MOLECULAR CHARACTERISTICS OF HIV-1 SUBTYPE C AND ITS IMPACT ON THERAPEUTIC OUTCOME IN ETHIOPIA

Amare Worku Kalu

Karolinska
Institutet

MOLECULAR CHARACTERISTICS OF HIV-1 SUBTYPE C AND ITS IMPACT ON THERAPEUTIC RESPONSE IN ETHIOPIA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Amare Worku Kalu

Principal Supervisor:
Professor Anders Sönnerborg
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Co-supervisor(s):
Gaetano Marrone
Karolinska Institutet
Department of Public Health Sciences (PHS)
Global Health

Opponent:
Professor Sören Andersson
Örebro University
Department of Microbiology
Division of School of Medical Sciences

Examination Board:
Mia Brytting
Public Health Agency of Sweden

Dr. Johan Lennerstrand
Uppsala University
Department of Medical Sciences
Division of Clinical Microbiology

Dr.Christer Lidman
Karolinska Institutet
Department of Medicine Huddinge
Division of Infectious Diseases

بِسْمِ اللهِ الرَّحْمَنِ الرَّحِيمِ

In the Name of Allāh, the Most Gracious, the Most Merciful

To my beloved ones; Zebiba (Hode), Musaab (Bebisha), Sofia (Mitta) and Maria (Super) who sacrificed all they have for the sake of this journey.

ABSTRACT

HIV-1 is characterized by a high genetic diversity which poses several challenges and implications with regard to disease progression, drug resistance and outcome of antiretroviral therapy (ART). HIV-1 subtype C (HIV-1C) is the most rapidly expanding subtype accounting for half of the global disease and nearly all infections in Ethiopia, Southern Africa and India which are the regions with the highest burden of HIV-1 infection. Molecular characteristics of the virus in such epidemic success need to be explored to better understand this subtype.

In the thesis, we analysed plasma samples and patient data collected during 2009-2011 in a large country-wide cohort, Advanced Clinical Monitoring of ART (ACM) which was established to evaluate the longitudinal effectiveness of ART as practiced in real life in Ethiopia. The overall aim was to investigate the molecular characteristics of HIV-1C and its impact on first line ART outcome in Ethiopia. Both genotypic and phenotypic molecular techniques were employed to characterize different regions of the viral genome. In **papers I and II**, population sequencing (PBSS) of the V3 loop of the HIV-1 envelope from therapy naïve, patients failing therapy, as well as HIV-1C sequences from Ethiopia dated 1984-2003 was used to assess the molecular epidemiology of HIV-1C in different geographic regions and the trend of viral tropism over the last decades. We also investigated the utility of different genotypic tropism prediction tools and the impact of the predicted viral co-receptor tropism on the outcome of standard first line ART. Our results showed that the Ethiopian epidemic is still monophylogenetic, exclusively dominated by HIV-1C, CCR5 tropic viruses. Furthermore, baseline tropism had an impact on outcome of standard first line ART. While each tool predicted tropism with comparable frequency, there was yet a large discordance between the tools. We elucidated this discordance further in **paper III** by employing an in-house phenotypic tropism method compared with the prediction by bioinformatics tools used in paper II as well as *in vitro* sensitivity of HIV-1C$_{Eth}$ strains for the co-receptor antagonist maraviroc. The results showed underestimation of R5 co-receptor usage by bioinformatics tools and effectiveness of maraviroc in HIV-1C. Expanding the exploration further to *pol* gene, we employed PBSS and next generation sequencing (NGS) to assess the prevalence of surveillance drug resistance mutations (sDRM) to reverse transcriptase- and protease-inhibitors as well as occurrence of DRM by NGS to the novel category of integrase strand inhibitors. The results in **paper IV** showed that NGS detected sDRM associated with RT- and PI- inhibitors more often than PBSS and major INSTI DRMs were found in minor viral variants. Furthermore, DRM identified before treatment was associated with a poorer treatment outcome.

In conclusion, viral tropism and drug resistance mutations at baseline have an impact on subsequent treatment outcome. Currently available genotypic tropism prediction tools need further improvement for use in HIV-1C. The Ethiopian epidemic remains uniquely dominated by R5 tropic HIV-1C since its introduction. Further investigations should be done to delineate associated molecular and epidemiological factors contributing to its uniqueness.

LIST OF SCIENTIFIC PAPERS

I. **Kalu, A. W**., Telele, N. F., Gebreselasie, S., Fekade, D., Abdurahman, S., Marrone, G., & Sonnerborg, A. (2017). Monophylogenetic HIV-1C epidemic in Ethiopia is dominated by CCR5-tropic viruses-an analysis of a prospective country-wide cohort. BMC Infect Dis, 17(1), 37. doi:10.1186/s12879-016-2163-1

II. **Kalu, A. W**., Telele, N. F., Gebreselasie, S., Fekade, D., Abdurahman, S., Marrone, G., & Sonnerborg, A. (2017). Prediction of coreceptor usage by five bioinformatics tools in a large Ethiopian HIV-1 subtype C cohort. PLoS One, 12(8), e0182384. doi:10.1371/journal.pone.0182384

III. **Kalu, A. W**., Telele, N. F., Aralaguppe, S., Gebre-Selassie, S., Fekade, D., Marrone, G., & Sonnerborg, A. (2018). Coreceptor tropism and maraviroc sensitivity of clonally derived Ethiopian HIV-1C strains using an in-house phenotypic assay and commonly used genotypic methods. Curr HIV Res. doi:10.2174/1570162X16666180515124836

IV. Telele, N. F., **Kalu, A. W**., Gebre-Selassie, S., Fekade, D., Abdurahman, S., Marrone, G., Neogi U., Tegbaru B., & Sonnerborg, A. (2018). Pretreatment drug resistance in a large countrywide Ethiopian HIV-1C cohort: a comparison of Sanger and high-throughput sequencing. Sci Rep, 8(1), 7556. doi:10.1038/s41598-018-25888-6

CONTENTS

LIST OF ABBREVIATIONS

3TC	lamivudine
ACM	Advanced Clinical Monitoring of ART
AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
ATV	atazanavir
AZT	zidovudine
CRF	circulating recombinant form
d4T	stavudine
DMEM	Dulbecco's Modified Eagle Medium
DRM	drug resistance mutation
DRV	darunavir
DTG	dolutegravir
EFV	efavirenz
env	envelope
Eth	Ethiopia
FPR	false positive rate
G2P	geno2pheno
GHOST	GFP-expressing Human Osteo Sarcoma T4
GTT	genotypic tropism testing
HIV-1	human immunodeficiency virus type 1
HIV-1C	HIV-1 subtype C
INSTIs	integrase strand transfer inhibitors
ITT	intention-to-treat
LPV	lopinavir
LTFU	lost to follow up
NGS	next generation sequencing
NNRTI	non-nucleoside analogue reverse transcriptase inhibitors
NRTI	nucleoside/nucleotide analogue reverse transcriptase inhibitors
NVP	nevirapine

OT	on-treatment
PBSS	population-based Sanger sequencing
PDR	pretreatment drug resistance
PI	protease inhibitor
RLS	resource limited settings
TDF	tenofovir disoprovil fumarate
TDR	transmitted drug resistance
VL	viral load
WHO	World Health Organization

1 INTRODUCTION

1.1 THE HIV/AIDS PANDEMIC

Human immunodeficiency virus (HIV) was identified as the causative agent of the acquired immunodeficiency syndrome (AIDS) in 1983 [1] and so far more than 70 million people have been infected globally among whom nearly half have died. At the end of 2016, about 36.7 million were estimated to live with HIV. In the same year, the number of the global annual new HIV infections was estimated to be 1.8 million while one million people died from AIDS-related illnesses [2]. Globally, 53% of all people living with HIV were reported to have access to antiretroviral therapy (ART).

According to recent regional HIV statistics by UNAIDS, the Eastern and Southern Africa region, which includes 21 African countries, was the most heavily affected by HIV, where 19.8 million people were living with the virus in 2016 and thus accounting for 53.9% of the global HIV burden. Although the estimated proportion of new infections showed a 29% decline between 2010 and 2016 in the region, 790,000 new infections were estimated in 2016, accounting for 43% of global annual new HIV infections. Similarly, despite a 42% decline in HIV related deaths between 2010 and 2016, yet this region accounted for 42% of the global AIDS related deaths (with 420,000 cases) [2]. Such decline in new infections as well as AIDS related deaths is attributed largely to the expanded access to ART in recent years: 11.7 million people (60% of those living with HIV in this region) were accessing ART, accounting for 60% of the global ART access in 2016.

1.2 THE HIV EPIDEMIC IN ETHIOPIA

Ethiopia, being one of the eastern African countries, is among the most seriously affected by HIV. The first HIV case was reported in 1986 and in a recent report, it is estimated that 710,000 people are living with HIV/AIDS [3].

Recent estimates show that the Ethiopian HIV epidemic is generalized and heterogenic with a high variation of prevalence between different regions, the highest being 5.2% (Gambella, western) and the lowest 0.7% (SNNP, Southern) [4]. According to recent Ethiopian Demographic and Health Survey (DHS) report, the national HIV prevalence is declining – in 2011 it was estimated to be 1.5% which dropped down to 0.9% in 2016 where females were twice affected by HIV compared to males (1.2% versus 0.6%) [5].

ART started in 2003 in very few facilities on out of pocket basis followed by the scale up of free ART services in 2005 and subsequent rapid expansion which resulted in the decline of AIDS related deaths and HIV incidence since 2005 [6]. Annual AIDS related deaths declined from 44,000 to 20,000 and new infections from 131,000 to 30,000 between 2007 and 2016 [3, 7]. By the end of 2016, about 420,000 (59% of those living with HIV) had access to ART. However, a national report from 2014 indicated that only 70.3% of those who ever started ART were on treatment at that time showing a significant number of patients lost to follow up (LTFU) , suggesting challenges in retention of patients in ART care [6]. Thus, while rapid

expansion of ART access contributed much for both the decline in new HIV infections and HIV related deaths, sub-optimal retention in care of ART exposed patients could serve as a source of transmission of drug resistant virus in the community.

1.3 HIV-1 BIOLOGY

1.3.1 HIV genetic diversity and phylogeny

The term "HIV" refers to a genetically diverse group of viral variants and consists of two phylogenetically distinct types, namely HIV-1 and HIV-2, each resulting from cross-species transmissions of the simian immunodeficiency viruses (SIV) [8]. While HIV-2 consists of nine groups, A-I, only group A and B are represented in the epidemic [8, 9]. HIV-1 comprises four distinct lineages, termed groups M, N, O, and P. Being discovered first among other groups, Group M (major) represents the pandemic form and is responsible for 95% of all HIV infections in virtually all countries of the globe [1, 10]. Group O is responsible for more than 100,000 cases in West-Central Africa, Cameroon [11, 12], and in some European countries with colonial ties to Cameroon; Group N is responsible for handful known cases mainly in Cameroon, France and possibly Togo [11, 13-15], and Group P was identified in 2009 with only two known cases in Cameroon [16].

The major HIV-1 Group M is the most diversified genetically and further classified into nine subtypes (or clades) A-D, F-H, J, and K. Subtypes A and F are again subdivided into sub-subtypes, A1-A4, and F1 and F2 [8] based on phylogenetic analysis. Furthermore, inter-subtype recombinant viruses are observed which, if documented in at least three individuals without any evident epidemiologic link, will be classified as a circulating recombinant form (CRF) or called unique (URF), if restricted in limited number of individuals without any further spread [17]. Currently, there are about 90 CRFs such as AB, AC, AE, and AG etc. and a steadily increasing proportion, accounting for nearly 20% of HIV-1 infections [13, 18], showing intersubtype recombination as a substantial force in generating further diversity in group M [18, 19].

Among the nine subtypes in Group M, subtype B predominates in North America and Australia. Initially subtype B also dominated in Western Europe but with time the diagnosis of non-B subtypes has increased substantially. However, it is in sub-Saharan Africa where most of the viral diversity is observed accounting for 70% of the global disease burden. The global HIV-1 Group M burden by subtype is: C (50%) followed by A (12%), B (10%), G (6%), AE (5%) and D (3%) [20]. Genetic variation at the amino acid level has been estimated at 8-17% within subtype and 17-35% between subtypes, dependent on the subtypes compared and the location of the HIV-1 genome examined [13]. Such extensive genetic diversity of HIV-1 poses several challenges and potential implications for viral diagnosis [21], monitoring of the infection [22], development of drug resistance, disease progression [23], viral transmission, response to ART, and effective vaccine development [22, 24, 25].

HIV-1C is the most prevalent variant among all other subtypes in group M due to its predominance in highly affected regions mainly southern Africa, east Africa and India, and

also to some extent Central Africa and Brazil [20]. It has also become the secondly most common subtype in e.g. Sweden and some other European countries. The first report of isolation of this subtype was in 1986 from an Ethiopian patient (HIV-1C$_{Eth}$) by Prof Anders Sönnerborg and colleagues [26], with two genetically distinct strains designated C and C′ reported to co-circulate in nearly similar prevalence in later studies [27-29]. It has been shown that one of the HIV-C$_{Eth}$-cluster is also found in other east African countries while C′ clade stands as an independent cluster associated to southern African strains [30]. Some unique features of HIV-1C which might explain its predominance in the global epidemic will be discussed later in this thesis.

While published studies (small studies, mainly from central and northern part of the country) unanimously have shown that the Ethiopian epidemic is overwhelmingly dominated by HIV-1C, a mix of different subtypes (A, C, D) as well as their recombinant forms with varying proportion has been reported to co circulate in other Eastern African countries including those neighbouring Ethiopia, namely Djibouti (subtype C (66%), CRF02_AG (20%), B (8.5%), CRF02_AG/C (2.9%) and K/C (2.9%) , Sudan (subtype A (46%), C (33%) and D (21%), and northern Kenya (subtype A (50%), C (39%), and D (11%) [31]. Thus, as increased human migration and mobility result in introduction of new subtypes and variants as well as intermixing with existing subtypes, one could hypothesize that HIV-1C dominated distribution in Ethiopia might have changed currently, at least in the border regions.

1.3.2 HIV-1 Genomic Organization and replication cycle

HIV-1 belongs to genus Lentivirus and family *Retroviridae* which are mainly distinguished by the presence of a reverse transcriptase enzyme. Its genetic material consists of two diploid strands of positive sense single-stranded RNAs, each approximately 10,000 nucleotides in length. Fifteen viral proteins are encoded in nine overlapping open reading frames as shown in figure 1 below.

Figure 1. The HIV-1 genome.

While *gag, pol* and *env* encode structural proteins as well as essential enzymes which are common to all retroviruses, *vif, vpr, tat, rev, vpu*, and *nef* encode regulatory or accessory proteins with the same names which are essential at various stages of the viral replication cycle which starts with binding to the target cell through CD4 receptor and co-receptors [32] (co-receptor usage is described in upcoming section).

The viral replication cycle consists of several steps as reviewed [33] and systematically depicted in figure 2 below. Different viral proteins interact with host immune mechanisms and play a decisive role at different steps for the successful completion of the replication cycle and survival.
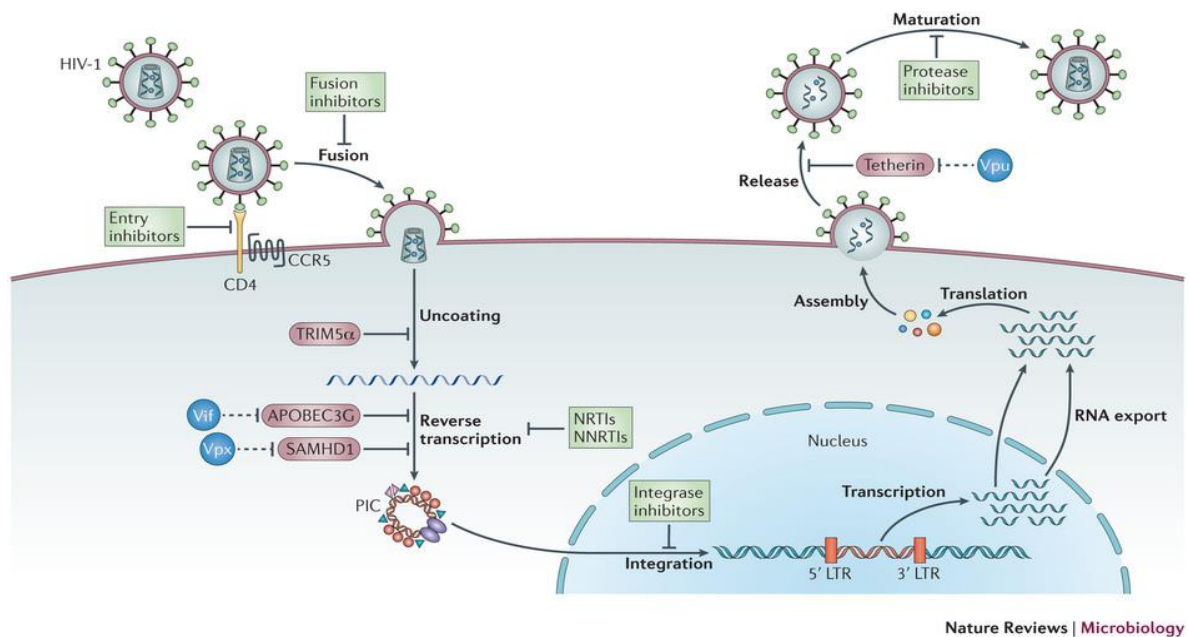

Figure 2. Main steps in HIV-1 replication cycle including key host HIV restriction factors and viral antagonists involved, and major class of antiretroviral drugs targeting various steps of replication cycle. Figure adapted from [33] with permission (License number 4343840892840). Details of antiretroviral drugs targeting various steps of replication cycle will be presented in a separate section below.

The host restriction APOBEC3G is a powerful inhibitor of reverse transcription but its inhibitory effect is antagonized by viral protein Vif [34] and Vpr [35], both mediating its proteasomal degradation and thus maintaining productive reverse transcription. Integration strongly favours transcriptionally active sections of the host genome [36] and once integrated, the provirus may remain transcriptionally inactive for years in a fraction of infected cells, a stage known as "latency" [37, 38]. Translated Nef and Vpr extensively modify the cellular environment to ensure efficient viral replication and persistence which includes the down-regulation of host cell-surface CD4 and HLA molecules by Nef [39, 40], and cell-cycle arrest and induction of apoptosis by Vpr [41-43], while the reverse transcription process by itself have been shown to induce apoptosis [43]. Budding is antagonized by host protein tetherin/BST-2 which prevent the release of virions by tethering onto cell surface [44] and shown to provide intrinsic herd immunity to group M HIV-1 epidemic [45], but it is counteracted by viral Vpu [46, 47]. Thus, the virus antagonizes the host cell's defence strategies using an array of proteins and establishes lifelong infection.

1.3.3 Co-receptor tropism and switch

In addition to the CD4 receptor, successful entry of HIV-1 to the target cell requires additional chemokine co-receptors, namely CCR5 and/or CXCR4. Some strains exclusively use CCR5 and are hence classified as R5- tropic; others exclusively use CXCR4 and hence

are classified as X4-tropic [48]. Individual virus variants with ability of utilizing both co-receptors (dual) and also a mixed viral population containing both R5- and X4-tropic strains are termed "dual/mixed or D/M [49]. The phenotypes are clinically relevant mainly in terms of the rate of disease progression [50].

1.3.3.1 Tropism in untreated infection (disease progression)

Transmission of both R5- and X4-tropic variants has been reported but the vast majority of primary infections are R5-tropic strains regardless of transmission route [51]. For instance, a French study (1996-2014) which determined co-receptor usage in primary infection showed that 94% of the infections were by R5-tropic strains and the prevalence of X4-tropic strains remained stable throughout the study period both in subtype B and non B infections [52]. A somewhat elevated prevalence (nearly 15%) of X4/DM-tropic virus has been reported recently in a large cohort of seroconverters, with the vast majority of subjects harbouring R5-tropic virus [53]. Such R5-tropic dominance during primary infection has been shown also by ultra-deep pyrosequencing, a method expected to detect X4-tropic minority variants [54]. Whether R5 dominance is due to its preferential transmission, availability of specific target cells, biased immune pressures limiting X4 virus, is not established yet. Nevertheless, the fact that in individuals with genetic CCR5 deficiency, where homozygosity is associated with strong resistance to HIV infection, and heterozygosity with a slower disease progression [55, 56], supports the predominant transmission and more efficient establishment of infection by R5-tropic viruses than X4-tropic ones. It should be noted that prevalence of X4-tropic virus during primary infection vary depending on the method used to determine the tropism (described separately in upcoming section below) where genotypic methods predict a higher prevalence in general.

In studies that have attempted to determine the prevalence of X4-tropic viruses in chronic HIV-1 infection, the results remain just a bit elevated than with primary infection, ranging up to 25% [57-59]. Also, difference in prediction by different methods remains minimal in chronic infection, both genotypic and phenotypic methods yielding comparable prevalence of X4-tropic viruses.

With disease progression however, nearly 50% of HIV-1B infected individuals will experience a tropism shift as increasing amounts of X4 virus emerge [60]. Studies have observed that co-receptor usage switch from R5 to X4 during late stage disease is usually associated with rapid CD4+ T cell depletion, rapid elevation of viral load (VL) (Figure 3) and occurrence of AIDS defining illness [61, 62]. Nevertheless, the proportion of X4 infected patients rarely exceeds 50%, even in patient cohorts of very advanced and final stage of disease [57]. Whether such evolution towards increased X4 usage is caused by disease progression or that increased usage of X4 leads to rapid disease progression, as well as the underlying mechanism is not established yet.
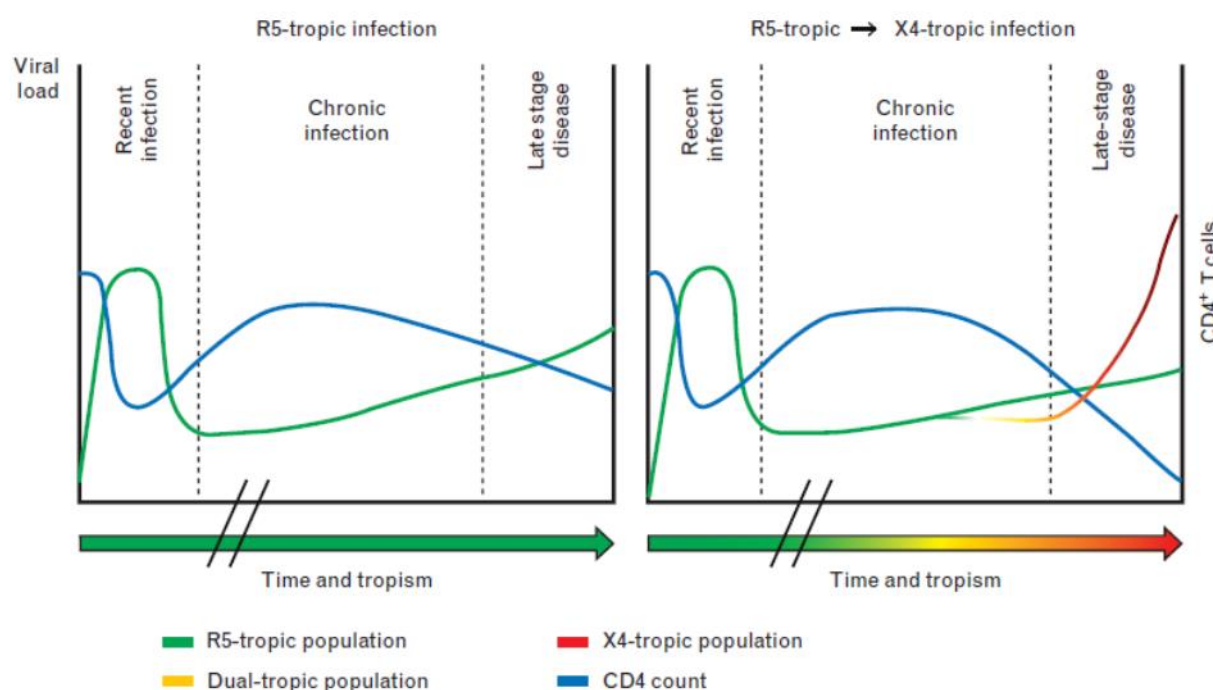
Figure 3. The three stages of HIV disease in relation to co-receptor usage: emergence of X4-tropic population associated with accelerated viral load increase and rapid CD4 decline (right panel) (Figure adapted from [57] with permission (License number 4343840188812).

Tropism data on non B subtypes are limited. In earlier studies, X4 using viruses were reported as rare in HIV-1C, even in late stages of disease [63, 64] including Ethiopia [65]. However, an increase in the incidence of X4-tropic HIV-1C has been reported recently in patients with advanced HIV disease from South Africa and India [66, 67] as well as from patients failing ART in Botswana [68] which might suggest a changing epidemiology and ongoing evolution of X4 tropic HIV-1C in Africa.

1.3.3.2 Tropism under ART

Thus, the relationship between tropism and disease progression seems established, at least in HIV-1B infections. Unfortunately, reports on viral tropism after initiation of standard first line ART are relatively scarce and effect of standard ART on co-receptor usage appears controversial. In some studies ART had no or a limited effect in the selection of X4 virus [69, 70]. In other studies preferential suppression of X4 viruses after ART has been reported [71, 72] while some other studies showed switch of tropism in both directions, R5 to X4 as well as X4 to R5, a switch independently associated with disease progression [73, 74] and still other studies reported switch during ART as rare [75].

Apart from their clinical relevance in terms of the rate of disease progression, studies also attempted to explore possible impact of these *env* phenotypes on treatment outcome of first line ART. Some claimed X4 tropism at baseline to differentially impact treatment outcome including rate of viral load suppression and CD4+ T cell gain [76-79] while others show similar rates between R5- and X4-tropic viruses at baseline [80, 81]. Difference in methods used to detect tropism, study design, patient population etc. imped comparison between the

above mentioned studies leading to controversial findings which need to be explored further. Moreover, most studies are based on HIV-1B and such data on HIV-1C is scarce.

1.3.4   HIV-1 subtype C (HIV-1C) unique features

In contrast to other subtypes in group M, HIV-1C is rapidly expanding, accounting for half of global disease and nearly all infections in Ethiopia, Southern Africa and India which are regions with a very high burden of HIV-1 infection. Such epidemic expansion of this subtype suggests that there might be some factors unique to this virus affecting its replication and/or transmission. Several studies have attempted to elucidate possible factors related to events during viral entry and replication. As mentioned above, HIV-1C strains predominantly use the CCR5 co-receptor, even in late infection [64, 65], and as CCR5 using virus are non-syncytium-inducing and hence display less cytopathogenicity it has been speculated that this might contribute to their rapid expansion and spread throughout the globe [82, 83]. Some studies have demonstrated a relatively high transmission fitness of this subtype compared to others in dendritic cells, increasing the frequencies of vaginal shedding and hence higher risks of heterosexual and mother-to-child transmission [84]. Other studies using PBMC models suggested similar transmission fitness but less fitness after transmission compared to other subtypes [85, 86] which might imply slow disease progression, prolonged asymptomatic infection and more opportunities for transmission favouring epidemic spread. This implies a slower rate of evolution and lower probability of accumulation of mutations that might lead to R5 to X4 transition. A study indeed showed that HIV-1C requires accumulation of more mutation in the *env* gene than other subtypes [87] in order to switch from R5 to X4 and a more recent study identified two distinct mutations in the V3 loop unique to HIV-1C [88].

Studies have further attempted to dissect the viral genome searching for an explanation of HIV-1C´s unique epidemic spread and success. Analysis of LTR in earlier studies revealed the presence of three instead of two or less NF-κB binding sites in HIV-1C than within other subtypes in group M [89, 90]. The extra NF-κB binding site may enhance viral gene expression, conferring highest transcriptional activity in HIV-1C [91] and hence higher replication and transmission capability. A recent study on Ethiopian HIV-1C isolates also revealed presence of three NF-κB binding sites irrespective of coinfection [92] and an earlier Indian study even demonstrated HIV-1C strains with multiple (four) NF-κB binding sites associated with higher plasma viral load when compared to isolates with three or less NF-κB binding sites and hence presumably more infectious [93]. Other studies observed a 5-amino-acid insertion in Vpu that could modulate its function and affect the virulence of HIV-1C viruses [94].

Furthermore, *Nef* sequences from HIV-1C display reduced ability of down-regulating CD4 and HLA-I compared to HIV-1B, a phenomena related to an escape mutation 'S88G' which is relatively more prevalent in HIV-1C [39]. The above mentioned molecular characteristics ought to result in enhanced viral replication, yet HIV-1C viruses displayed lesser replication fitness *in vitro* compared to other subtypes [85]. Such observations suggest presence of some other components of HIV-1C that might reduce the overall replication level without altering

an enhanced capacity of transmission. Analysis of *pol* gene products from HIV-1B and HIV-1C on overall viral replication suggested that the reverse transcription in HIV-1C results in a reduced accumulation of reverse transcripts and reverse transcription complexes compared to HIV-1B, which may lead to reduced viral replication [95]. Thus, further studies are needed to elucidate molecular basis of HIV-1C difference from other predominant HIV-1 subtypes.

1.4 SEQUENCING TECHNOLOGIES FOR CHARACTERIZATION OF HIV-1

Currently, two major sequencing technologies are in use for characterization of HIV, mainly focused on clinical HIV-1 drug resistance and tropism testing.

1.4.1 Sanger sequencing

Population-based Sanger sequencing (PBSS) has been the most used due to its relatively low cost and fast turnaround time. For the purpose of sequencing plasma-derived HIV-1 RNA, it is generally preceded by a reverse transcription with a gene-specific primer, which may be followed by a second-round "nested" PCR. The resulting library of double stranded PCR amplicon is the template for the sequencing reaction. This library is heterogeneous and contains a representation of the circulating HIV-1 quasispecies within a sample, which may be biased as a result of primer selection [96]. It is generally agreed that PBSS lacks sensitivity to detect minority variants that are less than 20% prevalent [97]. Conversely, minority drug resistance species and non-R5 viruses may have important impact on therapy outcome. Studies have shown that low prevalent drug resistance variants are associated with increased risk of treatment failure and are rapidly selected to represent the major virus population within weeks after starting ART [98-100], and patients with ≥2% non-R5 tropic variants have been reported to be associated with poorer maraviroc response [101]. Thus, a more sensitive sequencing approach could be beneficial for better clinical management, calling for the implementation of next generation sequencing (NGS) technology in the field of HIV research.

1.4.2 NGS/High throughput sequencing

To improve the sensitivity of detection of drug resistant variants, researchers have examined deep sequencing technologies such as NGS as an alternative to PBSS, where usually thousands of templates per sample from PCR amplification are clonally sequenced to obtain high depth coverage with thousands of reads per sample [102]. Due to their supreme sensitivity, platforms such as MiSeq (Illumina) can detect HIV-1 minority variants down to about 1% prevalence as well as reverse transcription and PCR errors caused by enzyme misincorporation. Using analysis of clonal samples and standard PCR conditions, Di Giallonardo *et al.* estimated such error rates at 0.08-0.16% [103]. Comparison of this method with conventional PBSS showed > 99% nucleotide concordance (sensitivity, 97.4%; specificity, 99.3%) [102]. Oversampling or redundant sampling of sequence variants derived from a low input copy numbers of HIV-1 RNA/DNA templates is a potential issue in all NGS methods [104], and the need for multidisciplinary team (wet-lab and *in silico*) is another bottleneck for implementation of NGS [100]. Despite the shortcomings, high throughput

NGS have been shown to be a promising approach for widespread individual drug resistance testing as well as surveillance in resource limited settings (RLS), with an added advantage of cost-effectiveness [100, 102, 105] and its application to multiple HIV subtypes [102]. Thus, we chose MiSeq (Illumina) for analysis of minority drug resistance variants in this thesis.

1.5 ANTI-HIV-1 DRUGS AND MONITORING OF ART

1.5.1 ART regimens

The discovery of drugs that suppress the HIV-1 replication has transformed the infection from a fatal to a chronic manageable disease [106]. Currently, there are 27 anti-HIV-1 drugs approved for clinical use and classified into six drug classes (NRTIs, NNRTIs, PIs, INSTIs, fusion inhibitor, CCR5-antagonist) among which 16 are more commonly used [107]: nucleoside or nucleotide analogue reverse transcriptase inhibitors (NRTIs) was the first drug class discovered and inhibits reverse transcription. The NRTIs mimic and compete with natural nucleotide substrates of RT and inhibit viral DNA synthesis. Commonly used NRTIs in RLS include: lamivudine (3TC), emitricitabine (FTC), stavudine (d4T), zidovudine (AZT), and tenofovir disoprovil fumarate (TDF) [108, 109]. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) also inhibit reverse transcription by allosteric binding of RT in non-competitive manner resulting in conformational changes and deactivation. Among the NNRTIs, efavirenz (EFV), and nevirapine (NVP), are the most commonly used in RLS, being the main and alternative first line agents, respectively [107, 110]. Protease inhibitors (PIs) inhibit Gag and Gag-Pol polyprotein cleavage by the protease enzyme. This class of drug was for many years part of the standard first line ART in high-income countries while it is reserved for second line in RLS where either lopinavir (LPV), atazanavir (ATV), or darunavir are used [111, 112]. Integrase strand transfer inhibitors (INSTIs) inhibit specifically the strand-transfer activity of the INT enzyme. Currently there are four INSTIs approved for HIV-1 treatment, namely raltegravir, elvitegravir, dolutegravir (DTG) and bictegravir (BIC) [113] and, the DTG is considered nowadays as a drug of choice among initial ART regimens because of the high efficacy and limited toxicity [114]. In 2017 World Health Organization (WHO) recommended that all first-line regimens should include the INSTI DTG if the pretreatment drug resistance (PDR) rate is >10% in a geographical region [115]. The only approved fusion inhibitor enfuvirtide T-20 is a gp41 inhibitor that binds to gp41 and inhibits host-viral membrane fusion [116]. It is indicated for treatment of therapy experienced patients not responding to other drugs despite ongoing therapy but is today hardly used [117]. The last drug class, CCR5-antagonists, targets the CCR5 receptor and specifically blocks the attachment of viruses which use CCR5 as a co-receptor for entry but does not affect viruses that use CXCR4 [108]. Maraviroc (MVC) is the only one approved in this class [116] and is used as an option for treatment of R5-tropic virus infection in high resource countries [118]. During the development of this PhD project, maraviroc's rollout to RLS was expected and thus study on the utility of this agent for HIV-1C was included as part of the project. Co-receptor tropism testing in connection with using this agent is discussed below.

In high-income countries, first-line standard ART combines two NRTIs with one of NNRTI, one boosted PI or one INI [119]. Subsequent regimen decisions are guided by routine VL testing, drug resistance monitoring and/or tolerability [120]. Since a few years the use of combination ART consisting of 2NRTI and DTG has increased substantially. In contrast, neither routine VL testing, drug resistance testing, tropism testing are usually available in RLS where non-subtype-B HIV-1 predominates. Also several drug options are lacking. Thus, WHO has until recently recommended a public health approach of ART using standardized first-line (NNRTI+ dual NRTI) and second line (boosted PI (LPV or ATV) + dual NRTI) regimens in RLS [112, 120]. The new WHO guidelines from 2017 recommend the use of the second generation INSTIs- DTG combined with 2 NRTIs as an alternative first line in RLS [115]. Clinical criteria and CD4+ count (see below for detailed discussion of CD4) are the main strategy for monitoring of ART in these setting which could result in switching to second-line therapy which might not be necessary as well as continuing on an already failed first-line ART, and consequently it might lead to an increased number of resistance mutations [121].

In Ethiopia, first line ART consisted for many years of two nucleoside analogues (AZT, 3TC, d4T and/or TDF) combined with either EFV or NVP [122, 123]. However in the last years d4T is avoided due to severe side effects. Patients are followed up on a monthly basis in the first three or more months of ART, until they show clinical stability and good adherence. Afterwards, the follow-up is scheduled on every three months basis, or as clinically required.

1.5.2 CD4+ cell count

The standard level for treatment initiation was recently updated to any CD4+ cell count instead of the previous of 500 cells/μl; this recommendation will eliminate CD4 count as a criteria for ART enrolment and allow initiation of ART for all people living with HIV [112, 124], while giving priority for those with CD4 cell count below 350 cells/μl. However, CD4+ count level is also useful for treatment efficacy monitoring in a setting lacking VL measurement. Flow cytometry counting with fluorescently labelled monoclonal antibodies is the conventional and most widely accepted choice for enumeration [124, 125] with the only challenge coming from the large machinery and high instrumental cost, which make its use difficult in RLSs.

National guidelines recommended CD4 monitoring of ART in Ethiopia every six months after initiation of therapy or when it deems necessary [122]. As availability of infrastructure and instruments is limited to ART centers located in urban areas, implementation has been attempted by establishing sample referral network where ART centers lacking CD4 enumeration instrument are linked to the nearby center with flow cytometry instrument. However it should be noted that there are bottlenecks in the implementation such as poor reagent supply chain, frequent breakage of equipment and, shortage of trained manpower and parts to maintain the instruments. Most recent Ethiopian guidelines (2017) recommend CD4 count assessment for patients on ART when indicated only [123].

1.5.3 HIV Viral Load

In high income countries, VL measurement is the gold standard for monitoring patients on ART [126]. VL monitoring is associated with favourable outcome compared to CD4 based monitoring to have a better early detection of treatment failure in RLS [127]. The most widely used method so far is the nucleic acid based assay. However, this assay is expensive and unsustainable, and is conducted centrally and occasionally in most of RLS as it requires a sophisticated laboratory infrastructure, highly skilled manpower and well established logistics [128]. Nevertheless, WHO hopes for availability of new point of care viral load measurement technologies and thus new WHO guidelines recommend routine VL monitoring at six month, 12 months, and every 12 months thereafter given that the patient remains clinically stable [112, 128].

VL monitoring facilities in Ethiopia are established at regional level where the regional laboratories are linked to referral hospitals in the region. Patients suspected of treatment failure are invited to visit referral hospitals where they are evaluated and sampled for VL measurement [129]. Samples are then transported to the regional laboratory where VL measurement is performed periodically, with turnaround time of several months. Furthermore, such approach is cumbersome given the rapid rollout of ART to remote areas of the country, with lack of infrastructure and resources. Thus, gaps in monitoring approaches might facilitate unnoticed development and spread of drug resistance to commonly used ART regimens, a scenario that necessitates drug resistance testing.

1.5.4 HIV-1 drug resistance testing

Assays for HIV-1 drug resistance are either phenotypic or genotypic.

Phenotypic assays measure the drug concentration that inhibits viral replication by 50% (IC_{50}) in cell cultures [130] compared to wild type control strains. Usually it requires production of recombinant virus by cloning of PCR amplified segment of HIV-1 gene of interest from a patient sample into a backbone of wild type clone lacking the gene of interest which is resource and labour-intensive as well as time-consuming. Thus, it is mostly reserved for drug development and resistance research [107]. Therefore, genotypic assays have become the preferred method over phenotypic assays.

Genotypic drug resistance testing depends on the detection of known drug-resistance mutations (DRMs), usually by PBSS of the pol region covering the 297 nucleotides of PR, and the 5′ polymerase coding region of RT (amino acid positions 40–240), where most of NRTI- and NNRTI-resistance mutations are found [130]. DRMs are usually unfit under natural conditions and arise as a result of selective drug pressure under suboptimal drug concentration. A mutation could be a primary directly reducing susceptibility to a given drug or accessory which enhance fitness of primary resistance variants or further reduce susceptibility [107].

Due to the varying impact and interactions among mutations, the genotypic method relies on the correct interpretation of the mutations detected. Among the several interpretation algorithms developed, Stanford HIV database is the most widely used one and provides scores for the mutations [131], which will be converted to various levels of susceptibility based on literature and expert's opinion [130, 131]. These algorithms were developed based on HIV-1B data. A recent study assessed the concordance between three interpretation methods and found significant discordances including subtype related differences, calling for a critical need for further development and improvement of the existing interpretation algorithms as ART and HIV genotyping becomes available in many African and Asian countries in connection with aiming at the new WHO 90-90-90 goals [132]. A version of Stanford HIVdb, calibrated population resistance tool (CPR), provides a standard list of surveillance drug resistance mutations (sDRMs) as indicator of transmitted drug resistance (TDR) which is based on the criteria that: i) the mutation should be recognized as causing or contributing to resistance; ii) being non-polymorphic and not appearing in polymorphic positions, and iii) applicable to eight common HIV-1 subtypes. This list is based on WHO's 2009 sDRM list [133]. The CPR tool was used to identify pre-treatment drug resistance mutations in this PhD project. There is no recommendation yet to use drug resistance testing as a monitoring tool of individuals on ART in RLS [134]; its availability is limited to regional or national reference laboratories due to its very high cost.

1.5.5 HIV-1 tropism testing

As mentioned in the tropism section above, HIV-1 tropism is related to disease progression but it has also been claimed to have an impact on treatment outcome of standard ART. Moreover, it affects response to the only currently licensed entry inhibitor targeting CCR5, maraviroc, where presence of X4-tropic viruses must be excluded prior to treatment with this drug. Both genotypic as well as phenotypic methods can be used to test for HIV-1 tropism.

1.5.5.1 Genotypic tropism testing

Genotypic tropism testing (GTT) offers a quicker and less expensive option and generally involve nucleotide sequencing of the gp120 third variable (V3) loop which is characterized by high variability including insertions, deletions and mutations [135]. Studies have shown that even a single change among the V3 loop amino acids could result in a tropism shift [136] and X4-tropic sequence characteristics include higher charge, increased genetic diversity and sequence length [135, 137]. These characteristics of X4-tropic sequences allow genotypic algorithms to draw sequence features for prediction of co-receptor usage [135]. The simplest prediction model has been the 11/25 charge rule where positively-charged amino acid residues at V3 loop codons 11 and 25 such as arginine (R) and lysine (K) have been strongly associated with a CXCR4-tropic phenotype [138]. However, positions outside 11 and 25 are also known to be associated with tropism [139] and as a result, bioinformatics approaches are required. Raymond *et al* developed a simple genotypic prediction combining 11/25 rule and the net V3 charge [140, 141]. There are also several bioinformatics based algorithms developed including position specific scoring matrix (PSSM) [142], geno2pheno (G2P)

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[143], and the most recent, PhenoSeq [144]. These algorithms were designed to infer phenotypic assay results from V3 loop nucleotide sequences using statistical techniques to weigh the prevalence of amino acid variants in each position. A score that estimates the probability of being associated with R5 phenotype is generated for amino acid variants in the V3 loop. In the case of G2P, this score is further transformed into a predicted false positive rate (FPR) [145] which indicates likelihood of falsely predicting a given V3 loop sequence as X4 using. To convert the spectrums of likelihood scores into a binary inference of tropism (i.e. "R5" or "non-R5"), appropriate cut-offs should be established. For instance, a G2P cut-off of 5.0% FPR is 92.6% specific and 67.4% sensitive against the original Trofile assay for detecting non-R5 [146]. As mentioned above, some clinical parameters, like CD4+ count and VL, are shown to be associated with co-receptor usage in chronic HIV infection. A clinical version of G2P has been developed and includes clinical parameters such as CD4 count and VL to improve the prediction [147].

Yet, there is a speculation that genetic determinants outside the V3 loop of HIV-1 *env* may affect the ability of GTT tools to precisely predict tropism [148-151]. Moreover, discordant prediction by genotypic algorithms arises because of different statistical models employed, the way of handling changes in the loop like insertions, deletions, and ambiguous amino-acid positions [152]. Also, GTT methods do not allow discriminating between pure X4 and dual/mixed R5X4 viruses [153].

Performance of the algorithms is influenced by the training data including the subtype of the V3 sequence used and most GTT methods have been developed using genetic data from HIV-1B [154] posing a question as to whether they have capacity to predict tropism in non-B HIV-1 subtypes. Studies that attempted to validate these utilities in different clinical setups and subtypes have shown a limited sensitivity of detecting X4-tropic strains as well as differences between subtypes [155] using phenotypic assays as gold standard even though 95% specificity of G2P has been reported for predicting X4-tropism in HIV-1C [156]. One recent study using a phenotypic assay reported an overestimation of X4 virus by G2P $_{fpr10\%}$ when compared with PSSM as well as commercial and non-commercial phenotypic assays in a cohort of acutely HIV-1 infected patients [152]. Thus, despite their ease of use and lesser expense, there are unresolved issues with GTT methods and hence, calls for more studies which evaluate further the utility of tropism tests in different subtypes and settings. The abovementioned genotypic tools were used in this thesis to predict tropism in HIV-1C and impact of the predicted tropism on the outcome of standard ART.

1.5.5.2 *Phenotypic tropism assays*

The oldest phenotypic assay to assess HIV tropism was the MT-2 assay which, based on the ability to induce formation of multinucleated giant cells, "syncytia", classified HIV into syncytium-inducing (SI) or non-syncytium-inducing (NSI) phenotypes [157]. NSI viruses are generally associated with CCR5 co-receptor usage, whereas SI viruses are generally associated with CXCR4 usage [158]. Over the last decade, various commercial phenotypic assays have been developed to measure tropism based on recombinant viruses, such as the

Enhanced-Sensitivity-Trofile-Assay (ESTA) [159], the Virco phenotypic test [160], the Phenoscript test [161] and the Toulouse Tropism Test [153]. Non-commercial assays utilizing recombinant particles or pseudovirions have been also shown to be good in measuring tropism [162-164] and has been claimed to even be able of differentiating between the dual-tropic viruses and a mixture containing both R5- and X4-tropic strains [165], which is one of the shortcomings of commercial assays. However, factors including higher cost and longer turn-around time made their usage in clinical setup limited although the non-commercial assays are relatively less expensive. In this thesis, an *in house* phenotypic assay was employed and compared with predictions by genotypic methods.

1.6 OUTCOMES OF ART

According to the US department of health, the goal of ART is "to achieve maximum and durable suppression of plasma HIV RNA; restoring and preserving immunological functions; reducing HIV-related comorbidities and prolonging and improving quality of life; and preventing transmission of the virus" [166]. Viral suppression indicates treatment success and lesser potential of transmitting the virus.

1.6.1 Definitions

According to WHO guidelines for RLS (2016), ART failure in adults and adolescents may be defined as clinical, immunological or virological.

Clinical failure is defined as ''a new or recurrent clinical event indicating severe immunodeficiency (WHO clinical stage 4 conditions) after six months of effective treatment. The condition must be differentiated from immune reconstitution syndrome (IRIS), which occurs after initiation of ART'' [112, 166].

Immunological failure is a situation when ''CD4 count falls to or below the baseline or persistent CD4 levels below 100 cells/mm. However, the event should be without concomitant or recent infection which may cause a transient decline in the CD4 cell count''.

Virological failure is defined by a ''persistently detectable viral load exceeding 1000 copies/mL (that is, two consecutive VL measurements within a three-month interval with adherence support between measurements) after at least six months of starting a new ART regimen'' [112].

1.7 THE ADVANCED CLINICAL MONITORING OF ART (ACM) PROJECT IN ETHIOPIA

Incepted in 2005 as a compendium of 10 national (Federal Ministry of Health, FHAPCO, EHNRI, and seven hospitals affiliated with medical universities in Ethiopia) and two international institutions (Johns Hopkins University – Bloomberg School of Public Health (JHU) and the United States Centers for Disease Control & Prevention (CDC), the ACM project in Ethiopia is a longitudinal cohort study designed to evaluate the effectiveness of the

national free ART program at the participating hospitals in diverse geographical locations (figure 4).

Established by considering age during the consent process, the ACM included an adult and adolescent cohort (age >= 14years), and a paediatric cohort (age < 14 years), each consisting of a database (retrospective data before enrolment) and repository (prospective data at enrolment and thereafter) sub cohorts. For the adult and adolescent sub-cohort, plasma samples were collected every six months at each study site, stored temporarily at -20°C, and transported regularly to EHNRI for storage at -80°C [167]. From January 1, 2005 to August 31, 2013, a total of 4339 participants were enrolled to the project among which 982 participants were enrolled from the multi-site adult and adolescent repository cohort between September 2009 and August 31, 2013 [167].
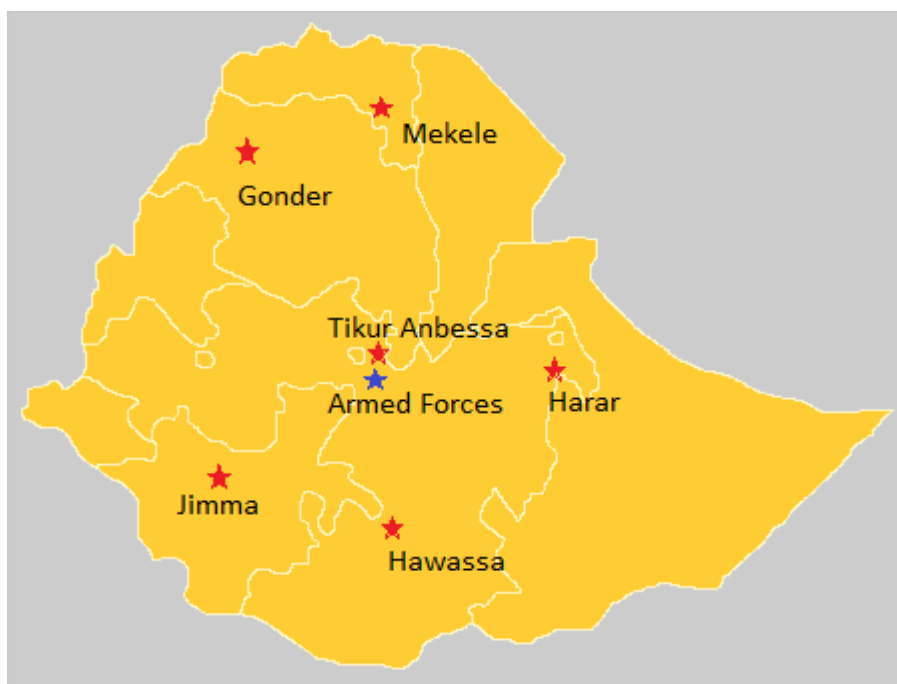

Figure 4. Study sites of the Advanced Clinical Monitoring (ACM) cohort: The Tikur Anbessa hospital is situated in the capital city, Addis Ababa. The clinical caring for the Armed Forces, the "Mobile Group", is also situated in Addis Ababa.

Although ACM project was terminated thereafter because of the discontinuation of funding from the donor (CDC), it is among the larger cohort studies in Africa, which included more than 4,000 patients. Such cohort studies have been critical in nourishing understanding of HIV and effectiveness of ART in a real-world setting. This thesis took an advantage of such research platform created by ACM and accessed adult and adolescent repository cohort specimens as well as associated data to achieve the aims which are described below.

2 OBJECTIVE AND AIMS

2.1 OBJECTIVE OF THE THESIS

Overall objective of the thesis was to investigate the molecular characteristics of HIV-1C and the impact on first line antiretroviral therapy (ART) outcome in Ethiopia.

2.2 SPECIFIC AIMS

Paper I

To assess the molecular epidemiology of the Ethiopian HIV-1C epidemic in different geographic regions of Ethiopia and the trend of viral tropism over the last two decades.

Paper II

To investigate the utility of genotypic co-receptor tropism prediction tools and evaluate the impact of the predicted viral co-receptor tropism on the outcome of non-maraviroc containing standard first line ART in Ethiopia.

Paper III

To analyse the phenotypic tropism of HIV-1C$_{Eth}$ strains in comparison with the genotypic prediction by five bioinformatics tools and compare the *in vitro* sensitivity of pure R5-tropic and dual-tropic HIV-1C$_{Eth}$ strains for the co-receptor antagonist maraviroc.

Paper IV

To assess the prevalence of surveillance drug resistance mutations (sDRM) to reverse transcriptase- and protease-inhibitors by population-based Sanger sequencing as compared to next-generation sequencing and evaluate their impact on first line ART outcome as well as analyse the occurrence of DRM by NGS to the novel category of integrase strand inhibitors.

3 MATERIAL AND METHODS

3.1 STUDY SUBJECTS

The samples and data used in this thesis have been mainly obtained from HIV-1 infected patients recruited to the ACM project, both at baseline (treatment-naïve) and/or at follow-up points- months six and twelve (treatment-experienced). Through October 2009 to December 2011, a total of 874 ART naïve patients were recruited to the ACM repository sub cohort, and started ART, as per the national guideline [122]. The subjects were from seven universities [167, 168] distributed geographically all over the country (figure 4): Tikur Anbessa Specialized Hospital in Addis Ababa- Central region; Gondar– Northwest; Jimma– West; Mekelle– North; Harrar– East; Hawassa– South; the Army unit providing service to mobile military staff and family, which is located in Addis Ababa (Figure 4). Plasma samples were temporarily stored at $-20^{o}C$ and transported thereafter to the central laboratory of the Ethiopian Health and Nutrition research institute (EHNRI) and stored at $-80^{o}C$. Historical sequence data were obtained from online databases for comparative analysis employed in some papers as described below.

In **Paper I and II,** plasma samples were obtained from 420 treatment-naïve patients of whom 41 also contributed with plasma while failing ART. In addition, a total of 387 historical V3 loop sequences from HIV-1C$_{ETH}$ dated from 1984-2003 were downloaded from the Los Alamos database (accessed on 23th January 2015) for **Paper I.**

For **Paper III**, plasma samples were obtained from 58 treatment-naïve subjects, who were selected based on a discordant co-receptor usage (n=42), a concordant CCR5 co-receptor usage (n=10) or a concordant CXCR4 co-receptor usage (n=6), as predicted by the five GTT tools in paper II.

For **Paper IV**, the study was conducted on 490 subjects (age ≥14 years), randomly selected after stratifying by study sites (70 from each site). In addition, baseline samples of 109 patients with virologic treatment failure (n=71) or with virologic suppression (n=38) patients were analysed by NGS.

3.2 CD4+ CELL COUNT AND VIRAL LOAD MEASUREMENT

CD4+ count was determined from patients' uncoagulated whole blood at laboratories within the participating health facilities using BD FACSCalibur machines (Becton Dickinson, San Jose, USA). Plasma VL was measured in the national reference laboratory at the Ethiopian Public Health Institute (EPHI) using NucliSENS easyQ® HIV-1 Nucleic Acid Sequence-Based-Amplification (NASBA) assay (BioMérieux Diagnostics).

3.3 RNA EXTRACTION

A summary of the experiments in the thesis is depicted in figure5 below. HIV-1 RNA was extracted from plasma by QIAamp Viral RNA kit (Qiagen, Hilden, Germany) 140ul of

plasma was used for patients having VL> 10,000 copies/ml, while 1ml of plasma was centrifuged at high speed to concentrate the virus for patients with VL< 10,000 copies/ml.
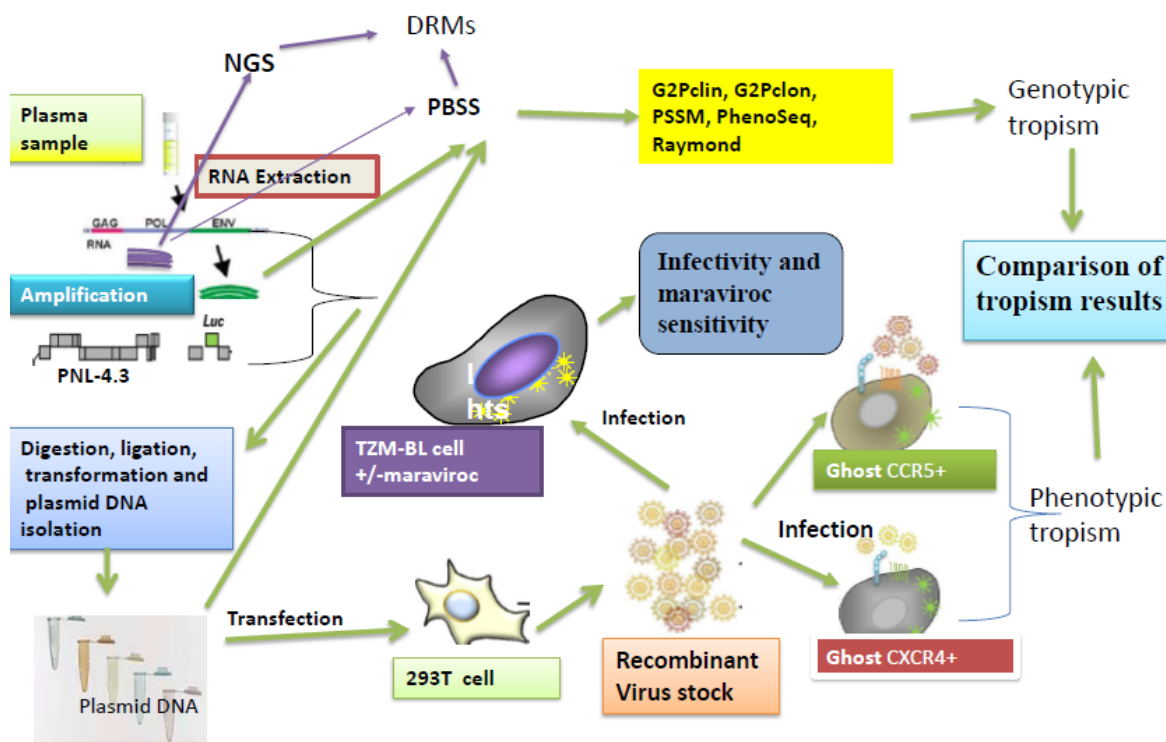

Figure 5: Summary of experiments in the thesis.

3.4   REVERSE TRANSCRIPTION AND NESTED PCR

In **Paper I, II** and **IV** cDNA was synthesized from extracted RNA using RevertAid H-minus reagents (Life technologies, UK) followed by subsequent amplification and nesting in two rounds by using the Applied Biosystem PCR system reagents and Taq-polymerase enzyme.

In **Paper III** cDNA was synthesized from extracted RNA using using SuperScript® III Reverse Transcriptase (Invitrogen/Life Technology) with Oligo (dT)18 primer (Thermo Scientific) followed by subsequent amplification and nesting using KAPA HiFi HotStart ReadyMix PCR kit (Kapa Biosystems, US). Table 1 below lists primers used for amplification and sequencing.

3.5   DNA PURIFICATION AND SEQUENCING

For **Paper I-III**, the amplicons were purified by QIAquick kit (Qiagen) and sequenced in automated sequencer (ABI 3130xl Genetic Analyser, Applied Biosystems). Sequences were aligned, edited, and analysed by the BioEdit software v. 7.0.9. The V3 loop sequence was derived by a gene cutter program (http://www.hiv.lanl.gov/content/sequence /GENE_CUTTER/cutter.html).

In **Paper IV**, the amplified fragments were purified (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany) and sequenced with PCR primers JA204F-C and JA205R-C plus PR2R

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and RT07. Sequences were aligned, edited and analysed using the BioEdit software version 7.2.6.1 (http://www.mbio.ncsu.edu/bioedit/bioedit.html)

Table 1: List of primers used for amplification and sequencing in each constituent paper**.**

Primer ID	Sequence	HXB2 Position	Used for	Used in paper
ED5-1F	5'-ATGGGATCAAAGCCTAAAGCCATGTG-3'	6556–6581	1st PCR	Paper I-II
ED12-1R	5'-AGTGCTTCCTGCTGCTCCCAAGAACCCAAG-3'	7822–7792	1st PCR	Paper I-II
ES7-2F	5'-TTRTTAAATGGTAGTATAGC-3'	7001–7020	2nd PCR, sequencing	Paper I-II
ES8-2R	5'-CACTTCTCCAATTGTCCCTCA-3'	7667–7647	2nd PCR, sequencing	Paper I-II
5550F	5'-AGARGAYAGATGGAACAAGCCCCAG-3'	5550–5574	1st PCR	Paper III
9555R	5'-TCTACCTAGAGAGACCCAGTACA-3'	9555–9533	1st PCR	Paper III
6433F	5'- CYACCAACGCGTGTGTACCCACAGA-3'	6433–6457	2nd PCR, sequencing	Paper III
8329R	5'-CCCTGCCGGCCTCTATTYAYTATAGAAA-3'	8356 – 8329	2nd PCR	Paper III
JA203F-C	5'-GAA AGA CTG TAC TGA GAG ACA GGC-3'	2058-2081	1st PCR	Paper IV
JA204F-C	5'-TTCAGAGCAGACCAGAGCCAACAG-3'	2135-2158	2nd PCR, sequencing	Paper IV
JA205R-C	5'-TTTTCCCACTAACTTCTGTATATC-3'	3338-3315	2nd PCR, sequencing	Paper IV
JA206R-C	5'-TTA ATC CCT GGG TAA ATC TGA CTT-3'	3373-3350	1st PCR	Paper IV
RT07	5'-AAGCCAGGAATGGATGGCCCA-3'	2586–2606	Sequencing	Paper IV
PR2R	5'-GGATTTTCAGGCCCAATTTTTG-3'	2691- 2713	Sequencing	Paper IV

3.6   SUBTYPING AND PHYLOGENETIC ANALYSIS

Subtyping was done by the REGA subtyping tool v2.0 [169], the RIP 3.0 (http://www.hiv.lanl.gov/content/sequence /RIP/RIP.html, and the COMET HIV [170]. Maximum likelihood phylogenetic analysis was performed using Molecular Evolutionary Generics Analysis version 7.0 (MEGA 7) software.

3.7 CELL CULTURE

TZM-bl, 293T, GHOST (GFP-expressing Human Osteo Sarcoma T4) (3) CXCR4+, and GHOST (3) CCR5+ Cells (Hi-5) cells were used in **Paper III**. TZM-bl and 293T cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, US), supplemented with 10% Fetal Bovine Serum (FBS) and 2mM L-glutamine while GHOST cells were propagated in high glucose DMEM supplemented with 10% FBS.

3.8 CLONING AND RECOMBINANT VIRUS PRODUCTION

In **Paper III**, QIAquickGel Extraction Kit (Qiagen, USA) was used for gel purification of PCR fragments and the purified products were cloned in pMN-K7-Luc-IRESs-NefΔgp120 plasmid following digestion with restriction enzymes NgoMIV and MluI-HF (New England Biolab, US) , ligation with T4 DNA ligase, transformation into competent DH5alpha *E.coli* cells (Invitrogen, Life Technologies, MA, USA) and subsequent colony screening + plasmid isolation. Recombinant viruses were produced by transfecting the plasmids using FuGENE® HD Transfection Reagent (Promega, US) in 293T cell line, incubation for 48 hours and lysing the cells followed by centrifugation to get cell free supernatants and stored at -80^0C if not used immediately. Infectivity of the recombinant viruses generated from individual clones was tested by infecting 10^4 TZM-bl cells with *Env*-recombinant viruses in DMEM containing 20ug/ml of DEAE-dextran and cultured for 48 hours, after which luciferase activity was measured using the Luciferase Bright Glo™ assay system (PROMEGA, USA). All infections were done in triplicate. Recombinant viruses were considered infective if the luciferase read were no less than 2.5x the background as described previously for TZM-bl cells [171].

3.9 TROPISM TESTING

Both genotypic (GTT) and phenotypic (PTT) methods were used to determine viral tropism in this thesis as described below.

3.9.1 Genotypic methods

In **Paper I** G2P clonal and clinical algorithms were used for prediction of tropism based on the V3 loop while in **Paper II** three additional bioinformatics tools- PhenoSeq-C, C-PSSM and Raymond's algorithm - were used for prediction of tropism based on the V3 loop. For GTT of cloned *env* in **Paper III**, same tools as in paper II were employed.

3.9.2 Phenotypic method

For phenotypic tropism testing in **Paper III**, GHOST indicator cells- GHOST (3) CXCR4+, and GHOST (3) CCR5+ Cells (Hi-5) were infected with viruses generated from individual clones in DMEM containing 10ug/ml polybrene and luciferase activity was measured using the Promega Luciferase assay kit as described above under recombinant virus production. Viruses were interpreted as R5 or X4-tropic if the luciferase read was more than 2.5x the background and dual tropic if luciferase read more than 2.5x in both GHOST cell lines.

3.10 MARAVIROC DRUG SENSITIVITY ASSAY

$TCID_{50}$ was determined for each recombinant virus as described elsewhere,[172] in hexaplicate, and using Spearman Karber formula [173]. The drug sensitivity of the recombinant viruses was measured by adding serial dilutions of drug spanning 10 μM to $1x10^{-6}$ μM, in 96-well plates containing TZM-bl cells in complete DMEM media followed by infection with reference virus (R5-tropic, MJ4 and X4-tropic, NL4-3) or the patient derived recombinant viruses, at a multiplicity of infection (MOI) of 0.01 IU/cells using 10μg/ml of DEAE-dextran and incubated for 48 hours. Luciferase activity was measured as described above. Dose response data was analysed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA.

3.11 GENOTYPIC DRUG RESISTANCE TESTING (PAPER IV)

Sequences generated from PBSS were aligned, edited and analysed using the BioEdit software version 7.2.6.1 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Primary DRM were identified using calibrated population resistance tool (http://cpr.stanford.edu/cpr.cgi) at Stanford HIVDR Database. DRMs associated with NRTI-, NNRTI-, and PI- drug classes are considered in this assay. For NGS, Gag-pol fragment (HXB2: 790 – 5096) was amplified, gel purified, and fragmented on the Coveris S200 followed by library preparation using NEBNext UltraTM DNA library Prep Kit. Forty-eight libraries were then pooled at equimolar (10nM each) and run on Illumina HiSeq 2500. The FASTQ file was demultiplexed and the consensus sequence was created for each sample followed by realignment again with the consensus sequence as input. The variant calling was performed at amino acid (AA) level. Only AA covering 5000X per position was considered quality passed. Based on the error calculation generated by PCR and NGS, any mutation >1% was considered. WHO list of DRM for surveillance of TDR was used to interpret sDRM for NRTIs, NNRTIs, PIs, and the Stanford drug resistance summaries for INSTIs (hivdb.stanford.edu).

3.12 STATISTICAL METHODS AND APPROACHES USED

We used descriptive statistics (mean, median, standard deviation, and percentiles for numerical variables, frequencies and percentages for categorical variables) to summarize sociodemographic, clinical, immunological, and virological parameters. Chi-square test or Fisher's Exact Test were used to test differences between categorical variables. Differences of numerical variables between two or more categories were assessed using Independent t-test, Mann-Whitney, Anova and Kruskal-Wallis test.

In **Paper I & II**, we used both intention-to-treat (ITT) and on-treatment (OT) analysis to assess treatment outcomes. ART failure was defined as either detectable VL (>1000 copies/ml), died or LTFU in the ITT analysis while only patients having VL data at a given follow up time point were included In the OT analysis. Logistic regression models were used for the multivariable analysis of virological responses to compare differences between R5 and X4 infected patients as tropism predicted by different methods, adjusting by age and gender,

baseline CD4+ T-cell count and VL. Results from regression models were presented using Odds Ratios (OR), 95% Confidence interval and p-values.

In **Paper III,** sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and Cohen Kappa coefficient (k) were calculated for the results obtained by the GTT methods using the *in house* phenotypic assay as a gold standard.

In **Paper IV,** the impact of pretreatment sDRM (RTI, PI) detected by PBSS and NGS assays on virologic treatment outcome at month six and 12 was assessed by using a multivariable logistic regression model testing for different confounding factors including gender, age, WHO clinical stage, functional status, TB, CD4 cell count, baseline VL, and NRTI regimens.

In all of the papers, p-value <0.05 was considered statistically significant. Data analysis was performed using STATA software 14 (Stata Corp. College Station, Texas, USA).

4 RESULTS

The overall aim of the thesis was to investigate the molecular characteristics of HIV-1C and its impact on first line antiretroviral therapy (ART) outcome in Ethiopia. We described the *env* (**Paper I-III**) and *pol* regions (**Paper IV**) using PBSS, NGS, as well as the functional characteristics of the *env* in a cloning based phenotypic assay, as described in the methods section, to characterize the virus and correlate with ART outcome. In this section, main results from the studies will be presented and summarized.

4.1 PAPER I

This paper described our attempt to analyse, using the countywide multi-site cohort, the current status of the Ethiopian HIV-1 epidemic which was the first HIV-1C epidemic reported globally and described by our group in 1980's. From 420 patients included in this study, sequencing was successful in 352 of the patients. Furthermore, 387 historical sequence data (dated 1984-2003) was included for comparison.

Subtyping and genotypic tropism prediction

Using the three subtyping tools, HIV-1C was found in 350 (99.4%) and A1 in two (0.6%) of the 352 patients. In addition to determining the subtype, we also analysed the predicted genotypic tropism of the 352 baseline V3-nucleotide sequences using the G2P bioinformatics tools. The G2P clinical model fpr10%, predicted the following: R5 - 285 (81.0%); X4 - 60 (17.0%); mixed (R5/X4) 7 (2.0%) (Figure 2a, Paper I). The G2P clonal model predicted: R5 - 291 (82.7%); X4 - 50 (14.2%); R5/X4 - 11 (3.1%) (Figure 2b, Paper I).

Altogether, 266 (75.6%) of the 352 predictions were concordant between the two models at fpr10%. No association was found between the predicted baseline tropism and age, gender or VL. There was no difference in occurrence of R5-virus across the geographical regions.

Temporal trend of viral tropism (1984-2011)

We assessed the temporal trend of viral tropism during the last two decades using 387 historical V3 loop sequences from HIV-1C$_{ETH}$ dated from 1984-2003 (1984-1993: n= 91; 1994-2003: n= 296), and sequences from our study (2009-2011). The proportion of X4-tropic/ mixed (R5/X4)-tropic virus increased from 5.6% (1984-1993), 7.1% (1994-2003), to 17.3% (2009-2011) (p<0.001) (Figure 6).

4.2 PAPER II

In Paper II, we analysed the sequences from Paper I further by employing more bioinformatics tools as there was a large discordance in tropism prediction between the methods used in Paper I. The aim was to investigate the utility of different bioinformatics prediction tools and evaluate impact of the predicted viral co-receptor tropism on the outcome of standard first line ART.
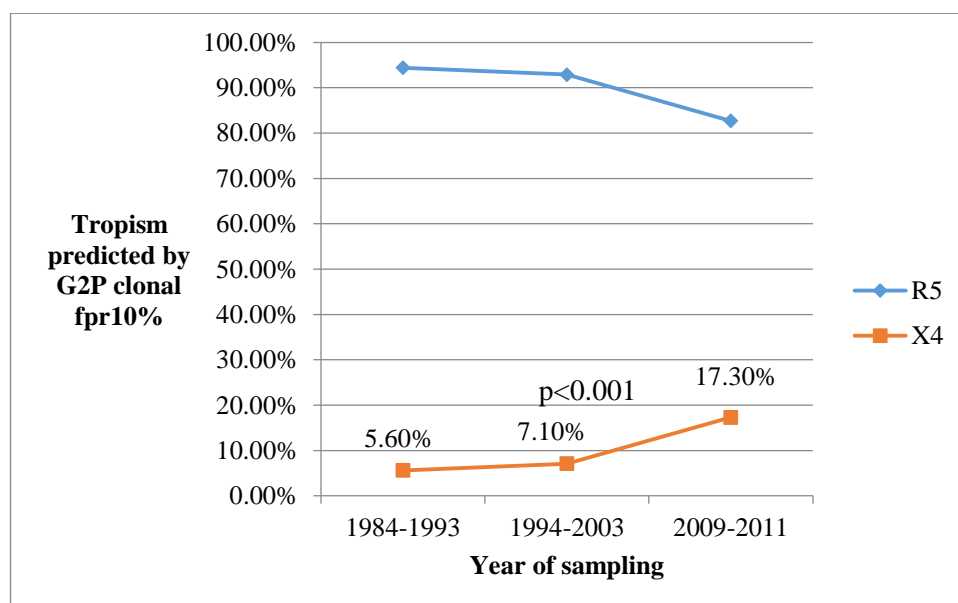
Figure 6. Temporal trend in the proportion of X4 tropic virus among Ethiopian HIV-1 isolates by Geno2Pheno clonal model (1984-2011).

R5 prediction at baseline

The proportion of patients predicted to harbour R5-tropic virus varied between the methods by 12.5%, (90.6%, Raymond's Vs 78.1%, PhenoSeq-C) as shown in Table 1 of Paper II. Altogether, only 205 (58.2%) of the predictions were concordant by the five tools used (Table 2 of Paper II).

Impact of baseline tropism on ART outcome

We assessed whether tropism as predicted by each method at baseline had an impact on treatment outcome both by OT and ITT analysis (as shown in table 3 and 4 of Paper II respectively). No difference was observed between R5 and X4 infected patients at months six and 12 in multivariable OT analysis. Tropism as predicted by C-PSSM had an impact on month 12 in multivariable ITT analysis, with patients harbouring R5 tropic virus at baseline having 2.47 higher odds to achieve VL suppression compared to those with X4 virus (p=0.04, OR 2.47, 95% CI 1.05-5.79) (Table 5 in Paper II).

Tropism switch at months six and 12

At month six, virological failure occurred in 37 of the 352 patients and seven additional patients who were included only for the study of co-receptor switch. Paired plasma samples were available for 41 patients out of which V3 sequencing was successful in 34 patients. The most frequent rate of tropism switch (7/34; 20.6%) was predicted by C-PSSM (R5 to X4: 4/26; X4 to R5: 3/8; p= 0.017), while 4/34 strains switched each (two R5 to X4 and two X4 to R5) as predicted by PhenoSeq-C and G2P clonal tools, and 4/34 strain switched (R5 to X4: 3/30; X4 to R5: 1/4) as predicted by Raymond's algorithm.

At month 12, virological failure occurred in 22 subjects in whom V3 sequencing was successful in 19. In most patients, tropism predicted at baseline was maintained at the month 12 prediction.

4.3 PAPER III

As a high discordance was observed between the genotypic tropism prediction tools used in previous papers, we moved next to employ a phenotypic assay and compare with the predictions obtained by the GTT tools.

Amplification of envgp120 was successful in 41 out of 58 (70.7%) and cloning in 35 of the 41 (85.4%) patients from which one-hundred-twenty clones were screened and 79 (65%) were infectious. Tropism was determined for all of the infectious clones by the phenotypic assay, while V3 sequencing for GTT prediction was managed in 70 of the clones.

Correlation between the phenotypic and genotypic methods

By the phenotypic assay, 73 out of the 79 clones were R5 tropic, six were dual tropic, and none were pure X4 tropic. A genotypic prediction was obtained for 70 of the infectious clones. The phenotypic results were compared with the genotypic predictions, obtained with the five algorithms as shown in Table 2 of Paper III. Only 30 out of 64 (46.9%) pure phenotypic R5 clones were predicted as R5 by all GTT tools.

Maraviroc drug sensitivity assay

The EC50 values for the six dual tropic and the six R5 tropic viruses are shown in Table 3 and the inhibition curves in Figure 1 of Paper III. There was no significant difference between the EC50 values of dual tropic and R5 tropic viruses (p=0.201).

4.4 PAPER IV

In order to explore a further region of the viral genome, we analysed the *pol* gene using PBSS and NGS with the aim to assess the prevalence of sDRM and evaluate their impact on first line ART outcome as well as analyse the occurrence of DRM by NGS to the novel category of integrase strand inhibitors.

Baseline sDRM detected by PBSS and impact on treatment outcome

At baseline, samples from 461 (94%) patients were successfully sequenced among which sDRM was detected in 18 (3.9%). (NRTI: n=9; NNRTI: n=7; PI: n=2) as shown in Figure 1 and Table 2 of Paper IV.

Patients harbouring baseline RTI-sDRM had higher odds of virologic failure considering both cut-offs (VL >150 copies/ml and VL> 1000 copies/ml) at month six (respectively OR (95% CI): 3.6 (1.2–11.1); and 9.00 (1.9–43.3)) as well as month 12 (respectively OR (95% CI): 6.5 (2.1–20.3); and 7.4 (1.5–35.0) compared to those without RTI-sDRM. In the ITT analysis, patients harbouring RTI-sDRM showed a significantly higher odds of treatment failure s at

month six, considering both viral failure cut-offs, than those without mutations (OR (95%CI): 2.9 (1.0–7.9) and 3.8 (1.4–10.5), respectively).

Baseline DRM detected by NGS

Baseline sequences were obtained from all of the 109 patients included for NGS. DRM was detected in 28 patients (Table of paper IV). NGS detected RTI or PI sDRM more often (23.9%) than PBSS (6.4%) (p<0.0001). The NGS DRMs were found in 32.4% of the 71 virologic failure patients at month six and/or 12 (>1000 copies/ml) (Table 3 of paper IV). In addition, INSTI DRMs-E138K, Q148R, Q148H, and T66I- were detected by NGS (Table 3 of Paper IV).

Impact of baseline DRM detected by NGS and correlation with PBSS

NGS detected any RTI sDRM significantly more often (28.2%; 20/71) than PBSS (8.54%; 6/71) (p=0.004) from baseline samples of ART failing patients (Table 3 Paper IV). Patients who failed ART with >150 copies/ml at month six and/or 12 had higher odds to have one or more NRTI, NNRTI and/or PI sDRM by NGS at baseline compared to the virologic suppressors (OR: 6.4; 95% CI: 1.6 – 26.4 adjusted for NRTI regimens and CD4 cell counts) (Table 3 Paper IV).

5 DISCUSSION

HIV-1 is characterized by extensive genetic variability dependent on the location of the genome examined [13]. Such extensive genetic diversity of HIV-1 pose several challenges and implications for viral diagnosis [21], monitoring of the infection [22], development and patterns of drug resistance, disease progression [23], viral transmission, response to ART, and effective vaccine development [22, 24, 25]. HIV-1C is the most rapidly expanding subtype accounting for half of global HIV disease and nearly all infections in Ethiopia, Southern Africa and India which are the regions with the highest burden of HIV-1 infection. The molecular characteristics of this subtype success need to be explored to better understand such epidemic.

In **Paper I** we attempted to describe the current status of the Ethiopian HIV epidemic which was the first HIV-1C epidemic reported globally. Studies have since then consistently described the Ethiopian epidemic as predominated (97%-100%) by HIV-1C [27, 28, 174-176]. As these studies were from limited regions of the country, and as various studies reported circulation of mixed subtypes in neighbouring countries including Djibouti (subtype C (66%), CRF02_AG (20%), B (8.5%), CRF02_AG/C (2.9%) and K/C (2.9%) [177], Sudan (subtype A (46%), C (33%) and D (21%), and northern Kenya (subtype A (50%), C (39%), and D (11%) [31], we hypothesized that regional differences may exist in the Ethiopian epidemic as a consequence or mirror of the situation in the neighbouring countries. We therefore investigated the subtype distribution in different geographical regions of Ethiopia. Results of our current study showed that the Ethiopian HIV-1 epidemic is still monophylogenetic, exclusively dominated by HIV-1C, even though its first introduction was estimated to have been four decades back, around 1970 [178]. Our finding calls for further molecular and epidemiological studies to unravel such uniqueness despite changing epidemiology in the neighbouring countries.

Viral tropism at baseline is of clinical relevance mainly in terms of disease progression and it has been claimed to impact standard first line ART [76, 79]. Again, the few old published studies from Ethiopia were conducted in limited geographical regions. Furthermore, recent studies from South Africa and India, countries where the epidemic is dominated by HIV-1C, have claimed an increase in the proportion of X4 viruses [66, 179]. Thus, we also analysed the temporal trend of co-receptor usage of HIV-C$_{ETH}$. We chose the G2P clonal and clinical models among several other tools available for genotypic tropism prediction (see below) as 95% specificity of G2P have been reported for predicting X4-tropism in HIV-1C [156]. Moreover, it is the most widely used one and the European Guidelines recommend its use [180]. In our study, both tools predicted R5 tropism at a comparable frequency yet with much discrepancy between the predicted tropisms. Although such prediction discrepancy could be declared as a potential shortcoming which we tried to address further in **Paper II**, in general, our results described an epidemic dominated by R5 tropic virus and yet an increasing trend in

X4 tropic strains over the last decades, in agreement with other studies from South Africa and India where the epidemics are dominated by HIV-1C. However to investigate the prediction discrepancy by G2P models, we decided to analyse the sequences using more prediction tools in **Paper II**.

Several genotypic bioinformatics algorithm based tools have been developed for prediction of tropism following the discovery of maraviroc, the only co-receptor antagonist licensed so far. These algorithms were designed to infer phenotypic results from V3 loop nucleotide sequences using statistical techniques to weigh the prevalence of amino acid variants in each position. A score that estimates the probability of being associated with R5 phenotype is generated for amino acid variants in the V3 loop. Performance of the GTT tools is influenced by the training data set employed and most have been developed using HIV-1 B data [154], although PhenoSeq-C and C-PSSM have been trained on HIV-1 C as well [142, 144]. In our study, each of the GTT tool predicted a somewhat similar prevalence of R5-tropic viruses (ranging from 78.1-90.6%), in agreement with several previous studies which claimed a reliable performance of GTT tools [159, 181, 182], with no one clearly showing better performance than the other [183] although the comparison in these studies was with phenotypic assays. However, studies comparing concordance between GTT tools are limited. A large discordance between the five bioinformatics tools in our study could be explained by difference in statistical models employed by each tool, and different approaches to handle changes in a given V3 sequence [152]. By most of the prediction tools we used, there was a bidirectional switch of tropism at therapy failure, X4 to R5 being more frequent than R5 to X4. HIV-1 tropism switch under ART pressure in HIV1B have been reported in previous studies [73, 184, 185] with no dominance in either of the directions. Such viral switch during rebound seemingly occurring biased towards R5-virus and whether it is a phenomenon that occurs only in HIV-1C patients remain to be established. In both cases, fewer number of patients analysed at follow-up could be a possible limitation to draw conclusions.

Published studies reporting impact of baseline tropism on standard first line ART outcome are scarce. While some studies showed patients harbouring X4 strains at baseline associated with poorer VL suppression or CD4+ T cell gain [76-79], few others reported similar rates [80, 81]. In our attempt to correlate the predicted tropism at baseline with outcome of standard ART at month six and 12, we did not find any significant association co-receptor for most comparisons. Thus, our study suggests that the clinical value for predicting outcome of ART by viral tropism as predicted by GTT is limited in an Ethiopian setting. We hypothesized that lack of a gold standard to compare with, which is usually a phenotypic test, could be a possible limitation of this study. We therefore decided to employ a phenotypic assay to compare with GTT predictions in **Paper III**.

Results of our phenotypic assay showed that more than 90% of the tested isolates were R5 tropic while no pure X4 tropic isolate was detected. When we compared the phenotypic results with GTT prediction by each of the five methods employed in previous paper, we found a varying degree of concordance. Moreover, early phenotypic studies reported that

HIV-1C$_{Eth}$ strains are almost exclusively R5, even in patients with advanced immunodeficiency [65, 83], in contrast to our results in **Paper I** and **II**, which are based on prediction by GTT methods. Thus, results from Paper **III** suggest that these GTT tools have a suboptimal performance in describing the co-receptor usage of HIV-1C$_{Eth}$ strains. Our result in **Paper I** might therefore have overestimated the increase in X4-tropic strains in the last two decades. Such overestimation is also supported by recent studies comparing GTT predictions with phenotypic results [152, 186]. Hence, available GTT algorithms need to be adapted further using larger phenotypic/genotypic data-set of HIV-1C to improve tropism prediction.

It has been suggested that the CCR-co-receptor antagonist maraviroc could be a suitable alternative in HIV-1C dominating countries, but the above reported increase in proportion of X4 using HIV-1C strains argues against that. In order to elucidate this further, we attempted to analyse, whether there was a difference in sensitivity to maraviroc *in vitro* between pure R5 tropic and the dual tropic viruses. Our result demonstrated a dose response similar to the MJ4 isolate, a prototype for R5 using viruses, showing maraviroc's *in vitro* effectiveness against R5 and dual-tropic HIV-1C$_{Eth}$ isolates. Our finding is supported by a previous study which also demonstrated maraviroc's effectiveness *in vitro* against viruses with dual-characteristics, suggesting that the CCR5-antagonists may be a therapeutic-option in patients with dual/mixed-tropic viruses [49]. We acknowledge that small number of isolates included in our comparison could be a shortcoming to derive such conclusion. Yet, it should be noted that only six clones were found to be dual tropic and we compared maraviroc sensitivity of those samples with equal number of R5 tropic samples and tried to match source sample (the patient from which a given clone was derived) whenever possible. Thus, our limited data on the *in vitro* activity of maraviroc in dual tropic viruses could support the use of maraviroc as an alternative regimen, where available, as the vast majority of HIV-1C$_{Eth}$ isolates are R5 tropic and also dual tropic isolates showed sensitivity to this drug.

Published studies showed TDR as a factor contributing to consequent virological failure [187-189], limiting first line treatment options. This is especially of concern after rapid rollout of ART in RLS. Although there are few published data from Ethiopia on the level of TDR, they are from small regional studies and countrywide data is lacking. We therefore attempted analysis of TDR, from patients enrolled to a first large nationwide ART cohort in Ethiopia [167, 168], employing both PBSS and NGS. Our results by PBSS using samples collected after six years of ART rollout (2005) showed a low TDR rate, with no difference between geographical regions. As the patients were recruited in 2009-2011, current situation of TDR may have been changed as prevalence of TDRMs has been shown to be directly correlated with length of ART availability in the area in question [190]. WHO's recent report indicated that TDR in RLS is increasing over time [191] and TDRMs are of public health concern mainly in RLS as ART is initiated mostly at CD4 counts lower than 350 cells/μL in these settings. At such delayed initiation of ART, those mutations might already become minority variants, constituting less than 20% of virus population [190], which could be detected only by sensitive methods such AS-PCR or NGS. As most of the patients in our cohort (ACM study) had a low CD4 level at start of ART, the TDR rate might be

underestimated by PBSS. Earlier smaller studies from limited regions of Ethiopia using PBSS have reported low frequencies, 3.3% in 2003 and 0% in 2005 [192], which somewhat increased in later studies, 5.6% in 2008 [193], and 7.2% in 2010 [194]. However, our result from a larger nation-wide Ethiopian ACM cohort did not suggest an increasing trend of TDR.

As mentioned above, most of our patients had a low CD4 during initiation of ART. Also, our earlier study based on sensitive allele-specific PCR detected NNRTI TDR at higher rate (6.5%) of in patients from one of the study sites for current study, Addis Ababa [195]. Hence, we decided to investigate for occurrence of minority variants by employing NGS in selected group of patients. As expected, higher number of sDRM was identified, where additional DRMs were detected in 17 patients. We acknowledge that selection of the patients included for NGS was biased. Yet, the observed discrepancy between PBSS and NGS indicates that NGS facilitates detection of sDRMs in RLS and reveals a higher prevalence of TDR to the same or lower cost [196].

Since integrase strand transfer inhibitors (INSTIs) are planned to be used in some African countries recently, we analysed our NGS data and major INSTI mutations (T66I, E138K, Q148R, and Q148H) were found in five patients. During the period when samples were collected (2009-2011), and until now, INSTIs are not an integrated part of the ART regimens in Ethiopia. Furthermore, no clustering of those strains with INSTIs DRM could be revealed by the phylogenetic analysis (figure 2, paper IV). Rather, the patients were from five different ACM study sites, rendering possible transmission of these strains from INSTI treated subjects unlikely. One explanation for this occurrence could be that wild-type HIV-1C strains might harbour low abundance of INSTI DRMs. Hence, this phenomenon needs further investigation of minor quasispecies with regard to INSTI DRMs in RLS.

Studies attempted to elucidate the impact of pre-existing INSTI DRMs on treatment outcome, where low abundance INSTI DRMs had no impact in earlier studies [197, 198], while the E157Q mutation has been reported in 1.7%- 5.6% of ART-naïve patients in recent studies, depending on subtype[199] and been implied to affect treatment response [200]. However, those earlier studies used AS-PCR with the sensitivity to detect a significantly lower proportion of mutants than our NGS method with 1% cut-off. Therefore, a potential clinical impact of our findings still remains to be evaluated.

In summary, this thesis provides key information about HIV epidemic in Ethiopia, where the most prevalent and successful HIV-1 subtype in terms of global spread, HIV-1 C, was described for the first time three decades back. Moreover, it correlates molecular characteristics of the virus, mainly co-receptor tropism and drug resistance mutations, at baseline with treatment outcome of standard ART. Although the thesis is based on analysis of samples and patient data from HIV-1C infected Ethiopians, the findings are of global importance as HIV-1C is rapidly spreading throughout the globe and changing the epidemiology in many countries, especially western countries previously dominated by HIV-1B as a result of recent migrant influx.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

Main conclusions of the thesis are:

I.   After its estimated introduction to Ethiopia in 1970's, the HIV-1 epidemic is still monophylogenetic in all geographical regions of Ethiopia, almost exclusively comprised of HIV-1C, and dominated by R5-tropic virus, even in patients with advanced immunodeficiency.

II.   Each of the commonly used GTT tools predicted co-receptor usage with comparable frequency nonetheless with large discordance between the methods underestimating the presence of R5 and overestimating X4 strains compared to a phenotypic assay. Available GTT algorithms need to be thus adapted further using HIV-1C phenotypic/genotypic data set to improve prediction.

III.   Baseline tropism predicted by C-PSSM method showed an impact on outcome of standard first line ART at month 12 and hence could be possibly used for prediction of ART outcome in HIV-1C infected Ethiopians.

IV.   Maraviroc has an *in vitro* activity against most HIV-1C viruses and could be considered as an alternative regimen in HIV-1C infected individuals if afforded.

V.   NGS detected sDRM more often than PBSS and major INSTI DRMs were identified in minor viral variants.

VI.   Pre-treatment DRMs were associated with a poorer treatment outcome. The high rate of TDR and the identification of pre-existing INSTI DRMs at baseline by NGS highlights the importance of TDR surveillance in RLS and shows added value of high-throughput NGS.

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