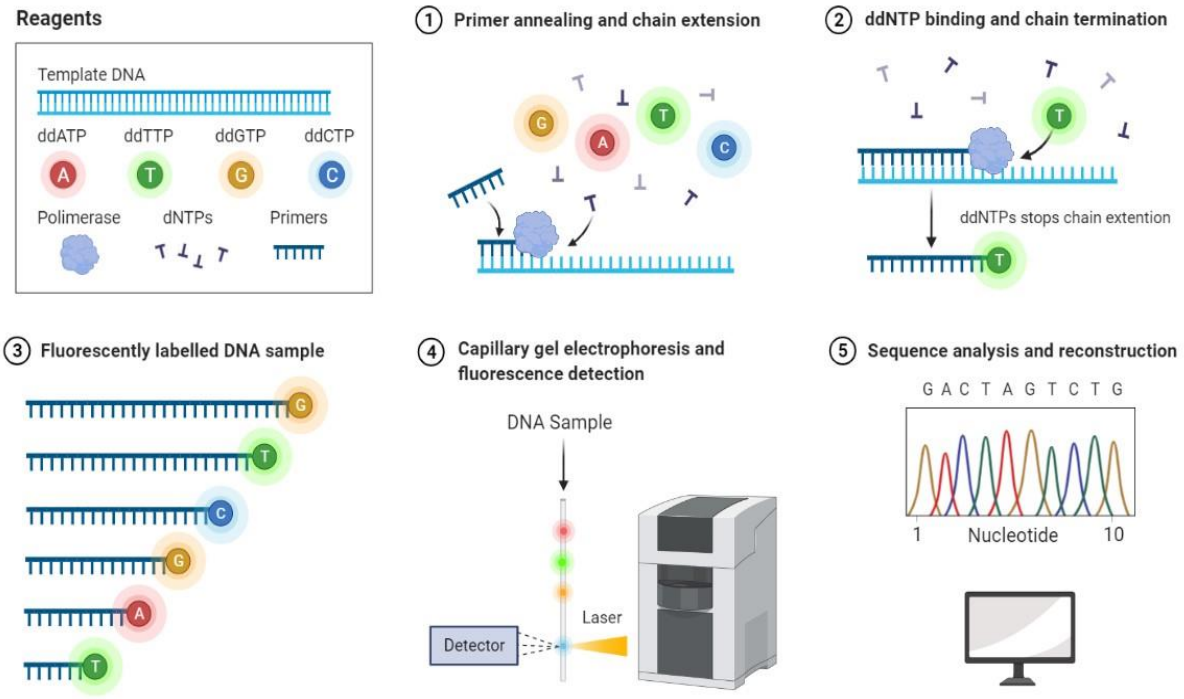


Figure 7: The Sanger (chain-termination) method for DNA sequencing ***(*GenScript, 2022)**.

6.2. Next Generation Sequencing (NGS)

The field of genomics has been revolutionized by the development of several next-generation sequencing (NGS), also known as high-throughput sequencing, platforms. While these platforms differ in their specifics, they all share a core set of steps. NGS has the ability to detect mutations present at lower percentages, typically down to 1% or even lower, depending on the sequencing depth and the specific variant calling algorithms used **(GenScript, 2022)**.

The first step involves **preparing a DNA library**, which is essentially a collection of fragmented DNAs representing the target genome. This library also allows for the simultaneous analysis of multiple samples through a technique called multiplexing. Multiplexing utilizes unique "barcode" sequences called MIDs (Multiplex Identifiers) to tag each sample within the library. Second, the DNA fragments within the library undergo clonal **amplification**, a process that generates multiple copies of each fragment. Some platforms, such as 454 and Ion Torrent®, employ emulsion PCR on microbeads, while Illumina®, utilize a solid surface for amplification. The final stage is **sequencing** itself, achieved by detecting signals emitted as nucleotides are incorporated. Illumina® platforms utilize fluorescence-based detection, while Ion Torrent® relies on detecting pH changes. Below is a description of some leading commercially available NGS platforms **(Satam *et al.,* 2023)**.

i. Illumina® Sequencing Technology:

Illumina sequencing, a dominant force in next-generation sequencing, operates on the principle of sequencing-by-synthesis. It involves immobilizing the DNA library on a flow cell, creating a dense array of individual DNA strands. During sequencing, fluorescently labelled *deoxynucleoside triphosphates (dNTPs)*, each bearing a distinct color representing its base (A, C, G, or T), are sequentially added to each strand. The key here is the fluorescently reversible terminator chemistry that removes the fluorescent groups and the terminators, and regenerates the 3’ OH-group, enabling only one nucleotide to be added at a time. A laser excites the fluorescent label, causing the dNTP to emit a specific color of light, which is captured by a camera. This process, repeated for each nucleotide addition, builds a sequence of bases one by one **(Illumina, 2013)**. Illumina sequencing boasts high throughput (it can sequence up to billions of sequences at a time in parallel), generating massive amounts of data at relatively low cost per base. This makes it ideal for large-scale projects and routine applications like genome sequencing. It also offers high

accuracy, particularly for shorter reads, making it suitable for many research and clinical applications. However, the chemistry isn't perfect and some clusters lag while other jump ahead. This causes a reduction in the true signal intensity with the number of cycles, limiting Illumina sequencing to about 300 bases for each read. The technology is, therefore, primarily focused on shorter read lengths, which can pose challenges for complex or repetitive genomic regions.

ii. Ion Torrent® Sequencing Technology:

Ion Torrent Sequencing relies on a direct detection approach called semiconductor sequencing. DNA fragments are immobilized on a chip containing millions of tiny wells, each containing a single DNA strand. Sequencing occurs by adding individual nucleotides, one type at a time, to each well. When a nucleotide is incorporated into a DNA strand, a hydrogen ion (H+) is released. This change in pH is detected by a sensor within the well, generating a signal that identifies the incorporated nucleotide. The sequence information is then compiled based on the order of detected signals, producing a continuous read for each DNA fragment. This method, known for its speed and simplicity, is particularly well-suited for applications that require rapid turnaround times, such as clinical diagnostics and pathogen identification. It can sequence millions of reads per run, but the reads length ranges from 200-400 bp **(Rothberg *et al.,* 2011)**.

iii. Pacific Biosciences® (PacBio) Sequencing Technology:

PacBio sequencing known as Single Molecule Real-Time (SMRT) sequencing, relies on a unique principle of directly observing the incorporation of nucleotides during DNA replication. Individual DNA molecules are loaded into tiny wells, each containing a polymerase enzyme. As the polymerase replicates the DNA strand, fluorescently labelled nucleotides are added one by one. The key of this technology lies in (i) the circular DNA template which enables the polymerase to go round it continuously, and (ii) the 5'-P position of the fluorescent label which causes it to be automatically float away after each nucleotide incorporation, and so does not require any washing chemistry. Each nucleotide carries a specific fluorescent label that emits a unique color when excited by a laser. A camera captures the emitted light, recording the sequence of colors as the DNA strand is synthesized. It has a high throughput proportional to the number of wells, and the reads reach up to 100kb. It however has a high error rate (10-15%), though these errors are random, making correction possible by comparison of same reads **(PacBio, n.d.)**.

iv. Oxford Nanopore Sequencing:

This technology utilizes nanopores, tiny holes in a membrane, to detect DNA molecules as they pass through. As DNA strands traverse the nanopore, changes in electrical current are measured, which correlate with the specific nucleotide sequence. Oxford Nanopore sequencing produces very long reads (up to millions of base pairs) and has a high throughput. However, it generally has a higher error rate compared to other NGS platforms **(Feng *et al.*, 2015).**

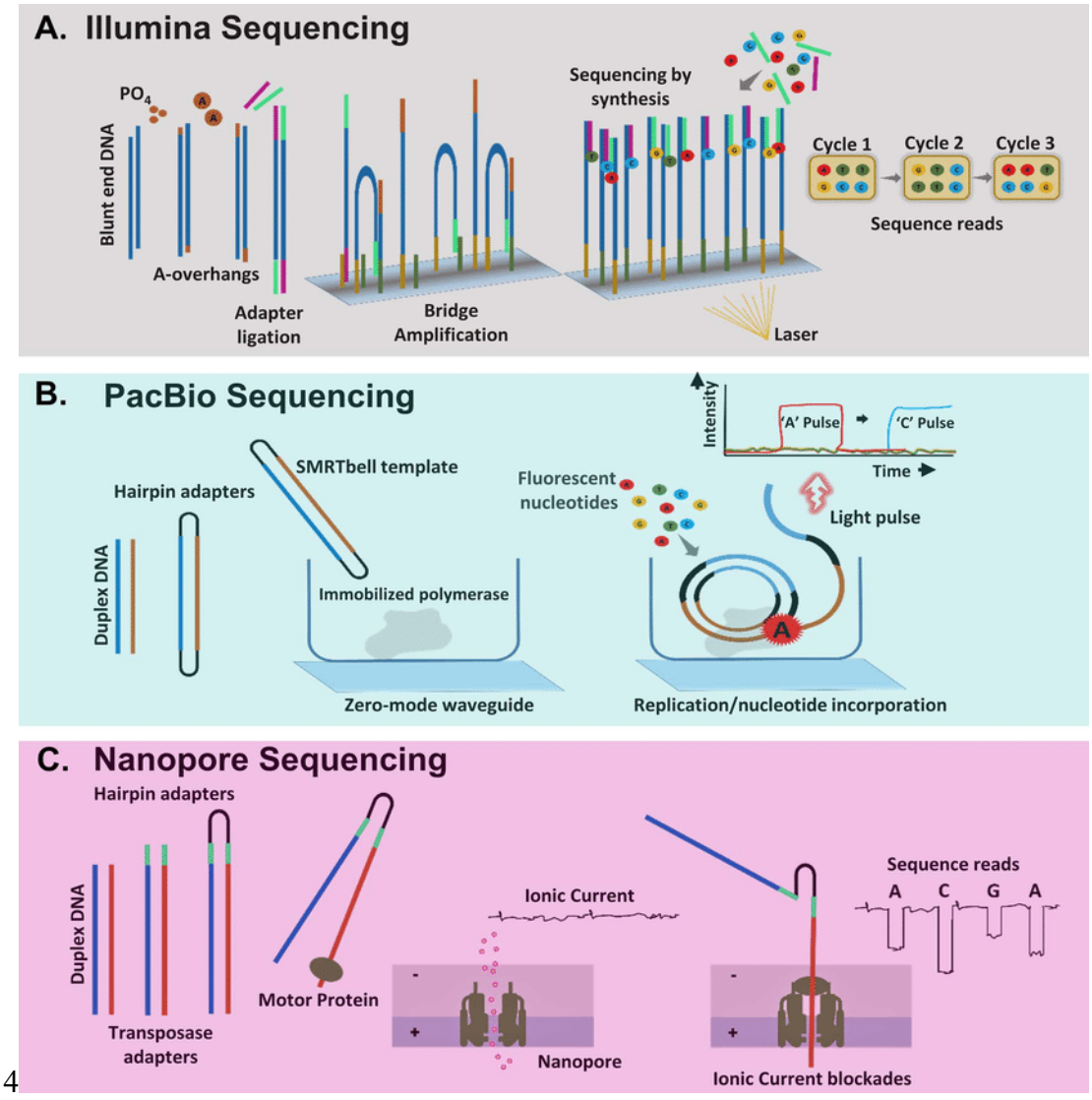


Figure 8: Major short-read and long-read sequencing technologies, (A) Illumina sequencing, (B) PacBio sequencing, (C) Nanopore sequencing **(Bharti & Grimm, 2019)**.

7. Mechanisms of Drug Resistance in HIV-1

HIV drug resistance is arises with changes in the genetic structure of HIV, caused by the error prone nature of RT, and recombination events that occur when more than one variant infect the same cell **(Abram *et al.,* 2010; Levy *et al.,* 2004)**, affecting the ability of medicines to block the replication of the virus. Drug resistance in viral infections is driven by a complex interplay of factors. The extent of viral replication when partially suppressed by treatment, the ease with which a virus can acquire specific resistance mutations (DRMs), and the impact of these DRMs on both drug susceptibility and viral replication itself directly influences the selection of resistant variants. Crucially, most clinically significant DRMs only arise in the presence of drug pressure, highlighting the importance of effective therapy and constant monitoring for resistance development **(Clutter *et al.,* 2016)**.

Antiretroviral therapy (ART) success hinges on balancing drug susceptibility and viral fitness. Some ARVs require multiple DRMs to become ineffective, while others need only one. The number and impact of these mutations determine the **genetic barrier** to resistance, which is, how difficult it is for HIV-1 to evolve resistance to a specific drug. DRMs can be categorized as **primary**, which directly reduce drug effectiveness, or **accessory**, which enhance the fitness of resistant variants or further reduce drug sensitivity. On the other hand, the **antiviral potency** of an ARV is determined by the extent to which it reduces plasma HIV-1 RNA levels, and this, combined with its genetic barrier to resistance affects its ability to protect an ART regimen from virologic failure **(Clutter *et al.,* 2016)** (Figure 9)**.**

There is generally no overlap in resistance between different classes of drugs. Viruses that are highly resistant to one class of antiretroviral drugs remain susceptible to drugs from other unused classes **(Larder, 1994)**. On the other hand, significant resistance can occur within the same drug class, as many drug resistance mutations reduce susceptibility to multiple drugs within that class **(Melikian *et al.,* 2012)**. However, there are exceptions where certain mutations can actually increase susceptibility to other drugs within the same class **(Parikh *et al.,* 2007)**. Understanding the cross-resistance profiles of antiretroviral drugs is crucial when using multiple drugs from the same class together or in succession.

Despite the successful implementation of WHO global plan for HIV drug resistance **(*WHO, 2016*)**, antiretroviral drugs, including those from newer drug classes, remain at risk of becoming

partially or fully inactive, due to the increase in the prevalence of acquired drug resistance (ADR) in treated individuals and transmitted drug resistance (TDR) in newly infected individuals **(Phillips *et al.*, 2014).**

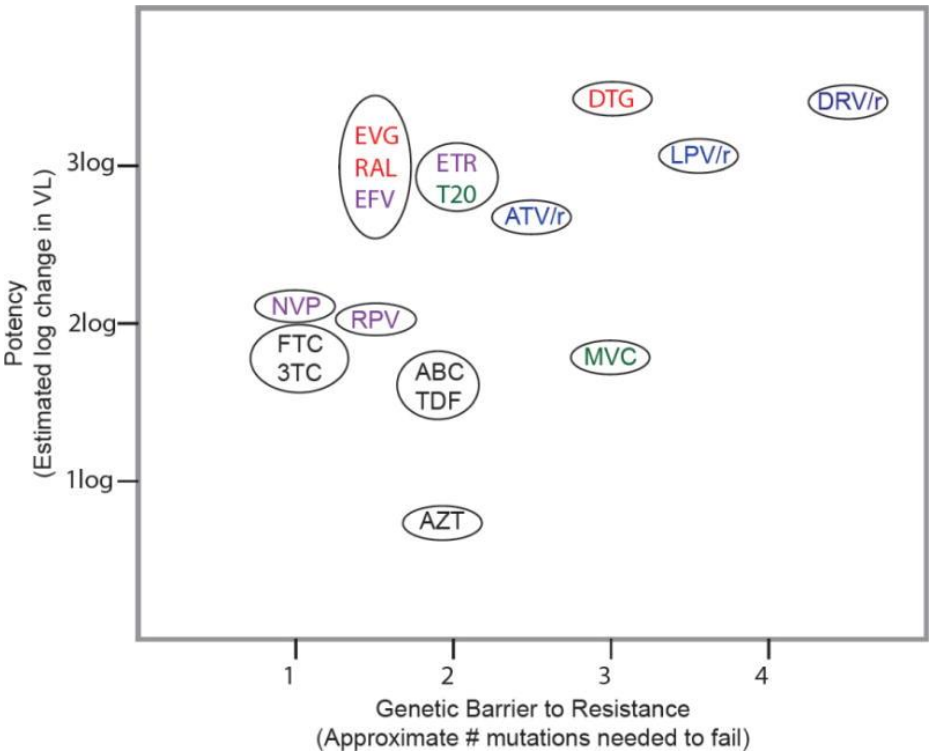


Figure 9: ARV potency versus genetic barrier to resistance ***(*Clutter *et al.*, 2016*).***

Lengend: *ARV: antiretroviral; VL: viral load; ABC: abacavir; ATV/r: boosted atazanavir; DRV/r: boosted darunavir; DTG: dolutegravir; EFV: efavirenz; FTC: emtricitabine; EVG: elvitegravir; T20: enfuvirtide; ETR: etravirine; 3TC: lamivudine; LPV/r: boosted lopinavir; MVC: maraviroc; NVP: nevirapine; RAL: raltegravir; RPV: rilpivirine; and TDF: tenofovir. ARVs in black font are nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs), those in purple font are non-NRTIs (NNRTIs), those in blue font are protease inhibitors (PIs), those in red font are integrase strand transfer inhibitors (INSTIs), and those in green font are entry inhibitors. ARVs appearing together in the same ellipse should be considered to have roughly equivalent potencies and genetic barriers to resistance* ***(Clutter et al., 2016)****.*

a. Resistance to NRTIs

NRTI resistance can occur through two mechanisms: (i) discriminatory mutations that allow the reverse transcriptase to differentiate between dideoxy-NRTI chain terminators and the cell's naturally produced dNTPs, preventing NRTIs from being integrated into a developing viral DNA chain; and (ii) primer unblocking mutations that aid in the removal of an NRTI-triphosphate added to the developing viral DNA chain through phosphorylytic excision. Mutations that unblock

primers are also known as thymidine analogue mutations (**TAMs**) because they are selected by the thymidine analogues zidovudine and stavudine (Tang & Shafer, 2012). The presence of these mutations on the TI gene increases the enzyme's capacity to remove the chain-terminating analogue (the antiretroviral molecule) recently integrated into the pro-viral DNA during elongation through a process known as phosphorolysis **(Meyer *et al.,* 1998)**. The TAMs (M41L, D67N, K70R, L210W, T215F/Y, and K219Q/E), associated with resistance to AZT and d4T, also lead to resistance to tenofovir, ABC, and ddI. They can be categorized into type I (M41L, L210W, T215Y) and type II (D67N, K70R, T215F, K219Q/E) patterns. The mutations at positions 67 and 70, on the fingers of the protein, increase its flexibility, while the mutation at position 215, in the palm, promotes ATP binding. Type I TAMs result in higher resistance to thymidine analogues and cross-resistance to ABC, ddI, and TDF compared to type II TAMs **(Marcelin, Delaugerre, *et al.,* 2004).**

The second mechanism works by enhancing RT's capability to distinguish between chain terminator analogs. As a result, TI incorporates terminators much less without greatly reducing its affinity for natural analogs. This is known as resistance through discrimination or loss of affinity **(Boyer *et al.,* 2000)**. The prevalent discriminatory mutations include M184V/I, K65R, K70E/G, L74V, Y115F and the Q151M complex of mutations (A62V, V75I, F77L, F116Y). M184V/I is selected by and leads to high-level phenotypic resistance to the cytosine analogs, 3TC and FTC, while also impacting abacavir and didanosine to a lesser extent. The reduction in incorporation is caused by steric hindrance between the enzyme carrying the M184V mutation and 3TC-TP, since the side chain of valine at position 184 prevents proper positioning of 3TC-TP for catalysis. However, M184V enhances susceptibility to tenofovir and zidovudine, and it viral fitness more than another RT mutation. **(V. Miller *et al.,* 2002)**.

Abacavir (ABC): The drug-selected mutations associated with resistance to Abacavir include K65R, L74V, Y115F, and M184V/I. In laboratory studies with wild-type HIV strains and ABC alone, the 184V mutation emerged first, leading to a modest less than 4-fold change in susceptibility to ABC compared to the wild type. This is in contrast to the over 100-fold change in susceptibility seen with 3TC. However, the combination of M184V with zidovudine mutations gives rise to high-level abacavir resistance, which may be clinically relevant **(Walter *et al.,* 2002)**. Y115F mutation is considered unique to ABC, while K65R was reported to emerge during therapy with ddI and TDF **(Moyle, 2001)**.

3TC and FTC: Both 3TC and FTC select for M184V/I, which is the most common mutation associated with high level resistance (>100-fold) to 3TC and FTC. While the M184I mutation emerges initially, it is eventually overtaken by the M184V mutation, which grants the virus a greater ability to replicate **(Frost *et al.,* 2000)**. K65R confers low-level resistance to 3TC and FTC. Even though the M184V/I mutation causes high-level resistance to 3TC and FTC, these drugs are often continued in treatment regimens, and if a patient's HIV treatment fails, but the M184V/I mutation isn't found in their resistance profile, it may be a sign of poor adherence to treatment. This is because maintaining these specific mutations actually hinders the virus's overall fitness, making it less effective at replicating and more susceptible to other NRTIs like AZT and TDF **(Eron *et al.,* 1995)**.

Tenofovir (TDF): The most important TDF resistance mutation is K65R. A study **(Miller *et al.,* 2004)** showed that patients with HIV-1 harboring 3 or more TAMs, particularly those with M41L or L210W, showed decreased response to TDF, and that, pre-existing K65R mutations were linked to reduced treatment effectiveness. Interestingly, the presence of the M184V mutation appeared to slightly improve treatment response. Another showed that K65R and TAMs exhibit a mutually antagonistic relationship, affecting each other's ability to inhibit HIV replication **(Parikh *et al.,* 2007)**. This suggests that therapies selecting for both TAMs and K65R could potentially extend treatment effectiveness. Since there is no differences in resistance patterns have been observed between TDF and TAF **(Margot *et al.,* 2015)**, both drugs are referred to as “tenofovir” in Figure 10 below.

Zidovudine (AZT): Several mutations in the HIV reverse transcriptase enzyme contribute to AZT resistance. Key mutations include M41L, D67N, and L210W, which significantly reduce AZT's effectiveness and often occur alongside other mutations. The K70R mutation further enhances resistance to AZT and other NRTIs, including ddI and d4T. Additional mutations, such as T215Y/F, V106A/I/M, and Q151M, can further enhance resistance, particularly when present with other resistance mutations. For antiretroviral drugs that are no longer recommended, the associated Figure Bars are listed at the bottom of the drug class and are shaded in gray. Their user notes are retained for historical significance***(Wensing et al., 2022)***.

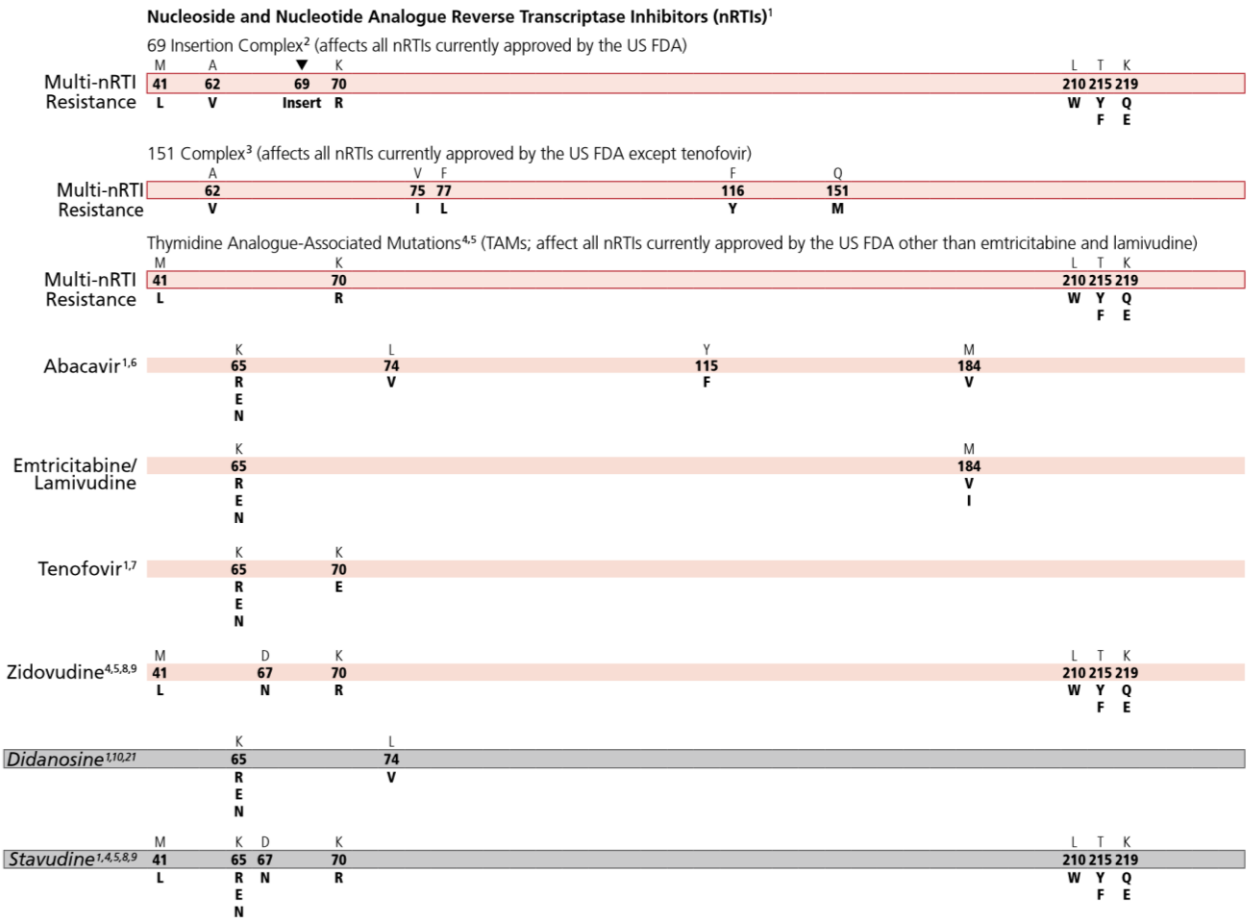
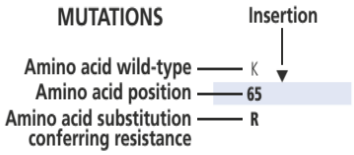


Figure 10: Mutations in the RT gene associated with resistance to NRTIs ***(*Wensing *et al.*, 2022)**

Amino acid abbreviations: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.



b. Resistance to NNRTIs

Relative to NRTIs, NNRTIs easily develop resistance with few genetic mutations (lower genetic barrier). One or two DRMs often lead to high-level resistance, though this varies: nevirapine (NVP) needs one, efavirenz (EFV) one or two, and etravirine (ETR) two, with an important level of cross-resistance **(Melikian *et al.*, 2014)**. Rilpivirine (RPV), despite structural similarity to ETR, has lower resistance due to its lower dosage **(Cohen *et al.*, 2011)**. Clutter *et al.* suggested that this high cross-resistance is as a result of (i) the ability of one NNRTI DRM to reduce potence to two or more NNRTI, and (ii) the fact that NNRTIs low genetic barrier enables the emergence of multiple independent NNRTI-resistant lineages **(Clutter *et al.*, 2016)**. Doravirine,

a more recent drug is seen to exhibit high potency and genetic barrier, maintaining its activity in the presence of NNRTI DRMs **(Brenner *et al.,* 2023)**. Therefore, Common NNRTI mutations (L100I, K101EP, K103NS, V106AM, Y181CIV, Y188L, G190ASE, M230L) generally cause high resistance to nevirapine (except L100I) and intermediate to high resistance to efavirenz (except V106A, Y181CIV) **(Vingerhoets *et al.,* 2010)**.

Doravirine (DRV): DRV, while effective against common NNRTI-resistant strains, can develop resistance. An *in vitro* DRM selection study, DRV after 8 weeks, selected for mutations V108I or V106A/I/M RAMs after 8 weeks, which conferred low-level (~2-fold) resistance to DRV. The development of high-level resistance (over 100-fold) to doravirine was observed after 24 weeks. This resistance was associated with the accumulation of three to six secondary RAMs, specifically F227L, M230L, L234I and/or Y318. Therefore, multiple mutations are needed for significant resistance **(Brenner *et al.,* 2023)**. Brenner *et al.* (2023) equally showed that, while doravirine resistance mutations emerged, they did not cause cross-resistance to rilpivirine or efavirenz unlike RPV DRMs, and also, DRV, especially when combined with islatravir or lamivudine, attenuated the development of both NRTI and NNRTI resistance.

Etravirine (ETR): While ETR commonly selects for K101E/P, Y181C/I/V, G190E and F227C. L100I (EFV DRM), K101E/P, Y188L and M230L are associated with decreased susceptibility to ETR and/or RPV. G190E causes significant resistance to ETR, while Y181I/V, though uncommon, is required for high resistance to ETR, as well as two or more NNRTI-resistance mutations **(Melikian *et al.,* 2014; Vingerhoets *et al.,* 2010)**.

Efavirenz (EFV): EFV selects for L100I, K101P, K103N, V106M, Y188C/L and G190A/E/Q/S). G190E causes high-level efavirenz resistance, while L100I, K101EP, K103NS, Y188L, G190ASE and M230L cause intermediate-level resistance to EFV **(Melikian *et al.,* 2014; Tang & Shafer, 2012)**.

Nevirapine (NVP): NVP selects for K101P, K103N/S, V106A/M, Y181C/I/V, Y188C/L and G190A/E/Q/S. K101EP, K103NS, V106AM, Y181CIV, Y188L, G190ASE and M230L mutations cause high-level resistance to nevirapine. E138G, V179F and M230L are associated with reduced susceptibility to NVP and/or EFV **(Melikian *et al.,* 2014; Tang & Shafer, 2012)**.

Rilpivirine (RPV): RPV selects for L100I, K101P, Y181I/V, G190E and F227C. L100I (EFV DRM), K101E/H/P, Y188Land M230L are associated with decreased susceptibility to ETR and/or RPV **(Melikian *et al.*, 2014; Tang & Shafer, 2012)**.

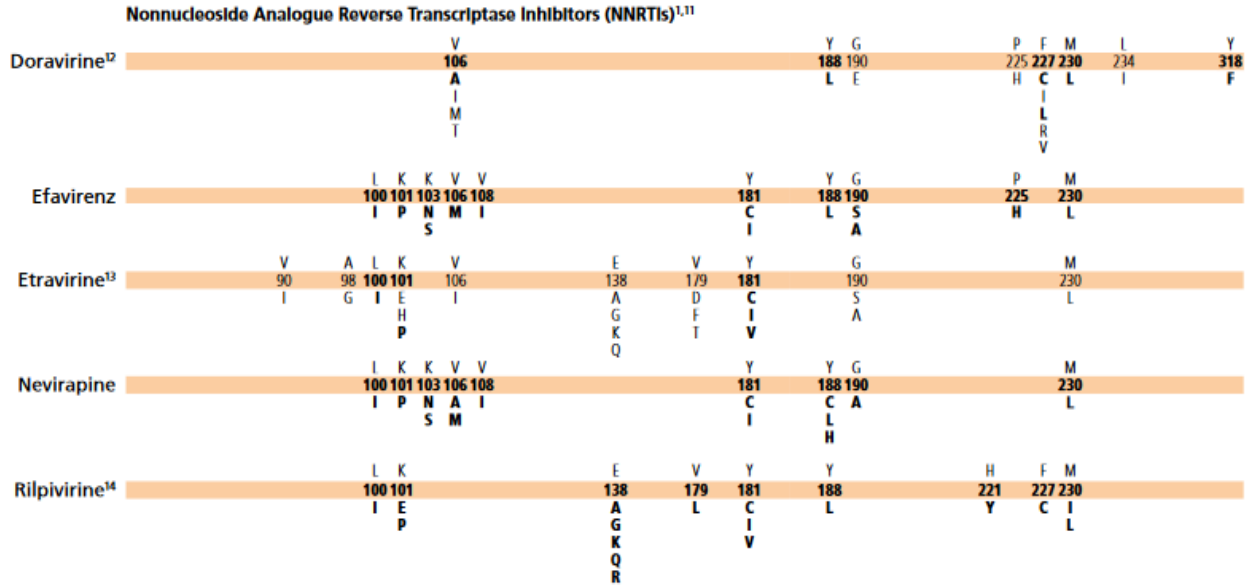


Figure 11: RT gene mutations associated with resistance to NNRTIs **(Wensing *et al.*, 2022)**.

c. Resistance to CAIs

Lenacapavir (LEN), the new and only HIV capsid inhibitor, demonstrates a distinct resistance profile compared to other antiretrovirals. Laboratory studies **(Yant *et al.*,)** using escalating doses of lenacapavir identified specific mutations (L56I, M66I, Q67H, K70N, N74D, N74S, and T107N) in the HIV capsid that confer reduced susceptibility to the drug, with Q67H and N74D being the most common. These mutations, except for Q67H, also impaired the virus's ability to infect cells and replicate. Importantly, viruses resistant to lenacapavir remained susceptible to other classes of antiretroviral drugs, including NRTIs, NNRTIs, INSTIs **(Yant *et al.*,)** and NRTTI **(Diamond *et al.*, 2024)**.

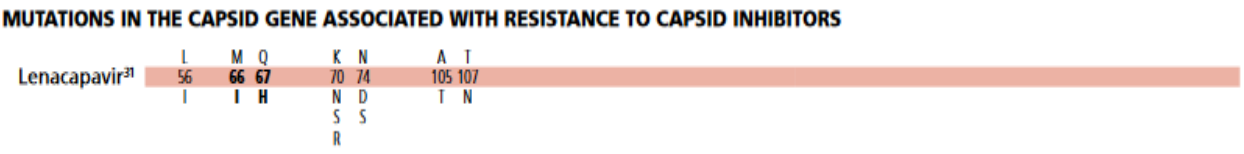


Figure 12: Mutations in the CA gene associated with resistance to CAIs **(Wensing *et al.*, 2022).**

d. Resistance to PIs

PIs block HIV maturation. While nine FDA-approved PIs exist, their usage varies. Ritonavir, often used at low doses (denoted '/r'), boosts other PIs' effectiveness by inhibiting their metabolism **(*HIV/AIDS Drugs | NIH*, n.d.)**. Nelfinavir (NFV), on the other hand, is rarely used due to its limited effectiveness. Indinavir, although a potent antiviral, carries a high risk of kidney toxicity, significantly limiting its use. This leaves six PIs commonly studied, with three, atazanavir (ATV), lopinavir (LPV), and darunavir (DRV), being widely used in clinical practice, typically in combination with ritonavir for enhanced effectiveness **(Thompson *et al.*, 2010)**. Current US HIV treatment guidelines recommend atazanavir/r and darunavir/r as first-line protease inhibitors (PIs), with lopinavir/r as an acceptable alternative **(*Guide to Protease Inhibitors for HIV*, 2023)**. While fosamprenavir/r (APV) and saquinavir/r are also considered options, they lack extensive clinical data. Tipranavir/r is reserved for heavily treatment-experienced patients, as it may be the only effective PI in cases with darunavir/r resistance **(*HIV/AIDS Drugs | NIH*, n.d.)**.

Analysis of the changes in structure and activity of the mutants suggested three categories of mutations. Mutations that enable proteases to: reduce the binding affinity of drugs yet still maintaining catalytic activity (D30N, V32I, I47V, G48V, I50V, Val82 and I84V), release of bound inhibitor (L24I, I50V and F53L), and then, distal mutations can cause PI resistance in unique ways, we have L76V which causes resistance to DOR, APV, IDV and LPV, while conferring susceptibility to the other PIs **(Weber *et al.*, 2015)**.

ATV/r: Multiple mutations can cause resistance to ATV/r, with I50L, I84V, and N88S being the most impactful. Interestingly, the presence of both M46I and L76V mutations might actually increase sensitivity to atazanavir in the absence of other resistance mutations. **(Young *et al.*, 2010)**.

DRV/r: DOR/r effectiveness decreases as the number of associated mutations increases. Specifically, I47V, I54M, T74P, and I84V negatively impact DOR/r response, while V82A is associated with a positive response. For DRV/r, the presence of two or more mutations from the following list at baseline (V11l, V32I, L33F, I47V, I50V, I54L/M, T74P, L76V, I84V, or L89V) was linked to decreased treatment response **(Sterrantino *et al.*, 2012)**.

LPV/r: The presence of three or more specific protease mutations (L10F/I/R/V, K20M/N/R, L24I, L33F, M36I, I47V, G48V, I54L/T/V, V82A/C/F/S/T, and I84V) at baseline is

linked to reduced virologic response to LPV/r. Additionally, the combination of V32I and 47A/V mutations confers high-level resistance **(Young *et al.*, 2010)**.

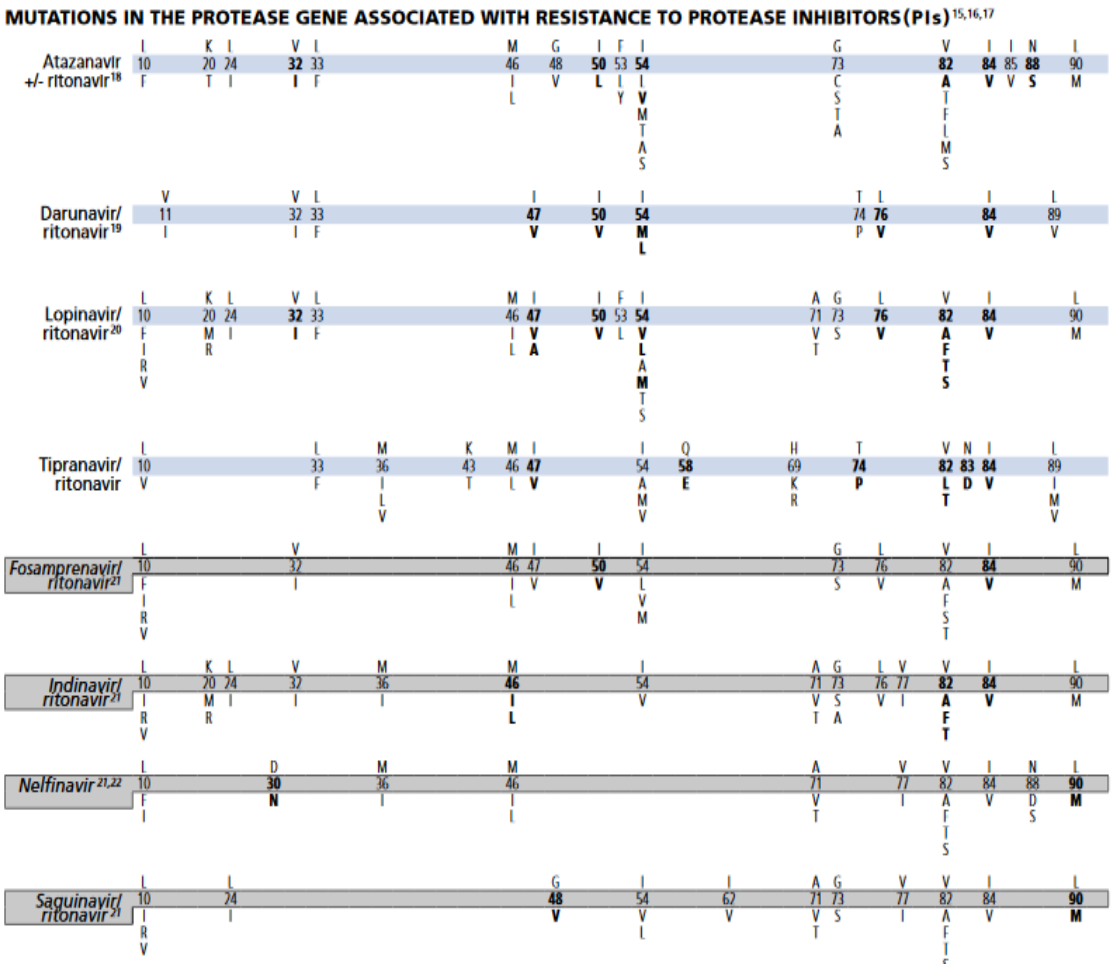


Figure 13: Mutations in the PR gene associated with resistance to PIs **(Wensing *et al.*, 2022).**

e. Resistance to EIs

Enfuvirtide (EFV) is highly susceptible to viral resistance. In patients with limited treatment options ("salvage therapy") who don't receive a sufficient number of other effective antivirals alongside enfuvirtide, resistance develops quickly. Mutations in the enfuvirtide target, specifically G36DEV, V38EA, Q40H, N42T and N43D, are the key drivers of resistance. A single mutation typically reduces enfuvirtide susceptibility by about 10-fold, while two mutations often result in a 100-fold reduction, making the drug significantly less effective **(Marcelin, Reynes, *et al.*, 2004)**.

Maraviroc (MRC): HIV can develop resistance to CCR5 antagonist drugs, but there is no clear pattern of mutations associated with this resistance according to **Wensing *et al.*, (2022)** update on HIV-1 DRMs, since some mutations occur in the V3 loop of gp120, allowing the virus to bind to CCR5 even with the drug present **(Ratcliff *et al.*, 2013)**, while other mutations have been observed in gp41 in lab settings, but their clinical relevance is unknown. More research is needed to identify specific mutations indicating CCR5 antagonist resistance **(Wensing *et al.*, 2022)**.

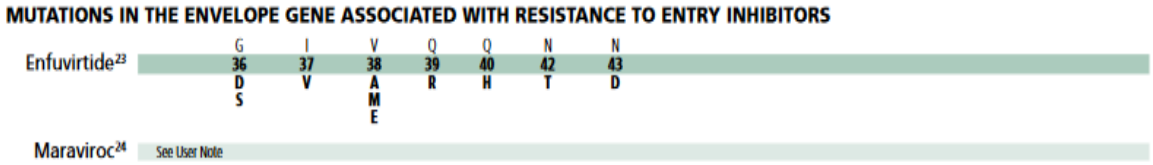


Figure 14: Mutations in the ENV gene associated with resistance to EIs ***(*Wensing *et al.*, 2022).**

f. Resistance to INSTIs

First-generation integrase strand transfer inhibitors (InSTIs) like elvitegravir and raltegravir are no longer recommended as first-line HIV treatment options for most individuals. This is due to their low barrier to resistance development and the high likelihood of cross-resistance among this class of drugs. Second-generation InSTIs (dolutegravir, bictegravir, and cabotegravir) are now the preferred choice for most treatment scenarios **(Saag *et al.*, 2020)**.

Dolutegravir (DTG) demonstrates a higher barrier to HIV resistance than older InSTIs like raltegravir and elvitegravir. It takes a combination of mutations (including Q148 and G140) to reduce its effectiveness, and complete resistance likely requires even more mutations **(Canducci *et al.*, 2011)**.

As for resistance to **cabotegravir** (a long-acting) and **bictegravir, Ndashimye *et al.*, (2021)** showed that while single or combined secondary mutations in HIV-1 did not significantly affect bictegravir and cabotegravir susceptibility, combinations of primary INSTI-resistance mutations (like E138A/G140A/G163R/Q148R or E138K/G140A/S147G/Q148K) significantly reduce susceptibility to both drugs, with fold change in EC50 values from 429 to 1000× for cabotegravir, and 60 to 100× for bictegravir**.** These results show that CAB and BIC remain an alternative treatment for resistant infections to first generation INSTIs but previous exposure to RAL may reduce therapy efficacy.

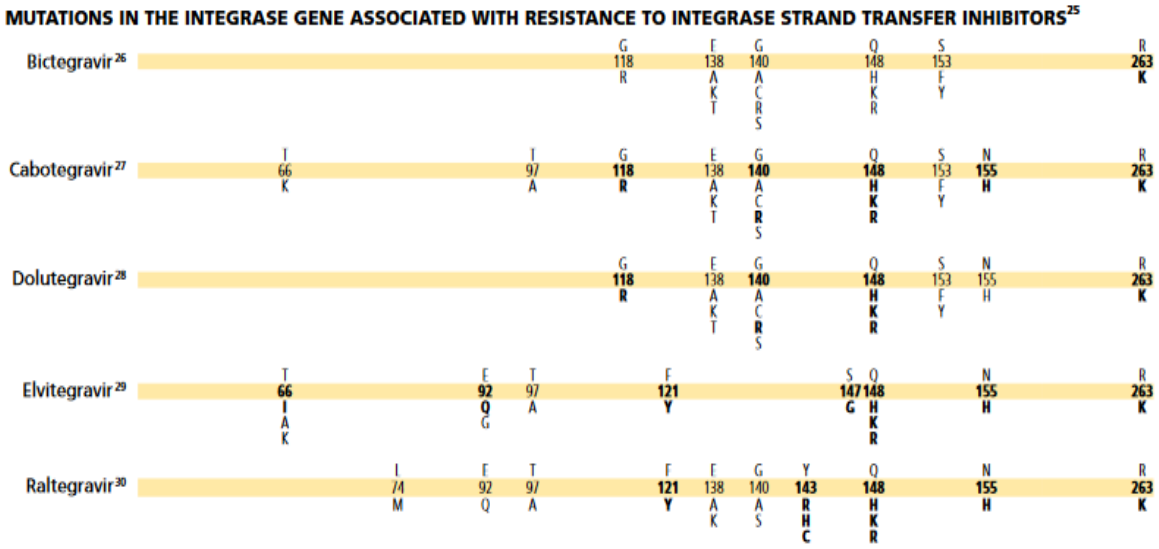


Figure 15: Mutations in the IN gene associated with resistance to INSTIs **(Wensing *et al.*, 2022).**

g. Resistance to NRTTIs

Viral resistance selection studies and antiviral assays with HIV-1 variants have been conducted to understand the mutations within RT that alter susceptibility to ISL and identified substitutions of M184 to V or I as the primary ISL resistance-associated variants **(Cilento *et al.*, 2021; Kawamoto *et al.*, 2008; Maeda *et al.*, 2014).** It is in this same light that a recent study **(Diamond *et al.*, 2022)** later performed additional viral resistance selection experiments with subtype A, B, or C wild-type (WT) viruses or subtype B viruses containing known resistance-associated mutations in RT. They identified RT mutations M184I and M184V as the most frequently observed changes. More detailly, the experiment revealed that: (i) resistance to ISL in vitro is associated with M184I or M184V in subtypes A, B, and C, (ii) Of all single-codon variants tested, M184I and M184V conferred the largest fold reductions in potency to ISL (FC of 6.2 and 6.8, respectively) compared to wild type (WT), (iii) only combinations of variants containing M184I or M184V conferred reduced potencies greater than 6.8-fold, (iv) A114S was an emergent substitution that when combined with other substitutions further reduced susceptibility >2-fold, and that ISL has a distinct resistance profile compared to approved NRTIs.

	ISL		TDF		AZT		3TC		FTC	
HIV-1 variant	IC_{50} (nM) (*n*)[b]	FC[c]	IC_{50} (nM) (*n*)[b]	FC[c]	IC_{50} (nM) (*n*)[b]	FC[c]	IC_{50} (nM) (*n*)[b]	FC[c]	IC_{50} (nM) (*n*)[b]	FC[c]
WT	0.80 ± 0.08 (11)	1	45.99 ± 11.56 (16)	1	9.99 ± 1.38 (16)	1	849.86 ± 265.52 (6)	1	291.28 ± 47.80 (17)	1
M41L	1.65 ± 0.11 (7)	2.1	97.16 ± 12.88 (8)	2.1	19.82 ± 2.50 (10)	2	1,481.14 ± 301.45 (8)	1.7	370.94 ± 54.58 (8)	1.3
K65R	0.34 ± 0.06 (6)	0.4	492.20; 580.36 (2)	11.6	7.79; 9.42 (2)	0.9	4,590.35; 4,787.03 (2)	5.5	NA	NA
L74I	1.08 ± 0.09 (5)	1.4	89.50 ± 25.02 (6)	1.9	20.83 ± 3.42 (7)	2.1	2280.97 ± 285.92 (5)	2.7	576.15 ± 86.19 (8)	2
A114S	1.74 ± 0.31 (4)	2.2	2.89 ± 2.01 (6)	0.1	4.67 ± 0.28 (7)	0.5	715.56 ± 84.49 (4)	0.8	217.75 ± 24.97 (8)	0.7
M184I	8.50 ± 0.59 (7)	10.6	44.96 ± 1.87 (4)	1	8.68 ± 0.55 (4)	0.9	>42,016.67 (6)	>49.4	>42,016.67 (4)	>144.2
M184V	9.89 ± 0.93 (7)	12.4	48.09 ± 10.38 (4)	1	9.40 ± 1.07 (4)	0.9	>42,016.67 (7)	>49.4	>42016.67 (8)	>144.2
T215Y	1.99 ± 0.33 (7)	2.5	192.86 ± 13.04 (3)	4.2	82.79 ± 8.07 (6)	8.3	4,045.70 ± 715.99 (5)	4.8	1184.01 ± 135.29 (7)	4.1
A114S/M184V	28.25 ± 6.93 (8)	35.3	1.81 ± 0.38 (8)	0.04	2.39 ± 0.40 (11)	0.2	>42,016.67 (10)	>49.4	>42,016.67 (12)	>144.2
M41L/A114S/M184V	32.25 ± 6.20 (4)	40.3	1.98 ± 0.25 (4)	0.04	3.82 ± 0.38 (4)	0.4	>42,016.67 (4)	>49.4	>42,016.67 (4)	>144.2
M41L/L210W/T215Y	2.95 ± 0.13 (4)	3.7	271.97 ± 26.11 (4)	5.9	73.80 ± 12.01 (4)	7.4	1,796.43 ± 249.18 (3)	2.1	764.22 ± 84.02 (7)	2.6
D67N/K70R/T215F/K219Q	3.05 ± 0.43 (7)	3.8	192.94 ± 7.21 (3)	4.2	78.19 ± 12.79 (5)	7.8	4,099.30 ± 508.53 (3)	4.8	3,354.82 ± 218.48 (4)	11.5
D67N/K70R/A114S/T215F/K219Q	2.72 ± 0.44 (6)	3.4	3.51 ± 0.40 (7)	0.1	6.73 ± 1.09 ()	0.7	969.34 ± 77.79 (3)	1.1	646.52 ± 138.82 (7)	2.2

[a]3TC, lamivudine; AZT, zidovudine; FC, fold change; FTC, emtricitabine; HIV-1, human immunodeficiency virus type 1; IC_{50}, half maximal inhibitory concentration; ISL, islatravir; NA, not available; TDF, tenofovir disoproxil fumarate; WT, wild-type.

[b]IC_{50} (half maximal inhibitory concentration; nM) displayed as geometric mean ± standard deviation.

[c]Fold change (FC) is calculated as (IC_{50} against mutant isolate) / (IC_{50} against WT) for each test article.

Figure 16: Antiviral activity of ISL and comparator molecules against a panel of HIV-1 variants with mutations in RT in a multiple cycle assay with 10% NHS **(Diamond *et al.*, 2022)**.

In figure 16 above, we as well identify the perfect match between ISL and TDF combination, since both drugs happen to keep their activity in the presence of the other'\s DRM (M184V/I for ISL and K65R for TDF). This was later confirmed by Cilento *et al.,* (2023)**,** and even more, they showed the opposing resistance profiles (antagonism) between ISL and TDF, highlighting the ability of K65R and M184V (which overrides M184I with time) to enhance susceptibility to ISL and TDF, respectively. Finally, the double mutant, K65R/M184, however conferred mild resistance to both ISL and TDF, without enhanced sensitivity to either drug **(Cilento *et al.*, 2023)**. Another study on cross resistance showed that of ISL and LEN showed no antagonism but equally no cross resistance. However, combining these two drugs was more effective at suppressing viral replication than either drug alone, even at multiple lower doses **(Diamond *et al.*, 2024)**. In a different study, DOR selection of NRTI and NNRTI DRMs was slowed down in the presence of ISL **(Brenner *et al.*, 2023)**. These findings support further exploration of ISL and TDF, LEN or DRV in a combination therapy for HIV-1.

Chapter Two:
Materials and Methods

CHAPTER 2: MATERIALS AND METHODS

I. STUDY DESIGN

1. Study design and setting

We used a retrospective, cross-sectional, analytical study design, at the Chantal Biya International Center for Research on HIV/AIDS prevention and management (CIRCB), located at Melen-Yaoundé.

2. Study duration

Our study effectively ran for six (06) months, from April to September 2024, and included archived patients received between 2016 and 2023 for genotypic resistance testing at CIRCB.

3. Target Population

The study targeted all PLHIV in Cameroon, who have been exposed to ART regimens containing RTIs.

4. Type of sampling

We carried out a consecutive and exhaustive sampling.

5. Sample size

The sample size was calculated using a statistical formula, considering a previous study of Takou *et al.,* 2019 which estimated M184VI prevalence at 83.2% (**Takou *et al.,* 2019**).

$$N = \frac{z^2 \times p\,(1 - P)}{d^2}$$

- **N:** Sample size
- **d:** Margin of error, 5% = 0,05
- **P:** prevalence, 83.2% (representing the prevalence of M184V/I in Cameroonian population estimated by **Takou *et al.,* 2019**)
- **Z:** Z is the Z-score corresponding to the desired level of confidence, 95% = 1,96

After numerical application, we obtained a minimum sample size of **219** participants (218.32 rounded-up).

6. Selection criteria

6.1. Inclusion criteria

- Confirmed diagnosis of HIV-1 infection

- History of exposure to ART regimens containing RTIs, documented in the database..
- Having performed genotypic resistance testing at CIRCB between 2016 and 2023.

6.2. Non-inclusion criteria

Any subject with non-confirmed HIV diagnosis, undocumented clinical follow-up data and history of exposure to RTIs was not included in this study.

6.3. Exclusion criteria

- DNA sequences of poor quality or that were uninterpretable
- Incomplete ART history.

II. MATERIALS

For our study, we used archived patient data and HIV sequences stored in a protected CARE database (CIRCB). All sociodemographic and clinical information of the patients was extracted using Microsoft Access, then cleaned and organized using Microsoft Excel. HIV sequences were aligned and gaps removed using BioEdit software (version 5.0.9). We used the Stanford HIVdb (version 9.7) to interpret drug resistance mutations and deduce viral susceptibility to antiretroviral drugs. Viral subtypes were also interpreted using the Stanford database, and a phylogenetic tree was constructed using Nextstrain-Nextclade software (https://clades.nextstrain.org). SeqScape Software (version 2.6) was used to calculate mutation prevalence, while covariation analyses, statistical tets and cluster analysis were performed using the Covarius package for R software (version 4.4.1).

III. METHODS

1. Origin of HIV RT sequences

The enrolled patients underwent an established in-house protocol for genotypic resistance testing. Initially, 10 ml of blood was drawn from those presenting for resistance testing at the CIRCB institute. Plasma was then separated from the whole blood and stored at temperatures between -20°C and -80°C if immediate manipulation was not feasible. Viral RNA extraction was performed using a commercially available protocol (Qiagen). The extracted viral RNA underwent a series of conventional PCR reactions (RT-PCR and Nested PCR), which allowed for exponential amplification of genetic material based on the initial RNA concentration. The quantity of genetic material obtained after the PCR reactions was estimated using the formula N = No x 2^n, where N represents the final concentration of genetic material, No is the initial concentration, and n is the

number of PCR cycles. The effectiveness and integrity of the PCR reactions were verified through agarose gel electrophoresis, after which the amplicons were column-purified. The products were quantified via spectrophotometry and subsequently subjected to a sequencing reaction, which is essentially another PCR that utilizes fluorescent ddNTPs to mark the DNA sequence. The sequencing products were purified using SEPHADEX gel (Separation Pharmacia Dextran) to eliminate excess reactants and ensure the purity of the DNA products. An automated sequence analyzer (AB3500) was used for nucleotide sequence determination, with sequence assembly and editing performed using SeqScape v2.6 or Recall v2.28. The sequences were interpreted using version 8.8 of the Stanford drug resistance database for mutation profiles and potentially active drugs.

2. Participant Enrollment

A total of 1170 participants were selected based on our study criteria from the patient pool in the CIRCB Antiviral Resistance (CARE) database. We extracted their socio-demographic, clinical, immuno-virological data, and therapeutic history using Microsoft access 2021.

3. Data entry and analysis

Data analysis was performed using Microsoft Excel 2021. Descriptive statistics were calculated for quantitative variables, including median and interquartile range, meanwhile qualitative variables were summarized using frequencies and proportions. All references were managed using Zotero software version 7.0.9.

4. Specimen used for analysis

This study analyzed 1170 patient's HIV-1 reverse transcriptase (RT) sequences obtained from the Chantal BIYA International Reference Centre for research on the HIV/AIDS prevention and management Antiviral Resistance Evaluation (CIRCB-CARE) database in Cameroon. These sequences were collected from patients failing first-line ART (2NRTI + NNRTI, n=671), second-line (2NRTI + PI/r, n=470), and third-line ART (2NRTI + INSTI = 29) between 2016 and 2023. Only patients with a clearly documented treatment history (available in their medical record) were enrolled; all participants on treatment were experiencing virologic failure (i.e. a sustained plasma viral load > 1000 copies/ml).

5. Subtyping and drug resistance determination

Nucleotide sequences were aligned with subtype/CRFs reference sequences from the Los Alamos National Laboratory (LANL) database using the CLUSTAL.W integrated into Bioedit version 5.0.9 software. Following comparison of each sequence to the subtypes and CRFs reference sequences (database accessed on 8/2/2024), gaps were removed from the final alignments. The phylogenic tree was constructed using Nextstrain-Nextclade software(https://clades.nextstrain.org).**(Aksamentov *et al.*, 2021)**

6. Mutation's prevalence

HIV-1 sequences were analyzed using SeqScape (V2.6 Initial License) and DRMs were interpreted using the Stanford HIVdb.v9 (https://hivdb.stanford.edu/hivdbby-mutations/) to determine the prevalence of M184V/I as well as the rate of co-occurrence with other major RTIs-DRMs.

7. Mutation's covariation (statistical analysis)

Pairwise interactions between the M184V mutation and other NRTIs and NNRTIs major DRMs were analyzed using the Covarius package for R software version 4.4.1. Fisher's exact test was employed to determine if the co-occurrence of these mutated residues differed significantly from what would be expected if they were independent events. Furthermore, the binomial correlation coefficient (phi) was calculated to quantify the strength of the correlation between M184V and other major RT drug mutations**(Teto *et al.*, 2022)**.

8. Deduction of potential ARV for combination

We utilized the Stanford HIVdb.v9 of major drug resistance mutations for NRTIs and NNRTIs to assess the susceptibility profiles of our study population to each antiretroviral (ARV). This assessment considered major mutations that exhibited significant correlation with M184V. For each drug, the impact of a mutation was categorized as follows: high-level reduced susceptibility, reduced susceptibility, reduced susceptibility in combination with other NRTI-resistance mutations, increased susceptibility, or no effect.

9. Cluster analysis

To visualize the covariation patterns of mutations in detail, we employed average linkage hierarchical agglomerative clustering, a technique commonly used in phylogenetic tree construction. This method constructs clusters of increasing size by iteratively merging pairs of clusters based on minimum average inter-cluster distances. The distance between mutation pairs

was derived from the phi correlation coefficient, where a value of 1 indicates a strong positive association and -1 a strong negative association. This measure was transformed into a distance metric, with linear interpolation between these extremes. To assess the robustness of the resulting dendrogram, a bootstrap analysis was performed by repeating the clustering process 100 times on randomly sampled subsets of the original sequence data. This analysis provides confidence values for each sub-tree within the dendrogram, indicating the stability of the observed clustering patterns.**(Sing *et al.,* 2005).** For example, a bootstrap value of 1 indicates that the two mutations (or groups of mutations) were consistently grouped together across all 100 bootstrap replications.

10. Ethical considerations

We also obtained a research authorization from the CIRCB directorate to carry out this research in their establishment. The Cameroon Regional Ethics Committee of the Center region approved the study protocol. Ethical clearance ***CE Nº 0946/CRERSHC/2024 (Appendix Nº 1)***

11. Study team and resources

- **Principal Investigator:** TSAPI LONTSI Wilfried Rooker; Masters II/Medical Microbiology/UY1.
- **Supervisors:** NJIKI BIKOÏ Jacky: Associate Professor (Immunology & Cancerology, Medical microbiology)/ University of Yaounde 1; Dr. FOKAM Joseph: Lecturer (Virologist, senior scientist, head of virology laboratory, Permanent Secretary CNLS), UB
- **Laboratory supervisor:** FOKAM Joseph; Dr. NKA Alex Durand; Virologist senior researcher, CIRCB.
- **Resources:** Chantal Biya International Reference Center

12. Overall analysis pattern of the HIV-RT sequences

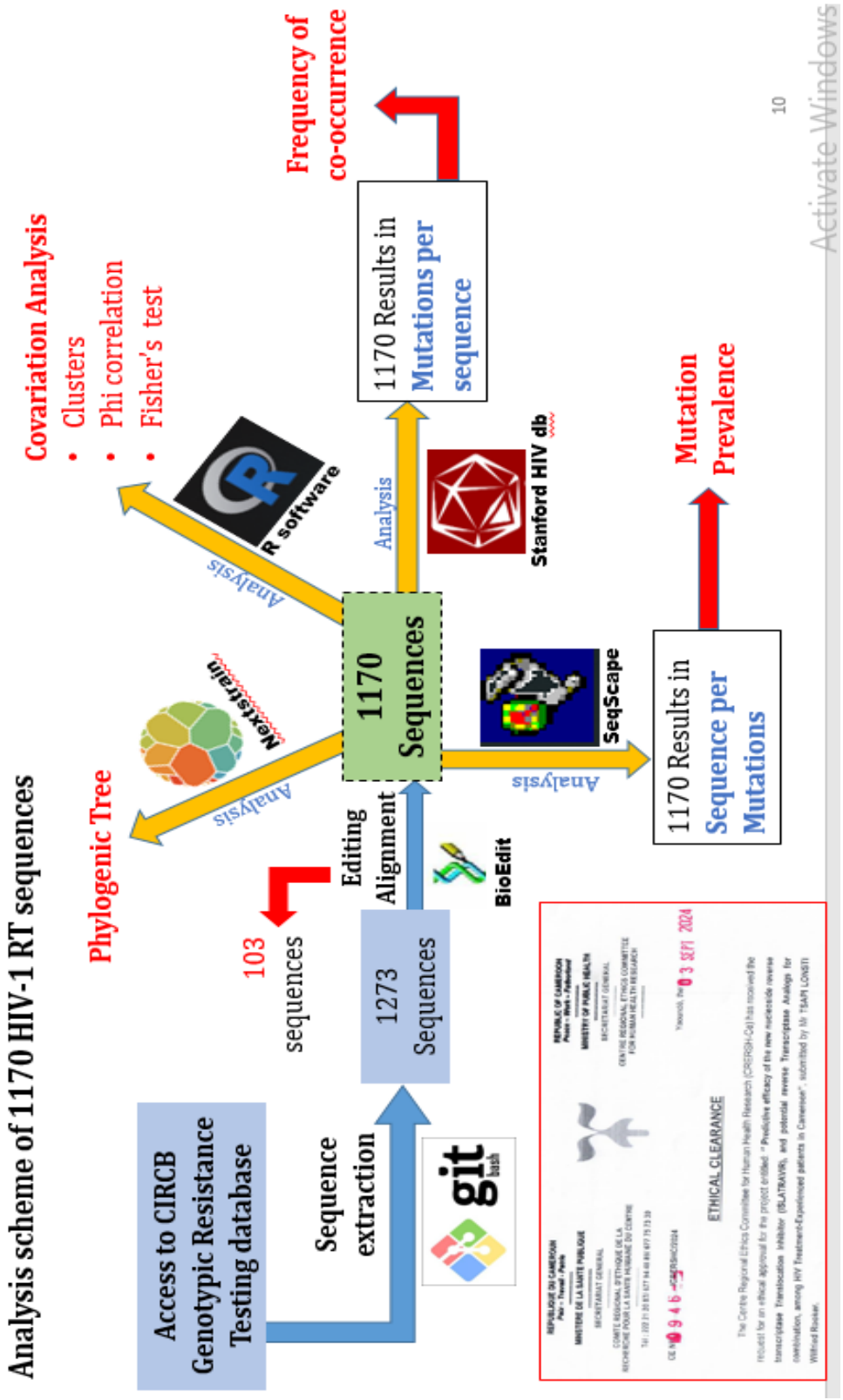


Figure 17: *Analysis pattern of 1170 HIV-RT sequencces*

13. General work flow

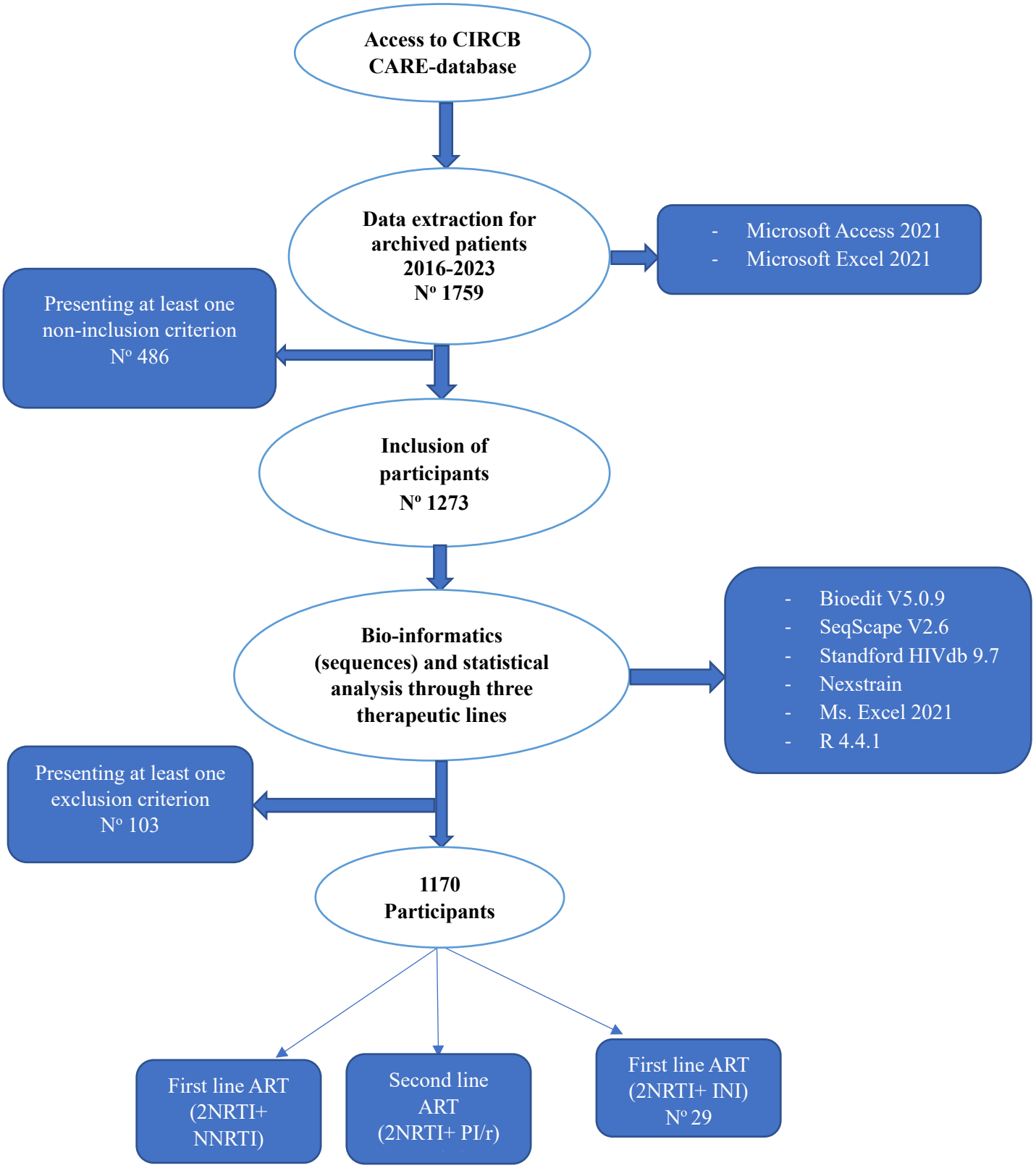


Figure 18: *General workflow*

Chapter Three: Results and Discussion

CHAPTER 3: RESULTS AND DISCUSSION

I. RESULTS

1. Demographic and clinical characteristics of study participants

Our study population consisted of 1170 PLHIV on ART, of whom 671 were on first line, 470 on second line and 29 on third line. Females were predominant (61.0%, 714/1170), and the overall median [IQR] age was 40 [28–47] years. The third line showed a lower age compared to those in first and second lines (median [IQR], years: 17 [9–38] vs. 41 [33–47] in first line-treated patients vs. 39 [20–38] in second line-treated patients). The overall median [IQR] CD4 cells count was 190 [63–665] cell/mm^3. Regarding viremia, the global median was 5 [3.5-5.3] log_{10} copies/mL, which was common throughout the three treatment lines, with varying interquartile ranges [IQR] (5 [4.2–5.5] log10 copies/mL, 5 [3.8–5.3] log10 copies/mL, and 5 [4.3–5.6] log10 copies/mL). According to ART regimen, the majority of patients on first line received TDF+3TC+EFV (63.2%), followed by AZT+3TC+NVP (31.3%); while for those on second line, the majority received LPV/r or ATV/r containing regimen (66.8% and 29.8%, respectively); among those on third line, the majority took DTG (75.9%) **(Table I).**

Table I: Socio-demographic and clinical characteristics of the study population.

		First Line(n=671)	Second Line(n=470)	Third Line(n=29)	Overall (N=1170)
Gender	Female (%)	437(65.1)	264(56.2)	13(44.8)	714 (61.0)
	Male (%)	234(34.9)	206(43.8)	16(55.2)	456 (39.0)
Age (years), median [IQR]		41 [33-47]	39 [20-48]	17 [9-38]	40 [28-47]
CD4 (cell/ul), median [IQR]		186 [63-361]	195 [58-374]	157 [63-665]	190 [63-665]
Viral load (Log_{10}copies/ml), median [IQR]		5 [4.2-5.5]	5 [3.8-5.3]	5 [3.4-5.3]	5 [3.5-5.3]
ART regimen n (%)	2NRTIs + EFV	424 (63.2)	1 (0.2)	-	425 (36.3)
	2NRTIs + NVP	210 (31.3)	-	-	210 (17.9)
	2NRTIs + ATV/r	2 (0.3)	314 (66.8)	-	316 (27.0)
	2NRTIs + LPV/r	-	140 (29.8)	-	140 (12.0)
	2NRTIs + DRV/r	-	3 (0.6)	-	3 (0.3)
	2NRTIs + NFV/r	-	1 (0.2)	-	1 (0.9E-1)
	2NRTIs + DTG	34 (2.9)	-	22 (75.9)	56 (4.8)
	Others	1 (0.1)	11 (2.3)	7 (24.1)	19 (1.6)

Note: IQR: Interquartile range; NRTIs: Nucleoside reverse transcriptase inhibitors; EFV: Efavirenz; NVP: Nevirapine; LPV/r: Lopinavir; ATV/r: Atazanavir; DRV/r: Darunavir; NFV/r:

Nelfinavir; DTG: Dolutegravir; PI/r: ritonavir boosted protease inhibitors; RTI: Reverse transcriptase inhibitors.

All regions in the country were represented in our study, with the majority of patients coming from the Center, Littoral and Northwest regions **(Fig. 18)**. The greatest majority lived in urban areas (82.6%, 966/1170)

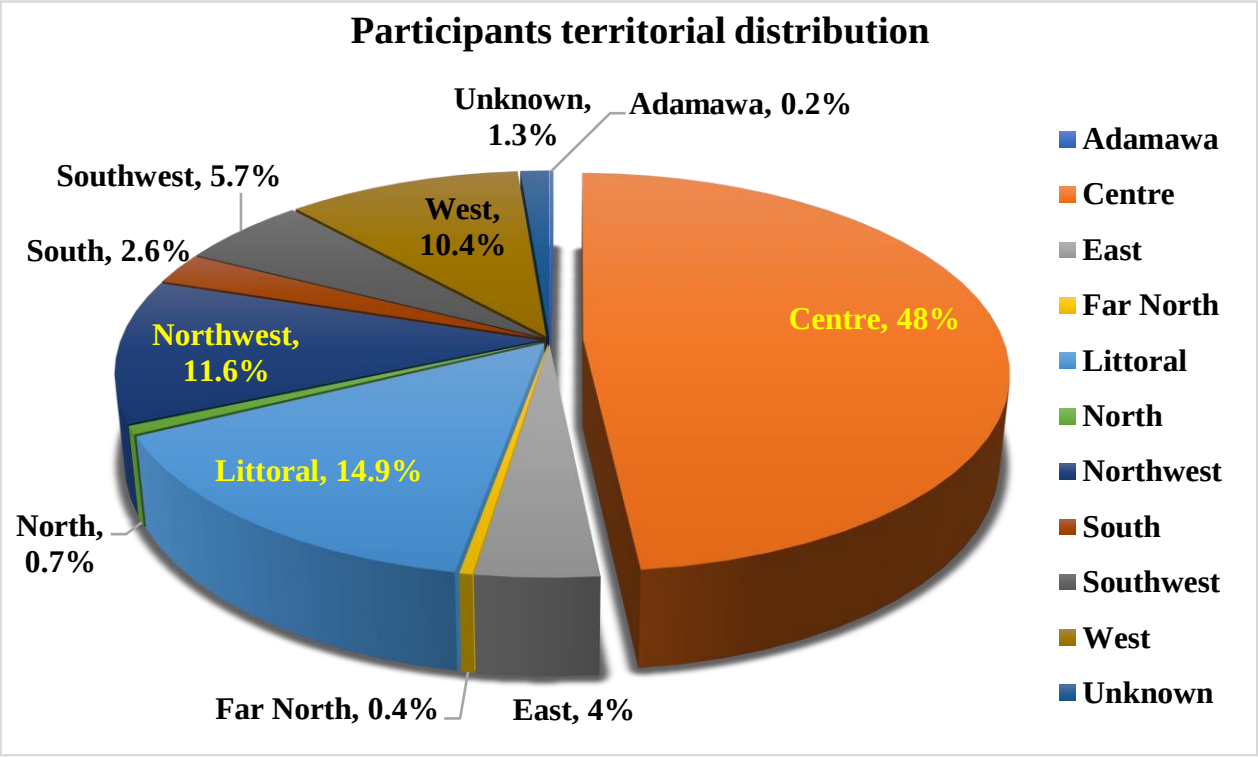


Figure 19: Territorial distribution of our study population according to Cameroon regional subdivisions

- **Viral subtypes distribution**

Among the 1170 study participants, a diverse distribution of HIV-1 non-B clades was observed, with CRF02_AG being the most prevalent (60.6%), followed by subtypes A1 (9.49%), G (5.81%), F2 (4.44%), and CRF11_cpx (2.14%). Additional combinations included A1+G (2.48%), A1+J (1.54%), CRF18_cpx (1.54%), and CRF01_AE (1.03%). Additionally, eleven participants (0.94%) were found to carry the B subtype **(*Fig. 20*).**

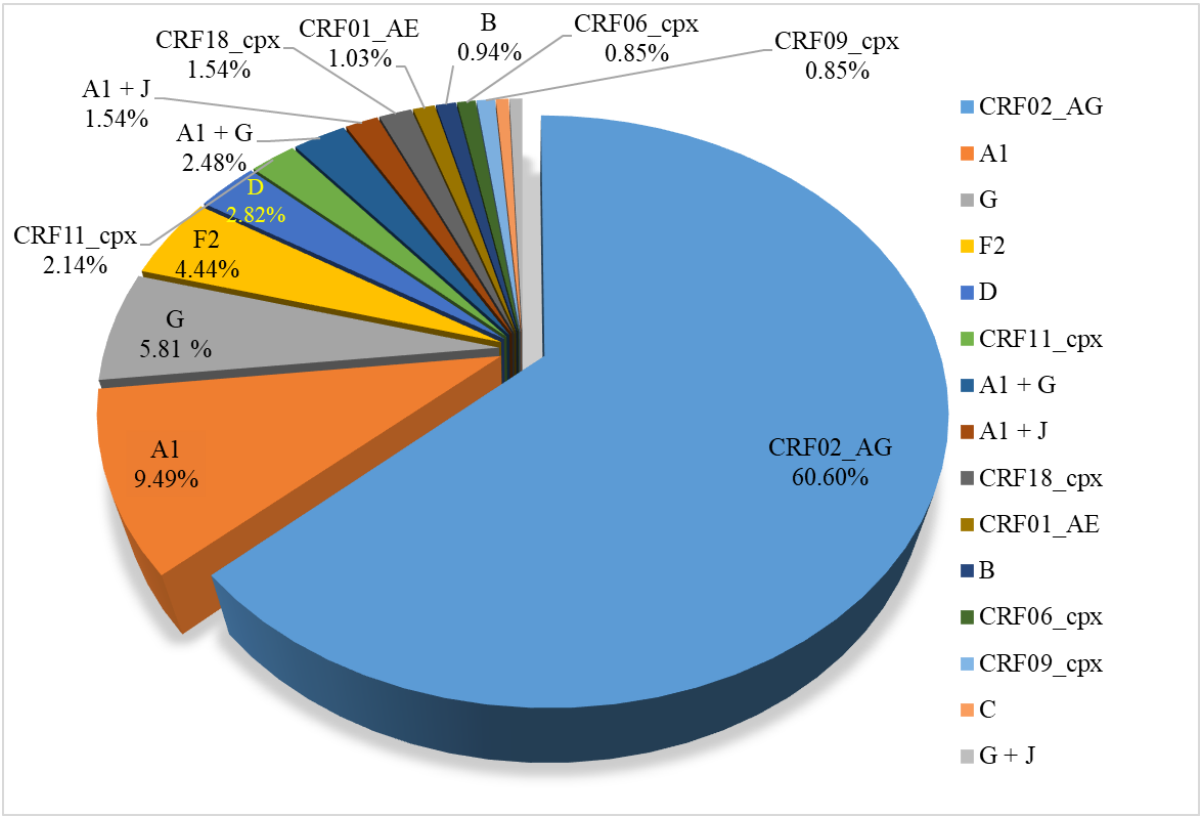
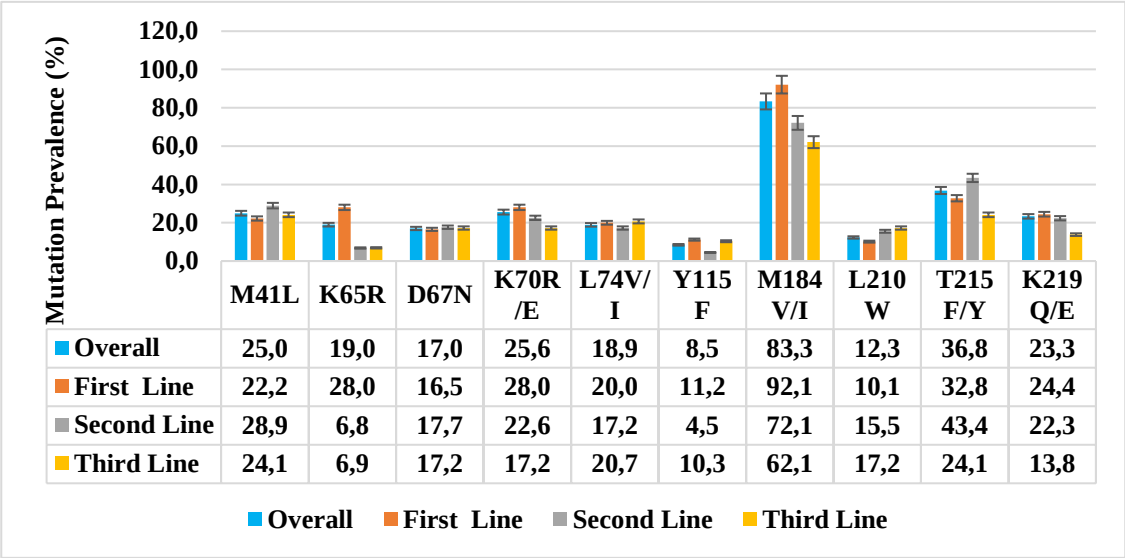


Figure 20: Overall rates of HIV subtypes.

2. Patterns of Reverse Transcriptase Drug Resistance Mutations

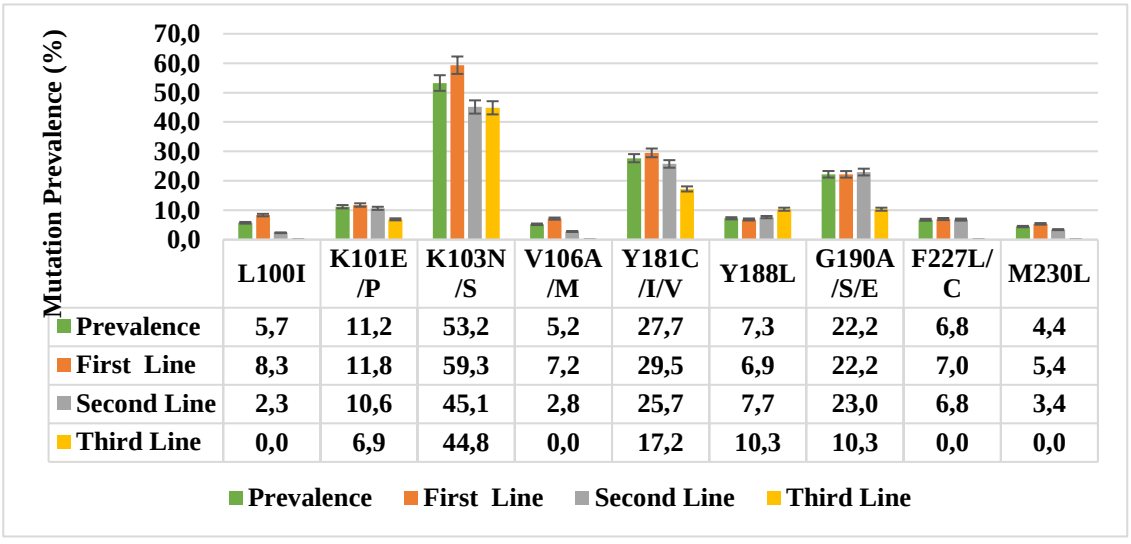
By analyzing the reverse transcriptase portion among patients failing first-, second-, and third-line ART, we identified 15 NRTI and 17 NNRTI major drug resistance mutations (DRMs) **(Fig. 21)**. The overall prevalence of reverse transcriptase drug resistance mutation was 77.4% for NRTI DRMs and 49.1% for NNRTI DRMs. NRTI DRM prevalence decreased across treatment lines, with 83.6%, 69.6%, and 62.1% observed in first-, second-, and third-line patients, respectively. Similarly, NNRTI DRM prevalence decreased across treatment lines, with 56.3%, 39.4%, and 41.4% observed in first-, second-, and third-line patients, respectively. The most frequent NRTI DRMs were M184V/I (83.3%), which was most prevalent among first-line patients; M41L (25.0%), highest among second-line patients; and T215F/Y (36.8%), most prevalent among second-line patients as well. For NNRTIs, the most frequent DRMs were K103N/S (53.2%), most prevalent among first-line patients; Y181C/I/V (27.7%), also highest among first-line patients; and

G190A/S/E (22.2%), which was most prevalent among second-line patients. Overall, both NRTI and NNRTI mutations showed decreasing rates from first to the third line through the second line **(Fig. 21)**.



	M41L	K65R	D67N	K70R /E	L74V/ I	Y115 F	M184 V/I	L210 W	T215 F/Y	K219 Q/E
Overall	25,0	19,0	17,0	25,6	18,9	8,5	83,3	12,3	36,8	23,3
First Line	22,2	28,0	16,5	28,0	20,0	11,2	92,1	10,1	32,8	24,4
Second Line	28,9	6,8	17,7	22,6	17,2	4,5	72,1	15,5	43,4	22,3
Third Line	24,1	6,9	17,2	17,2	20,7	10,3	62,1	17,2	24,1	13,8

Figure 21a: NRTI major drug resistance mutations' prevalence, with varying tendencies throughout first, second and third line.



	L100I	K101E /P	K103N /S	V106A /M	Y181C /I/V	Y188L	G190A /S/E	F227L/ C	M230L
Prevalence	5,7	11,2	53,2	5,2	27,7	7,3	22,2	6,8	4,4
First Line	8,3	11,8	59,3	7,2	29,5	6,9	22,2	7,0	5,4
Second Line	2,3	10,6	45,1	2,8	25,7	7,7	23,0	6,8	3,4
Third Line	0,0	6,9	44,8	0,0	17,2	10,3	10,3	0,0	0,0

Figure 21b: NNRTI major drug resistance mutations' prevalence, with varying tendencies throughout first, second and third line.

3. Antiretroviral drugs overall susceptibility rate

Based on the mutation profile and their prevalence in our study population, we assessed the overall susceptibility of each antiretroviral agent according to the Stanford list of mutations(*HIV Drug Resistance Database*, n.d.). For NRTIs, the highest overall susceptibility was observed for ABC (91.5%), followed by TDF (81.4%) and AZT (65.2%). In contrast, 3TC and FTC both exhibited a susceptibility of 24.1%. For NNRTIs, the leading agents were DOR (93.7%), RPV (88.8%), and ETR (72.5%), while EFV and NVP both had a susceptibility of 49.9% **(Fig. 22)**.

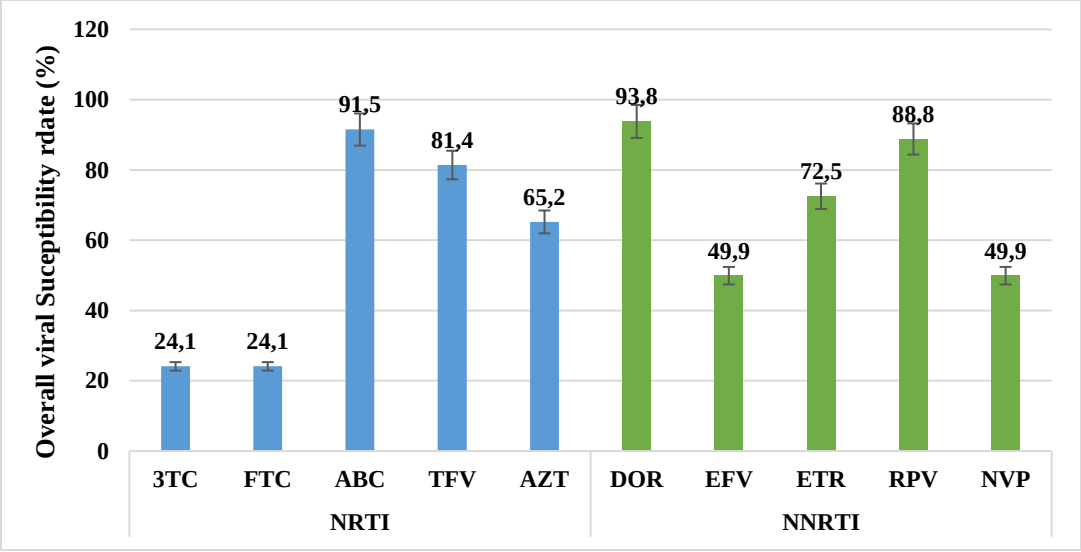


Figure 22: Predictive antiretroviral susceptibility in Cameroon, derived from mutation prevalences. blueNRTIs; greenNNRTIs.

4. Covariation of major reverse transcriptase DRMs with M184V

Another goal of our study was to assess the covariation of HIV RT-M184V mutations with other mutations observed in the reverse transcriptase gene of 1,170 RTI-treated patients, focusing on RT major drug resistance mutations according to the Stanford list of mutations **(*HIV Drug Resistance Database*, n.d.)**. To identify significant patterns of pairwise correlations between M184V and other major RT mutations observed in isolates from RT-treated patients, we calculated the binomial correlation coefficient (phi) and its statistical significance for each pair of mutations**.**

a. Reverse transcriptase major DRMs involved in positive correlations with M184V

In the first line, eight mutations associated with RTI exposure showed significant (adjusted $p < 0.001$) positive correlations with M184V. In particular, NRTIs were; K70R (phi = 0.19, $p < 0.001$);

L74I (phi = 0.18, p < 0.001), M41L (phi = 0.19, p < 0.001), T215Y (phi = 0.18, p < 0.001), T215F (phi = 0.18, p < 0.001), and K219E (phi = 0.14, p < 0.008), while NNRTIs were; K103N (phi = 0.16, p < 0.004), and G190A (phi = 0.12, p < 0.05). In the second line, M184V showed significant positive correlations with eleven mutations; T215Y (phi = 0.34, p < 0.001), and M41L (phi = 0.33, p < 0.001), D67N (phi = 0.28, p < 0.001), T215F (phi = 0.28, p < 0.001), L210W (phi = 0.26, p < 0.001), K70R (phi = 0.26, p < 0.001), L74I (phi = 0.20, p < 0.001), K219E (phi = 0,19, p < 0.001), and K219Q (phi = 0.19, p < 0.001) for NRTIs; and G190A (phi = 0.22, p < 0.001), K103N (phi = 0.16, p < 0.05), and K103S (phi = 0.14, p < 0.05) for NNRTIs. Among all 29 participants in third line, no mutation showed a significant correlation with M184V **(*Table II*)**.

Table II: Significantly correlated pairs of M184V with HIV-1 RT major or accessory resistance mutations

ART Line	Frequency M184V (%)[a]	Covariated Mutations	Frequency (%)[b]	Covariated frequency (%)	phi	p-value[c] (0.001)
I	**561 (83.61)**	K70R	122 (18.18)	84 (12.52)	0.19	6.69E-06
		L74I	111 (16.54)	74 (11.03)	0.18	6.69E-06
		T215F	109 (16.24)	88 (13.11)	0.18	1.89E-05
		M41L	149 (22.21)	120 (17.88)	0.19	2.60E-05
		T215Y	111 (16.54	92 (13.71)	0.18	4.79E-05
		K219E	97 (14.46)	68 10.13)	0.14	7.38E-03
		K65R	188 (28.02)	108 (16.10)	-0.26	2.15E-07
		K103N	378 (56.33)	304 (45.31)	0.16	3.37E-03
		G190A	135 (20.12)	106 (15.80)	0.12	4.91E-02
		Y181I	9 (1.34)	1 (0.15)	-0.16	3.09E-02
II	**327 (69.57)**	T215Y	110 (23.40)	94 (20.00)	0.34	9.73E-14
		M41L	136 (28.93)	114 (24.26)	0.33	1.08E-11
		D67N	83 (17.66)	75 (15.96)	0.28	1.73E-09
		T215F	94 (20.00)	87 (18.51)	0.28	2.06E-09
		L210W	73 (15.53)	63 (13.40)	0.26	4.93E-08
		K70R	93 (19.79)	81 (17.23)	0.26	2.01E-07
		L74I	61 (12.98)	50 (10.64)	0.20	2.35E-04
		K219E	53 (11.28)	45 (9.57)	0.19	3.31E-04
		K219Q	52 (11.06)	43 (9.15)	0.19	5.25E-04
		G190A	97 (20.64)	84 (17.87)	0.22	3.49E-05
		K103N	185 (39.36)	136 (28.94)	0.16	1.59E-02
		K103S	27 (5.74)	21 (4.47)	0.14	2.77E-02
III	**18 (62.1)**	T215Y	5 (17.24)	4 (13.79)	0.36	0.13
		D67N	5 (17.24)	2 (6.90)	0.36	0.13

	M41L	7 (24.14)	3 (10.34)	0.27	0.20
	K70R	4 (13.79)	3 (10.34)	0.31	0.27
	Y115F	3 (10.34)	3 (10.34)	0.27	0.27
	L210W	3 (10.34)	2 (6.90)	0.27	0.27
	T215F	2 (6.90)	1 (3.45)	0.21	0.27
	K103N	12 (41.38)	10 (34.48)	0.37	0.06
	Y188L	3 (10.34)	2 (6.90)	0.27	0.27
	G190A	3 (10.34)	4 (13.79)	0.27	0.27

Note: . [a]M184V frequency was determined in 671, 470, and 29 isolates from first-, second-, and third-lines ART respectively. [b]Percentages were calculated for patients containing each specific mutation. [c]All p-values for covariation were significant at a false discovery rate of 0.001

b. RT major DRMs involved in negative correlations with M184V

Two mutations showed negative correlations in first line; K65R (phi = -0.26, $p < 0.001$) for NRTIs, and Y181I (phi = -0.16, $p < 0.05$) for NNRTIs. We observed no negative correlations in the second and third lines **(*Table II*)**.

5. Clusters of correlated mutations

Because pairwise analysis suggested that M184V is significantly correlated with many RTI-major resistance mutations, we performed average linkage hierarchical agglomerative cluster analysis **(Ceccherini-Silberstein *et al.*, 2007)** to visualize this hypothesis more precisely.

In the first-line, the dendrogram **(Fig. 23)** shows that; M184V significantly clustered (bootstrap value = 0.62) with T215Y and M41L (covariation frequency: 13.7% and 17.9%, respectively) **(*Table II*)**,

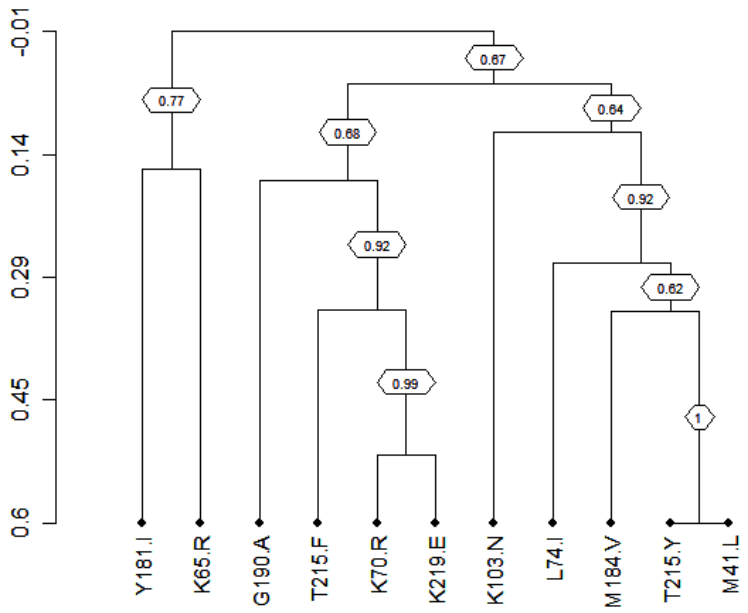


Figure 23: Clusters of correlated mutations in first line ART. Dendrogram obtained from average linkage hierarchical agglomerative clustering, showing clusters of RT mutations. The length of branches reflects distances between mutations in the original distance mutations in the original distance matrix. Bootstrap values, indicating the significance of clusters (≥ 0.2), are reported in the boxes.

While in second-line (bootstrap value = 0.64) **(Fig. 24)** with M41L, L210W and T215Y (covariation frequency: 24.26%, 13.4%, and 20.0% respectively) **(*Table II*)**.

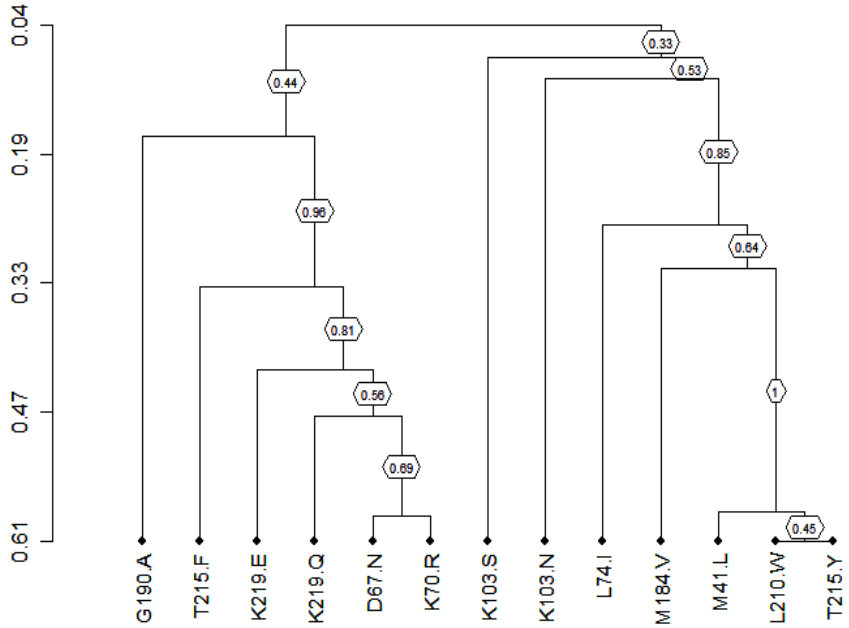


Figure 24: Clusters of correlated mutations in second line ART

Among patients on the third line, M184V significantly clustered (bootstrap value = 0.53) **(Fig. 25)** with K103N (covariation frequency: 28.9%) **(*Table II*)**.

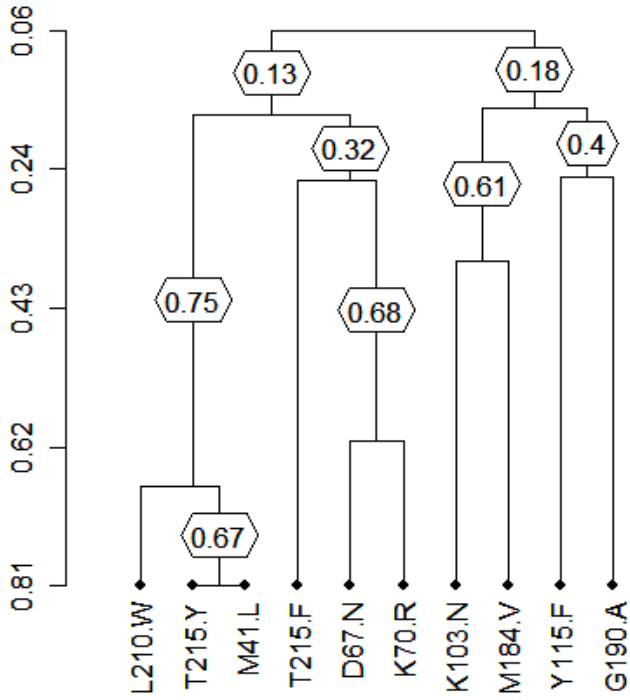


Figure 25: Clusters of correlated mutations in third line ART.

II. DISCUSSION

Our study population comprised 1170 PLWH, with a median age of 40 years (IQR 28-47), representing a predominantly middle-aged cohort but also including a significant younger population segment. Women constituted the majority (61.0%) across all treatment lines, consistent with findings from previous studies demonstrating a higher prevalence of HIV among women in similar settings [e.g., **(Ceccarelli *et al.,* 2012)**]. The higher proportion of women in our study likely reflects the greater burden of HIV among women in sub-Saharan Africa, attributable to socioeconomic and biological factors **(Griesbeck *et al.,* 2016)**. This disparity contributes to a higher representation of women among treatment-failure patients. While patients from all regions of Cameroon were represented in our study **(Fig. 18)**, the majority originated from the Center, Littoral, and Northwest regions. This reflects the high concentration of genotypic resistance testing at the CIRCB, suggesting that our findings accurately represent the national situation in Cameroon.

NRTIs have played a crucial role in HAART **(Lu *et al.,* 2018)**. However, their widespread use has led to a rise in NRTI resistance **(NIH, 2022)**. Our findings highlight the substantial burden of drug resistance among treatment-experienced patients in Cameroon, with high prevalence rates for both NRTIs and NNRTIs, an overall prevalence of 77.4% and 49.1% respectively. The high rates of resistance observed, particularly the dominance of the M184V/I mutation which was present in 83.3% of NRTI-resistant cases, similar to the 83.2% observed by Takou and collaborators in 2019 **(Takou *et al.,* 2019),** suggest that previous treatment regimens have profoundly impacted the viral landscape, notably lamivudine and emtricitabine which are highly affected by this mutation **(Tzou *et al.,* 2020)**. Therefore, given this high resistance, closely monitoring the tendency and co-occurrence between M184V and major reverse transcriptase mutations is a strong point for the development and strengthening of new treatment strategies such as combination therapies to combat evolving HIV drug resistance especially in low- and middle-income countries (LMICs) like Cameroon, given the current treatment landscape which is rapidly changing with the introduction of new antiretrovirals like Islatravir (ISL) **(Fokam *et al.,* 2019; Ismail *et al.,* 2021)**. This study provides valuable insights into the potential efficacy of ISL in combination with other ARVs within this context. Our analysis indicates that while ISL shows promise as a novel antiretroviral, the prevalence of the M184V/I mutation, which is known to reduce ISL's in vitro activity, is concerning**(Cilento *et al.,* 2021, 2023).** This emphasizes the importance of considering this mutation in designing future treatment strategies.

Our results revealed significant positive covariation between M184V and other major NRTI and NNRTI DRMs especially in first and second line ART, suggesting that long-term exposure to specific antiretroviral classes, particularly NRTIs, might contribute to the emergence of M184V and potentially reduce the efficacy of ISL. Specifically, the positive correlation of M184V with several NRTI DRMs among which a cluster is seen with L74I, T215F, and M41L on first-line and second lines, and additionally with T215Y and L210W on second-line ART, strongly suggests high-reduced susceptibility to AZT and its disqualification for combination with ISL.**(Cilento *et al.,* 2023)** Abacavir despite keeping the second most important susceptibility rate (91.5%) in our study population, it remains hugely affected by L74I and K65R, which happened to show significant positive correlations with M184V.

Concerning NNRTI DRMS, positive correlations were equally seen on clusters with K103N on first line and K103S on second line**.** This correlation eliminates EFV and NVP as treatment options for combination with ISL.**(Cilento *et al.,* 2023)** Doravirine on the other hand had the highest susceptibility rate (93.7%) in our study population, though Y188L which is known to reduce viral susceptibility to DOR, was present (7.26%), it showed no significant correlation with M184V throughout all three treatment lines. This renders DOR the best potential NNRTI for combination with ISL **(Brenner *et al.,* 2023; Lai *et al.,* 2022)**.

On the other hand, M184V showed negative correlation with K65R (NRTI) and Y181I (NNRTI) among first line ART patients. This indicates the antagonistic relationship between M184V and K65R, showing the potential of K65R to enhance the activity of ISL **(Diamond *et al.,* 2022)** while reducing the susceptibility to TDF. M184V on the other hand enhances susceptibility to TDF while reducing the susceptibility to 3TC/FTC and ISL **(Cilento *et al.,* 2023; Diamond *et al.,* 2022)**, and the combined effect of M184V and K65R maintains the optimal activities of both drugs if administered together **(Diamond *et al.,* 2022)**. Despite Y181I showing antagonism with M184V in our analysis, it is known to reduce the efficacy of ETR, RPV and NVP. Abacavir despite keeping the second most important susceptibility rate (91.5%) in our study population, it remains hugely affected by K65R and L74I, which happened to show significant correlations with M184V.

There were no significant correlations among patients among patients failing third line, which could be due to the low group size (29/1170). The mutation clusters among these patients show an M184V cluster similar to those of first and second lines with K103N and G190A, further supporting the elimination of EFV and NVP for combination to ISL.**(Cilento *et al.,* 2023)**

Estimating ISL predictive efficacy solely by deducing it from the prevalence of known resistance mutations, such as M184V/I, is inaccurate. This approach neglects ISL's high genetic barrier to resistance. Unlike lamivudine and emtricitabine, which exhibit reduced susceptibility in the presence of M184V, ISL requires a combination of multiple mutations, such as M184V/A114S, to confer significantly reduced susceptibility or outright resistance(>40x fold change)**(Diamond *et al.,* 2022).** However none of our 1170 participants present with A114S mutation, which can be justified by the fact that A114S is exceptionally rare in clinical samples, primarily because it occurs in a highly conserved region of reverse transcriptase (RT) enzymes, resulting in a slight reduction of RT activity in both biochemical and viral assays, attributed to its decreased binding affinity for incoming dNTPs**(Larder *et al.,* 1989)**. This reduced affinity can lead to diminished viral replicative capacity, particularly at low dNTP concentrations, as can be observed in certain infected cells**(Van Cor-Hosmer *et al.,* 2012)**. As a result, the occurrence of A114S in combination with other mutations is also expected to be very uncommon. While M184V affects the activity of Lamivudine(>49.1 x fold change) and Emtricitabine(>144.2x fold change), the degree of impact differs significantly compared to ISL**(Diamond *et al.,* 2022)**. Therefore, simply correlating the prevalence of M184V with reduced ISL efficacy would be a misrepresentation of its potent antiviral activity and the significant number of mutations required to overcome its genetic barrier.

Study Limitations

Though this approach has the potential to improve treatment outcomes and address the evolving landscape of HIV drug resistance in LMICs facing similar challenges, further research is needed to validate these findings in large cohorts and assess the clinical efficacy of these proposed combinations, given that we used archived data.

Conclusion, Recommendations and Perspectives

CONCLUSION

Our analysis of 1170 HIV-1 sequences from treatment-experienced patients in Cameroon revealed significant covariation patterns between the M184V mutation and key NRTI and NNRTI drug resistance mutations. These highlight the complex interplay of drug resistance development and underscore the urgent need for novel treatment strategies in low- and middle-income countries (LMICs) like Cameroon.

- High rate of resistance to nucleos(t)ide and non-nucleoside reverse transcriptase inhibitors was observed in people failing first second and third line ART in Cameroon, with overall high prevalence of M184V/I (83.3%) mutation throughout three treatment lines
- M184V associated to Islatravir resistance positively correlated with NRTI/NNRTI major resistance associated mutations (K70R; L74I; M41L; D67N; T215F; K219E; K65R; K103N; G190A; Y181I)
- Islatravir would keep its optimal efficacy rate, due to its high genetic barrier and potency, particularly when combined with other NRTIs and NNRTIs.
- Islatravir would serve as an additional therapeutic weapon, if combined preferencially with Tenofovir and Doravirine after first second or third line failure in LMICs sharing similar programmatic challenges with Cameroon.

RECOMMENDATIONS

For Clinicians:

Optimize ART Management: Continue managing HIV according to national guidelines, with a strong emphasis on timely resistance testing and appropriate treatment switches to prevent further resistance mutation accumulation.

Strategic Regimen Switching: Consider switching patients on TDF-monotherapy regimens to AZT-containing second-line regimens, even in the absence of genotypic resistance testing, while carefully monitoring plasma viral load. Prioritize genotypic testing for patients failing first-line therapy who have prior exposure to AZT/d4T. These actions are crucial given the high prevalence of M184V and associated resistance we observed (Objective 1).

For Researchers and Virologists:

Increase Awareness: Raise awareness among clinicians and the Cameroonian population about HIV-1 drug resistance, genotypic resistance testing, and its impact on epidemic control.

Validate Islatravir Combinations: Conduct clinical trials evaluating Islatravir combined with TDF or, preferentially, Doravirine in treatment-experienced patients with M184V and other resistance mutations (Objectives 2, 3, & 4). This is critical given the identified covariation patterns (Objective 2) and the observed potential for Islatravir efficacy when combined with Doravirine.

For the Ministry of Public Health:

Review Treatment Guidelines: Revise national guidelines on Tenofovir-based regimens, acknowledging their potential sub-optimality in later-line treatments and considering the high prevalence of resistance identified in this study.

Increase Doravirine Access: Increase the availability of Doravirine in routine clinical practice, due to its potential effectiveness even after second-line failure with PI/r regimens, as suggested by our findings (Objective 4).

PERSPECTIVES

As perspectives, further research should prioritize clinical trials evaluating this promising combination therapy in larger and more diverse populations in Cameroon and other LMICs to address the complex issue of drug resistance and improve treatment outcomes for patients failing ART in these settings. The high prevalence of M184V mutations in our study is a significant consideration for the selection of future therapeutic regimens, underscoring the need for further investigation of Islatravir susceptibility in similar contexts.

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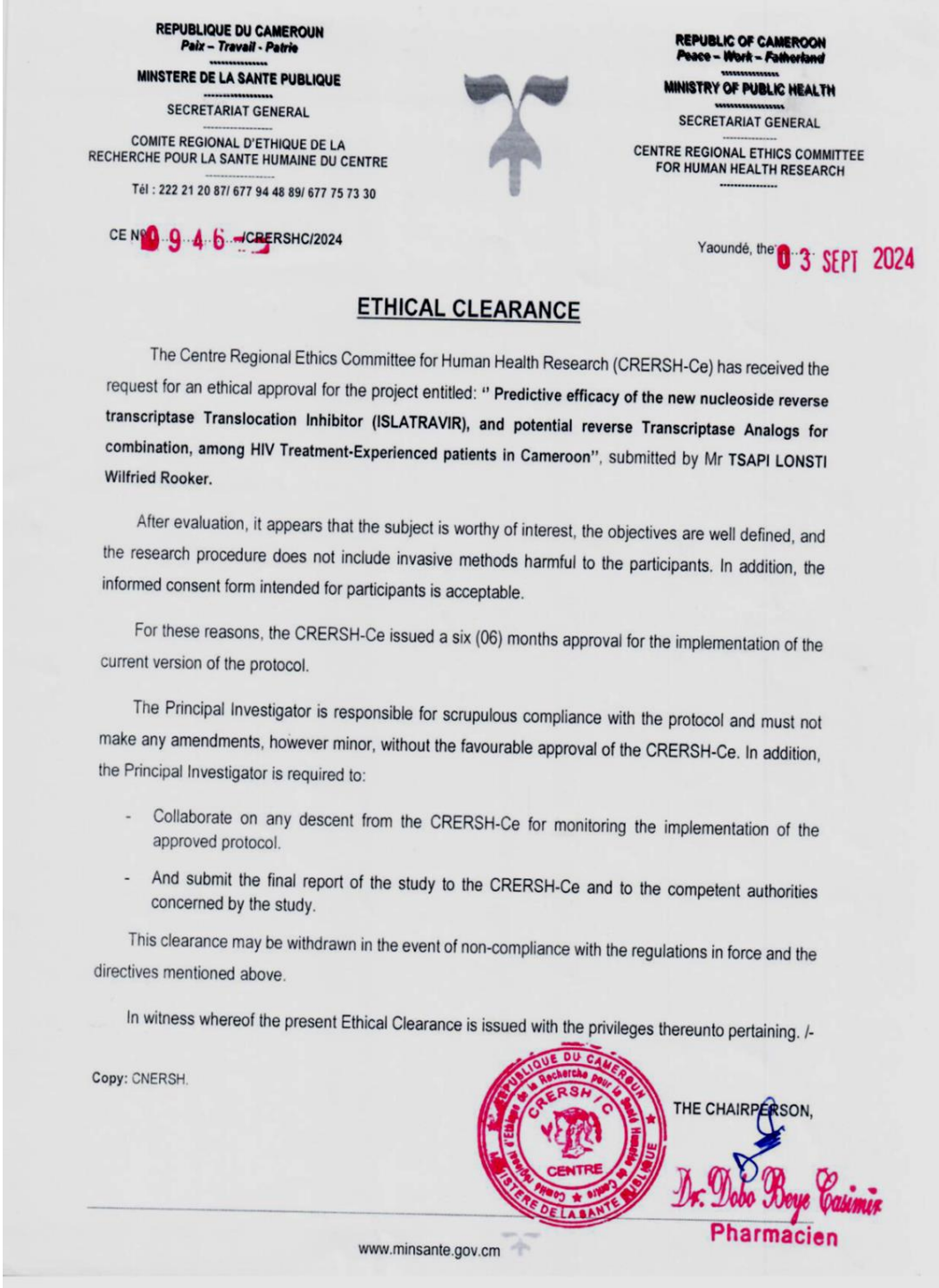
Appendix

APPENDIX

1. Ethical Clearance



REPUBLIQUE DU CAMEROUN
Paix – Travail - Patrie
MINSTERE DE LA SANTE PUBLIQUE
SECRETARIAT GENERAL
COMITE REGIONAL D'ETHIQUE DE LA RECHERCHE POUR LA SANTE HUMAINE DU CENTRE
Tél : 222 21 20 87/ 677 94 48 89/ 677 75 73 30

REPUBLIC OF CAMEROON
Peace – Work – Fatherland
MINISTRY OF PUBLIC HEALTH
SECRETARIAT GENERAL
CENTRE REGIONAL ETHICS COMMITTEE FOR HUMAN HEALTH RESEARCH

CE N° 0946 /CRERSHC/2024

Yaoundé, the 03 SEPT 2024

ETHICAL CLEARANCE

The Centre Regional Ethics Committee for Human Health Research (CRERSH-Ce) has received the request for an ethical approval for the project entitled: " **Predictive efficacy of the new nucleoside reverse transcriptase Translocation Inhibitor (ISLATRAVIR), and potential reverse Transcriptase Analogs for combination, among HIV Treatment-Experienced patients in Cameroon**", submitted by Mr **TSAPI LONSTI Wilfried Rooker.**

After evaluation, it appears that the subject is worthy of interest, the objectives are well defined, and the research procedure does not include invasive methods harmful to the participants. In addition, the informed consent form intended for participants is acceptable.

For these reasons, the CRERSH-Ce issued a six (06) months approval for the implementation of the current version of the protocol.

The Principal Investigator is responsible for scrupulous compliance with the protocol and must not make any amendments, however minor, without the favourable approval of the CRERSH-Ce. In addition, the Principal Investigator is required to:

- Collaborate on any descent from the CRERSH-Ce for monitoring the implementation of the approved protocol.
- And submit the final report of the study to the CRERSH-Ce and to the competent authorities concerned by the study.

This clearance may be withdrawn in the event of non-compliance with the regulations in force and the directives mentioned above.

In witness whereof the present Ethical Clearance is issued with the privileges thereunto pertaining. /-

Copy: CNERSH.

THE CHAIRPERSON,

Dr. Dobo Boye Casimir
Pharmacien

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Figure 26: Ethical clearance CE N° 0946/CRERSHC/2024.

2. Laboratory research authorization

REPUBLIQUE DU CAMEROUN
Paix –Travail – Patrie

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Peace – Work - Fatherland

CIRCB
CENTRE INTERNATIONAL DE RÉFÉRENCE "CHANTAL BIYA"
POUR LA RECHERCHE SUR LA PRÉVENTION ET LA PRISE EN CHARGE DU VIH/SIDA

La Direction Générale

N°25 1 7 / 23 /L/CIRCB/DG/SAA/BRH

Yaoundé, le 27 DEC 2023

Le Directeur Général
A
Monsieur TSAPI LONTSI Wilfried Rooker
-Université de Yaoundé I-

Objet: V/autorisation pour travaux de recherche.

Monsieur,

Dans le cadre de vos travaux de recherche intitulés " ***Résistance du VIH aux antirétroviraux chez les patients Camerounais",***

J'ai l'honneur de marquer mon accord pour la realisation desdits travaux au Laboratoire de Virologie du CIRCB, pendant une période de six (06) mois allant du **27 décembre 2023 au 31 mai 2024 .**

Par conséquent, je vous prie de bien vouloir prendre attache avec le Chef dudit laboratoire en vue des modalités pratiques et du planning de ces travaux.

Veuillez agréer, **monsieur**, l'assurance de ma parfaite considération.

LE DIRECTEUR GENERAL

Dr. Alexis NDJOLO

Siège : BP / P.O BOX : 3077 Yaoundé - Messa Tél. : (237) 222 31 54 50 / Fax : (237) 222 31 54 56 E-mail : contacts@circb.cm
Site web : www.circb.cm Décret présidentiel N° 2018/507 du 20 septembre 2018

Figure 27: Laboratory research authorization